Ethanol Production from Oil Palm Empty Fruit Bunch by Simultaneous Saccharification and Fermentation (SSF) with *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*

Suwanan Sukhang

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Engineering in Chemical Engineering

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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Candidate

ชื่อวิทยานิพนธ์ การผลิตเอทานอลจากทะลายปาล์มเปล่าด้วยวิธีการหมักรวม (SSF) โดยใช้ยีสต์ *Kluyveromyces marxianus* และ *Saccharomyces cerevisiae*

ผู้เขียน นางสาวสุวนันท์ สุขัง

สาขาวิชา วิศวกรรมเคมี

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บทคัดย่อ

งานวิจัยนี้เป็นการศึกษาการผลิตเอทานอลจากทะลายปาล์มเปล่าด้วยวิธีการหมักรวม Simultaneous Saccharification and Fermentation (SSF) โดยใช้ยีสต์ *K. marxianus* เปรียบเทียบกับใช้ยีสต์ *S. cerevisiae* ซึ่งทะลายปาล์มจัดอยู่ในประเภทวัสดุลิกโนเซลลูโลสและโครงสร้างหลักของทะลายปาล์มคือ เซลลูโลส เฮมิเซลลูโลส และลิกนิน ซึ่งเชื่อมโยงด้วยพันธะเคมี และขั้นตอนหลักของการผลิตเอทานอลจากวัสดุลิกโนเซลลูโลสมี 3 ขั้นตอนคือ 1. ขั้นตอนการปรับสภาพวัตถุดิบเพื่อกำจัดลิกนินออก ซึ่งเป็นตัวขัดขวางการย่อยของเอนไซม์ 2. ขั้นตอนการย่อยคือการย่อยเซลลูโลสและเฮมิเซลลูโลสเป็นน้ำตาลโมเลกุลเดี่ยวเพื่อเป็นวัตถุดิบหลักในการหมัก 3. ขั้นตอนการหมักคือใช้ยีสต์ในการเปลี่ยนน้ำตาลเป็นเอทานอล ดังนั้น**ส่วนแรก**เป็นการศึกษาขั้นตอนการปรับสภาพด้วยกรดซัลฟิวริกและตามด้วยโซเดียมไฮดรอกไซด์ โดยออกแบบการทดลองด้วยวิธีการพื้นผิวตอบสนองในการขั้นตอนการปรับสภาพด้วยกรด ศึกษาทั้งหมด 3 ตัวแปร คือ ปริมาณวัตถุดิบเริ่มต้นร้อยละ 15-25 โดยน้ำหนักต่อปริมาตรของกรด เวลาในการทำปฏิกิริยา (30 - 90 นาที) และความเข้มข้นของกรดซัลฟิวริก 0.2 - 1.0 โมลาร์ หลังจากนั้นปรับสภาพต่อด้วยสารละลายโซเดียมไฮดรอกไซด์ที่ความเข้มข้นร้อยละ 5 โดยน้ำหนักต่อปริมาตร เป็นเวลา 20 นาที พบว่า สภาวะที่ดีที่สุดในปรับสภาพคือ ปริมาณวัตถุดิบเริ่มต้นร้อยละ 15 โดยน้ำหนักต่อปริมาตรของกรด เวลาในการทำปฏิกิริยา 53 นาที และความเข้มข้นของกรดซัลฟิวริก 0.2 โมลาร์ และปรับสภาพต่อด้วยสารละลายโซเดียมไฮดรอกไซด์ความเข้มข้นร้อยละ 5 โดยน้ำหนักต่อปริมาตร ให้ปริมาณเซลลูโลสมากที่สุดคือ ร้อยละ 72.10 โดยน้ำหนัก ส่วนเฮมิเซลลูโลสและลิกนินได้น้อยที่สุดคือ ร้อยละ 3.24 และ 17.60 โดยน้ำหนักตามลำดับ มีอัตราการย่อยด้วยเอนไซม์ร้อยละ 83.50 นอกจากนี้เมื่อวิเคราะห์ด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด พบว่าการปรับสภาพด้วยกรดตามด้วยด่างสามารถทำลายโครงสร้างผลึกบนเส้นใยโดยการขจัดผนังเซลล์ ส่งผลให้ปริมาณเฮมิเซลลูโลสและลิกนินลดลง ใน**ส่วนที่สอง**เป็นการศึกษาการหมักเอทานอลด้วยวิธีการหมักรวม (SSF) โดยการใช้ยีสต์ *K. marxianus* เปรียบเทียบกับการใช้ยีสต์ *S. cerevisiae* โดยออกแบบการ

ทดลองด้วยวิธีการพื้นผิวตอบสนองและในกระบวนการหมัก ศึกษาทั้งหมด 4 ตัวแปรคือ อุณหภูมิในการหมัก 30 - 45 องศาเซลเซียส ปริมาณวัตถุดิบที่ผ่านการปรับสภาพเริ่มต้นร้อยละ 5 - 15 โดยน้ำหนักต่อปริมาตร ค่าความเป็นกรด-ด่าง 4 - 6 และปริมาตรเชื้อยีสต์เริ่มต้นร้อยละ 1 - 5 โดยปริมาตร พบว่าสภาวะที่เหมาะสมที่สุดโดยใช้ยีสต์ *K. marxianus* คือ อุณหภูมิในการหมัก 36.94 องศาเซลเซียส ปริมาณวัตถุดิบที่ผ่านการปรับสภาพเริ่มต้นร้อยละ 12.24 โดยน้ำหนักต่อปริมาตร ค่าความเป็นกรด-ด่าง 4.5 และปริมาตรเชื้อยีสต์เริ่มต้นร้อยละ 2.04 โดยปริมาตร ผลิตเอทานอลได้สูงสุดที่ 0.281 กรัมเอทานอลต่อกรัมวัตถุดิบ ที่เวลาการหมัก 48 ชั่วโมง และ สภาวะที่เหมาะสมที่สุดโดยใช้ยีสต์ *S. cerevisiae* คือ อุณหภูมิในการหมัก 35.03 องศาเซลเซียส ปริมาณวัตถุดิบที่ผ่านการปรับสภาพเริ่มต้นร้อยละ 8.16 โดยน้ำหนักต่อปริมาตร ค่าความเป็นกรด-ด่าง 4.91 และปริมาตรเชื้อยีสต์เริ่มต้นร้อยละ 3.38 โดยปริมาตร ผลิตเอทานอลได้สูงสุดที่ 0.320 กรัมเอทานอลต่อกรัมวัตถุดิบ ที่เวลาการหมัก 48 ชั่วโมง ใน**ส่วนสุดท้าย** ทำการศึกษาการหมักเอทานอลด้วยวิธีการหมักแยก Separated Hydrolysis and Fermentation (SHF) โดยเลือกสภาวะการหมักจากการศึกษาในส่วนที่สอง พบว่า การหมักโดยใช้ยีสต์ *K.marxianus* ผลิตเอทานอลได้ 0.258 กรัมเอทานอลต่อกรัมวัตถุดิบ และโดยใช้ยีสต์ *S.cerevisiae* ผลิตเอทานอลได้ 0.302 กรัมเอทานอลต่อกรัมวัตถุดิบ แสดงให้เห็นว่า การผลิตเอทานอลด้วยวิธีการหมักรวม (SSF) สามารถผลิตเอทานอลได้มากกว่าวิธีการหมักแยก (SHF) และใช้ระยะเวลาในการผลิตน้อยกว่า ซึ่งเวลาที่ใช้ในกระบวนการผลิตแบบแยกใช้เวลาทั้งหมด 120 ชม.ส่วนกระบวนการผลิตแบบรวมใช้เวลาทั้งหมด 48 ชม.จึงใช้เวลาน้อยกว่า 72 ชม.

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Academic Year	2018

ABSTRACT

This research purpose is to study the ethanol production from Oil Palm Empty Fruit Bunch (OPEFB) by Simultaneous Saccharification and Fermentation (SSF) with *Kluyveromyces marxianus* and *Saccharomyces cerevisiae.* OPEFB is lignocellulosic biomass and their main components are cellulose, hemicellulose and lignin which cross linked into chemically complex. The basic steps for production of ethanol from lignocellulosic biomass is through three major operations; pretreatment for delignification is necessary to liberate cellulose and hemicellulose before hydrolysis of cellulose and hemicellulose to produce fermentable sugars and fermentation of reducing sugars to ethanol. **The first section** was the study of pretreatment step with sulfuric acid followed by sodium hydroxide and employing Response Surface Methodology (RSM) for designing experiment and optimization. Three factors including, substrate loading (5 – 25 % w/v), reaction time (30 - 90 min) and acid concentration (0.2 - 1 M) were optimized after that pretreated with sodium hydroxide 5 % (w/v) for 20 min. The optimum condition of pretreatment step was substrate loading (15 % w/v), reaction time 53 min and concentration of sulfuric acid 0.2 M gave the highest cellulose yield of 72.10 %wt. and the lowest hemicellulose and lignin yield of 3.24 %wt. and 17.60 %wt. respectively. In addition, the enzyme digestibility of the treated OPEFB 83.5 %. Scanning Electron Microscope (SEM) analysis showed that the acid pretreatment followed by alkali caused great disruptions on the fiber structure by removing the cell wall, hydrolyzing both hemicellulose and lignin. **The second section** was to study ethanol fermentation with SSF was conducted by using *K. marxianus* and *S. cerevisiae* yeasts and employing RSM for designing experiment and optimization. Four factors including, temperature (30 - 45 ºC), substrate loading (5 - 15 % w/v), pH (4 - 6) and yeast concentration (1 – 5 % v/v) were optimized. It was found that the optimum condition of *K. marxianus* yeast was the

fermentation temperature of 36.94 °C, substrate loading (12.24 % w/v), pH 4.5 yeast concentration (2.04 % v/v). The ethanol production was 0.281 g/g biomass at 48 h and the optimal condition of *S. cerevisiae* was the fermentation temperature is 35.03 °C substrate loading (8.16 % w/v), pH 4.91 and yeast concentration (3.38 % v/v). The ethanol production was 0.320 g/g biomass at 48 h. **The final section** to study, ethanol fermentation with Separated Hydrolysis and Fermentation (SHF) by select the fermentation conditions from the study in the second section. It found that fermentation by *K. marxianus* produced ethanol 0.258 g/g biomass and *S. cerevisiae* produced ethanol 0.302 g/g biomass. Show that production of ethanol by SSF gives ethanol yield more than SHF and decrease fermentation time. Due to Total time in SHF process was used 120 h and SSF process was used 48 h. SSF process used less time 72 h of SHF process.

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Suwanan Sukhang

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ABBREVIATION

AP	Alkaline Peroxide
BL	Black Liquor
CA	Calcium Alginate
CAB-OH	Acidic-Alkaline Pretreated Cashew Apple Bagasse
CBP	Consolidated Bioprocess
CCD	Central Composite Design
DAA-AA	Sequential Dilute Acid Autoclaving and Alkaline Autoclaving Pretreatment
EFB	Empty Fruit Bunch
EPFBF	Empty Palm Fruit Bunch Fiber
FPU	Filter Paper Unit
GU	Goedae-Uksae
LHW	Liquid Hot Water
LOF	Lack of Fit
OA	Oxygen – Alkali
OD yeast	Optical Density yeast
OPEFB	Oil Palm Empty Fruit Bunch
OPF	Oil Palm Frond
OPT	Oil Palm Trunk
PA	Peracetic Acid
PPF	Palm Pressed Fiber
PVA	Polyvinyl Alcohol
RSM	Response Surface Methodology
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
TISTR	Thailand Institute of Scientific and Technology Research
U or UI	Unit or Internation Unit
WIS	Water-insoluble solids
WO	Wet - Oxidation

CHAPTER 1

INTRODUCTION

1.1 Source of problems and significance

Nowadays, Thailand is having a large economic growth. Energy is important in industrial production systems, transportation and activities. Due to increasing energy use, rising crude oil prices and increasing fossil fuel consumption, we need to develop renewable energy sources. In Thailand, ethanol is one of the most attractive alternative energy sources because it is clean energy and is supported by the Thai government (Bloyd and Foster, 2014). Thailand is eager to expand its biofuel production sector, increase its use of renewable energy, reduce fossil energy use, reduce energy intensity, and greenhouse gas emissions. Ethanol (Ethanol or Ethanol) is a renewable energy source used in gasoline, a common fuel produced from agricultural raw materials, which can be divided into three categories of agricultural raw materials: sugar materials, starch materials and lignocellulosic materials, biomass is one of the new forms of biomass energy with appropriate administration and technologies (Preechajarn and Ponnarong, 2014). Most lignocellulosic biomass products are by-products of agricultural products such as rice straw, sugar cane and palm bunches. However, ethanol production by fermentation of raw materials has a great influence on economics, accounting for more than half of the cost of production (Redding *et al.*, 2011). To obtain cost-effective for the production, the supply of agricultural residual and inexpensive raw materials specifically from lignocellulosic biomass is a good alternative substrate in the future. Besides, they do not demand divdide land, water, and energy desires and do not have food value as well (Sarkar *et al.,* 2012). Since Thailand is an agricultural country, the most suitable raw material for renewable energy is lignocellulosic material, which reduces environmental problems and builds energy security.

Due to the problem mentioned by the raw material substitute food crops to produce ethanol. Based on estimates of agricultural wastes in the south, most of them are waste from the palm oil industry. The oil palm empty fruit bunch are the most disposable 32 % of fresh palm fruit

bunch. On the other hand, the chemical composition of OPEFB with high cellulose content of 40 – 70 % (Triwahyuni *et al.,* 2015) that is be decent to ethanol production by hydrolysis of cellulose to sugar then ferments to ethanol. Ethanol production from lignocellulosic material has three main stages: pretreatment, hydrolysis and fermentation. In addition, during the production process there is a by-product of xylose (Duangwang *et al.,* 2016). If return of xyloses increases the value of investment. It found that pretreatment had different advantages and disadvantages. Steam explosion is the most popular method. The advantage is that it destroys the complex structure of the fiber, making the enzyme hydrolysis easier because it has very little inhibitory effect (Kossatz *et al.,* 2017). But the disadvantage is that it can bring the xylose back from process very little. In addition, acid treated can remove hemicellulose and increase cellulose. If used with alkaline conditions can remove lignin. In this way, xylose is a valuable by-product. There are two ways of hydrolysis: hydrolysis with acid, the disadvantage is that many side effects are inhibitors of microorganisms in the fermentation process. In addition, hydrolysis with enzyme will not cause any side effects, resulting in more ethanol fermentation. The fermentation process has 2 ways: SHF and SSF. SSF has the advantage of producing less glucose, less toxic to yeast, less time to ferment and increased ethanol. *Kluyveromyces marxianus* is a yeast resistant to heat up to 45 °C (Nachaiwieng *et al.,* 2015), but most research in the past used *Saccharomyces cerevisiae* to produce ethanol produced high ethanol, but the temperature is only 35 °C, suitable for SHF. The variables that effect the SSF are temperature, substrate loading, yeast concentration, and pH.

Therefore, this study is interested in the optimal conditions for ethanol production with SSF by *S. cerevisiae* and *K. marxianus* fermented comparing with SHF. The two types of fermentation and two yeasts have different advantages and disadvantages. However, it is necessary to have an economic evaluation and increase the value of investment. So, ethanol production from biomass is the second generation fuel that will play a role in the future.

1.2 Research objectives

1.2.1 To study the factor affecting the pretreatment of OPEFB with dilute-acid (H_2SO_4)/alkaline (NaOH).

1.2.2 To investigate the optimum condition for SSF process for ethanol production from OPEFB by *K. marxianus* and *S. cerevisiae*.

1.2.3 To compare ethanol production process by Simultaneous Saccharification & Fermentation (SSF) and Separated Hydrolysis & Fermentation (SHF).

1.3 Scopes of research work

1.3.1 Dilute-acid/alkaline pretreatment by using H_2SO_4/NaOH

1.3.1.1 Study the factors affecting pretreatment of OPEFB with dilute sulfuric acid and sodium hydroxide solution. The parameters: substrate loading, sulfuric acid concentration and reaction time.

1.3.1.2 Using dilute sulfuric acid (0.2 – 1 M) and sodium hydroxide solution (5 % w/v) for OPEFB pretreatment. Design Experiment with Design Expert 8.0.7.1 using Response Surface Methodology (RSM) and Central Composite Design (CCD).

1.3.2 Fermentation

1.3.2.1 Study the optimum condition for ethanol production via SSF from treated OPEFB by using *K. marxianus* and *S. cerevisiae*. Design Experiment with Design Expert 8.0.7.1 using RSM and CCD.

1.3.2.2 Study the factor effect of increase ethanol content are four variables: pH 4 - 6, substrate loading 5 - 15 % (w/v), yeast concentration 1 - 5 % (v/v) and the fermentation temperature 30 - 45 °C.

1.3.2.3 Comparing the yield of ethanol production between SSF and SHF.

1.4 Expected benefits

1.4.1 Using OPEFB as a raw material for ethanol production.

1.4.2 Achieve the sequential acid/alkaline pretreatment of OPEFB for maximized cellulose.

1.4.3 Obtain fermentation of OPEFB with *K. marxianus* and *S. cerevisiae* for maximized ethanol.

CHAPTER 2

THEORIES AND LITERATURE REVIEWS

2.1 Oil palm

Elaeis guineensis, generally called oil palm which configuration is a species of palm being the main source of oil palm from southwest Africa and west. The oil palm is generally grown in three south part of Thailand is Krabi, Chumporn and Suratthani, where more than 70 % of southern Thailand planted with palm oil.

2.2 Types of raw materials for ethanol production

Ethanol production can use a variety of raw materials through alcoholic fermentation and other processes to maximize ethanol production. The raw materials of ethanol production can be classified into 3 types as shown in Figure 2.1.

1. Sugar; such as sugar cane, molasses, beetroot and sweet sorghum.
2. Starch; such as rice, wheat, corn, cassava, potatoes and sweet potatoes.
3. Cellulose; such as rice straw, bagasse, sawdust, and oil palm empty fruit bunches.

Figure 2.1 Ethanol production process from each raw material

Source: Slade, R.B., 2009, *Prospects for cellulosic ethanol supply-chains in Europe: a techno-economic and environmental assessment, in Centre for Process Systems Engineering and Centre for Environmental Policy*., Univiersity of London. p. 170.

2.3 Lignocellulose material

Lignocellulosic material is a carbohydrate organic compound that is an important component of plant cells. It is made of single molecule sugar, connected by a long chain or polymer of single molecule sugar composed of cellulose, hemicellulose and lignin. In general, lignocellulosic materials such as bagasse, rice straw and oil palm empty fruit bunch contain 40 - 60 % cellulose, 20 - 30 % hemicellulose, and 15 – 30 % lignin as shown in Figure 2.2. Lignocellulosic substance can be used to produce ethanol. Cellulose and hemicellulose may be separated before being hydrolyzed to a single molecule of sugar for ethanol production. The main components of lignocellulosic materials structure are shown in Figure 2.3.

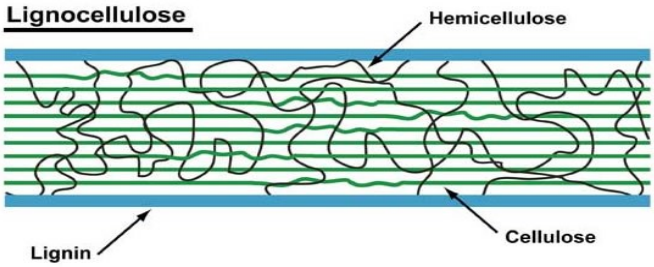


Figure 2.2 Main components of lignocellulosic materials

Source: USDA Agricultural Research Service,

http://www.sfi.mtu.edu/FutureFuelfromForest/LignocellulosicBiomass.htm

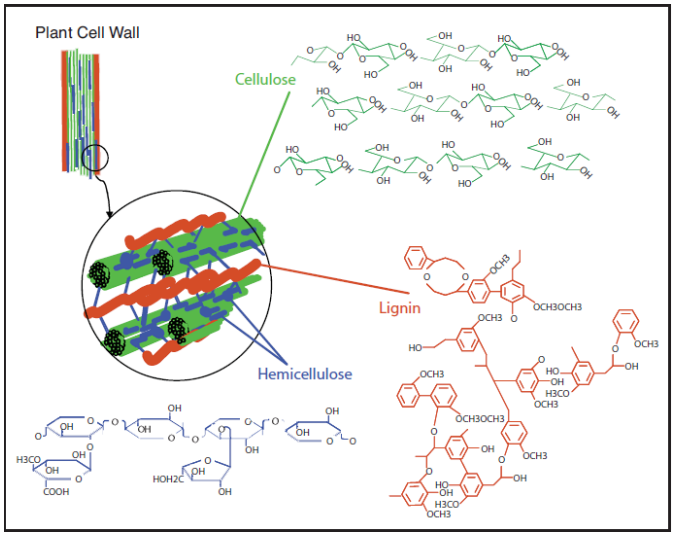


Figure 2.3 Lignocellulosic materials structure

Source: https://biofuel.webgarden.com/sections/blog/pictures-for-lignocellulose

2.3.1. Cellulose

Cellulose is the most abundant element in lignocellulosic material. It is found in the cell walls of plants and is combined with hemicellulose and lignin. The amount found varies depending on the species and composition of the plant such as 40 – 50 % of wood and 98 % of cotton fiber (Eriksson, 1990). Cellulose is a homopolymer that has a straight line with no branches. It contains about 50,000 molecules of glucose. The basic subunit is β - D - Glucopyranose, which is linked by (β - 1, 4 - glycosidic bond is formed polymer glucan of about 10,000 units, bonded by hydrogen bonds. The chemical structure formula of cellulose is $(C_6H_{10}O_5)_n$ and the structure of one shackle of the polymer is offer in zero (Harmsen *et al.,* 2010). Two types of cellulose are found in nature: crystalline cellulose and amorphous cellulose. The crystalline cellulose is degraded by enzymes harder than amorphous cellulose for the cellulose chemistry. Cellulose is likewise insoluble in dilute acid solutions at low temperature. The solubility of the polymer is stably related to the quality of hydrolysis accomplish. So the result, factors that influence the hydrolysis rate of cellulose also influence its solubility that obtain place, yet, with the molecule being in a dissimilar form than the inherent one. At higher temperatures it transforms into soluble, forasmuch the energy providing is sufficient to break the hydrogen bonds that hold the crystalline structure of the molecule. Concentrated acids can also dissolve cellulose. However, severe polymer degradation occurs in the alkali solution, resulting in significant cellulose swelling, similar to the low molecular weight fraction of the polymer. As shown in Figure 2.4.

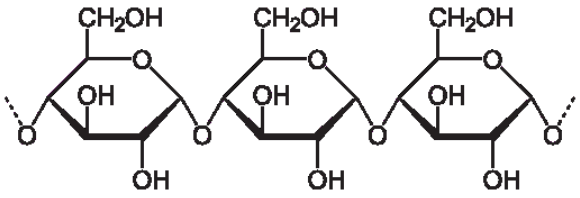


Figure 2.4 Cellulose structure formulation

Source: https://lv.wikipedia.org/wiki/Att%C4%93ls:Amylose3.svg

2.3.2. Hemicellulose

The hemicellulose is a collective of sugar type. It is used to supersede a clan of polysaccharides such as galactans, gluco-mannans, arabino-xylans, and others that are found in the plant cell wall and have dissimilar composition and structure depending on their the extraction method and source. Hemicellulose is a heteropolymer of each type sugar of which is mixed, such as glucose, mannose, xylose and arabinose. It is found in the polymers xylan, mannan, galektan and arabinan with an average length of about 200 units (Bastawde, 1992). In Polymerwilan found the greatest amount of D-xylose is 85 – 93 %, other components such as glucose, glucuronic acid, galacturonic acid is found in small amounts (Browing, 1963) by xylose will linked with β 1, 4 - glycosidic bonds (Altıntas *et al.,* 2002). Water at low temperatures cannot dissolve hemicellulose. However, its hydrolysis process starts at a lower temperature than the cellulose, which dissolves at higher temperatures. The chemical structure of xylan is shown in Figure 2.5.

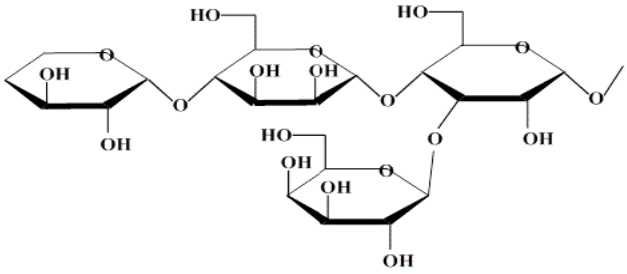


Figure 2.5 Hemicellulose structural formulation

Source: Sajith, S., Priji, P., Sreedevi, S., Benjamin, S., 2016, *An Overview on Fungal Cellulases with an Industrial Perspective*. Journal of Nutrition & Food Sciences, 6:1.

2.3.3. Lignin

Lignin is an aromatic compound found in plant cell walls, found in varying amounts by plant type. Lignin is a cellulose defense that cannot be hydrolysis by microbial enzymes. Lignin is heteropolymer has structural 3D, and consists of three aromatic compounds, namely tran-p-coumaryl alcohol, trans-coniferyl alcohool and trans-p-sinapyl alcohol (Cheng *et al.,* 2008). Lignin molecules are also linked to many other aromatic compounds, such as vanillin and syringaldehyde (Yudkin and Offord, 1973). Structural formula trans-p-coumaryl alcohol, trans-coniferyl alcohool and trans-p-sinapyl alcohol are shown in Figure 2.6. Lignin in lignocellulosic biomass performs as an insoluble three-dimensional network. It is behavior as fastening between

cells establishing a composite material that has a specific resistance to impact, bending and compression.

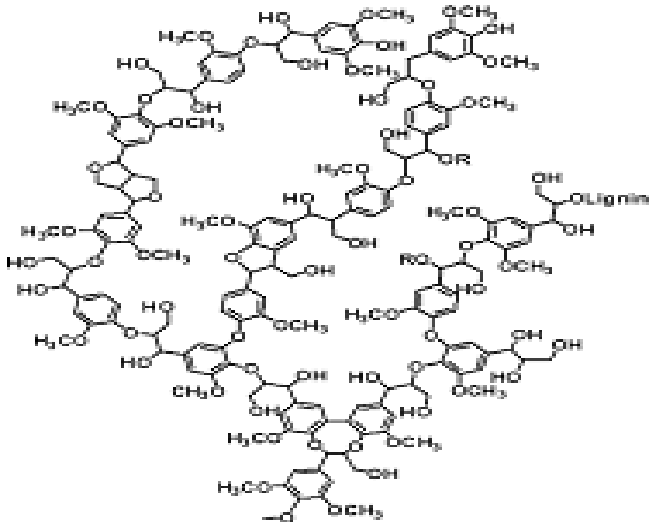


Figure 2.6 Lignin structure formulation

Source: https://www.biobasedpress.eu/2015/12/lignin-makes-a-take-off-across-the-board-says-ludo-diels-vito/lignin-structure/

2.4 Steps to produce ethanol from lignocellulosic material

Lignocellulosic materials can be used to hydrolysis the cellulose to glucose for ethanol fermentation. Ethanol production from lignocellulosic materials can be divided into 3 steps: 1. Pretreatment step 2. Hydrolysis step 3. Fermentation step (Margeot *et al.,* 2009). The ethanol production from lignocellulosic material is shown in Figure 2.7.

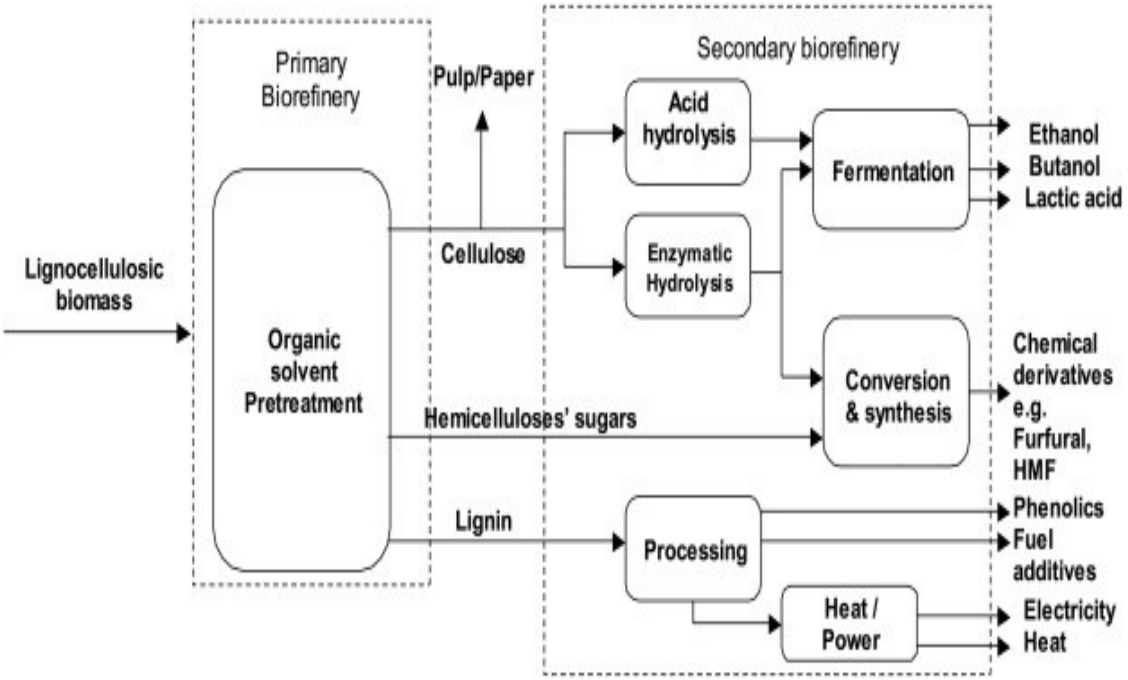


Figure 2.7 Steps to produce ethanol from lignocellulosic material

Source: Dimitrios, K.S. and Ioanna S.S., 2015, Organosolv pretreatment as a major step of lignocellulosic biomass refining. Engineering Conferences International ECI Digital Archives.

2.4.1 Pretreatment step

Pretreatment is the process of removing lignin compounds encapsulated in hemicellulose and cellulose shown in Figure 2.8, because these compounds affect the hydrolysis step. If lignin compounds are not removed, it occurrence the effect of hydrolysis or by-product likely to affect the fermentation step. Factor affecting the pretreatment step include temperature, substrate loading, reaction time, solution concentration and particle size. The ensuing criteria lead to an improvement in (enzymatic) hydrolysis of lignocellulosic biomass:

- Increasing of the porosity and surface area
- Alteration of lignin structure
- Removal of lignin
- Curtailment of hemicellulose
- (Partial) depolymerization of hemicellulose
- Relieve the crystallinity of cellulose

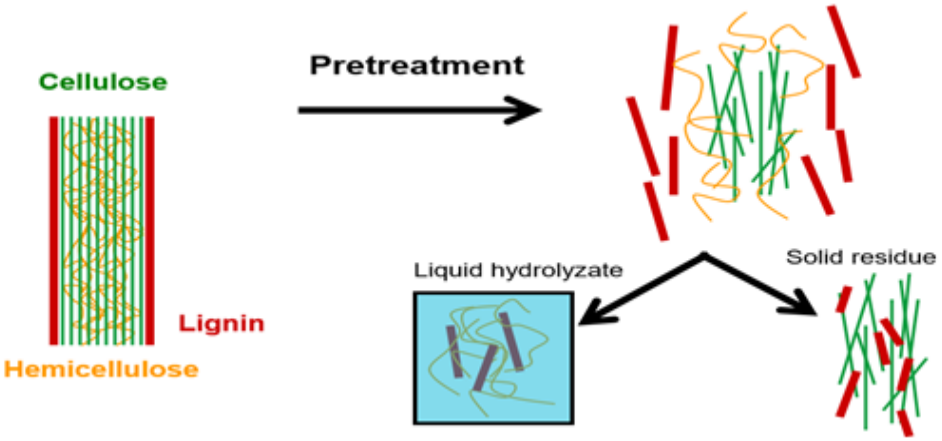


Figure 2.8 Pretreatment step of lignocellulosic material

Source: https://www.e-education.psu.edu/egee439/node/653

Type of pretreatment basic

Physical pretreatment

Mechanical commination

Reduction of the particle size is required to facilitate material handling and to increase the surface/volume ratio. This can be achieved by milling or grinding, chipping. Mechanical pretreatment is normally carried out prior to further processing step, and the desired particle size is required are based on the following steps. For mechanical pretreatment factors such as capital costs, operating expenses, scalability and depreciation of equipment, it is very important.

Chemical pretreatment

1) Liquid hot water

Liquid hot water (LHW) processes are lignocellulose biomass pretreatments with water at high pressure and temperature. During the process can be decreased hemicellulose but can be increased cellulose. Hemicellulose can be recovered monomeric which results in the formation of sugar monomers that may decompose into furfural (fermentation inhibitors).

2) Acid pretreatment

Using of dilute acid is the most popular studied methods because the effect is the best for pretreatment step. Normally, there are two types of pretreatment:

1. High temperature and low-solids loading (T > 160 °C, 5 – 10 %wt. substrate concentration).

2. Low temperature and high-solids loading (T $\leq$ 160 °C, 10 - 40 %wt. substrate concentration).

Most use of dilute acid is sulfuric acid. Sulfuric acid can be removed hemicellulose for the purpose of increasing porosity and improving enzymatic hydrolysis. Hemicellulose is hydrolysate as xylose and some part of them became furfural and hydroxymethyl furfural, which inhibit the growth of microorganisms. This way is not suitable for lignin removal.

3) Alkaline pretreatment

Pretreatment step must remove lignin was the important purpose of pretreatment process. From research show that alkaline can be lignin effectively remove and can be enhanced reaction of enzymatic hydrolysis in the hydrolysis process. Because of lignin inhibits enzymatic hydrolysis. Examples of alkaline used Calcium or Sodium Hydroxide and Ammonia.

Combined chemical and Physical pretreatment

1) Steam explosion

Steam explosion is the most of applied pretreatment processes belated to its limited energy consumption and low use of chemicals. Steam explosion method, high-pressure saturated steam is injected into reactor filled with biomass. During the steam injection, the temperature increases to 160 - 260 ºC. After that, pressure is abruptly decreased and the biomass under-goes an explosive decompression with removal hemicellulose and lignin disruption.

Biological pretreatment

Biological pretreatment method will use microorganisms in lignocellulose pretreatment. In this group of pretreatments microorganisms such as bacteria, fungi and enzymes are employed to degrade cellulose, hemicellulose and lignin. Type of fungi such as white-rot, brown-rot and soft-rot are employed to degrade cellulose, hemicellulose and lignin are white-rot. However, the rate of biological hydrolysis is usually very low, so this pretreatment requires long times.

The study research of the pretreatment step, Kim *et al.,* (2012) studied sequential acid/alkali pretreatment of empty palm fruit bunch fiber. Total pretreatment process three step, first step was solid (20 % w/v) with soaked in H_2SO_4 solutions within the concentration range of 0.1 – 8.0 % (v/v) at 121 °C, 15 psi for 1 h. the second was alkali pretreatment with soaked in NaOH 10 N at ambient temperature for 4 h and the last was thermal pretreatment at 121 °C for 15 min. The result found that dilute sulfuric acid in first step, which can removed hemicellulose 90 % and lignin 32 %, but increase cellulose under the optimum condition. Sodium hydroxide in the second step,

which can removed lignin effectively with a 70 % delignification yield, this is an inhibitor of enzyme digestion.

Tan *et al*., (2013) studied pretreatment of empty fruit bunch from oil palm for fuel ethanol production, pretreatment was conducted at 180 °C for 30 min with sodium bisulfite and sulfuric acid dosages of 8 % and 1 %, respectively. The result found that from total 664 kg solid composition was 353 kg of glucan, 95 kg of xylan and 107 kg of lignin.

Muryanto *et al.,* (2015) studied pretreatment step of EFB using black liquor and alkaline delignification. The experiment used black liquor (BL) and its mixture with NaOH as pretreatment solution at condition was 100 % BL, 50 % BL mixing 2.5 M NaOH and 2.5 M NaOH. The reactor was controlled at 4 bar, 150 °C with reaction time 30 and 60 min. It was found that the best of increase of cellulose 63.82 % and decrease of hemicellulose and lignin was 12.14 %, 12.56 % respectively under pretreatment condition 2.5 M NaOH at 150 °C for 30 min. Hydrolysis with two kinds of enzymes, CTec2 30 FPU/g EFB and HTec2 6 U/g EFB or the ratio 5:1 at pH 4.8 and temperature 50 °C, 150 rpm for 72 h, which achieved the maximum glucose yield 93.80 g/L from the maximum theoretical glucose yield 106.35 g/L.

Palamae *et al*., (2017) studied cellulose and hemicellulose production from oil palm empty fruit bunch (EFB) fibers by pretreatment with peracetic acid (PA) and alkaline peroxide (AP) at temperatures (20 – 35 °C) can removed lignin more than 98 % of lignin from (EFB). The totaltreatment time was 21 h (a 9 h PA treatment at 35 °C, a 12 h treatment with AP (20 °C, 4 % NaOH)). The result found that the post-treatment composition was 11.2 ± 0.5 % of hemicellulose, 81.9 ± 0.7 % of cellulose and 2.8 ± 0.0 % of lignin.

Duangwang and Sangwichien, (2013) studied optimizing alkali pretreatment of Oil Palm Empty Fruit Bunch by analysis optimizations with response surface methodology. The reactive conditions tested were NaOH concentration (1 – 15 % (w/v), temperature (100 – 130 °C) and reaction time (15 – 90 min). The result found that under optimum conditions, cellulose yield was 68.8 % when operating temperature, reaction time and NaOH concentration were 130 °C, 40 min and 15 %, respectively.

2.4.2 Hydrolysis step

Hydrolysis step is digestive cellulose become polymer of C5 sugar covert to glucose, and digestive hemicellulose become co-polymer of C5 or C6 sugar covert to mannose, arabinose, xylose and glucose. The production of each type of sugar depends on the type of plant (Bosch *et al*., 2010). Hydrolysis can be separated into 2 group as follows (Niwaswong *et al*., 2012).

1) Acid Hydrolysis

Acid hydrolysis of lignocellulosic biomass by using dilute acid under high pressure and temperature or low pressure and temperature then increase the concentration of acid. Hydrolysis with acid will provide high volume of sugar but, it occurred several inhibitory compounds, such as furfural, hydroxymethyl furfural and heavy metal ions that from lignin degradation products. Their toxicity is a major factor affect fermentation processe. General, acid hydrolysis frequently used sulfuric acid and hydrochloric acid because of low cost but it occurred by-products that high toxic. (Mussatto and Roberto, 2004)

2) Enzymes Hydrolysis

Hydrolysis of lignocellulosic biomass has been minutely researched since the 1970. Enzymatic hydrolysis, which use enzyme to digest cellulose and hemicellulose into glucose and xylose respectively by cellulase enzyme and cellobiose (β-glucosidse), which this reaction will occur under temperature around 40 - 50 °C. Enzymatic hydrolysis has a number of advantages such as; high yield of pure glucose, non-toxic to the environment and mild reactive conditions when it compared with acid hydrolysis. Reaction step need to react under temperature of 50 °C and pH 5 without by-products. Current, enzyme hydrolysis is the most popular method due to the high sugar content (Hamzah *et al.,* 2011).

The study research of the hydrolysis step, Hamzah *et al.,* (2011) studied hydrolysis of enzymatic on treated EFB by using combination of cellulase and β 1-4 glucosidase. EFB was soaked with 2.5 M NaOH after that autoclaved for 15 min at 121 °C in pretreatment step. The composition of the treated EFB was 66.77 ± 1.22 % of cellulose, 24.5 ± 1.28 % of hemicellulose and 7.25 ± 0.98 % of lignin. Factor study in the hydrolysis step was ratio of cellulase and β 1-4 glucosidase (5 : 1, 2 : 1, 1 : 2, 1 : 1 and 1 : 5), pH (4 - 6), temperature (30 – 60 °C) and substrate loading (2 - 8 % w/v). It found that the best of condition hydrolysis was ratio of cellulase and β 1-

4 glucosidase (5 : 1), pH 4.8 at 50 °C and substrate loading (8 % w/v) gave highest glucose concentration up to 2.7 g/L.

Boonsawang *et al.,* (2012) ethanol production from palm pressed fiber by (SSF). PPF was pretreated with 2.5 M NaOH (solid:liquid ratio of 100 kg : 1 m^3) at 100 °C for 15 min. They studied the effect of concentration of enzyme range (10 and 20 FPU/g PPF) and temperature range (35 and 50 °C). It found that the best of condition hydrolysis with cellulase (10 FPU/g PPF) and β-glucosidase (10 U/g PPF) gave the higher reducing sugar production than using cellulase alone at 50 °C and ethanol yield was 195 ± 9.00 g/kg cellulose.

Dahnum *et al*., (2015) studied optimization of bioethanol production from empty fruit bunch using enzyme and dry yeast. The experimental was connected to evaluate the effect of hydrolysis methods and enzyme concentration for producing ethanol. Pretreatment used 10 % NaOH at temperature 150 °C for 30 min. Four concentration of enzyme Cellic® CTec2, 10, 20, 30, 40 FPU/g biomass were performed in SHF and SSF processes respectively, while Cellic® HTec2 was added 20 % from Cellic®CTec2. The best of condition was 40 FPU of enzyme concentration, it could be produce 6.05 % of ethanol in 24 h fermentation by SSF process and 4.74 % of ethanol in 72 h by SHF process.

Cha *et al.,* (2015) Ethanol production from *Miscanthus sacchariflorus* with *S.cerevisiae* KCTC 7928. They studied the amount of enzymes using biomass hydrolysis for the best production ethanol, which containing 10 – 30 FPU/g cellulose. Biomass were treated with 0.5 M NaOH at 140 °C and 8 min. Thus, 20 FPU/g cellulose should be used for bioethanol production from Goedae-Uksae (GU) for practical reasons and theoretical ethanol from 1 Kg GU (dry base) was estimated at 0.17 g ethanol/g GU.

Akhtar and Idris, (2017) studied effect of different enzymes ratios on enzymatic hydrolysis. Pretreatment two step, step I EFB (20 g) was soaked in 2.5 M NaOH (20 % w/v) at 121 °C for 2.0 h under 0.12 MPa. Step II EPB (20 g) was soaked in 8.0 % (v/v) H_2SO_4 at 121 °C for 1 h. The composition of the treated EPB was 86.8 ± 1.4 % of cellulose, 3.4± 1.5 % of hemicellulose and 5.3 ± 0.16 % of lignin. They studied the effect of ratios of cellulase and cellobiase 1 : 0, 1 : 1, 1 : 2, 2 : 1, 5 : 1, 7 : 1 and 10 : 1 on enzymatic hydrolysis for the best glucose production. It found

that the best of glucose yield 31.4 g/L under mixing of cellulase and cellubiase in a ratio of 7:1 and ethanol production was 0.47 g/g EPB.

2.4.3 Fermentation step

Fermentation step is a sugar digestion into bioethanol with using microorganism, it can grow well optimum at 24-72 h. Fermentation processes esteem using in ethanol production are SHF and SSF. The judgment of ethanol production and the process configurations of cellulose hydrolysis and ethanol fermentation depend on each type, Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF) show in Figure 2.9.

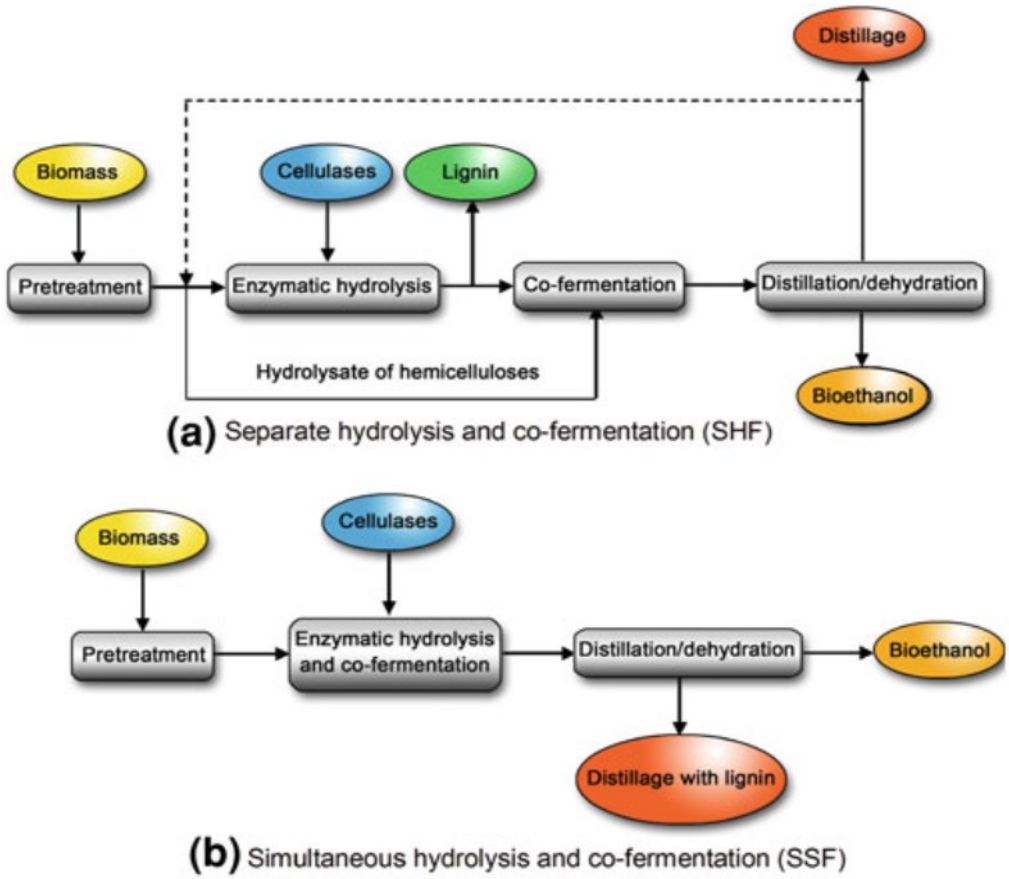


Figure 2.9 The ethanol production by (a) SHF process (b) SSF process from lignocellulose biomass

Source: Zhao, X.Q., *et al.,* 2012, *Bioethanol from Lignocellulosic Biomass,* Adv Biochem Engin/Biotechnol, 128: 25–51.

1) Separate Hydrolysis and Fermentation (SHF) process

Separate Hydrolysis and Fermentation (SHF) process, used cellulase hydrolysis of cellulose to glucose under optimum conditions, temperatures around 50°C, followed by fermentation using yeast for conversion glucose was ethanol. Hydrolysis and fermentation use a different temperature, because some yeast cannot fermentation at temperature the same with hydrolysis. Yeast cannot withstand temperatures of hydrolysis. While another concern about the SHF process is the contamination of yeasts during transportation of hydrolysates through fermentation equipment, which may deteriorate during ethanol fermentation and decreased produce ethanol (Zhao *et al*., 2012).

2) Simultaneous Saccharification and Fermentation (SSF) process

Simultaneous Saccharification and Fermentation (SSF) process, used cellulase hydrolysis of cellulose to glucose and used yeast for conversion glucose was ethanol in the same reactor under temperatures optimum, of yeast at withstand high temperature. The SSF process is easy in operate and easy to design. The most important is the higher ethanol yield as it reduces of product inhibition in cellulases, which makes the cellulases more complete hydrolysis of the cellulose component. However, the temperature used for hydrolysis and fermentation were significantly different, so it was required to operate at low temperatures to accommodate yeast growth and ethanol fermentation at 30 – 35 °C (Zhao *et al*., 2012).

The study research of the fermentation step, Wirawan *et al.,* (2012) studied ethanol production from sugarcane bagasse with *Zymomonas mobilis*. Pretreatment of cellulosic treated with phosphoric acid and hydrolysis with cellulolytic enzymes. Comparison of SHF and SSF processes of ethanol production, SHF process was operated concentration 20 g/L of substrate, pH 6 and enzymatic hydrolysis at 45 °C and fermented a temperature controlled at 30 °C, which achieved the maximum ethanol yield 0.403 g/g substrate. SSF process was operated with an agitation rate of 100 rpm and a temperature controlled at 30 °C, which achieved the maximum ethanol yield 0.357 g/g substrate.

Ohgren *et al.,* (2007) studied a comparison ethanol production between SSF and SHF using corn stover, at 8 % water-insoluble solids (WIS), regarding ethanol production from steam pretreated corn stover at 190 °C for 5 min. It was found that ethanol production with *Saccharomyces cerevisiae* by a comparison between ethanol yield from SSF (after 120 h) and SHF (after 120 h hydrolysis and 24 h fermentation), ethanol yield was 20.5 and 16.8 g/L respectively.

Loaces *et al.,* (2017) studied a comparison ethanol production between SSF and SHF by *Escherichia coli* MS04 from *Arundo donax* biomass. Pretreatment of cellulosic treated with dilute acid and liquid hot water and hydrolysis with cellulolytic enzymes. SHF process was operated concentration 5 % (v/v) CellicCTec2 enzymatic cocktail, pH 5 at 50 °C for 72 h and fermented a temperature controlled at 40 °C at pH 7 for 24 h, which achieved the maximum ethanol yield 24 g/L or 0.44 g/g. SSF process was operated with an agitation rate of 400 rpm and a temperature controlled at 40 °C pH 6.2 for 96 h, which achieved the maximum ethanol yield 25 g/L.

2.4.4 Yeast strain

According to research, yeast grow an optimal temperature of 30 - 35 °C and some yeasts strain can grow at high temperature over 40 °C, which demarcate in thermo tolerance species. Most of thermo tolerance yeasts are found in the genus *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* and *Candida tropicalis*.

The general characteristics of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* are as follows.

Saccharomyces cerevisiae

Kim *et al.,* (2013) Bioethanol production using empty palm fruit bunch fiber, treatment were soaked in 4 % (v/v) H_2SO_4 solution, heated in an autoclave at 121 °C, 15 psi for 60 min after that soaked in 10 N NaOH solution, stirred at ambient temperature for 4 h, which was consisted of 85.2 ± 1.9% cellulose, 1.8 ± 0.5% hemicellulose and 9.2 ± 0.3% lignin. Under optimal conditions for enzyme hydrolysis, 10 % (w/v) of biomass was hydrolyzed completely and converted to 70.8 ± 0.8 g/L glucose and 1.8 ± 0.1 g/L xylose. SSF process of EPFBF by *S. cerevisiae* W303-1A produced 37.8 g/L ethanol in 1.5 L fermented medium containing 10 % (w/v) substrate at 30 °C, 200 rpm after 60 h. The ethanol productivity was 0.378 g/g biomass and 0.45 g/g glucose after fermentation.

Kumneadklang *et al*., (2015) Bioethanol production from OPF by SSF with *S. cerevisiae* TISTR5048, OPF (20 % w/v) was pretreated by 2 % H_2SO_4, 2 % NaOH and 2 % NaOH in H_2O_2 presoaking at room temperature for 24 h contained 37 %, 42 % and 49 % of cellulose respectively. Hydrolysis process used cellulase enzyme hydrolysis cellulose into sugar contained 45.72, 55.73 and 56.94 g/L of sugar concentration respectively. Ethanol concentration of 2 % H_2SO_4, 2 % NaOH and 2 % NaOH in H_2O_2 presoaking was 14.5, 15.0 and 17.2 g/L respectively. The condition in SSF process was 10 % (v/v) yeast inoculum at 30 °C with shaking at 150 rpm for 24 h and incubated for 96 h.

Kluyveromyces marxianus

Tomás-Pejó *et al*., (2009) studied bioethanol production from wheat straw by SSF with *Kluyveromyces marxianus* CECT 10875. Dried wheat straws were pretreated with steam explosion at 220 °C for 2.5 min. WIS composition after pretreatment was glucan (79.2 %). SSF step used cellulase 15 FPU/g cellulose and β-glucosidase 15 U/g cellulose. SSF process obtained WIS content ranging from 5 % to 12.5 % (w/v). pH was adjusted to 5.5 with NaOH 4 M, temperature at 42 °C for 48 h with 1 g/L of yeast at 150 rpm. Fed-batch experiments were added 2 % (w/v) and 4 % (w/v) of WIS at 12, 24 and 40 h. The highest ethanol concentration (36.2 g/L) of condition was initial WIS content of 10 % (w/v) and 4 % (w/v) of substrate addition at 12 h at 42 °C and 150 rpm.

Nachaiwieng *et al.,* (2015) studied bioethanol production from rice husk by SSF with *Kluyveromyces marxianus* CK8. Dried rice husks were pretreated with 2.0 % (w/v) of NaOH at 130 °C for 30 min, which can removed 14 % of Klason lignin and the ratio of sugar to 76.91 % glucose, 16.38 % xylose and 6.71 % arabinose. Hydrolysis with enzyme adjusted to be 20 FPU/g substrate at 45 °C and 150 rpm for 72h of SHF process. SSF process conditions testing were substrate loading (3.29 - 11.7 % w/v), pH (3.32 - 6.68) and temperature (18.18 - 51.82 °C). It found that response surface plots predicted an ethanol yield of 15.40 g/L from the condition of 9.44 % (w/v) substrate loading, 43 °C, and pH 4.2 for 96 h. Ethanol yield from SSF process was higher than 10.8 g/L obtained from the SHF process at the same substrate loading.

Meneses *et al.* (2017) Comparison of SSF and SHF for ethanol production from cashew apple bagasse using *Kluyveromyces marxianus* ATCC 36907. The raw material was pretreated with acid/alkaline pretreatment. The first step was carried out at 121 °C for 15 min using

0.6 M H_2SO_4 and 30 % w/v CAB. In the second step, was carried out solid fraction of 7.5 % w/v at 121 °C for 30 min using 1.0 M NaOH. The yield of pretreated solid (CAB-OH) was 9.3 % composed of 74.72 % ± 1.2 % cellulose, 5.58% ± 0.5 % hemicellulose, 12.04 % ± 0.1 % lignin plus ash and 0.93 % ± 0.2 % extractives. Ethanol production used cellulase 30 FPU/g glucan and cellobiase 60 U/g glucan in hydrolysis of cellulose into glucose. SSF process were conducted at solid loadings 7.5 %, 10 % and 15 % w/v, pH 4.5 - 5, adding yeast concentration 5 g/L at 40 °C, 150 rpm for 72 h. The highest ethanol produce was 58.7 g/L under solid loadings 15 % CAB-OH. SHF process were conducted at solid loadings 7.5 %, 10 %, 15 % w/v and 20 %, pH 4.5 - 5 under hydrolysis at 45 °C for 72 h after that adding yeast concentration 5 g/L at 30 °C, 150 rpm for 24 h. The highest ethanol produce was 50.1 g/L under solid loadings 15 % CAB-OH.

2.4.5 Response Surface Methodology (RSM)

Response Surface Methodology is process of mathematical and statistical techniques for finding response of optimal condition. It is used to study many independent variables that affect, certain properties or the amount of results.

Aim of RSM is to estimate response surface and optimize the response. The relation between the independent variables and the response show in Eq. 2.1

$$y = f(x_1, x_2, x_3, ..., x_n) + \varepsilon \quad (2.1)$$

when y is the response result, f is the unknown function of response result, x_1, x_2, x_3,…, x_n is the factors, also called independent variables, n is the number of the factor and ε is the statistical error that represents other factor of variance not accounted for by function. It is generally set up as zero. In most RSM process, it is presented relationship between the response result and the every factor. So, the first stage of RSM is to derive a appropriate approximation for the true functional relationship between the response result and the factor. Normally, a low-order polynomial in some model of the independent variables is employed. If the response result is well modeled by the first-order model of the independent variables, then the approximating function is a linear function.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + ... + \beta_k x_k + \varepsilon \quad (2.2)$$

If there is curvature in model, then a higher-order polynomial must be used, such as the second-order model, also called quadratic function.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + ... + \sum_{i<j} \sum \beta_{ij} x_i x_j + \varepsilon \quad (2.3)$$

Almost all RSM problems use second-order models in function prediction of research this. Of course, it is likely that a second-order model will be a suitable of the true functional relationship of the independent variables (Steppan *et al.,* 1998).

Chongkhong, (2017) Ethanol production from banana peels by RSM using Baker's yeast. Hydrolysis step, the independent variable of this work were vinegar amount (0.74-1.47 %w/w), microwave power (100-800 Watt) and reaction time (1-10 min). Fermentation step, the independent variable were yeast (2-6 %w/w), pH (4.5-6.5), temperature 28-40 ℃ and time 24-192 h. The optimal condition of hydrolysis step was 1.47 %w/w vinegar and 465 W for 10 min, give maximum reducing sugar content was 15.3 g/L. The optimal condition of fermentation step was 3% w/w yeast pH 4.8 at 28°C for 192 h, give maximum ethanol content was 72.6 g/L.

Lavudi *et al.,* (2017) Ethanol production from sweet sorghum bagasse using *Pichia kudriavzevii* HOP-1. Pretreatment step, the independent variable of this work were alkali concentration (1.5–4%), temperature (125–140 ℃) and time (10–30 min). Hydrolysis step, the independent variable were substrate (10–15 %w/v), time (24–60 h), temperature (40–60 ℃) and Celluclast (10–20 IU/g-dwt). The optimal condition of pretreatment step was 4% alkali concentration, 125 ℃ and 30 min, give maximum glucose and xylose were 57 and 10 g/L, respectively. The optimal condition of hydrolysis step was Substrate (15% w/v) temperature of 60 ℃, Celluclast (20 IU/g-dwt) for 58 h, give maximum glucose concentration was 68.58 g/l. Fermentation step, the highest ethanol concentration was 26.81 g/L(SSF) at 48 h of fermentation time and 26.02 g/L(SHF) at 24 h of fermentation time.

Table 2.1 Summary of literature review

No.	Title	Authors	Material	Method	Set up experiment	Yield	Comments
1.	Sequential acid/alkali-pretreatment of empty palm fruit bunch fiber	Kim *et al.,* (2012)	EPFBF	Sequential acid/alkali pretreatment	- 0.0 - 8.0 % (v/v) H_2SO_4 at 121 ℃ for 1 h. - 10 N NaOH at ambient temperature for 4 h.	Cellulose: Hemicellulose: Lignin %wt. - 1.0 % H_2SO_4 51.5 : 4.0 : 19.8 - After 10 NaOH 61.8 : 9.2 : 10.2	- Acid removed 90 % hemicellulose and 32 % lignin. - Alkali used in the second step, removed 70 % lignin. -The condition was 1.0 % H_2SO_4 and 10 N NaOH.
2.	Pretreatment of empty fruit bunch from oil palm for fuel ethanol production and proposed	Tan *et al.,* (2013)	EFB	- WO pretreatment[1]	- Oxygen pressure of 0.6 MPa at 120 ℃ for 30 min	Glucan: Xylan: Lignin %wt. (1) 39.58 : 20.36 : 20.10 (2) 39.15 : 19.17 : 19.75 (3) 36.24 : 11.76 : 19.88 (4) 45.44 : 20.80 : 17.11	The optimal condition was considered from higher cellulose and lower lignin

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
	biorefinery process			WO + Fe^{3+} pretreatment[2]	-0.5 % $Fe_2(SO_4)_3$ on WO treated at 120 ℃ for 30 min		
				OA pretreatment[3]	-1.6 %NaOH on Oxygen pressure of 0.6 MPa at 120 ℃ for 30 min		
				OA+H_2O_2 pretreatment[4]	-10 % NaOH and 0.5 % H_2O_2 on Oxygen pressure of 0.6 MPa at 120 ℃ for 30 min		
3.	Cellulose and hemicellulose recovery from oil palm empty fruit bunch (EFB) fiber and	Palamae *et al.,* (2017)	EFB	First step, Peracetic acid pretreatment (PA)	5 % (w/v) of EFB was carried out at 35 ℃, 150 rpm for 9 h.	Cellulose: Hemicellulose: Lignin - Non-treated 28.3 : 36.6 : 35.1	A sequential PA/AP pretreatment at mild temperature (20 – 35 ℃) removed more than 98 % of the lignin from EFB

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
	production of sugars from the fibers			Second step, Alkaline peroxide (AP)	2 g EFB with 5.2 mL H_2O_2 and 34.8 mL NaOH was carried out at 20 ℃ or 40 ℃, 90 rpm for 12 h.	- PA (35 ℃, 9 h) 42.5 : 37.5 : 15.7 - AP (20 ℃, 4 % NaOH) 81.9 : 11.2 : 2.8 - AP (40 ℃, 4 % NaOH) 82.5 : 9.4 : 3.3 - AP (20 ℃, 8 % NaOH) 84.2 : 8.6 : 2.9 - AP (40 ℃, 8 % NaOH) 85.0 : 7.0 : 3.4	
4.	Optimizing alkali pretreatment of oil palm empty fruit bunch for ethanol production by application of	Duangwang and Sangwichien , (2013)	OPEFB	NaOH pretreatment	Analysis by RSM method - 1 – 15 % (w/v)NaOH - Temperature at 100-130 ℃ - Reaction time 15 - 90 min	Cellulose: Hemicelllulose : Lignin 68.8 % : 23.4 % : 7.6 %	The optimal condition pretreatment was 15 % (w/v) NaOH at 130 ℃ for 40 min.

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
	response surface methodology.			H_2SO_4 hydrolysis	-6 %H_2SO_4 at 130 ℃ for 90 min	Glucose concentration was 19.96 g/L	
5.	Alkaline delignification of oil palm empty fruit bunch using black liquor from pretreatment	Muryanto *et al.,* (2015)	OPEFB	NaOH pretreatment	- 2.5 M NaOH - 150 ℃ for 30 min	Cellulose: Hemicellulose : Lignin 63.83 % : 12.14 % : 12.56 % Maximum delignification was 58.36 %	-The optimum condition was 2.5 M NaOH in 150 ℃ for 30 min -Theoretical glucose yield was 106.35 g/L
				Black liquor pretreatment	- 50 – 100 % (BL) - 150 ℃ for 30,60 min	Glucose yield was 93.8 g/L -Basic at 500 g/2.5 L	
				Enzymatic hydrolysis	- Substrate 15 % (w/v) - pH 4.8 - 50 ℃ for 72 h.		

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
6.	Pretreatment of oil palm empty fruit fiber with aqueous ammonia for high production of sugar	Zulkiple *et al.,* (2016)	OPEFB	NH_4OH pretreatment	- CTec2 : HTec2, 5 : 1 - 6.25 % NH_4OH, 24 h. - 13.13 % NH_4OH, 14 h. - 20 % NH_4OH, 24 h. - Room temperature	Glucose : Xylose (mg/ml) - 290.28 : 126.57 - 439.90 : 171.59 - 168.58 : 74.5 - 125.32 : 45.3 - 134.89 : 51.1 - 351.51 : 83.7	The optimum condition for pressurize chamber pretreatment was solid loading 1 : 30 at 3 h.
				Pressurize chamber pretreatment	- solid loading 1 : 30 at 1 h. - solid loading 1 : 30 at 3 h.		
				NaOH pretreatment	10 % NaOH at 120 ℃ for 2 h.		
				Enzymatic hydrolysis	-pH4.8, 50 ℃ at 150 rpm for 24 h. - CTec2 : HTec2, 1 : 1		

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
7.	Sulfite pretreatment to overcome recalcitrance of lignocellulose for enzymatic hydrolysis of oil palm trunk	Noparat *et al.,* (2017)	OPT	-H_2SO_4 : Na_2SO_3 pretreatment	- 3, 5, 7 % H_2SO_4 - 2, 4, 6 % Na_2SO_4 - Temperature 170, 180, 190 ℃	Maximum cellulose to glucose conversion yield % : glucose yield % were 92 % : 66.6 %	The optimum condition was 7 % H_2SO_4: 6 % Na_2SO_3 at 190 ℃ -Hydrolysis at 48 h.
				Enzymatic hydrolysis	- pH 4.8, 50 ℃ at 200 rpm - CTec2 : HTec2, 15 FPU : 30 CBU		
8.	Use of empty fruit bunches from the oil palm for biomass production : A thorough comparison between dilute acid and dilute	Chiesa and Gnansounou, (2014)	OPEFB	H_2SO_4 pretreatment	-0.05 – 2 % (v/v) H_2SO_4 -140 - 210 ℃ for 1 - 20 min	Lignin : Glucan : Xylan -59.1 : 38.4 : 1.2 -9.9 : 61.3 : 25.1	The optimum condition was 1.51 % v/v H_2SO_2 at 161.5 ℃ for 9.44 min
				NaOH pretreatment	-0.04 – 2 % (w/v) NaOH -121 - 195 ℃ for 1 - 20 min		The optimum condition was 2 % w/v NaOH at 195 ℃ for 10.5 min

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
	alkali pretreatment			Enzymatic hydrolysis	- 50 ℃ at 150rpm for 72 h. - CTec2 : HTec2, 40 FPU:60 IU		
9.	Continuous alkaline pretreatment of Miscanthus sacchariflorus using a bench – scale single screw reacter	Cha *et al.,* (2015)	M.sacchari florus GU	NaOH Pretreatment	-0 - 1.5 M at 70 ℃	Cellulose : Hemicellulose : Lignin -62.6 : 24.1 : 8.3 %	The optimum condition was 0.5 M NaOH
				Enzymatic hydrolysis	-pH 4.8 at 50 ℃ for 72 h. -10 – 30 FPU cellulose/gcellulose	Glucose yield was 76.4, 85.0, 88.2 respectively	Maximum glucose yield at cellulose 30 FPU/g cellulose
10.	Alkaline deacetylation as a strategy to improve sugars	Castro *et al.,* (2017)	Rice straw	NaOH pretreatment	-20 – 80 mg NaOH/g biomass -temperature (50 – 70 ℃)	Glucan : Hemicellulose : Lignin -43.3 : 27.3 : 12.5 Glucose : xylose 1.8 : 20.3	Condition was 80 (mg NaOH/g biomass) at 70 ℃ Condition was 1.5 % w/v H_2SO_4 at 90 min

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
	recovery andethanol production from rice straw hemicellulose and cellulose			H_2SO_4 pretreatment	- 0.5 – 1.5 % w/v H_2SO_4 - 30 – 90 min		
				Enzymatic hydrolysis	- CellicCTec2 20 FPU/g cellulose - 8 % (w/v) cellulignin content - pH 4.8 at 43 °C and 100 rpm		
11.	Effect of severity on dilute acid pretreatment of lignocellulosic biomass and the following hydrogen fermentation	Gonzales *et al.,* (2016)	- EPFB - Rice husk - Pine tree wood	Dilute acid pretreatment	- 5 % (v/v) H_2SO_4 - 10 % (w/v) S/L ratio at 121 ℃ for 30, 60, and 90 min.	Glucose : Xylose : Total sugar recovery - 45.9 : 97.5 : 57.4 - 46.4 : 99.0 : 60.7 - 39.0 : 92.0 : 56.6	-The condition of pretreatment was 5 % (v/v) H_2SO_4, 10 % (w/v) S/L ratio at 121 ℃ for 60 min.

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
12.	Fractionation of oil palm empty fruit bunch by bisulfite pretreatment For the production of bioethanol and high value products	Tan *et al.,* (2016)	-EFB	Bisulfite pretreatment ($NaHSO_3$ dosages $+H_2SO_4$ dosages)	- 8 %, 10 % $NaHSO_3$ - 0.25 – 1.5 % H_2SO_4 - 1:4 S/L ratio at 180 ℃ for 30 min	Glucose : Xylose : lignin 52.0 % : 11.4 % : 20.9 %	The optimum condition was 8 % $NaHSO_3$: 1 % H_2SO_4
13.	Importance of acid or alkali concentration on the removal of xylan and lignin for enzymatic cellulose hydrolysis	Martínez *et al.,* (2015)	-OPEFB Sugarcane bagasse -Barley straw	H_2SO_4 pretreatme-NaOH pretreatment	-0 - 6 % (w/w) H_2SO_4 at 140 ℃, 30 min -0 – 12 % (w/w) NaOH at 120 ℃, 60 min	Glucose:Xylose: Lignin OPEFB -69.5 : 48.5 : 30.4 Sugarcane bagasse - 60.6 : 63.1 : n.a. Barley straw - 77.0 : 68.2 : 10.0	Pretreatment condition of OPERB, Sugarcane bagasse and Barley straw were 12, 8 and 10 % w/w NaOH) respectively at 120 ℃ for 60 min
				Enzymatic hydrolysis	-pH 5.5 CellicCTec2 : CellicHTec (ratio 10 : 1) at 55 ℃ for 72 h.		

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
14.	Lignin preparation from oil palm empty fruit bunches by sequential acid/alkaline treatment – A biorefinery approach	Medina *et al.,* (2015)	OPEFB	Sequential acid/alkaline pretreatment (H_2SO_4, and NaOH)	-1 % (w/w) H_2SO_4 at 121 ℃ for 60 min -0.5 -5.5 % (w/w) NaOH at 121 ℃ for 60 min	Cellulose : Hemicellulose : Lignin 54.52 : 6.93 : 0.28	The optimum condition was 5.5 % (w/w) NaOH at 121 ℃ for 60 min
15.	Preliminary study on enzymatic hydrolysis of treated oil palm (Elaeis) empty fruit bunches fibre (EFB) by using combination of	Hamzah *et al.,* (2011)	EFB	-Hot water pretreatment	Operate at 80 ℃ for 60 min	Cellulose : Hemicellulose : Lignin (%wt.) 66.77 : 24.5 : 7.25 - Maximum glucose concentration of 2.4 g/L	The optimum ratio of Cellulase: β 1-4 glucosidase of 5:1 which glucose produced also increased until 8 % (w/v) solid loading, pH 4.8 and 50 ℃.

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
	cellulase and β 1-4 glucosidase			- NaOH pretreatment	2.5 M NaOH at 121 ℃ for 15 min		
				Enzymatic hydrolysis	The condition was Cellulase : β 1-4 glucosidase at ratio of 5 : 1, 2 : 1, 1 : 2, 1 : 1 and 1 : 5 -pH 4 - 6 at 30 – 60 ℃ for 72 h. -2 – 8 % (w/v) solid loading		
16.	Ethanol production from palm pressed fiber by prehydrolysis prior to	Boonsawang *et al.,* (2012)	PPF	NaOH pretreatment	Operate at 100 ℃ for 15 min	- Reducing sugar concentration was 46 % for the period of 96 h. -Ethanol yield of 193 g/Kg cellulose.	The optimal condition was 10 FPU cellulase: 10 U β- glucosidase at 50 ℃ for 96 h higher than at 35 ℃.

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
	simultaneous saccharification and fermentation (SSF)			Enzymatic hydrolysis	(1) 10 FPU cellulose/g biomass (2) 20 FPU cellulose/g biomass (3) 10 FPU cellulose : 10 U β- glucosidase -At 35 ℃ or 50 ℃ and 5.7 Hz for 96 h.		
17.	Comparison of SHF and SSF processes using enzyme and dry yeast for optimization of bioethanol production from empty fruit bunch	Dahnum *et al*., (2015)	EFB	NaOH pretreatment	- 10 % NaOH at 150 ℃ for 30 min	- 4.74 % of Ethanol in SHF process - 6.05 % of ethanol in SSF process	The best of condition was 40 FPU of Cellic® CTec2.

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
			Dry yeast	Enzymatic hydrolysis	-10, 20, 30 and 40 FPU of Cellic® CTec2 : 20 % Cellic® CTec2 of Cellic® HTec2 at pH 4.8		
				SHF and SSF fermentation	- SHF process hydrolysis at 50 ℃for 72 h and fermented at 32 ℃ for 72 h. -SSF operated at 32 ℃ for 72 h.		
18.	Oil palm empty fruit bunches a promising substrate for succinic acid production via	Akhtar and Idris, (2017)	OPEFB	Pretreatment two step Step I, NaOH pretreatment	-20 % (w/v) OPEFB soaked in 2.5 M NaOH at 121 ℃ for 2 h	Cellulose : Hemicellulose : Lignin 86.8 : 3.4 : 5.3 %wt.	The optimum ratio of cellulase and cellobiase was 7 : 1.

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
	simultaneous saccharification and fermentation			Step II, H_2SO_4 pretreatment	-8 % (v/v) H_2SO_4 at 121℃ for 1 h	The best glucose yield was 31.4 g/L and 0.47 g ethanol/g OPEFB	
				Enzymatic hydrolysis	Ratio of cellulase and cellobiase 1 : 0, 1 : 1, : 2, 2 : 1, 5 : 1, 7 : 1 and 10 : 1		
19.	Cellulosic ethanol production performance with SSF and SHF processes using immobilized *Zymomonas mobilis*	Wirawan *et al.,* (2012)	Bagasse	Phosphoric acid pretreatment	Phosphoric acid at 50 ℃ for 30 - 60 min	- Ethanol concentration was 6.24 g/L and ethanol yield 79.09 % of PVA and 5.52 g/L, 69.96 % of CA in SHF process. -Ethanol concentration was 5.53 g/L and ethanol yield 70.09 % of PVA and 5.44 g/L,	The comparison with suspended cells shows that the immobilized cells of *Z. mobilis* are feasible for ethanol production via SSF and SHF.

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
			Z.mobilis	SHF and SSF fermentation	- Cellulase 100 FPU at pH 6 - SHF process hydrolysis at 45 ℃ for 72 h and fermented at 30 ℃ for 72 h. - SSF operated at 30 ℃ for 72 h.	68.95 % of CA in SSF process.	
20.	A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using	Ohgren *et al.,* (2007)	Corn Stover	Steam pretreatment	8 % (WIS) at 190 ℃ for 5 min	-Ethanol concentration was 20.5 g/L and ethanol yield 72.4 % in SSF process. -Ethanol concentration was 16.8 g/L and ethanol yield 59.3 % in SHF process.	SSF process was concluded to be a better process configuration than SHF process when the whole slurry was used.

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
	steam-pretreated corn stover		*S.cerevisiae*	Enzymatic hydrolysis	Cellulase 10 FPU/g WIS at pH 5		
				SHF and SSF fermentation	-SHF process hydrolysis at 45 ℃ for 72 h and fermented at 35 ℃ for 120 h. -SSF operated at 35-45 ℃ for 120 h.		
21.	Ethanol production by Escherichia coli from Arundo donax biomass under SSF, SHF or CBP process configurations	Loaces *et al.,* (2017)	*Arundo donax*	-Acid pretreatment -Hot water pretreatment	-10 % (w/v) solid loading soaked 2 % H_2SO_4 -1.1 bar at 121 ℃ for 20 or 30 min	-SHF process achieved ethanol yield 24 g/L or 0.44 g/g biomass. -SSF process achieved ethanol yield 25 g/L.	In situ expression of a multifunctional enzyme increase ethanol yield under SHF and SSF, but also under CBP configuration showing out a potential decrease of costs.

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
	and in situ production of a multifunctional glucanase and xylanase		*Escherichia coli*	SHF and SSF fermentation	- 5 % (v/v) Cellic CTec2 enzymatic cocktail at pH 5 - SHF process hydrolysis at 50 ℃ for 72 h and fermented at 40 ℃, pH 7 for 24 h. -SSF operated at 40 ℃, pH 6.2 for 96 h.		
22.	Bioethanol production using the sequential acid/alkali-pretreated empty palm fruit bunch fiber	Kim *et al.,* (2013)	EPFBF	Sequential acid/alkali pretreatment	- 4 % (v/v) H_2SO_4 at 121 ℃ for 60 min - 10 M NaOH at ambient temperature for 4 h	Cellulose : Hemicellulose : Lignin 85.2 : 1.8 : 9.2	These research confirm that sequential acid/alkali pretreatment effectively remove hemicellulose and lignin components and

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
				SSF fermentation	- Cellulase 50 FPU/g biomass - SSF process operated 10 % (w/v) substrate at 30 °C, 200 rpm for 60 h.	The ethanol productivity was 0.378 g/g biomass and 0.45 g/g glucose.	increases enzymatic digestibility and ethanol yield.
23.	Bioethanol Production from Oil Palm Frond by Simultaneous Saccharification and Fermentation	Kumnead-klang *et al.,* (2015)	OPF	-H_2SO_4 pretreatment[1] -NaOH pretreatment[2] -NaOH mix H_2O_2 pretreatment[3]	(1) 2 % H_2SO_4 (2) 2 % NaOH (3) 2 % NaOH and H_2O_2 at room temperature for 24 h	Cellulose %wt. (1) 37 % (2) 42 % (3) 49 %	Sodium hydroxide in hydrogen pretreatment was an efficient pretreatment method of OPF for its ethanol production.
			S.cerevisiae	Enzymatic hydrolysis	-15 % (w/w) OPF -pH 4.8 -2 mL cellulase enzyme at 50 °C, 150 rpm for 72 h.	Sugar concentration (1) 45.72 g/L (2) 55.73 g/L (3) 56.94 g/L	

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
				SSF fermentation	10 % (v/v) yeast at 30 ℃, 150 rpm for 96 h	Ethanol concentration (1) 14.5 g/L (2) 15.0 g/L (3) 17.2 g/L	
24.	Bioethanol production from wheat straw by the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875 in a simultaneous saccharification And fermentation fed-batch process	Tomás-Pejó *et al.,* (2009)	wheat straw *K.marxianus* CECT 10875	steam-explosion pretreatment	-At 220 ℃ for 2.5 min	Component was 79 % of Glucose, 7.9 % of Xylose and 21.4 % of Lignin	The optimal condition was initial WIS content of 10 % (w/v) and 4 % (w/v) of substrate addition at 12 h, 42 ℃ and 150 rpm.

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
				SSF fermentation	- Cellulase 15 FPU/g cellulose and β-glucosidase 15 IU/g cellulose - 5 - 12.5 % (w/v) WIS - 1 g/L of yeast pH 5.5 at 42 ℃, 150 rpm for 48 h	The highest ethanol concentration was 36.2 g/L.	
				Fed-batch	Add 2 or 4 % (w/v) WIS at 12, 24 and 40 h.		
25.	Bioethanol production from rice husk under elevated temperature simultaneous saccharification	Nachai-wieng *et al.,* (2015)	Rice husk *K.marxianus* CK8	NaOH pretreatment	-2 % (w/v) NaOH at 130 ℃ for 30 min	Component was 76.91 % of Glucose, 16.38 % of Xylose and 6.71 % of arabinose.	-The optimal condition was 9.44 % (w/v) substrate loading, 43 ℃and pH 4.2 for 96 h. -Ethanol yield from SSF process was higher than 10.8 g/L

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
	and fermentation using *Kluyveromyces marxianus* CK8			SSF process	Design by RSM - cellulase 20 - 60 FPU/g - substrate loading 1 – 5 % (w/v) - temperature 30-45 ℃ - incubation time 48 - 96 h - agitation speed 100 - 200 - initial seed inoculums 2 – 10 % (v/v) - pH 4 - 6	The highest ethanol concentration was 15.40 g/L	obtained from the SHF process at the same substrate loading.

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
26.	Comparison of strategies for the simultaneous saccharification and fermentation of cashew apple bagasse using a thermotolerant *Kluyveromyces marxianus* to enhance cellulosic ethanol production	Meneses *et al.,* (2017)	cashew apple bagasse *K.marxianus*	Acid/alkaline pretreatment	The first step was carried out 0.6 M H_2SO_4 and 30 % (w/v) CAB at 121 ℃ for 15 min. The second step was carried out 1 M NaOH and 7.5 % (w/v) CAB at 121 ℃ for 30 min.	Cellulose : Hemicellulose : Lignin 74.72 : 5.58 : 12.04 The highest ethanol produce was 58.7 g/L in SSF process and 50.1 g/L in SHF process.	The optimal condition was 15 % (w/v) of solid loading
				SSF and SHF fermentation	- Cellulase 30 FPU/g glucan and cellobiase 60 CBU/g glucan		

No	Title	Authors	Material	Method	Set up experiment	Yield	Comments
					- SSF process was conducted at (7.5, 10, 15 % w/v) solid loading, pH 4.5-5 and 5 g/L of yeast at 40 ℃, 150 rpm for 72 h. -SHF process was conducted at 7.5, 10, 15, 20%w/v) solid loading, pH 4.5-5 at 45 ℃ for 72 h, fermented at 30 ℃ for 24 h.		

CHAPTER 3

METHODOLOGIES, RESULTS AND DISCUSSION

Bioethanol production from acid and alkaline pretreated oil palm empty fruit bunches by simultaneous saccharification and fermentation (SSF) using *Kluyveromyces marxianus* yeast

Introduction

Alternative fuels currently will present a crucial role in the future and current of human life. The use of bioethanol as a renewable for fossil energy resources has become more popular in worldwide due to the high of fuel prices and environmental problems with fossil fuels. Ethanol (ethyl alcohol or bioethanol), is a substitute for gasoline fuel and is generally produced from various raw materials, primarily biomass with proper management and technologies (Preechajarn and Prasertsri, 2014). The agricultural raw materials fall into three categories: sucrose-containing sugars, starch materials, and lignocellulosic materials (Sarkar *et al*., 2012). To achieve cost-effective ethanol production, inexpensive agricultural residual raw materials, especially from lignocellulosic biomass, seem to be good substrate alternatives in the future. Especially locally available agricultural residues will be used in bioethanol production (Mojovic *et al*., 2009).

Oil Palm Empty Fruit Bunch (OPEFB) is an agricultural lignocellulosic residue (Correia *et al*., 2004). The OPEFB has cellulose content of 40 – 50 %, hemicellulose content of 20 – 35 % and lignin content of 16 – 29 % (Folakemi *et al*., 2008), these being the major constituents. In 2015, Thailand's oil palm plantations covered an area of 4.7 million hectares, and palm oil processing industries generate waste by-products at about 70 – 75 % of the oil palm fruit bunches produced, mainly in the form of OPEFB (Ministry of Agriculture and Cooperatives (Thailand), 2015). Lignocellulosic residues are inexpensive and attractive renewable resources for the production of renewable energy. However, lignocellulosics have complex structures with lignin binding carbohydrates. Ethanol production from lignocellulosic biomass by Simultaneous Saccharification and Fermentation (SSF) has two major processing stages: (1) delignification

pretreatment to liberate cellulose and hemicellulose, and (2) hydrolysis of cellulose and fermentation of reducing sugars to ethanol (Renewable Fuels Association (RFA), 2017).

Sulfuric acid (H_2SO_4) at concentrations usually below 4 %wt has received the most attention in delignification studies, as it is inexpensive, effective, and gives low acid consumption with high rates of conversion of cellulose to glucose (Nguyen *et al*., 2000). Alkaline pretreatment is considered the most effective pretreatment, characterized by low use of chemicals and low energy consumption, and is frequently tested as biomass pretreatment (Wang *et al*., 2010).

Dilute acid and alkali pretreatments have been successfully developed for a wide range of feedstocks, ranging from hardwoods to grasses and agricultural residues. Sulfuric acid (H_2SO_4) and NaOH at low concentrations are both inexpensive, effective, with low chemicals consumption and high rates of removing hemicellulose and lignin.

Response Surface Methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the interactions between multiple experimental factors, and searching for the optimum conditions (Bujang *et al.,* 2013). This methodology has already been successfully applied to optimize chemical pretreatments of several substrates, including lignocellulosic biomass (Montgomery, 2001). In this study, ethanol production from OPEFB with dilute acid and alkaline autoclaving pretreatments, and enzymatic prehydrolysis of treated biomass residues using cellulase and ß- glucosidase were investigated. Then, ethanol fermentation by SSF using *Kluyveromyces marxianus* was compared with SHF for the biomass residues, and RSM was applied to chemical pretreatment conditions to maximize cellulose and glucose selectivity (Hsu *et al*., 2010).

3.1 Materials and Methods

3.1 1 Pretreatment step

Materials

Oil palm empty fruit bunch residues

The oil palm empty fruit bunch was obtained from Trang Palm Oil Industry Co., Ltd. (Trang, Thailand). They were washed with piped water several times for removal of soil and other particles, and were dried in the sunlight for a day. OPEFB was ground into a particle size of about 3 – 5 mm, and then dried in an oven at 105 ºC for 24 h. The dried OPEFB was stored in

sealed plastic bags and kept in desiccators until utilization. OPEFB components was 41.11 % cellulose, 30.03 % hemicellulose, 26.36 % lignin and others and were analyzed by AOAC method.

Methods

Sequential Dilute Acid Autoclaving and Alkaline Autoclaving pretreatment (DAA-AA)

DAA-AA pretreatment was operated in two steps; First step is to study the effect of the reaction time, sulfuric acid concentration and substrate loading in dilute acid pretreatment on hemicellulose yield, cellulose yield, lignin yield and enzymatic digestibility of cellulose pulps were evaluated through RSM. Response Surface Methodology (RSM) is normally used to inspect collective effects of several manipulated factors, and to find the optimum conditions based on optimality criteria (Kim *et al*., 2008). The Central Composite Design (CCD) is a commonly used experimental design in RSM (Gao *et al*., 2006).

The pretreatment step was carried out in a 150 mL Duran bottle. The three independent variables, namely substrate loading (A, 5 - 25 % w/v), sulfuric acid concentration (B, 0.2-1.0 M), and reaction time (C, 15-90 min), had each five factor levels, coded with (-1.68, -1, 0, +1, +1.68) shown in Table 3.1.

Table 3.1 Summary of the coded level of the three factors for each trial with the central composite design

Independent variable	Unit	Symbol	Code				
			-1.68	-1	0	1	1.68
Substrate loading	% w/v	A	5	9	15	21	25
Sulfuric acid conc.	M	B	0.2	0.4	0.6	0.8	1.0
Reaction time	min	C	15	30	53	75	90

A 2^3 factorial central composite experimental design with four duplicates at the central point had 18 experimental runs (Table 3.2).Second step, after the acid treatment, treated OPEFB after drying was further pretreated with 5 % w/v NaOH in an autoclave at 121 ºC for 20 min.

Table 3.2 Experimental conditions of sequential dilute acid autoclaving and alkaline autoclaving pretreatment

Substrate loading	H_2SO_4 conc.	Reaction time	NaOH conc.
% (w/v)	M	min	% (w/v) at 20 min
9	0.40	30	5
21	0.40	30	5
9	0.40	75	5
21	0.40	75	5
9	0.80	30	5
21	0.80	30	5
9	0.80	75	5
21	0.80	75	5
5	0.60	53	5
25	0.60	53	5
15	0.60	15	5
15	0.60	90	5
15	0.20	53	5
15	1.00	53	5
15	0.60	53	5
15	0.60	53	5
15	0.60	53	5
15	0.60	53	5

The conduct of every variable, their relations, and statistical analysis to get predicted responses were clarified by the resulting second-order polynomials (quadratics), shown that in Eq. (3.1)

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j}^{k} \sum_{j}^{k} \beta_{ij} x_i x_j \qquad (3.1)$$

where Y represents the response observed experimentally) hemicellulose yield, cellulose yield, lignin yield and enzymatic digestibility of cellulose pulps residual(; β_o is a constant coefficient; i, j

and k are indexes (i from 1 to 3, j from 2 to 3, and k is the total number of parameters investigated equal to 3); $\beta_i, \beta_{ii}, \beta_{ij}$ are, respectively, coefficients of linear, quadratic and interaction effects; and x_i and x_j represent the independent variables or factors (substrate loading, sulfuric acid concentration, and reaction time). The statistical software package Design Expert (Trial version 10.0) was used to analyze the results. The goodness of fit by the models was assessed from R^2 and adjusted R^2. Validation experiments at the model predicted optimal points were carried out. After then the DAA-AA treated OPEFB was dried at 105 ºC for 24 h. and analyze the chemical composition of OPEFB by AOAC methods (AOAC, 1995).

3.1.2 Fermentation step

Materials

Enzymes

The cellulase enzyme used in hydrolysis step was a commercial product from *Trichoderma reesei* (Sigma–Aldrich, Co. LLC.) with a filter paper activity of 531.0 FPU/Kg enzyme at 4.8 and 50 ºC according to the method used by (Pan *et al*., 2005). The cellobiase enzyme was a commercial product derived from *Aspergillus niger* (Sigma–Aldrich, Co. LLC.) with a cellubiose assay of 3324.8 U/L for 1.0 mL of enzyme to 10 mL citrate buffer at pH 4.8 and 50 ºC according to (Merino and Cherry, 2007).

Microorganism and cultural conditions

Kluyveromyces marxianus (TISTR5116) and *Saccharomyces cerevisiae* (TISTR5606) used in SHF and SSF experiments were obtained from the archives of Thailand Institute of Scientific and Technology Research (TISTR). The culture of *K. marxianus* and *S. cerevisiae* were maintained on YM agar slants consisting 20 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 1.5 g/L agar at 4 °C. An inoculum was prepared by transferring a loop of cells to 100 mL of YM medium broth; and was incubated in a shaker at 30 ± 2°C and 150 rpm for 24 h before inoculating the reactor**.** This was done to get a yeast inoculum with 10^8 CFU/mL cell concentration before fermentation**.**

Methods

Simultaneous Saccharification and Fermentation (SSF) using response surface methodology

The OPEFB treated optimally with sequential dilute acid and alkaline pretreatments was used as the substrate in ethanol fermentation by *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* yeast. Optimizing fermentable ethanol production from OPEFB was targeted using RSM. Four independent variables, namely temperature (A, 30 - 45 °C), substrate loading (B, 5 - 15 % w/v), pH (C, 4 - 6) and yeast concentration (D, 1 - 5 % v/v) were assigned five coded levels (-1.68, -1, 0, +1, +1.68) as show in Table 3.3.

Table 3.3 Summary of the coded level of the four factors for each trial with the central composite design

Independent variable	Unit	Symbol	Code				
			-1.68	-1	0	1	1.68
Temperature	ºC	A	30	33.75	37.50	41.25	45
Substrate loading	% w/v	B	5	7.5	10	12.5	15
pH	-	C	4	4.5	5	5.5	6
Yeast concentration	% v/v	D	1	2	3	4	5

A 2^4 factorial central composite experimental design with four duplicates at the central point gave 28 experimental runs (Table 3.4). The SSF experiment was carried out in a 25 mL Erlenmeyer flask, with the yeast feed containing 3.0 g/L yeast extract, 0.25 g/L $(NH_4)_2HPO_4$, 0.025 g/L $MgSO_4.7H_2O$ and citrate buffer (pH, 4 - 6), autoclaved at 121°C for 15 min and cooled down to about 35°C. After that, cellulase and ß-glucosidase in 5 : 1 ratio (20 FPU/g and 4 U/g of substrate, respectively) were loaded in the Erlenmeyer flask. Afterwards the yeast inoculum was added in volume fraction assigned by the RSM design. Three replicates of fermentation in Erlenmeyer flask were incubated with shaking at 150 rpm for 96 h. Samples for ethanol yield, glucose concentration and cell growth of organisms were taken at the start and every 24 h during the 96 h of fermentation.

Table 3.4 Experimental conditions of Simultaneous Saccharification and Fermentation (SSF)

Temp. (ºC)	Substrate loading (% w/v)	pH	Yeast conc. (% v/v)
33.75	7.5	4.5	2
41.25	7.5	4.5	2
33.75	12.5	4.5	2
41.25	12.5	4.5	2
33.75	7.5	5.5	2
41.25	7.5	5.5	2
33.75	12.5	5.5	2
41.25	12.5	5.5	2
33.75	7.5	4.5	4
41.25	7.5	4.5	4
33.75	12.5	4.5	4
41.25	12.5	4.5	4
33.75	7.5	5.5	4
41.25	7.5	5.5	4
33.75	12.5	5.5	4
41.25	12.5	5.5	4
30.00	10.0	5.0	3
45.00	10.0	5.0	3
37.50	5.0	5.0	3
37.50	15.0	5.0	3
37.50	10.0	4.0	3
37.50	10.0	6.0	3
37.50	10.0	5.0	1
37.50	10.0	5.0	5
37.50	10.0	5.0	3
37.50	10.0	5.0	3
37.50	10.0	5.0	3
37.50	10.0	5.0	3

Separate Hydrolysis and Fermentation (SHF)

The fermentation of glucose from treated OPEFB to produce ethanol by (SHF) with *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* was assessed in these experiments. The OPEFB was given optimum condition pretreatment from section of (*3.2.2.1*) and the glucose was given optimum condition fermentation from section of (*3.3.2.1*) but ferments by SHF. Hydrolysis step operate for 72 h. After enzymatic hydrolysis then filter the liquid for fermentation step operate for 96 h. Garnering for ethanol yield, reducing sugar concentration and cell growing of organisms were taken at the start and every 24 h of a 96 h fermentation process.

3.1.3 Analytical methods

The cellulose, hemicellulose and lignin contents were determined followed by standard AOAC methods (AOAC, 1995).

Reducing sugar concentration was determined with 3, 5-dinitrosalicylic acid (DNS) method using UV - visible spectrophotometer at 540 nm (Miller, 1959).

The enzymatic hydrolysis experiments were performed in test tubes with screw caps with a total working volume of 10 mL at 1 % w/v substrate loading of treated OPEFB. The test tube contained 50 mM sodium citrate buffer (pH = 4.8) and enzyme loading of 20 FPU cellulase/g of substrate and 4 U cellobiase/g of substrate, then the mix was digested at 50 ºC and 160 rpm for 48 h according to (Hamzah *et al*., 2011). The enzymatic digestibility of treated OPEFB expressed as percentage was calculated as follows Eq.3.2 (Zhou *et al.,* 2015):

$$\text{Enzymatic digestibility} = [\text{Reducing sugar (g/L)} \times 0.9 / \text{Initial cellulose pulps (g/L)}] \times 100 \quad (3.2)$$

Ethanol was estimated by Gas Chromatography (GC) with an HP-FFAP polyethylene glycol column (30 m × 0.25 mm) at 120 ºC, and Flame Ionization Detectors (FID) at 250 ºC with injector set at 150 ºC. The carrier gas was helium with flow rate set at 2 mL/min (Duangwang *et al*., 2016).

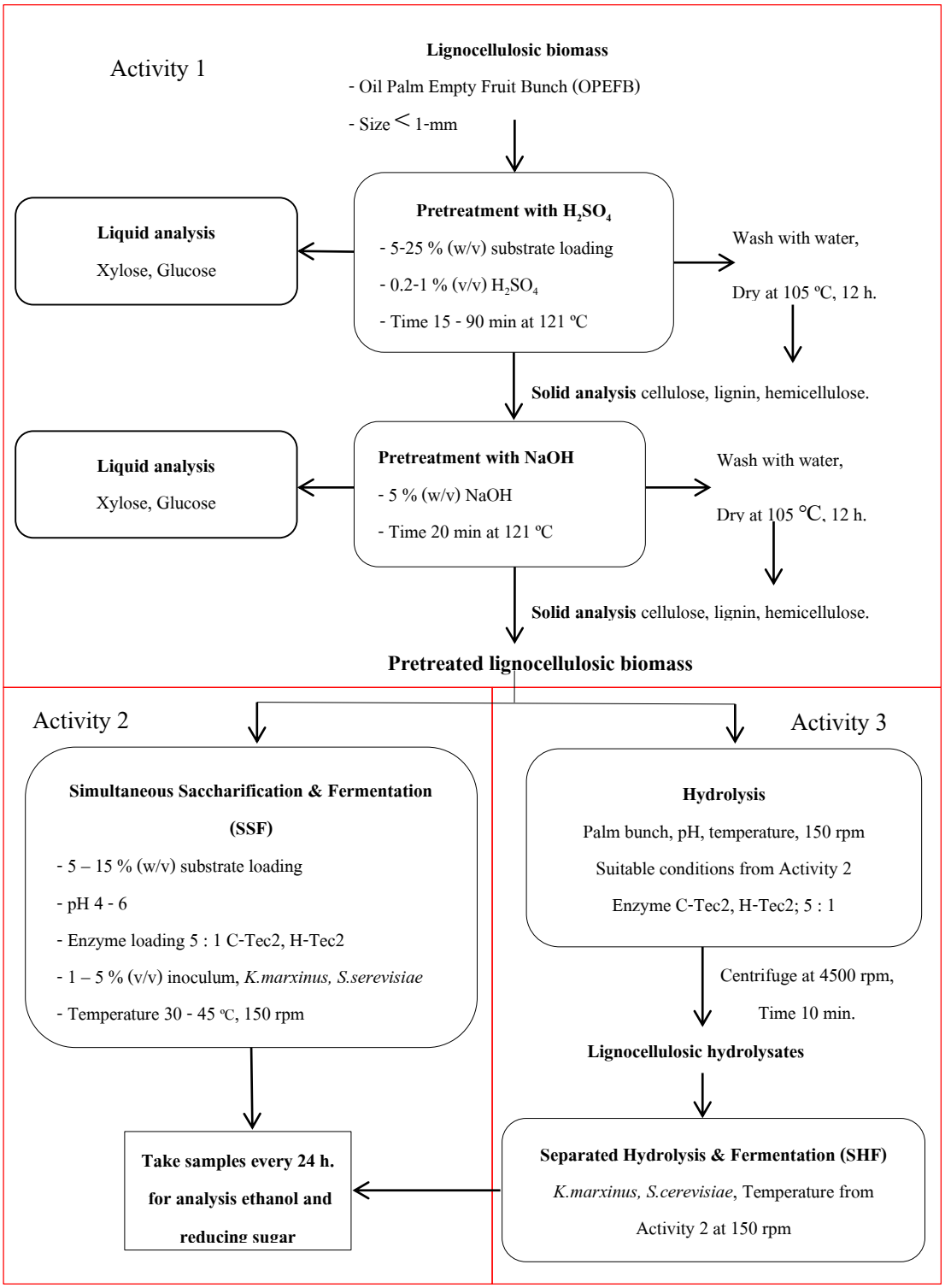


Figure 3.1 Charts of step of ethanol production from OPEFB with SSF and SHF by *K.marxinus* or *S.cerevisiae*

3.2 Results and Discussion

Bioethanol production from OPEFB has 3 steps; pretreatment, hydrolysis and fermentation. Pretreatment of biomass increases cellulose fraction by removing hemicellulose and lignin. SSF combines hydrolysis and fermentation for simpler and faster processing.

3.2.1 Characteristics of raw OPEFB

Lignocellulose contents of raw OPEFB used in this study before chemical pretreatment were determined according to AOAC methods. The cellulose, hemicellulose, lignin and other contents were 41.11 ± 0.80 %, 30.03 ± 0.65 %, 26.36 ± 0.60 % and 2.5 ± 0.10 %, respectively, by mass in dry matter. These contents match well a prior study (Medina *et al*., 2016). However, cellulose and hemicellulose contents were slightly below those obtained by (Hamzah *et al.,* 2011), who reported 43.8 ± 0.02 % of cellulose and 35.0 ± 0.59 % of hemicellulose. The differences may be due to variety, growth conditions, and maturity level of the plant produce (Siti Sabrina *et al*., 2013). Additionally, different methods were employed in determining the compositions (Zakaria *et al.,* 2014). The high hemicellulose and lignin contents would make bioethanol production difficult, so that dilute acid and alkaline pretreatments were required to remove these components partly, and to increase the cellulose content.

3.2.2 Pretreatment steps

3.2.2.1 Effect of sequential dilute acid autoclaving and alkaline autoclaving treatment on OPEFB

The pretreatment with dilute acid and alkaline can efficiently dissolve hemicellulose and lignin from the biomass (Kim S and Kim CH, 2013). The first and second pretreatment were introduced to improve the enzymatic digestibility of OPEFB for bioethanol production. The acid pretreatment was performed using dilute sulfuric acid, and experiments were designed to determine the optimal concentration using Response Surface Methodology (RSM). The manipulated process variables were substrate loading (5 – 25 % w/v), sulfuric acid concentration (0.2 – 1 M) and reaction time (15 – 90 min) and processing was performed in an autoclave at 121 ºC. Statistical data were analyzed using Design Expert software to find the regression equations, and the regression coefficients for the estimation of variance (ANOVA). After that *P-value* was

used to analyze of independent variables affect to hemicellulose, cellulose and lignin significant when *P-value* lower than 0.05. The experimental conditions and chemical composition data of acid pretreatment show in Table 3.5.

Table 3.5 Experimental conditions and chemical composition of the OPEFB of dilute acid autoclaving pretreatment step

Run	Substrate loading % (w/v)	H_2SO_4 conc. (M)	Reaction time (min)	Component of OPEFB		
				Hemicellulose (g/g biomass)	Cellulose (g/g biomass)	Lignin (g/g biomass)
1	9	0.40	30	0.071	0.575	0.269
2	21	0.40	30	0.070	0.562	0.278
3	9	0.40	75	0.091	0.622	0.247
4	21	0.40	75	0.066	0.609	0.271
5	9	0.80	30	0.085	0.595	0.283
6	21	0.80	30	0.087	0.610	0.273
7	9	0.80	75	0.079	0.620	0.261
8	21	0.80	75	0.018	0.627	0.283
9	5	0.60	53	0.080	0.600	0.268
10	25	0.60	53	0.064	0.600	0.280
11	15	0.60	15	0.071	0.555	0.274
12	15	0.60	90	0.057	0.612	0.251
13	15	0.20	53	0.038	0.657	0.278
14	15	1.00	53	0.041	0.635	0.304
15	15	0.60	53	0.077	0.611	0.260
16	15	0.60	53	0.078	0.605	0.260
17	15	0.60	53	0.079	0.619	0.243
18	15	0.60	53	0.076	0.607	0.258
Untreated				0.300	0.411	0.264

Table 3.6 Statistical analysis with ANOVA showed the effect of various factors by dilute acid autoclaving treatment on OPEFB

	***P* value**		
Effect	Hemicellulose	Cellulose	Lignin
Model	0.0085*	0.0059*	0.0019*
A : Substrate loading (% w/v)	0.0159*	0.9326	0.0236*
B : Sulfuric acid conc. (M)	0.5975	0.4181	0.0086*
C : Reaction time (min)	0.0501	0.0008*	0.0089*
AB	0.2681	0.1970	0.2695
AC	0.0133*	0.8213	0.0294*
BC	0.0115*	0.1560	0.3600
A^2	0.9706	0.2370	0.0098*
B^2	0.0031*	0.0096*	0.0002*
C^2	0.3654	0.0187*	0.3221
LOF	0.0025*	0.0963	0.8699

*Significant $P \leq 0.05$

From Table 3.6 shows the variables that affect the hemicellulose, cellulose and lignin by dilute acid pretreatment on OPEFB. It was found that result of hemicellulose cannot use predict the quadratic equation due to LOF significant but cellulose and lignin can predict the quadratic equation as Eq. 3.3-3.4 in Table 3.7. Statistical significance of factor effect to model equation was analyzed by *P*-value < 0.05. From prediction of cellulose found that regression of linear term C was the most significant factor for cellulose, giving a *P*-value of 0.0008. Interaction terms has not term significant. For the quadratic terms, B^2, C^2 were significant. Moreover, prediction of lignin found that regression of quadratic term B^2 was the most significant factor for lignin, giving a *P*-value of 0.0002, followed by the quadratic term A^2. The linear terms of A, B and C significantly influenced the lignin. For interaction terms, only AC was significant.

Table 3.7 Equation showing the influence of factors on cellulose and lignin from dilute acid treatment

Result	Eq. Quadratic	Eq. no.	R^2	R^2_{adj}
Cellulose (g/g biomass)	$54.89 + 1.06x10^{-3}A + 24.08B + 3.89x10^{-3}C + 5x10^{-3}AB - 7.37x10^{-6}AC - 1.48x10^{-3}BC - 1.24x10^{-4}A^2 - 21.38B^2 + 2.03x10^{-5}C^2$	3.3	0.8860	0.7593
Lignin (g/g biomass)	$39.65 - 5.26x10^{-3}A - 22.4B - 1.59x10^{-3}C - 2.19x10^{-3}AB + 4.34x10^{-5}AC + 4.77x10^{-4}BC + 1.69x10^{-4}A^2 + 21.57B^2 + 3.78x10^{-6}C^2$	3.4	0.9171	0.8237

In order to visualize more obviously the interaction terms effects to the three parameters on cellulose in Figure 3.2 and lignin in Figure 3.3, Both Figure, RSM plot ranges have been supported from the derived equation: from the 9 - 21 % w/v experimental range of substrate loading, range 0.4 – 0.8 M of acid concentration; and the 30-75 min of reaction time. From all the graphs shown that blue with low yield and red with high yield.

From Figure 3.2(a) a central point of 52.5 min was treated all over for the reaction time, whereas in Figure 3.2(b) it was a central point of acid concentration at 0.6 M, and for Figure 3.2(c) the central point of substrate loading was 15% w/v. In both Figure 3.2(b) and 3.2(c) cellulose increases with increasing reaction time. In Figure 3.2(a), cellulose gradually decreases with increasing acid concentration from 0.45 to 0.75 M due to cellulose was hydrolyzed with acid at high concentration.

From Figure 3.3(a) a central point of 52.5 min was treated all over for the reaction time, whereas in Figure 3.3(b) it was a central point of acid concentration at 0.6 M, and for Figure 3.3(c) the central point of substrate loading was 15% w/v. In both Figure 3.2(b) and 3.2(c) lignin decreases with increasing reaction time from 52.5 to 75 min due to the reaction time increases, the lignin bond is weakened and easily released. In Figure 3.2(a), lignin gradually decreases with increasing acid concentration from 0.4 to 0.6 M.

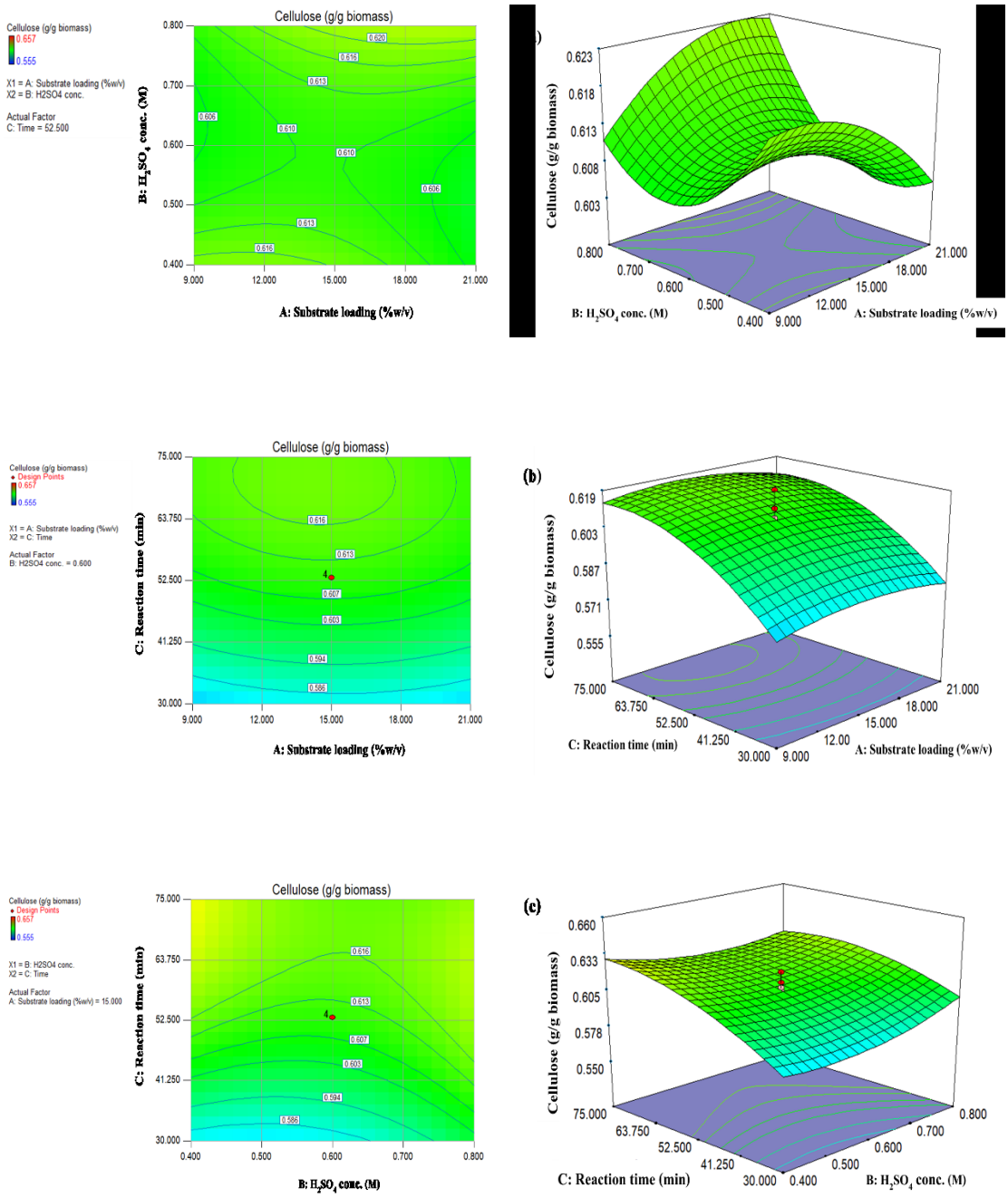


Figure 3.2 Contour and 3D response surface plot between variables that affect cellulose on dilute acid autoclaving treatment on OPEFB

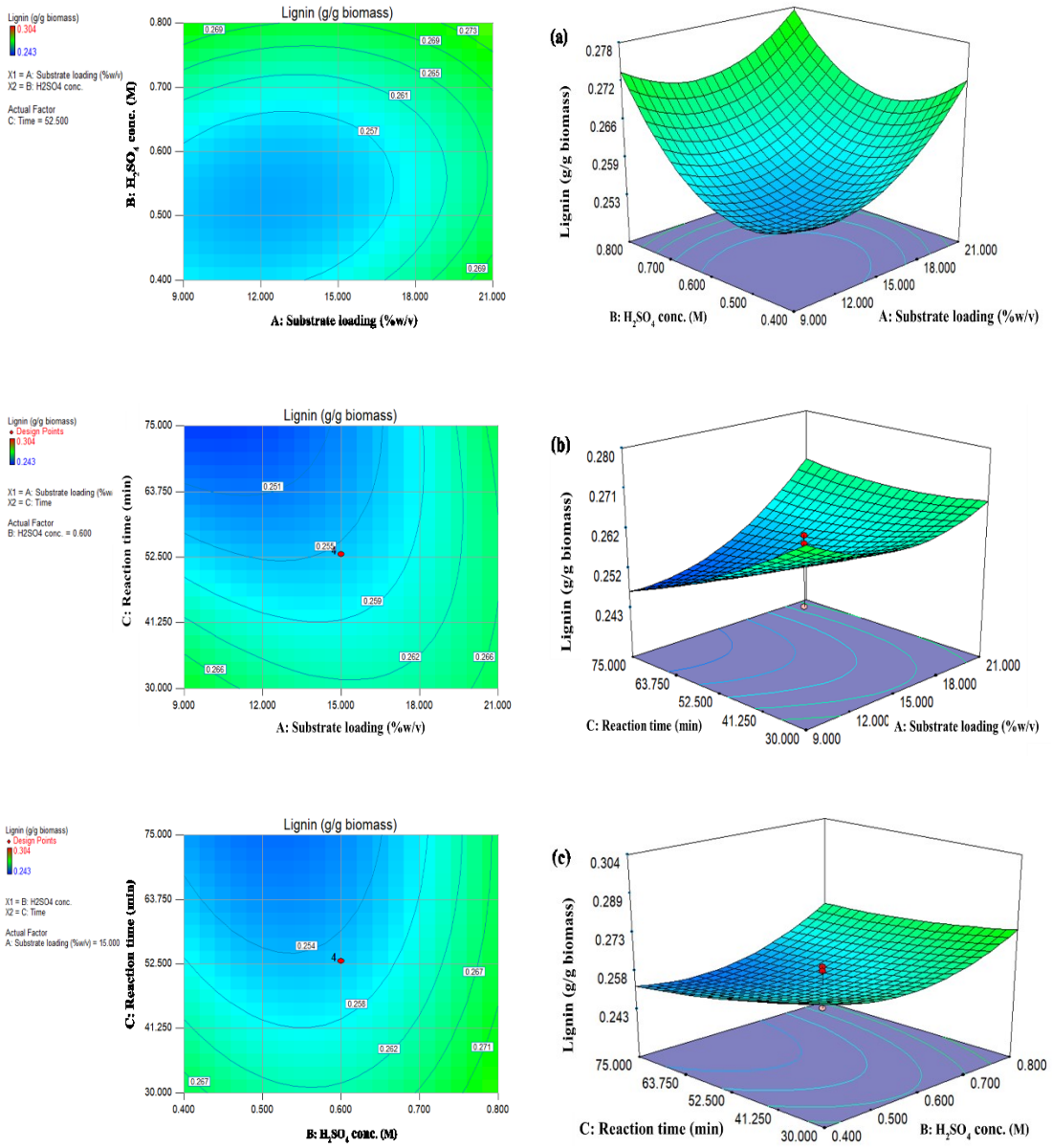


Figure 3.3 Contour and 3D response surface plot between variables that affect lignin on dilute acid autoclaving treatment on OPEFB

Sequentially, the dry acid-treated OPEFB was pretreated to remove lignin using 5 % w/v NaOH at 121 ºC and 15 psi for 20 min. The chemical composition after alkaline treatment was presented in Table 3.8 where lignin decreased up to 10.22 %, and hemicellulose slightly decreased about 0.53 %, while cellulose content increased. Which contained with 72.10 % cellulose, 3.24 % hemicellulose and 17.60 % lignin at Run 13 on condition substrate loading=15 % w/v, reaction time = 53 min and concentration of H_2SO_4= 0.2 M.

Table 3.8 Experimental conditions and enzymatic digestibility, chemical composition of the OPEFB of sequential dilute acid autoclaving and alkaline autoclaving pretreatment step

	Conditions				Component of OPEFB			
Run	Substrate loading (% w/v)	H_2SO_4 conc. (M)	Reaction time (min)	NaOH conc. at 20 min (% w/v)	Hemicellulose (g/g biomass)	Lignin (g/g biomass)	Cellulose (g/g biomass)	Enzymatic digestibility (%)
1	9	0.40	30	5	0.064±0.002	0.162±0.002	0.711±0.002	69.76±0.59
2	21	0.40	30	5	0.034±0.003	0.274±0.013	0.610±0.014	68.58±0.16
3	9	0.40	75	5	0.046±0.004	0.183±0.015	0.662±0.004	73.98±0.33
4	21	0.40	75	5	0.056±0.001	0.224±0.004	0.640±0.012	72.79±1.12
5	9	0.80	30	5	0.045±0.006	0.261±0.003	0.679±0.008	71.55±0.47
6	21	0.80	30	5	0.042±0.006	0.282±0.006	0.630±0.011	72.88±0.19
7	9	0.80	75	5	0.069±0.005	0.250±0.009	0.632±0.007	73.80±0.89
8	21	0.80	75	5	0.025±0.004	0.243±0.006	0.621±0.003	74.39±0.29
9	5	0.60	53	5	0.054±0.004	0.240±0.004	0.674±0.006	72.00±0.20
10	25	0.60	53	5	0.077±0.002	0.219±0.004	0.640±0.009	72.00±0.98
11	15	0.60	15	5	0.069±0.003	0.144±0.013	0.578±0.019	67.95±1.04

Table 3.8 Experimental conditions and enzymatic digestibility, chemical composition of the OPEFB of sequential dilute acid autoclaving and alkaline autoclaving pretreatment step (cont.)

	Conditions				Component of OPEFB			
Run	Substrate loading (% w/v)	H_2SO_4 conc. (M)	Reaction time (min)	NaOH conc. at 20 min (% w/v)	Hemicellulose (g/g biomass)	Lignin (g/g biomass)	Cellulose (g/g biomass)	Enzymatic digestibility (%)
12	15	0.60	90	5	0.047±0.002	0.237±0.012	0.641±0.006	73.07±0.49
13	15	0.20	53	5	0.032±0.002	0.176±0.007	0.721±0.002	75.15±0.75
14	15	1.00	53	5	0.032±0.001	0.288±0.014	0.650±0.033	70.83±1.66
15	15	0.60	53	5	0.075±0.004	0.193±0.014	0.688±0.016	72.63±0.27
16	15	0.60	53	5	0.077±0.003	0.172±0.010	0.651±0.003	72.45±0.50
17	15	0.60	53	5	0.069±0.005	0.218±0.007	0.667±0.020	72.98±0.10
18	15	0.60	53	5	0.065±0.004	0.209±0.017	0.656±0.002	73.69±1.08
Untreated					0.300±0.017	0.264±0.039	0.411±0.004	36.68±0.47

From Table 3.9 shows the variables that affect the hemicellulose, cellulose and lignin by sequential dilute acid and alkaline treatment. From statistical analysis with ANOVA cannot predict the quadratic equation due to *P-value* of the model higher 0.05 not significant.

Table 3.9 Statistical analysis with ANOVA showed the effect of various factors by sequential dilute acid autoclaving and alkaline autoclaving treatment on OPEFB

Effect	***P* value**			
	Hemicellulose	Cellulose	Lignin	Enzymatic digestibility
Model	0.3193	0.0695	0.1844	0.1336
A: Substrate loading (% w/v)	0.6429	0.0276*	0.3337	0.9386
B: Sulfuric acid conc. (M)	0.6398	0.7356	0.5672	0.8682
C: Reaction time (min)	0.7509	0.0806	0.0183*	0.0056
AB	0.9903	0.1258	0.3430	0.3413
AC	0.5694	0.3880	0.1969	0.8635
BC	0.9494	0.6020	0.8363	0.2844
A^2	0.5420	0.6358	0.1729	0.6104
B^2	0.2442	0.0184*	0.9295	0.8432
C^2	0.0097*	0.3531	0.1513	0.1201
LOF	0.0299*	0.2020	0.1347	0.0367*

*Significant $P \leq 0.05$

The optimal condition for both models was predicted with the software by RSM. In the second case (Table 3.10) a maximum cellulose and enzymatic digestibility of 0.721 (g/g biomass) and of 75.15 % respectively, a minimum hemicellulose of 0.032 (g/g biomass) and lignin of 0.176 (g/g biomass) was predicted at substrate loading = 15 % w/v, reaction time = 53 min, concentration of H_2SO_4 = 0.2 M and concentration of NaOH = 5 % w/v.

Table 3.10 Optimum condition results to obtain high cellulose and enzymatic digestibility

Solution No.	Substrate loading (%w/v)	H_2SO_4 conc. (M)	Reaction time (min)	Hemicellulose (g/g biomass)	Cellulose (g/g biomass)	Lignin (g/g biomass)	Enzymatic digestibility (%)	Desirability
1	15	0.20	53.00	0.032	0.721	0.176	75.15	1.000
2	16	0.40	43.48	0.062	0.672	0.185	71.98	0.609
3	16	0.40	45.47	0.062	0.672	0.188	72.18	0.608
4	17	0.40	49.06	0.062	0.672	0.194	72.49	0.606
5	18	0.40	54.61	0.061	0.673	0.197	72.95	0.600

*Optimum conditions for high cellulose, enzymatic digestibility, and low hemicellulose and lignin.

In each pretreatment step, compare the chemical composition of OPEFB the Table 3.11 at optimal condition was substrate loading = 15 % w/v, reaction time = 53 min, concentration of H_2SO_4 = 0.2 M and concentration of NaOH = 5 % w/v of No.1 of Table 3.10.

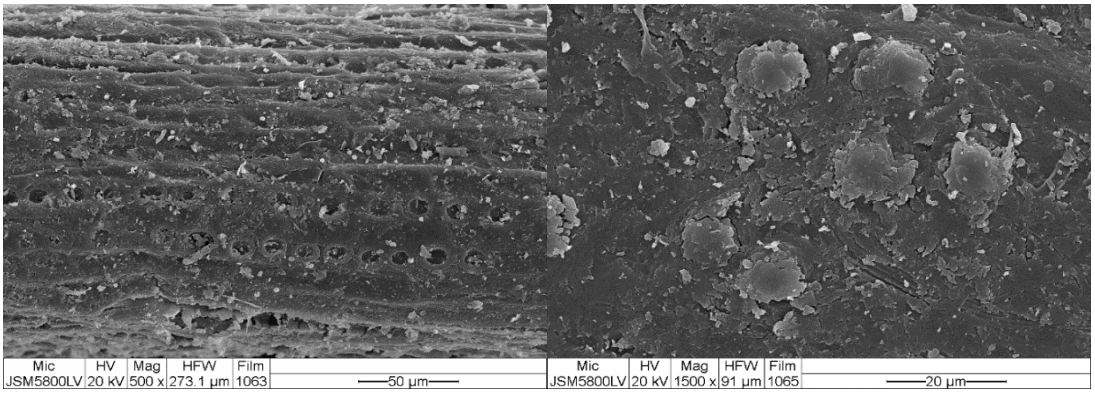
Table 3.11 The chemical composition of the OPEFB that has pretreatment each step

Component of OPEFB	Non-treatment	1st treatment	2nd treatment
% Cellulose	41.11	65.7	72.1
% Hemicellulose	30.03	3.77	3.24
% Lignin	26.36	27.82	17.6

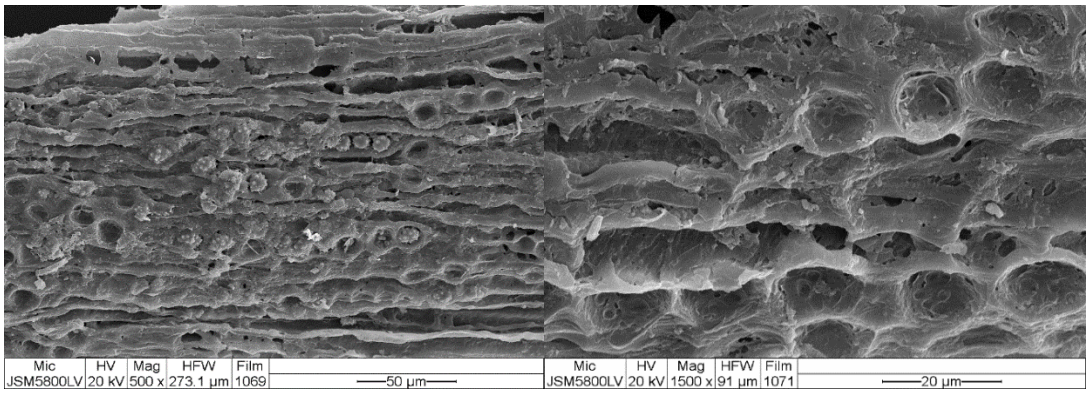
The increment in cellulose content is due to alkaline pretreatment can efficiently penetrates and swells both the accessible amorphous and crystalline regions of cellulose (Aziz *et al*., 2002). These results are in agreement with sequential acid/alkaline pretreatment of empty palm fruit bunch fiber as presented by (Kim *et al*., 2012&2013). Comparing to other studies about alkaline pretreatment, the cellulose and hemicellulose recoveries in this study was higher and lower than that obtained in (Medina *et al*., 2015; Muryanto *et al*., 2015; Ishola *et al*., 2014), respectively, but cellulose content was slightly lower than in reported by (Triwahyuni *et al*., 2015). While lignin content was higher than that obtained in (Medina, 2015; Triwahyuni, 2015; Muryanto, 2015) using 10 – 20 % w/v NaOH concentration. This is due to the increment of NaOH concentration caused to efficiently remove lignin through saponification reaction of ester bonds (Barlianti *et al*., 2015; Sun and Chang, 2002). Thus, acid/alkaline-treated OPEFB that contained high cellulose and low lignin contents was conducted to use as substrate further sugar fermentation.

3.2.2.2 Scanning Electron Microscope (SEM) analysis of the OPEFB by sequential acid/alkaline treatment.

(a)



(b)



(c)

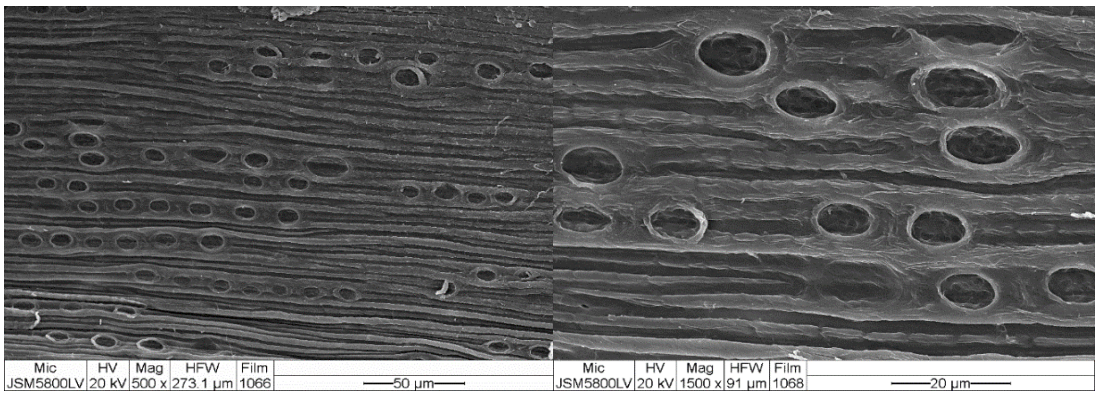


Figure 3.4 Analysis with SEM micrograph of (a) Untreated OPEFB (b) 0.2 M H_2SO_4 Pretreatment (c) Sequential 0.2 M H_2SO_4/ 5 % (w/v) NaOH treatment.

Figure 3.4 illustrates the surface image of untreated, acid treated and acid/alkaline-treated OPEFB analyzed under the Scanning Electron Microscope (SEM). The untreated OPEFB was showed in Figure 3.4a had a smooth surface with the external layer composed of wax and lignin that protected the fiber against rupture. Additionally, the outer surface also had a deposition of hemicellulose and other inorganic components such as Na, K and Ca as reported by (Nazir *et al*., 2013). Morphological changes after dilute acid pretreatment were observed in Figure 3.4b, which have rough surface due to erosion of sulfuric acid under high temperature. Additionally, sulfuric acid pretreatment also could separate fibers from pith and loosening of the fibrous network, and remove hemicellulose from biomass disrupting the cell wall with a loose matrix (Chandel *et al*., 2014). Figure 3.4c was showed surface changes after the alkaline treatment in second pretreatment. The OPEFB surface had more holes, cracks and erosion troughs, because NaOH pretreatment under high pressure and temperature collapsed fiber into a pliable fiber, clean up the fiber surface and thus exposed more cellulose component in the EFB fiber (Hamzah *et al*., 2011). During the pretreatment process, NaOH penetrates and swell both the accessible amorphous and crystalline region of cellulose, which caused to remove hemicellulose and lignin effectively as cellulose exposure increased (Hamzah *et al*., 2011; Aziz *et al*., 2002). Thus, the sequential acid/alkaline pretreatment can successfully disrupt the OPEFB surface, remove other components and break bone between lignin and the complex carbohydrates for further improving the subsequent enzymatic hydrolysis.

3.2.3 Fermentation steps

3.2.3.1 Design of experiments and RSM of SSF with *K. marxianus*

The optimization of fermentation parameters viz., fermentation temperature, substrate loading, pH and yeast concentration was selected on the basis of effected ethanol product of fermentation and optimized using a Central Composite Design (CCD). Design of experiment with CCD of RSM consisted 28 trials for which the ethanol production ranged between 0.108 g/g biomass and 0.292 g/g biomass. Summarize the level of the four factors in the experimental design shown in Table 3.12. The results for the experimental were used as responses quadratic models for ethanol yield, reducing sugar and theoretical efficiency

Table 3.12 Experimental conditions and experimental results of fermentation step by *K.marxianus* at 48 h

Condition				*K. marxianus*		
Temperature (ºC)	Substrate loading (% w/v)	pH	Yeast conc. (% v/v)	Ethanol Yield (g/g biomass)	Reducing sugar (g/g biomass)	Theoretical efficiency (%)
33.75	7.5	4.5	2	0.206	0.017	55.90
41.25	7.5	4.5	2	0.238	0.077	64.58
33.75	12.5	4.5	2	0.259	0.019	70.28
41.25	12.5	4.5	2	0.249	0.059	67.57
33.75	7.5	5.5	2	0.267	0.023	72.45
41.25	7.5	5.5	2	0.219	0.083	59.43
33.75	12.5	5.5	2	0.263	0.038	71.37
41.25	12.5	5.5	2	0.189	0.088	51.29
33.75	7.5	4.5	4	0.173	0.028	46.95
41.25	7.5	4.5	4	0.213	0.126	57.80
33.75	12.5	4.5	4	0.273	0.011	74.08
41.25	12.5	4.5	4	0.240	0.074	65.13
33.75	7.5	5.5	4	0.243	0.015	65.94
41.25	7.5	5.5	4	0.218	0.133	59.16
33.75	12.5	5.5	4	0.241	0.031	65.40
41.25	12.5	5.5	4	0.180	0.074	48.85
30	10	5	3	0.169	0.134	45.86
45	10	5	3	0.091	0.305	24.69
37.5	5	5	3	0.193	0.014	52.37
37.5	15	5	3	0.233	0.025	63.23
37.5	10	4	3	0.263	0.005	71.37
37.5	10	6	3	0.259	0.013	70.28

Table 3.12 Experimental conditions and experimental results of fermentation step by *K. marxianus* at 48 h (cont.)

Condition				*K. marxianus*		
Temperature (ºC)	Substrate loading (% w/v)	pH	Yeast conc. (% v/v)	Ethanol Yield (g/g biomass)	Reducing sugar (g/g biomass)	Theoretical efficiency (%)
37.5	10	5	1	0.281	0.005	76.28
37.5	10	5	5	0.255	0.012	69.20
37.5	10	5	3	0.248	0.035	67.30
37.5	10	5	3	0.250	0.040	67.84
37.5	10	5	3	0.243	0.038	65.94
37.5	10	5	3	0.245	0.039	66.48

Table 3.13 Statistical analysis with ANOVA showed the effect of various factors ethanol fermentation by SSF with *K. marxianus*

	***P* value**		
Effect	Ethanol yield	Reducing sugar	Theoretical efficiency
Model	< 0.0001*	< 0.0001*	< 0.0001*
A : Temperature (^{o}C)	< 0.0001*	< 0.0001*	< 0.0001*
B : Substrate loading (% w/v)	0.0055*	0.1871	0.0055*
C : pH	0.5225	0.1687	0.5225
D : Yeast conc.(% v/v)	0.0177*	0.1225	0.0176*
AB	0.0029*	0.0157*	0.0029*
AC	0.0003*	0.8459	0.0003*
AD	0.6718	0.0447*	0.6716
BC	0.0001*	0.2406	0.0001*
BD	0.2605	0.0386*	0.2606
CD	0.9516	0.3782	0.9512
A^2	< 0.0001*	< 0.0001*	< 0.0001*
B^2	0.0213*	0.0370*	0.0213*
C^2	0.0434*	0.0053*	0.0434*
D^2	0.0114*	0.0048*	0.0112*
LOF	0.0163*	0.0050*	0.0164*

*Significant $P \leq 0.05$

Table 3.14 Equation showing the influence of factors on ethanol yield, reducing sugar and theoretical efficiency from ethanol production on OPEFB by SSF with *K. marxianus*

Result	Eq. Quadratic	Eq. no.	R^2	R^2_{adj}
Ethanol yield (g/g biomass)	$-4.33 + 0.19A + 0.13B + 0.20C - 0.08D - 1.18x10^{-3}AB - 7.90x10^{-3}AC + 3.50x10^{-4}AD - 0.01BC + 1.42x10^{-3}BD - 3.75x10^{-4}CD - 1.94x10^{-3}A^2 - 1.04x10^{-3}B^2 + 0.02C^2 + 7.28x10^{-3}D^2$	3.5	0.9578	0.9123
Reducing sugar (g/g biomass)	$2.91 - 0.22A - 0.05B + 0.32 + 0.04D - 9.33x10^{-4}AB + 3.33x10^{-4}AC + 1.87x10^{-3}AD + 3.10x10^{-3}BC - 2.90x10^{-3}BD - 5.75x10^{-3}CD + 3.13x10^{-3}A^2 - 9.57x10^{-4}B^2 - 0.03C^2 - 8.73x10^{-3}D^2$	3.6	0.9802	0.9588
Theoretical efficiency (%)	$-1174.11 - 52.02A + 35.33B + 55.73C - 20.62D - 0.32AB - 2.14AC + 0.10AD - 3.60BC + 0.39BD - 0.10CD - 0.53A^2 - 0.28B^2 + 6C^2 + 1.98D^2$	3.7	0.9578	0.9124

These three models the data with a R^2 equal to 0.9578 for ethanol yield, 0.9802 for reducing sugar and 0.9578 theoretical efficiency. The adjusted R^2 for the responses were 0.9123, 0.9588 and 0.9124 respectively. Analyze with ANOVA were quadratic models to test the effects of factors on the responses and the possible interaction between factors. As for ethanol yield, A *P-value* < 0.0001 shows that the model was statistically valid. The reversal of linear term A (temperature, ºC) was the most significant factor for ethanol production, having a less *P-value* of 0.0001. After of fermentation 48 h. in the experiment, the maximum ethanol yield of 0.281 g/g biomass was operate in the condition using substrate loading of 10 (% w/v), pH of 5 and yeast concentration of 1 (% v/v) at 37.5°C for 48 h. while the predicted value was 0.290 g/g biomass (0.9 % higher than the predicted value). The significance and effects of each variable on SSF of OPEFB and ethanol yield are present in Eq.3.5 (Table 3.14). By applying multiple regression analysis on

the experimental data, the following quadratic equation (Eq.3.5-3.7) account ethanol yield, reducing sugar and theoretical efficiency of fermented OPEFB. Where A, B, C and D are fermentation temperature, substrate loading, pH and yeast concentration, respectively.

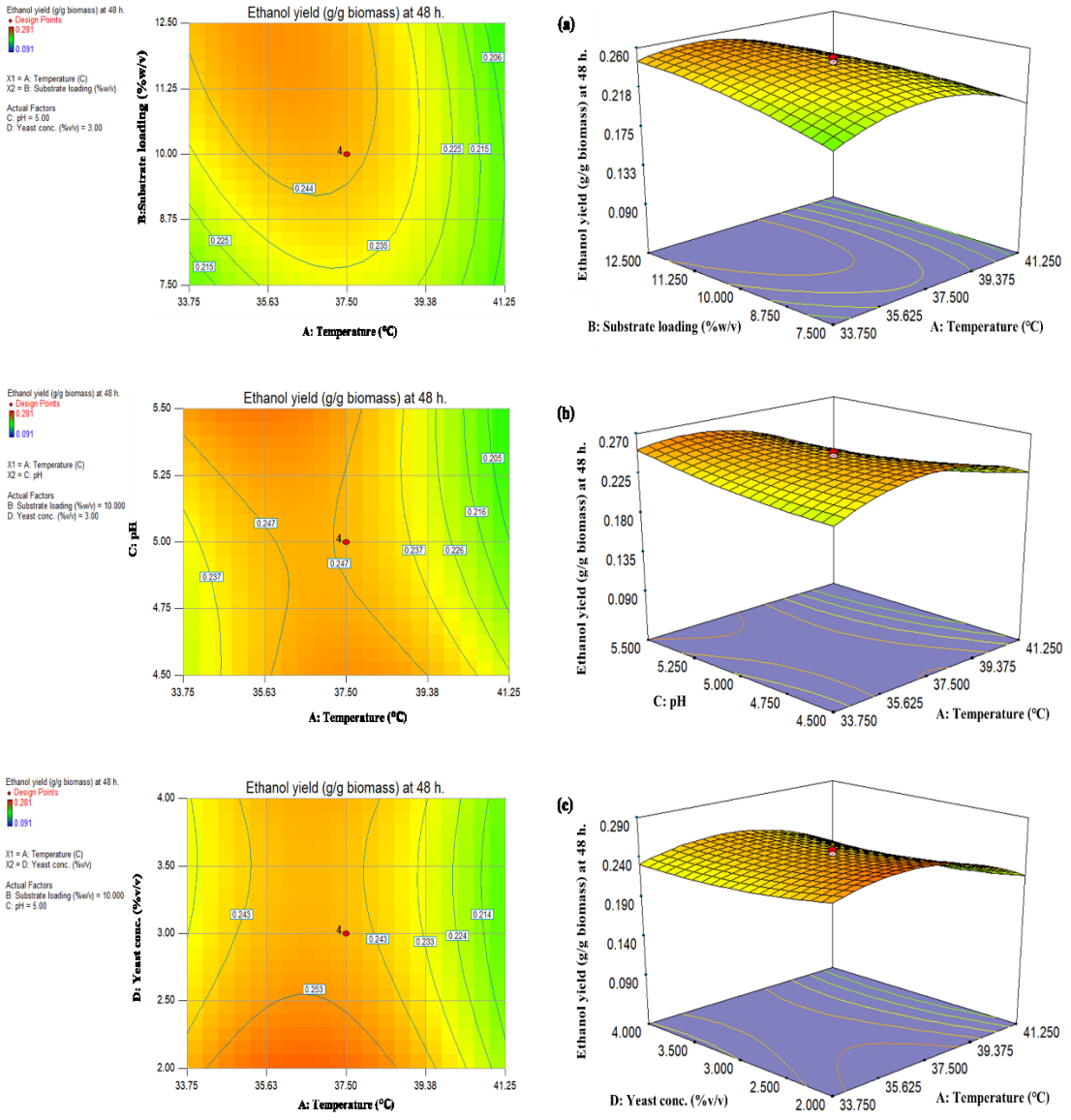


Figure 3.5 Contour and 3D response surface plots interaction between (a) temperature and substrate loading: (b) temperature and pH and (c) temperature and yeast concentration on ethanol yield (g/g biomass) by fermentation with *K. marxianus*

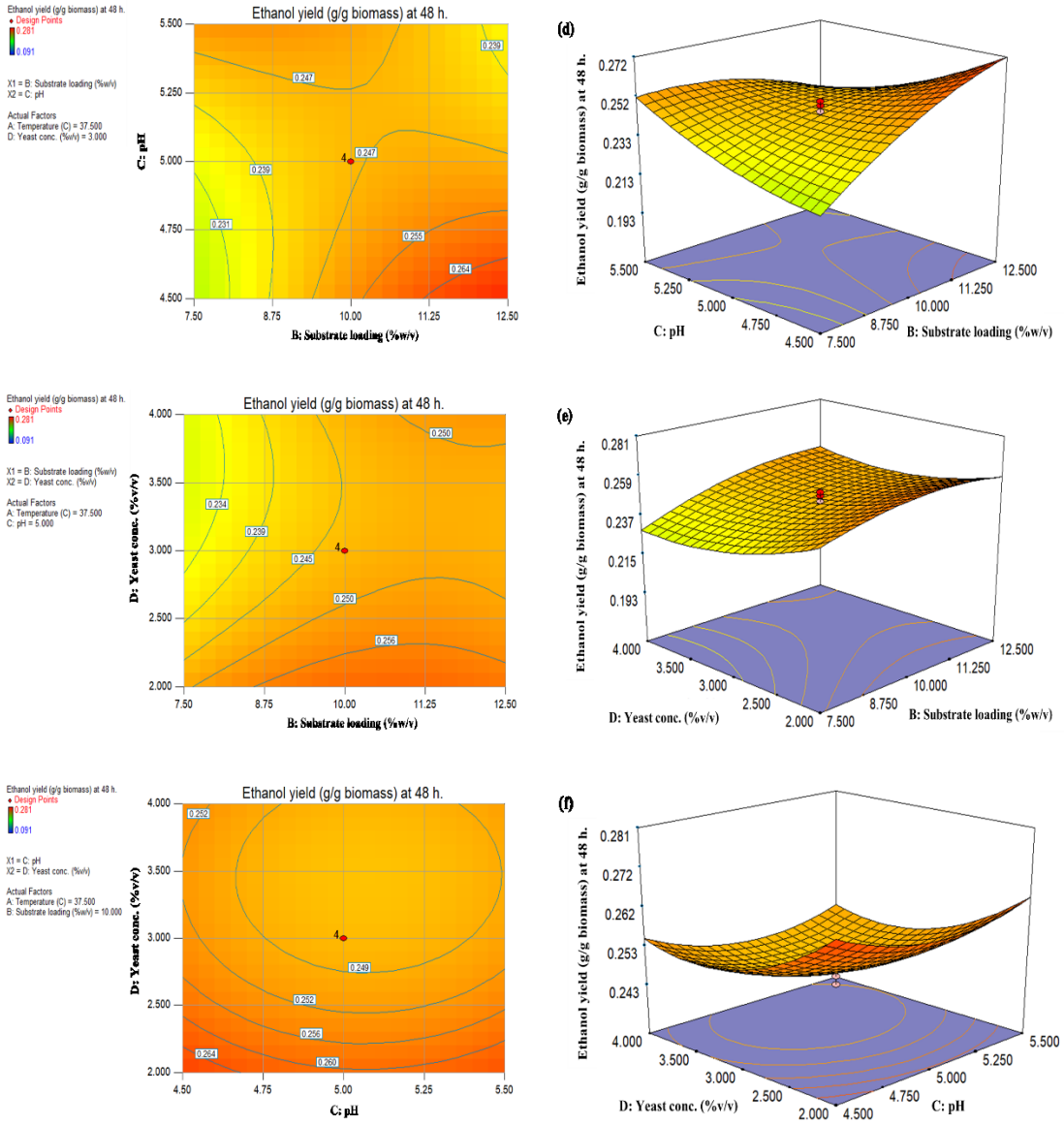


Figure 3.5 Contour and 3D response surface plots interaction between (d) substrate loading and pH: (e) substrate loading and yeast concentration and (f) pH and yeast concentration on ethanol yield (g/g biomass) by fermentation with *K. marxianus* (cont.)

In order to visualize more obviously the interaction terms effects to the four parameters on ethanol yield in Figure 3.5, Both Figure, RSM plot ranges have been supported from the derived equation: from the 33.75 – 41.25 ℃ experimental range of temperature, range 7.5 – 12.5 (%w/v) of substrate loading; range 4.5 -5.5 of pH and the 2 – 4 min of yeast concentration. From all the graphs shown that blue with low yield and red with high yield.

In both Figure 3.5 (a), 3.5 (b) and 3.5 (c) ethanol yield gradually decreases with increasing temperature from 39 to 41.25 ℃ due to high temperatures cause the yeast to not grow, resulting in less ethanol. In both Figure 3.5(d) and 3.5 (e), ethanol yield decreases with decreasing substrate loading lower than 7.5 (%w/v) due to high substrate loading give high glucose content therefore receive high ethanol yield.

Table 3.15 Optimum condition results to obtain high ethanol yield by *K. marxianus* at 48 h

No.	Temperature (℃)	Substrate loading (%w/v)	pH	Yeast conc. (%v/v)	Ethanol yield (g/g biomass)	Theoretical efficiency (%)	Desirability
1	36.94	12.24	4.5	2.04	0.281	76.363	1.000
2	37.01	12.50	4.5	4.00	0.277	75.162	0.979
3	35.84	8.65	5.5	2.00	0.274	74.484	0.965
4	36.76	12.50	4.5	2.86	0.273	74.216	0.96

*Optimum conditions for high ethanol yield and theoretical efficiency

Table 3.15 tabulates the optimal conditions for maximizing ethanol yield and theoretical efficiency. No. 3 yielded a maximum ethanol yield of 0.274 (g/g biomass) and theoretical efficiency of 74.48% obtaining at 35.84 ℃ temperature, 8.65 (% w/v) substrate loading, 5.5 pH and 2 (% v/v) yeast concentration. If the use of substrate loading is to be maximized, No. 4 would be more desirous; a considerably high ethanol yield of 0.281 (g/g biomass) and theoretical efficiency of 76.36 % was obtained at a higher substrate loading of 12.24 % w/v, although with a little increase of fermentation temperature from 35.84 to 36.94 ℃ and a little decrease of pH from 5.5 to 4.5.

The optimal point for both models was predicted with the software by RSM. In the first case (Table 3.15) a maximum ethanol yield of 0.281 (g/g biomass) was predicted at temperature = 36.94 °C, substrate loading = 12.24 (% w/v), pH = 4.5 and yeast concentration = 2.04 (% v/v). High temperature resistant yeasts strains can increase ethanol yields, which are important because yeast grows and ferments at high temperatures (Limtong *et al.,* 2007).

Results of ANOVA listed in Eq.3.5 revealed that the Second-order polynomial models adequately represent the responses of ethanol yield with coefficients of determination R^2, which indicates that 95.78 % of the variability of response might be explained by the model. These values are in accordance with the adjusted coefficient of determination R^2_{adj} 0.9123.

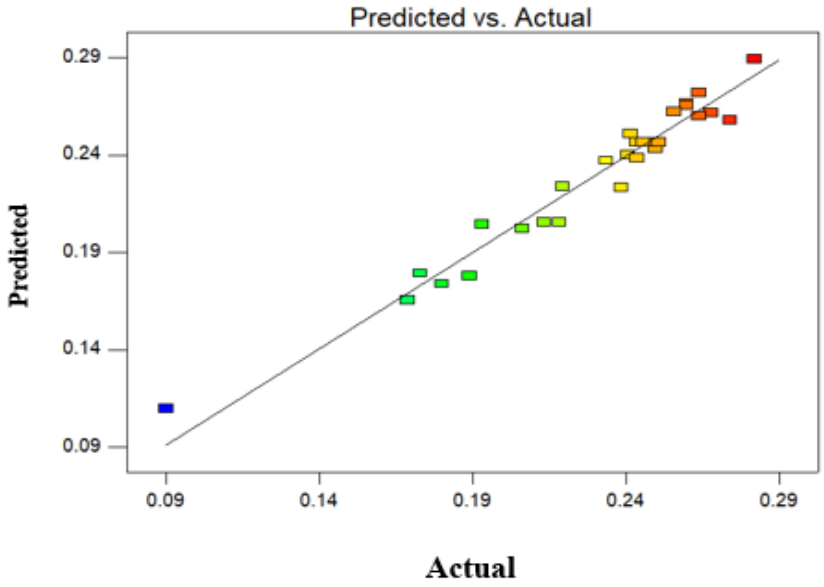


Figure 3.6 Charts of predicted values vs. actual values of Ethanol yield by SSF with *K. marxianus*

According to ANOVA results for ethanol yield in SSF process by *K.marxianus* the linear terms of A, B, D, the quadratic terms of A^2, B^2, C^2,D^2 and the interaction terms of AB, AC, BC have a significant effect on ethanol yield responses with p-value under a significance level of $\alpha = 0.05$. The effects can be visualized in Figure 3.5 and it is observed that the variables of temperature, substrate loading, yeast concentration and temperature-substrate loading, temperature-pH, yeast concentration-pH interaction and double temperature, substrate loading, pH, yeast concentration are important in a confidence level of 95 % on the ethanol yield and that the effect of temperature, substrate loading are positive and yeast concentration is negative, when increased from lower to higher values. Furthermore, the predicted values versus observed values by the application of the model for multiple regressions are shown in Figure 3.6 and evidence the good quality of fit. Woottichai *et al.* showed that the statistical analysis of the factors affecting the production of ethanol in the SSF process was significant, namely substrate concentration, pH,

temperature and incubation period (Woottichai *et al*., 2015). Generally, ethanol fermentation used *S. cerevisiae* strains is known to be an excellent strains. However An interesting alternative to ethanol fermentation SSF is *K. marxianus* strains that has the ability to ferment at higher temperatures and to the optimum temperature of the cellulase enzyme from the fungus in hydrolysis steps (Eklund and Zacchi.,1995; Kádár *et al*.,2004; Ohgren *et al*.,2007; García-Aparicio *et al*.,2011).

To obtain this result the model suggested a severe SSF, at the top of our range. The temperature, which is the most variable in this process, was found to be optimal at a value between the medium of our range. Regarding the effect of the substrate loading and pH, as expected the optimal output was at the medium of the range. And regarding the effect of the yeast concentration, as expected the optimal output was at the minimum of the range.

3.2.3.2 Design of experiments and RSM of SSF with *S. cerevisiae*

The optimization of fermentation parameters viz., fermentation temperature, substrate loading, pH and yeast concentration was selected on the basis of effected ethanol product of fermentation and optimized using a Central Composite Design (CCD). Design of experiment with CCD of RSM consisted 28 trials for which the ethanol production ranged 0.145 g/g biomass and 0.306 g/g biomass. Summarize the level of the four factors in the experimental design shown in Table 3.16. The results for the experimental were used as responses quadratic models for ethanol yield, reducing sugar and theoretical efficiency.

Table 3.16 Experimental conditions and experimental results of fermentation step by *S. cerevisiae* at 48 h

Condition				*S. cerevisiae*		
Temp. (ºC)	Substrate loading (% w/v)	pH	Yeast conc. (% v/v)	Ethanol yield (g/g biomass)	Reducing sugar (g/g biomass)	Theoretical efficiency (%)
33.75	7.5	4.5	2	0.257	0.034	69.74
41.25	7.5	4.5	2	0.081	0.188	21.98
33.75	12.5	4.5	2	0.257	0.007	69.74
41.25	12.5	4.5	2	0.011	0.363	2.98
33.75	7.5	5.5	2	0.268	0.025	72.73
41.25	7.5	5.5	2	0.075	0.059	20.35
33.75	12.5	5.5	2	0.262	0.024	71.10
41.25	12.5	5.5	2	0.042	0.369	11.40
33.75	7.5	4.5	4	0.279	0.039	75.71
41.25	7.5	4.5	4	0.057	0.280	15.47
33.75	12.5	4.5	4	0.277	0.030	75.17
41.25	12.5	4.5	4	0.012	0.339	3.26
33.75	7.5	5.5	4	0.271	0.029	73.54
41.25	7.5	5.5	4	0.087	0.155	23.61
33.75	12.5	5.5	4	0.283	0.041	76.80
41.25	12.5	5.5	4	0.028	0.347	7.60
30	10	5	3	0.265	0.041	71.91
45	10	5	3	0.003	0.414	0.81
37.5	5	5	3	0.239	0.021	64.86
37.5	15	5	3	0.213	0.080	57.80
37.5	10	4	3	0.205	0.081	55.63
37.5	10	6	3	0.217	0.054	58.89

Table 3.16 Experimental conditions and experimental results of fermentation step by *S. cerevisiae* at 48 h (cont.)

Condition				*S. cerevisiae*			
Temp. (ºC)	Substrate loading (% w/v)	pH	Yeast conc. (% v/v)	Ethanol yield (g/g biomass)	Reducing sugar (g/g biomass)	OD yeast	Overall yield (%)
37.5	10	5	1	0.212	0.087	0.042	57.53
37.5	10	5	5	0.217	0.096	0.089	58.89
37.5	10	5	3	0.293	0.035	0.045	79.51
37.5	10	5	3	0.288	0.030	0.059	78.15
37.5	10	5	3	0.290	0.034	0.055	78.70
37.5	10	5	3	0.291	0.036	0.068	78.97

Table 3.17 Statistical analysis with ANOVA showed the effect of various factors ethanol fermentation by SSF with *S. cerevisiae*

	***P* value**		
Effect	Ethanol yield	Reducing sugar	Theoretical efficiency
Model	< 0.0001*	< 0.0001*	< 0.0001*
A : Temperature (^{o}C)	< 0.0001*	< 0.0001*	< 0.0001*
B : Substrate loading (% w/v)	0.1934	0.0015*	0.1934
C : pH	0.5677	0.1901	0.5677
D : Yeast conc. (% v/v)	0.7882	0.3291	0.7882
AB	0.1879	0.0006*	0.1879
AC	0.7134	0.1629	0.7134
AD	0.5592	0.5900	0.5592
BC	0.8414	0.0839	0.8414
BD	0.9228	0.2493	0.9228
CD	0.9845	0.9953	0.9845
A^2	< 0.0001*	< 0.0001*	< 0.0001*
B^2	0.0129*	0.2545	0.0129*
C^2	0.0051*	0.1154	0.0051*
D^2	0.0063*	0.0330	0.0063*
LOF	0.0002*	0.0002*	0.0002*

*Significant $P \leq 0.05$

Table 3.18 Equation showing the influence of factors on ethanol yield, reducing sugar and theoretical efficiency from ethanol production on OPEFB by SSF with *S. cerevisiae*

Result	Eq. Quadratic	Eq. no.	R^2	R^2_{adj}
Ethanol yield (g/g biomass)	$-6.74 + 0.23A + 0.11B + 0.96C + 0.2D - 1.41x10^{-3}AB + 1.9x10^{-3}AC - 1.52x10^{-3}AD + 1.55x10^{-3}BC + 3.75x10^{-4}BD + 3.75x10^{-4}CD - 3.22x10^{-3}A^2 - 3.57x10\text{-}3B^2 - 0.1C^2 - 0.03D^2$	3.8	0.9381	0.8713
Reducing sugar (g/g biomass)	$7.3 - 0.28A - 0.27B - 0.45C - 0.12D + 5.07x10^{-3}AB - 8.3x10^{-3}AC + 1.55x10^{-3}AD + 0.02BC - 5.08x10^{-3}BD - 1.25x10^{-4}CD + 3.87x10^{-3}A^2 + 1.64x10^{-3}B^2 + 0.06C^2 + 0.02D^2$	3.9	0.9505	0.8971
Theoretical efficiency (%)	$-1828.48 + 61.19A + 30.13B + 261.63C + 55.51D - 0.38AB + 0.52AC + 0.41AD + 0.42BC + 0.1BD + 0.1CD - 0.87A^2 - 0.97B^2 - 28.3C^2 - 6.84D^2$	3.10	0.9381	0.8714

These four models the data with a R^2 equal to 0.9381 for ethanol production, 0.9505 for reducing sugar and 0.9381 theoretical efficiency. The adjusted R^2 for the responses were 0.8713, 0.8971 and 0.8714 respectively. Analyze with ANOVA were quadratic models to test the effects of factors on the responses and the possible interaction between factors. As for ethanol production, A *P-value* < 0.0001 shows that the model was statistically valid. The reversal of linear term A (temperature, ℃) was the most significant factor for ethanol production, having a less *P-value* of 0.0001. After of fermentation 48 h. in the experiment, the maximum ethanol yield of 0.293 g/g biomass was operate in the condition using substrate loading of 10 (% w/v), pH of 5 and yeast concentration of 3 (% v/v) at 37.5 °C for 48 h while the predicted value was 0.295 g/g biomass (0.2 % higher than the predicted value). The significance and effects of each variable on SSF of OPEFB and ethanol yield are present in Eq.3.8 (Table 3.18). By applying multiple regression analysis on the experimental data, the following quadratic equation (Eq.3.8 - 3.10) account ethanol yield,

reducing sugar and theoretical efficiency of fermented OPEFB. Where A, B, C and D are fermentation temperature, substrate loading, pH and yeast concentration, respectively

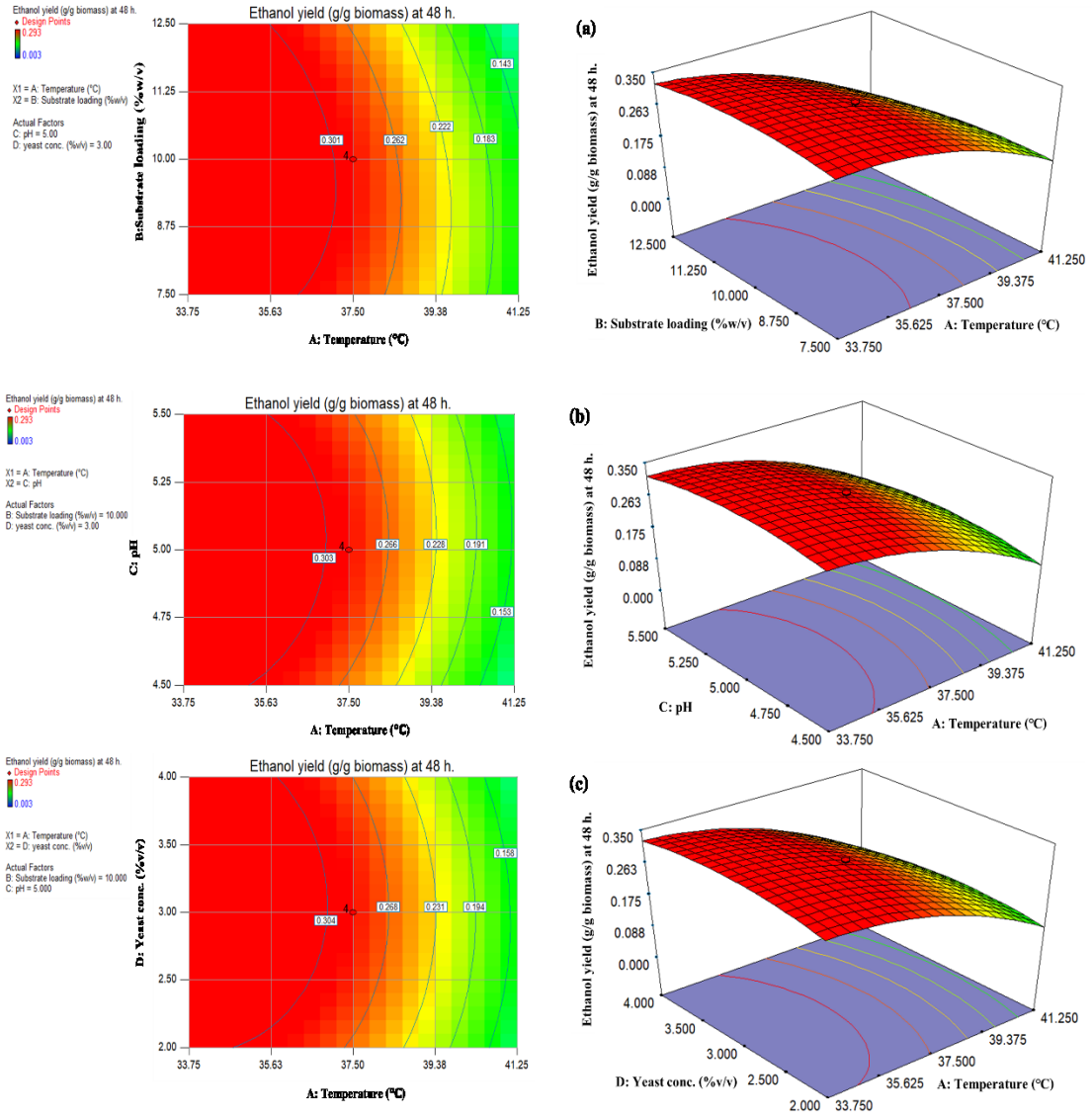


Figure 3.7 Contour and 3D response surface plots interaction between (a) temperature and substrate loading: (b) temperature and pH and (c) temperature and yeast concentration on ethanol yield (g/g biomass) by fermentation with *S. cerevisiae*

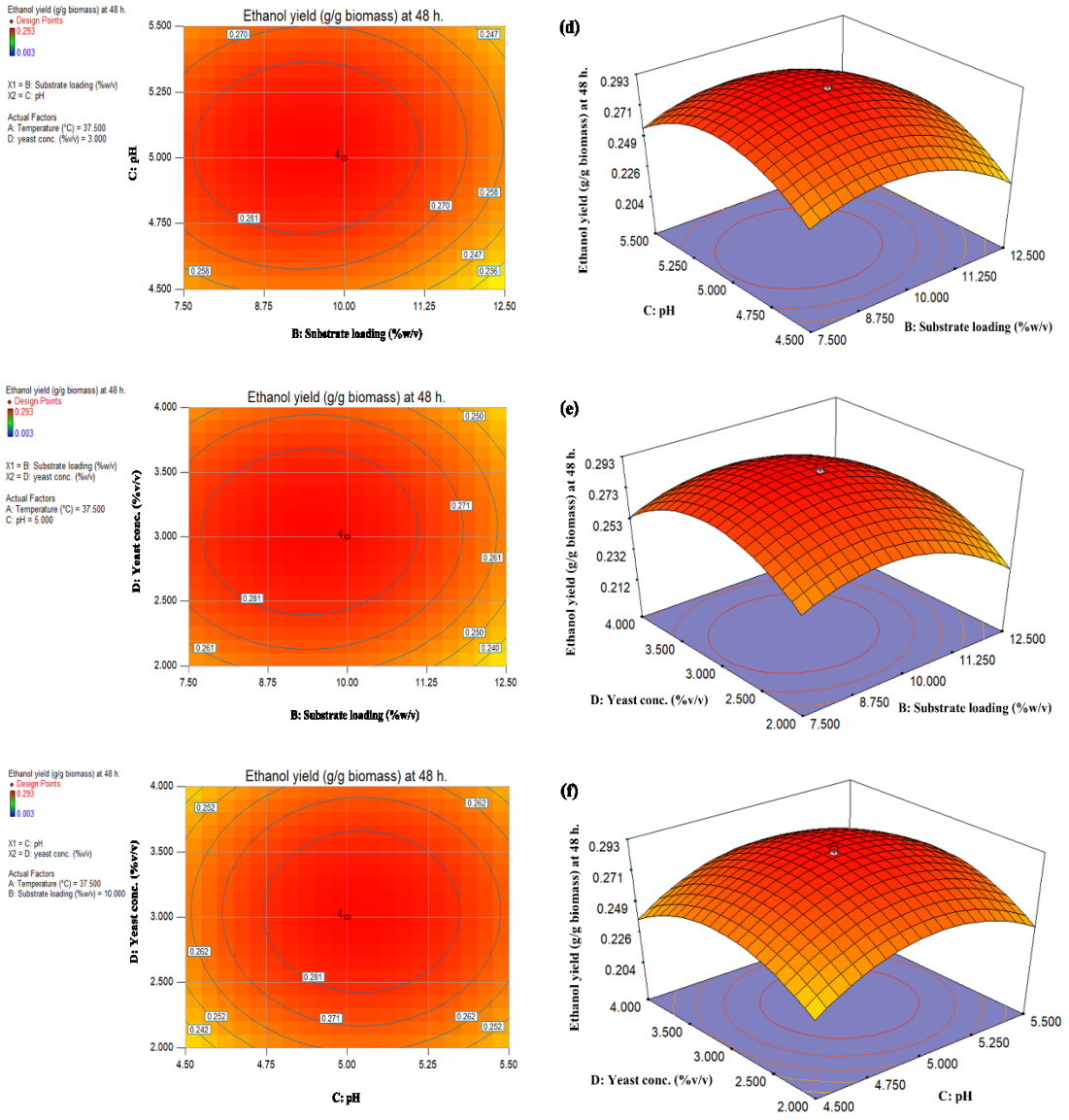


Figure 3.7 Contour and 3D response surface plots interaction between (d) substrate loading and pH: (e) substrate loading and yeast concentration and (f) pH and yeast concentration on ethanol yield (g/g biomass) by fermentation with *S. cerevisiae* (cont.)

In order to visualize more obviously the interaction terms effects to the four parameters on ethanol yield in Figure 3.7, Both Figure, RSM plot ranges have been supported from the derived equation: from the 33.75 – 41.25 ℃ experimental range of temperature, range 7.5 – 12.5 (%w/v) of substrate loading; range 4.5 -5.5 of pH and the 2 – 4 min of yeast concentration. From all the graphs shown that blue with low yield and red with high yield.

In both Figure 3.7 (a), 3.7 (b) and 3.7 (c) ethanol yield gradually increases with decreasing temperature from 37.50 to 33.75 ℃ due to low temperatures cause the yeast to grow well, resulting in more ethanol yield.

Table 3.19 Optimum condition results to obtain high ethanol yield by *S. cerevisiae* at 48 h

No.	Temperature (°C)	Substrate loading (%w/v)	pH	yeast conc. (%v/v)	Ethanol yield (g/g biomass) at 48 h.	Theoretical efficiency (%)	Desirability
1	36.83	10.32	5.21	3.24	0.301	81.77	1
2	36.46	10.31	5.30	2.45	0.296	80.2	1
3	35.66	7.93	4.80	3.38	0.308	83.49	1
4	35.03	8.16	4.91	3.38	0.320	86.88	1
5	36.18	7.92	4.67	3.34	0.293	79.59	1

*Optimum conditions for high ethanol yield and theoretical efficiency

Table 3.19 tabulates the optimal conditions for maximizing ethanol yield and theoretical efficiency. No. 3 yielded a maximum ethanol yield of 0.308 (g/g biomass) and theoretical efficiency of 83.49% obtaining at 35.66 ℃ temperature, 7.93 (% w/v) substrate loading, 4.8 pH and 3.38 (% v/v) yeast concentration. If the use of substrate loading is to be maximized, No. 4 would be more desirous; a considerably high ethanol yield of 0.320 (g/g biomass) and theoretical efficiency of 86.88 % was obtained at a higher substrate loading of 8.16 % w/v, although with a little decrease of fermentation temperature from 35.66 to 35.03 ℃ and a little increase of pH from 4.8 to 4.91.

The optimal point for both models was predicted with the software by RSM. In the first case (Table 3.19) a maximum ethanol yield of 0.320 (g/g biomass) was predicted at Temperature = 35.03 ℃, Substrate loading = 8.16 (% w/v), pH = 4.91 and Yeast concentration = 3.38 (% v/v). The SSF process is easy to operate and easy in design. Most importantly, higher ethanol yield and inhibitor of hydrolysis cellulose decreases. However, the optimum temperature for cellulose hydrolysis and ethanol fermentation is different because the optimum temperature for hydrolysis is 50 ℃, but normal yeast grows at 30 - 35 °C (Zhao *et al*, 2012). Thus, *S. cerevisiae*

strains is not suitable for SSF process because *S. cerevisiae* strains cannot grow at temperatures higher 40 ºC.

Results of ANOVA listed in Eq.3.8 revealed that the Second order polynomial models adequately represent the responses of ethanol yield with coefficients of determination R^2, which indicates that 93.81 % of the variability of response might be explained by the model. These values are in accordance with the adjusted coefficient of determination R^2_{adj} 0.8713.

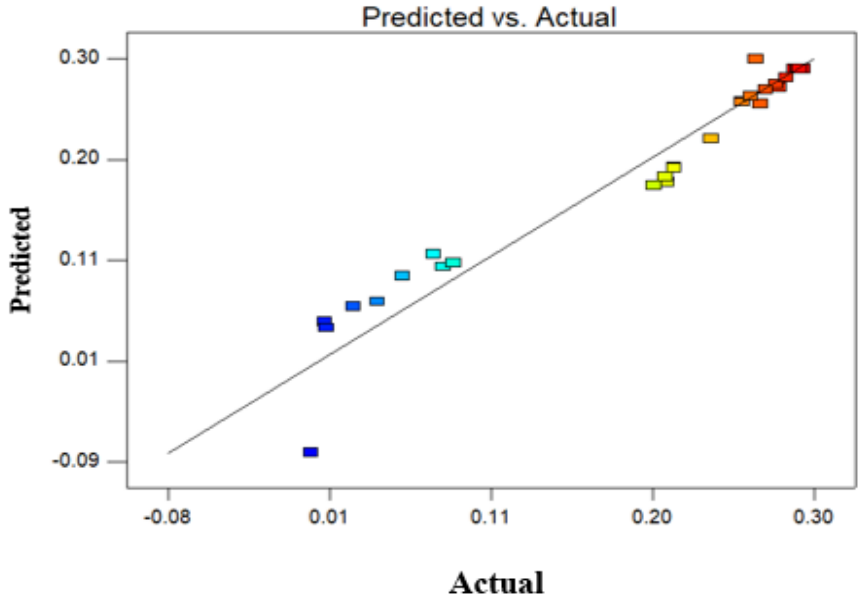


Figure 3.8 Charts of predicted values vs. actual values of ethanol yield by SSF with *S. cerevisiae*

According to ANOVA results for ethanol yield in SSF process by *S. cerevisiae* the linear terms of A, the quadratic terms of A^2, B^2, C^2,D^2 and no the interaction terms have a significant effect on ethanol yield responses with p-value under a significance level of $\alpha = 0.05$. The effects can be visualized in Figure 3.7 and it is observed that the variables of temperature and double temperature, substrate loading, pH, yeast concentration are important in a confidence level of 95 % on the ethanol yield and that the effect of temperature and double temperature, substrate loading, pH, yeast concentration are negative, when variables effect increased from lower to higher values. Furthermore, the predicted values versus observed values by the application of the model for multiple regressions are shown in Figure 3.8 and evidence the good quality of fit. Generally, ethanol fermentation used *S. cerevisiae* strains is known to be an excellent strains and widely used variety in Thailand.

To obtain this result the model suggested a severe SSF, at the top of our range. The temperature, which is the most variable in this process and substrate loading, pH and yeast concentration, was found to be optimal at a value between the medium of our range.

3.2.3.3 Comparison ethanol production by SSF and SHF processes

Two processes for ethanol production from OPEFBs by *K.marxianus* and *S.cerevisiae* was estimated: namely, Simultaneous Saccharification and Fermentation (SSF) and Sseparate Hydrolysis and Fermentation (SHF). The productivity and ethanol yield of these two cellulosic ethanol fermentation processes were compared. Figure 3.9 shows the measured reducing sugar concentration and ethanol concentration during the SHF and SSF process by *K marxianus*. It shows that after the fermentation process (24 and 48 h, respectively, for the test with *K.marxianus*), the ethanol yield increase because sugar was used ethanol production by yeast. Both ethanol production process yields ethanol yield at 48 h. was 25.82 g/L of SHF and 28.10 g/L of SSF from condition for 10 % w/v of substrate loading, pH 5, 1 % v/v of yeast concentration of *K.marxianus* at 37.5 ºC. It shows that ethanol production with SSF yields more ethanol production than ethanol production with SHF. Figure 3.10 shows the measured reducing sugar concentration and ethanol concentration during the SHF and SSF process by *S.cerevisiae*. It shows that after the fermentation process (24 and 48 h, respectively, for the test with *S.cerevisiae*), the ethanol yield increase because sugar was used ethanol production by yeast. Both ethanol production process yields ethanol yield at 48 h. was 27.19 g/L of SHF and 29.28 g/L of SSF from condition for 10 % w/v of substrate loading, pH 5, 3 % v/v of yeast concentration of *S.cerevisiae* at 37.5 ºC. It shows that ethanol production with SSF yields more ethanol production than ethanol production with SHF. Summary of the comparison of ethanol production is shown in Table 3.20. The result from this work are included and are in agreement that ethanol production by SSF is better than SHF according to giving more ethanol, less production time and less costs (Ohgren *et al.* 2007., Elia *et al*. 2008., Franco *et al*. 2015).

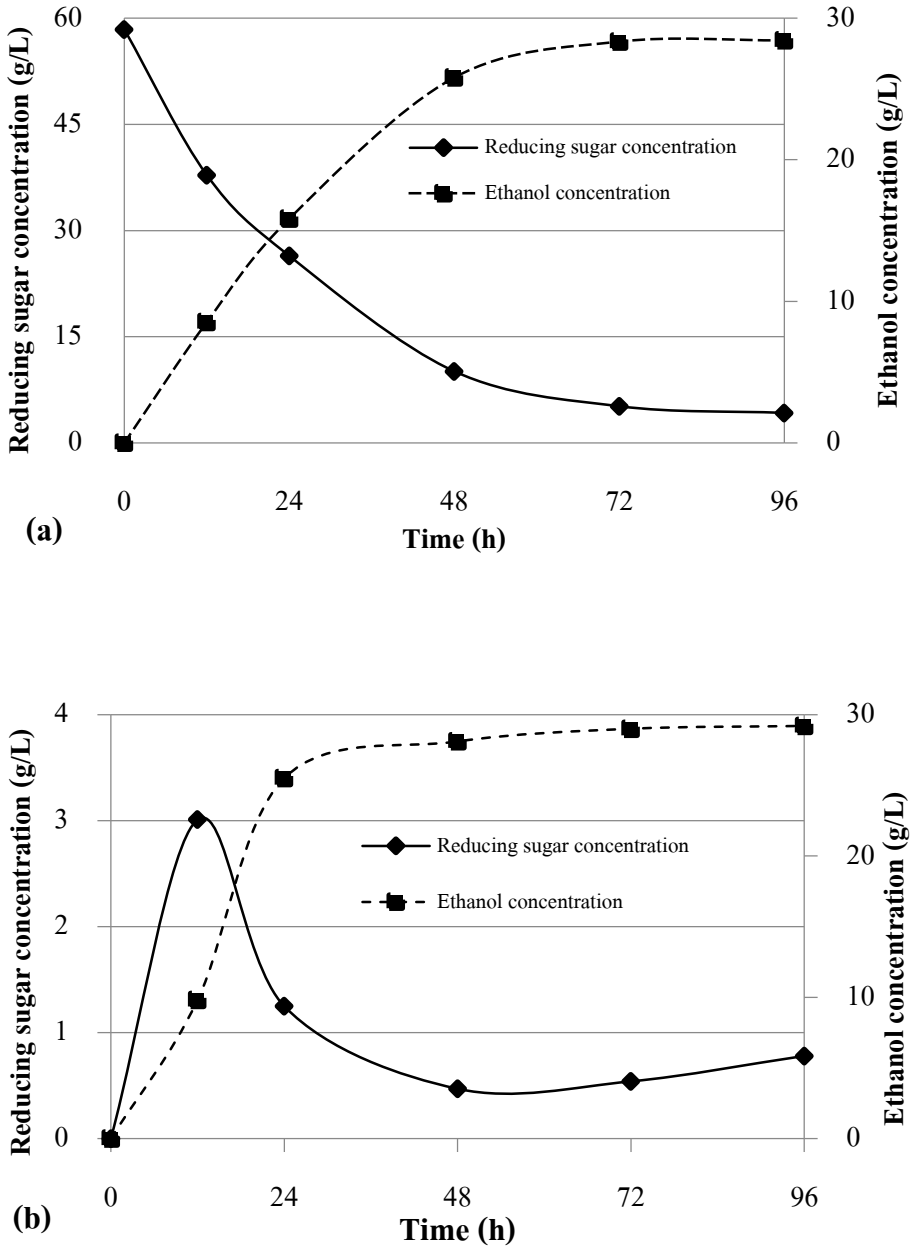


Figure 3.9 Two ethanol production process of (a) SHF process (b) SSF process on DAA-AA pretreatment OPEFB by *K. marxianus*

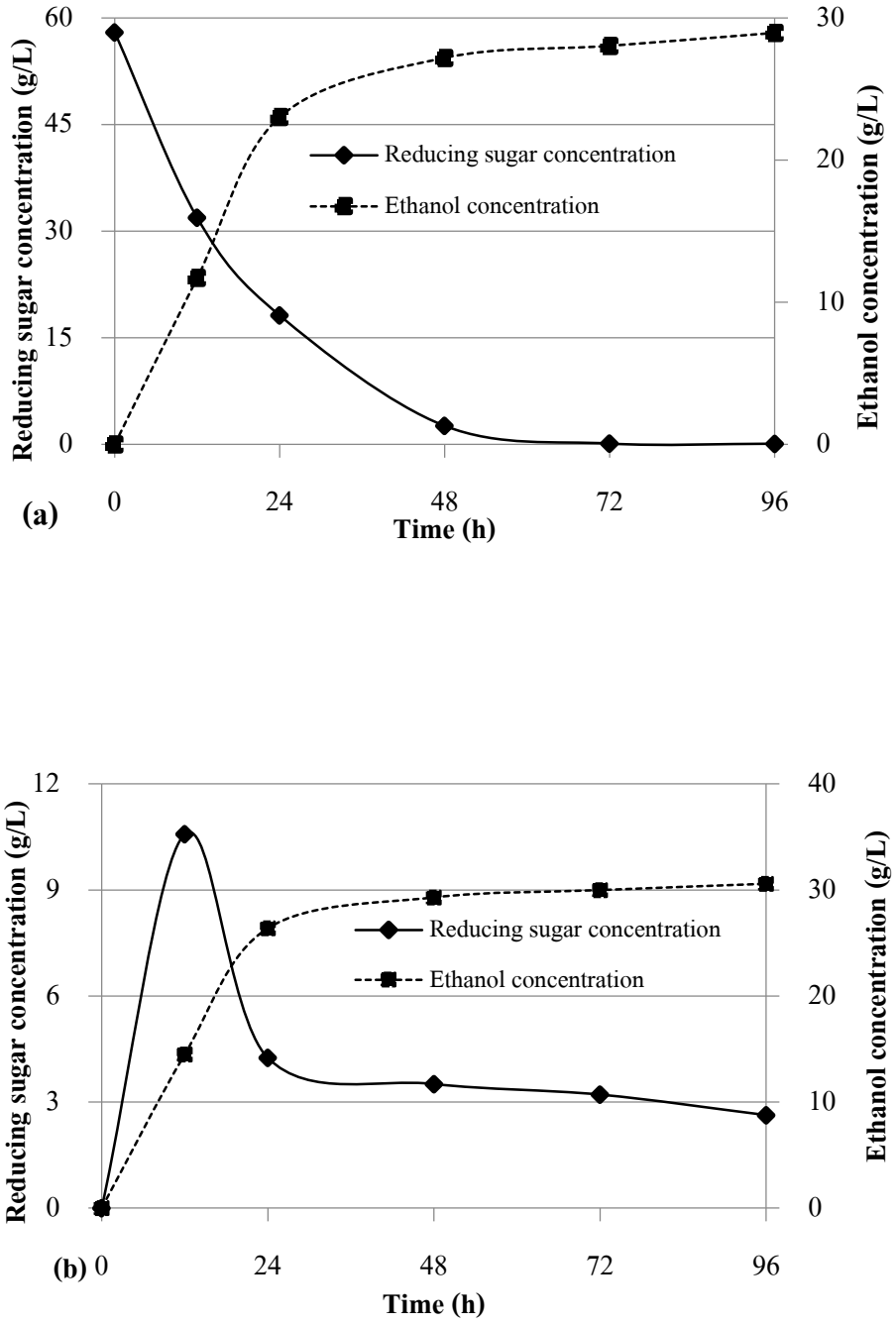


Figure 3.10 Two ethanol production process of (a) SHF process (b) SSF process on DAA-AA pretreatment OPEFB by *S. cerevisiae*

Table 3.20 Recent reports of ethanol production via SSF and SHF using pretreated OPEFB as feedstock

Substrate type	Microorganism	Operation mode	Performance	Reference
Sugarcane bagasse	*Z.mobilis*(immobilized by Ca-alginate)	SHF	0.356 g/g	Wirawan *et al*., (2012)
		SSF	0.351 g/g	
Corn stover	*S.cerevisiae* after 120/144 h.	SHF	20.5 g/L	Ohgren *et al.,* (2007)
		SSF	16.8 g/L	
Barley straw	*K.marxianus CHY1612* (35-45 ºC)	SSF	34.3 g/L	Kang *et al*., (2012)
Poplar wood	*S.cerevisiae* (48 h.)	SHF	14.6 g/L	Cantarella *et al.*,(2004)
	S.cerevisiae (38 h.)	SSF	22.9 g/L	
Wheat straw	*S.cerevisiae (F12)*	SHF	22.6 g/L	Elia *et al*., (2008)
		SSF	23.7 g/L	
	S.cerevisiae (Red Star)	SHF	17.2 g/L	
		SSF	16.8 g/L	
EFB	*S.cereviceae* (72 h.)	SHF	4.74%ethanol	Dahnum *et al*., (2015)
	S.cereviceae (24 h.)	SSF	6.05%ethanol	
Arundo donax	*E.coli* (144 h.)	SHF	24±1 g/L	Loaces *et al.,* (2017)
	E.coli (96 h.)	SSF	25±0.8 g/L	
OPEFB	*K.marxianus* (TISTR5116)	SHF	0.258 g/g	In this work
		SSF	0.281 g/g	
OPEFB	*S.cereviceae*	SHF	0.272 g/g	In this work
		SSF	0.293 g/g	

3.2.3.4 Mass output analysis for ethanol production

In this research, an overall mass output was prepared to explain the stages from pretreatment to SSF and to SHF, which gave the best ethanol production from OPEFB by *K.marxianus* and *S.cerevisiae* showed Figure 3.10 - 3.11. The mass output analysis for OPEFB 100 g. Pretreatment step, Step I, Dilute acid pretreatment under condition H_2SO_4 0.2 M at 121 ºC for 53 min resulted in a loss of 12.50 % (w/w) of solids from OPEFB. Following alkaline pretreatment under condition NaOH 5 % (w/v) at 121 ºC for 20 min resulted in a loss of 35.08 % (w/w) of solids from OPEFB. Cellulose increase in average at 75.38 %, whereas hemicellulose and lignin removal were 89.21 % and 33.23 % respectively.

In SSF step of *K. marxianus*, biomass residue was subjected to unification of cellulase and β-glucosidase at 20 FPU/g of biomass and 4 U/g of biomass under condition pH 5, 1 % v/v of yeast concentration at 37.5 ºC, 150 rpm after 48 h resulted in reducing sugar concentration 0.005 g/g of biomass. The highest ethanol concentration was 0.281 g/g of biomass. In SHF step of *K.marxianus*, after enzymatic saccharification at 72 h, gave about 0.584 g/g of biomass sugar was produced through this process as sugar recovery. Fermentation process resulted in remaining sugar 0.010 g/g of biomass after 48 h. The highest ethanol yield from experiment was 0.258 g/g of biomass.

In SSF step of *S.cerevisiae*, biomass residue was subjected to unification of cellulase and β-glucosidase at 20 FPU/g of biomass and 4 U/g of biomass under condition pH 5, 3 % v/v of yeast concentration at 37.5 ºC, 150 rpm after 48 h resulted in reducing sugar concentration 0.035 g/g of biomass. The highest ethanol yield was 0.293 g/g of biomass. In SHF step of *S.cerevisiae*, after enzymatic saccharification at 72 h, gave about 0.580 g/g of biomass sugar was produced through this process as sugar recovery. Fermentation process resulted in remaining sugar 0.026 g/g of biomass after 48 h. The highest ethanol yield from experiment was 0.272 g/g of biomass. Hence, pretreatment process with sequential acid/alkaline treatment can increase sugar production to generate bioethanol from OPEFB. The concentration of pH and yeast influences the ethanol production of the SSF process and SHF process. Thus, future studies could examine the effects of glucose concentration, or xylose fermentation yeast, to further optimize the ethanol production.

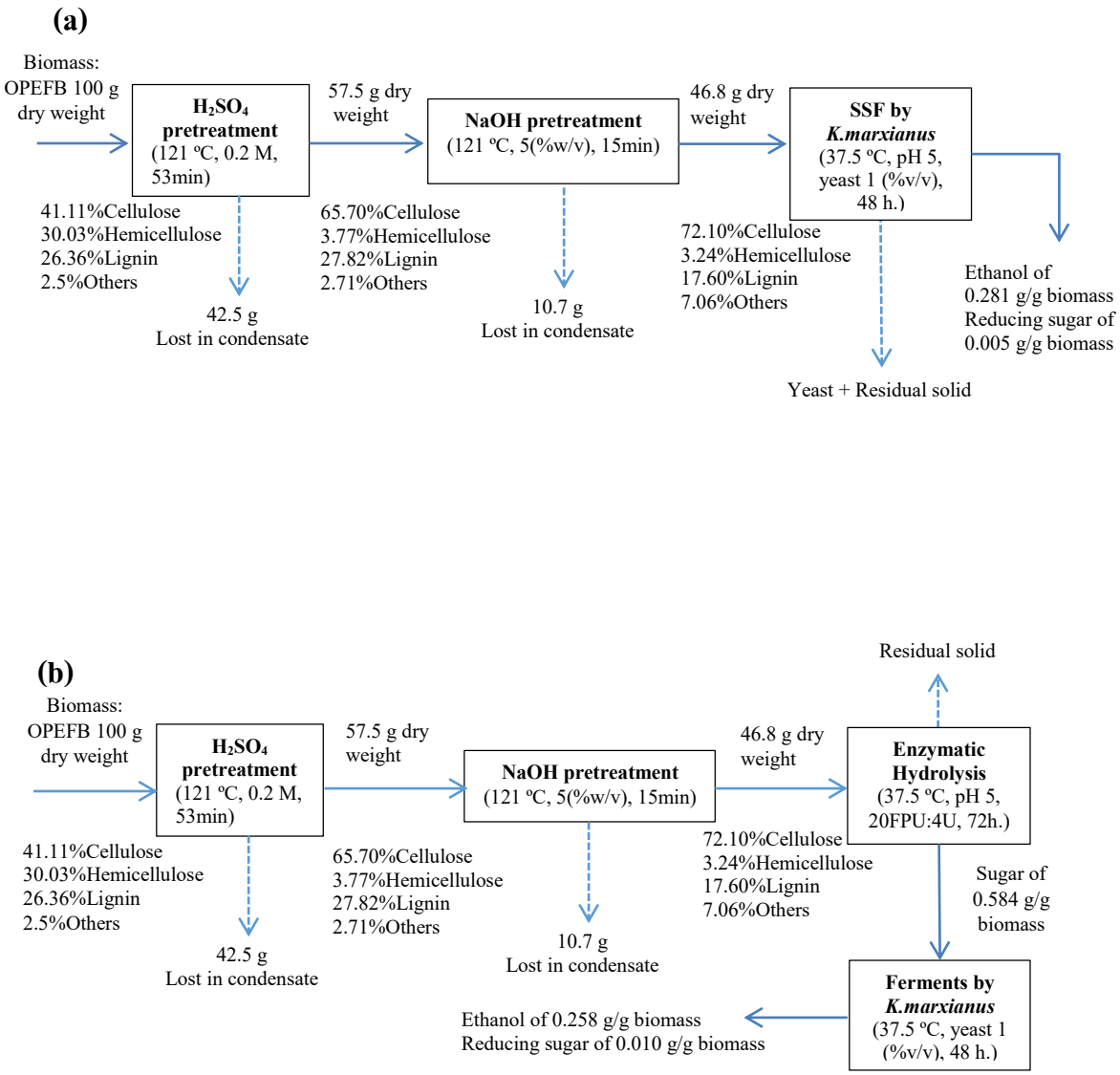


Figure.3.11 Mass output for ethanol production process from OPEFB with *K.marxianus* by (a) SSF process (b) SHF process

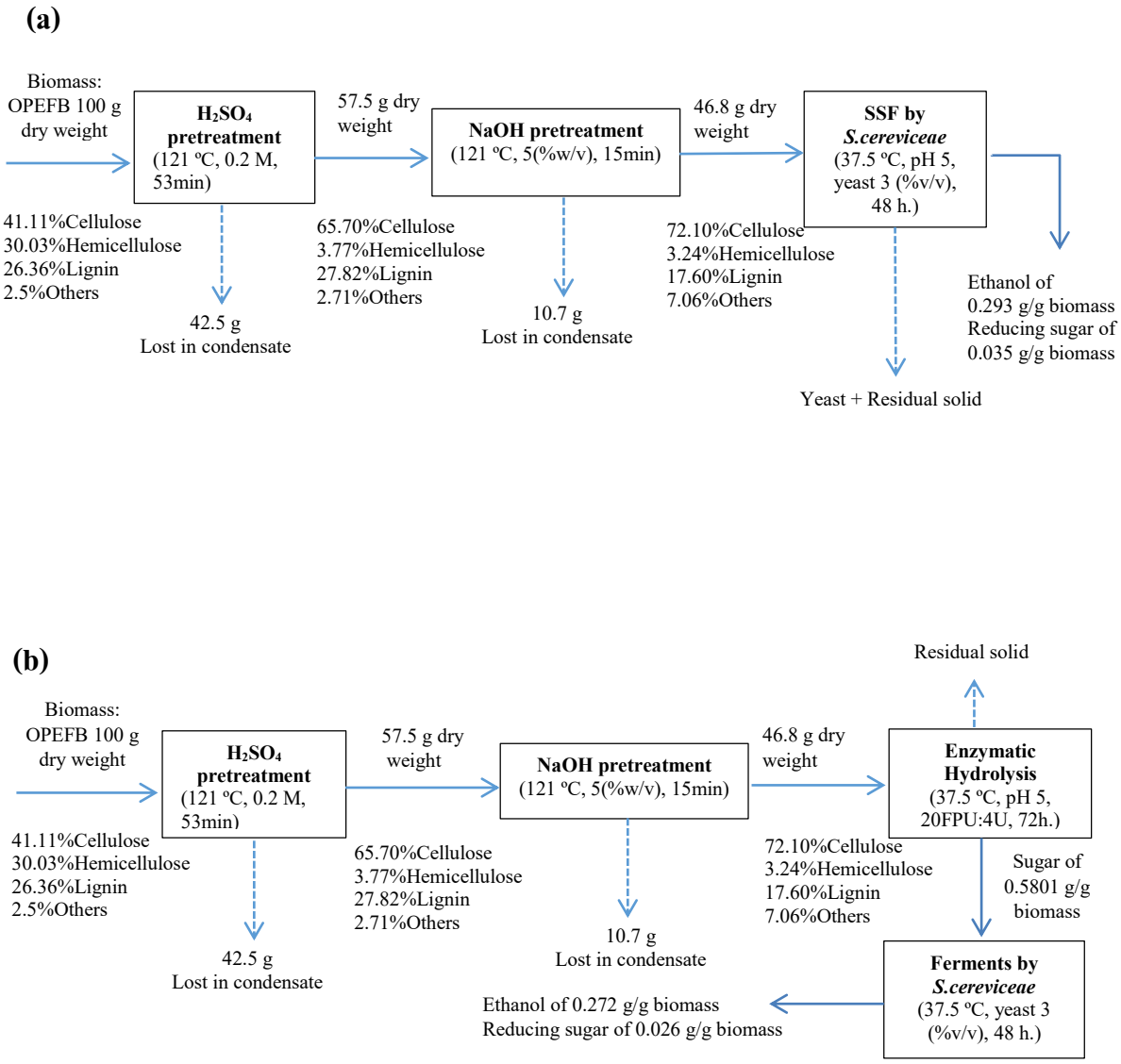


Figure.3.12 Mass output for ethanol production process from OPEFB with *S.cereviceae* by (a) SSF process (b) SHF process

3.3 Costs analysis

This study of economic possibility for ethanol production from oil palm empty fruit bunches was calculated basic on 100 g OPEFB using the optimal conditions as shown in Figures 3.11-3.12. The costs for ethanol production calculated from chemical for pretreatment, commercial enzymes, electricity for heating and yeast. From Table 3.21 – 3.22, the optimal conditions of ethanol production since pretreatment step until fermentation step of each yeast strain, found that have the high cost of production, and mostly of it caused from electrical and enzymatic cost which were variable and fixed costs. However, this research studied for comparison ethanol production between SSF and SHF process could improve to be much higher ethanol yield. From the calculation, it was found that ethanol production with SSF production costs less than SHF, and also SSF ethanol yields more than SHF. Therefore SSF is more cost-effective choice. In order to reduce production costs, future research suggests that reusing of enzymes should be investigated which can reduce enzyme costs by 70%.

Table 3.21 Costs analysis of chemical for ethanol production from 100 g of OPEFB by SSF with *K.marxianus*

		Amount	Chemical usage (Baht)	Enzyme and Medium usage (Baht)	Total Chemical (Baht)	Cost per unit (Baht/g ethanol) SSF	Cost per unit (Baht/g ethanol) SHF
Pretreatments	0.2 M H_2SO_4	1L	0.29		0.29	0.02	0.02
	5% w/v NaOH	1L	22.50		22.50	1.71	1.86
	Total				**22.79**	**1.73**	**1.88**
Hydrolysis and Fermentation	Cellulase (20FPU/g OPEFB)	1.56g		81.74	81.74	6.21	6.77
	Cellobiase (4U/g OPEFB)	0.66mL		60.72	60.72	4.62	5.03
	Medium	0.40 g		1.32	1.32	0.10	0.11
	Citric acid 50mM	5.2g/500 mL	9.36		9.36	0.71	0.78
	Tri-Sodium citrate 50mM	7.4g/500 mL	29.60		29.60	2.25	2.45
	Total				**182.74**	**13.89**	**15.14**
Total cost						**15.62**	**17.02**

Note: summary based on 100 g OPEFB treated gave 13.15 g ethanol by SSF and 12.07 g ethanol by SHF.

Table 3.22 Costs analysis of chemical for ethanol production from 100 g of OPEFB by SSF with *S.cereviceae*

		Amount	Chemical usage (Baht)	Enzyme and Medium usage (Baht)	Total Chemical (Baht)	Cost per unit (Baht/g ethanol) SSF	Cost per unit (Baht/g ethanol) SHF
Pretreatments	0.2 M H_2SO_4	1L	0.29		0.29	0.02	0.02
	5% w/v NaOH	1L	22.50		22.50	1.64	1.76
	Total				**22.79**	**1.66**	**1.78**
Hydrolysis and Fermentation	Cellulase (20FPU/g OPEFB)	1.56g		81.74	81.74	5.96	6.42
	Cellobiase (4U/g OPEFB)	0.66mL		60.72	60.72	4.43	4.77
	Medium	0.68g		2.24	2.24	0.16	0.18
	Citric acid 50mM	4.20g/ 500mL	7.56		7.56	0.55	0.59
	Tri-Sodium citrate 50mM	8.82g/ 500mL	35.28		35.28	2.57	2.77
	Total				**187.54**	**13.67**	**14.73**
Total cost						**14.99**	**16.13**

Note: summary based on 100 g OPEFB treated gave 13.71 g ethanol by SSF and 12.73 g ethanol by SHF.

CHAPTER 4

CONCLUSIONS AND SUGGESTIONS

The aim of this research was the optimum condition of DAA-AA pretreatment on OPEFB to maximize cellulose, and to reduce lignin and hemicellulose and studied enzymatic digestibility of pretreated OPEFB for ethanol production were studied. Pretreatment step was studied effect of three factors, which were substrate loading, acid concentration and reaction time, employing RSM for design experiment in research and in all experiments, followed by alkaline pretreatment. Second, research the optimum condition ethanol production by SSF was investigated to find ethanol yield and productivity from OPEFB by DAA-AA pretreatment step. So, fermentation step studied four factors included of temperature, substrate loading, pH and yeast concentration. Finally, ethanol production by SHF was investigated to find ethanol yield and productivity select condition from SSF process and comparison ethanol yield between SSF and SHF. Conclusion of all investigation was described in three parts as show below.

4.1 Conclusion

4.1.1 Optimizing sequential Dilute Acid Autoclaving and Alkaline Autoclaving pretreatment (DAA-AA) of oil palm empty fruit bunches for production of maximum cellulose with high enzymatic digestibility

Optimal conditions for increase cellulose and decrease hemicellulose, lignin from Oil Palm Empty Fruit Bunches (OPEFBs) by (DAA-AA) pretreatment were determined in this study. Between the parameters; substrate loading, sulfuric acid concentration and reaction time by sulfuric acid concentration and reaction time is the most significant parameter that define the performance of the process. Low sulfuric acid concentration renders a high cellulose with a high cellulose yield, while high sulfuric acid concentration gives a low cellulose yield and with a low cellulose. Based on the desirous maximum enzymatic digestibility, low acid concentration (0.2 M or less) with reaction time (53 min or more) are proper to increase cellulose, decrease hemicellulose and that acid concentration and reaction time is the significant factor. In addition, if high of alkaline concentration can decrease lignin to improve enzymatic digestibility. Furthermore, it was

concluded that the optimum condition of DAA-AA pretreatment, 15 % w/v of substrate loading, 0.2 M of sulfuric acid concentration at 121 ºC for 53 min gives maximum cellulose, enzymatic digestibility and to reduce hemicellulose and lignin in OPEFB for sugar production.

4.1.2 Ethanol production from acid/alkaline-treated oil palm empty fruit bunch by Simultaneous Saccharification and Fermentation (SSF) using *Kluyveromyces marxianus*

This study found that pretreatment of OPEFB with dilute sulfuric acid in the first step then followed sodium hydroxide treatment in the second step can effectively remove hemicellulose and lignin, reduced crystallinity of cellulose, and increase cellulose exposure after pretreatment. Furthermore, it was concluded that the optimum condition of SSF and SHF fermentation was 12.24 % w/v of substrate loading, pH 4.5, 2.04 % v/v of yeast concentration of *K. marxianus* at 36.94 ºC. The maximum ethanol concentration by SSF and SHF at 48 h was 34.39 g/L (0.281 g/g biomass) and 31.58 g/L (0.258 g/g biomass), respectively. The SSF process gave higher ethanol yield than SHF. These results confirm that efficiency of ethanol production increased when used substrates through removal of hemicellulose and lignin using sequential acid/alkali pretreatment.

4.1.3 Ethanol production from acid/alkaline-treated oil palm empty fruit bunch by Simultaneous Saccharification and Fermentation (SSF) using *Saccharomyces cerevisiae*

This study found that pretreatment of OPEFB with dilute sulfuric acid in the first step then followed sodium hydroxide treatment in the second step can effectively remove hemicellulose and lignin, reduced crystallinity of cellulose, and increase cellulose exposure after pretreatment. Furthermore, it was concluded that the optimum condition of SSF and SHF fermentation was 8.16 % w/v of substrate loading, pH 4.91, 3.38 % v/v of yeast concentration of *S. cerevisiae* at 35.03 ºC. The maximum ethanol concentration by SSF and SHF at 48 h was 26.11 g/L (0.320 g/g biomass) and 24.64 g/L (0.302 g/g biomass), respectively. The SSF process gave higher ethanol yield than SHF. These results confirm that efficiency of ethanol production increased when used substrates through removal of hemicellulose and lignin using sequential acid/alkali pretreatment.

4.2 Suggestions

4.2.1 After NaOH pretreatment in solid phase should washed intensively and should use by Stirrer in order to reduce time, the enzymatic digestibility might be improved.

4.2.2 Larger equipment for acid and alkaline pretreatment would reduce the cost of energy for pretreatment.

4.2.3 The temperature during SSF process and SHF process should be controlled.

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APPENDICES

APPENDIX A

Statistical analysis of OPEFB pretreatment with H_2SO_4

Statistical analysis of <u>hemicellulose</u> of OPEFB pretreatment with H_2SO_4

Statistical analysis of <u>lignin</u> of OPEFB pretreatment with H_2SO_4

Statistical analysis of <u>glucose concentration</u> of OPEFB pretreatment with H_2SO_4

Statistical analysis of <u>xylose concentration</u> of OPEFB pretreatment with H_2SO_4

Statistical analysis of <u>cellulose</u> of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

Statistical analysis of <u>reducing sugar</u> from OPEFB with SSF by *K.marxianus*

Statistical analysis of <u>ethanol yield</u> from OPEFB with SSF by *K. marxianus*

Statistical analysis of <u>reducing sugar</u> from OPEFB with SSF by *S. cerevisiae*

Statistical analysis of <u>ethanol yield</u> from OPEFB with SSF by *S. cerevisiae*

APPENDIX B

Raw data for cost calculation

APPENDIX C

Analysis methodology

APPENDIX D

Chemical preparations

APPENDIX E

Calculations

APPENDIX F

Standard curve

APPENDIX A

Statistical analysis

Statistical analysis of cellulose of OPEFB pretreatment with H_2SO_4

Table A-1 Fit summary analysis of variance for independent variables on cellulose of OPEFB pretreatment with H_2SO_4

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	Selection
Linear	0.0612	0.0250	0.3989	0.2701	
2FI	0.2425	0.0190	0.4624	0.1692	
Quadratic	0.0059	0.0963	0.8867	0.7593	Suggested
Cubic	0.0049	0.4134	0.9855	0.9382	Aliased

Table A- 2 Regression coefficients on cellulose of OPEFB pretreatment with H_2SO_4

Source	Cellulose (g/g OPEFB)	
	Coefficients	P-value
Model		0.0059
Intercept	+0.54892	
A-Substrate loading	+1.05656E-003	0.9326
B-Acid concentration	-0.24083	0.4181
C-Reaction time	+3.88783E-003	0.0008
AB	+5.00000E-003	0.1970
AC	-7.37218E-006	0.8213
BC	-1.48358E-003	0.1560
A^2	-1.23906E-004	0.2370
B^2	+0.21383	0.0096
C^2	-2.02781E-005	0.0187

Statistical analysis of hemicellulose of OPEFB pretreatment with H_2SO_4

Table A-3 Fit summary analysis of variance for independent variables on hemicellulose of OPEFB pretreatment with H_2SO_4

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	Selection
Linear	0.2717	0.0005	0.2366	0.0730	
2FI	0.0860	0.0008	0.5804	0.3516	
Quadratic	0.0085	0.0025	0.8752	0.7348	Suggested
Cubic	0.0596	0.0011	0.9453	0.7675	Aliased

Table A- 4 Regression coefficients on hemicellulose of OPEFB pretreatment with H_2SO_4

Source	Hemicellulose (g/g OPEFB)	
	Coefficients	P-value
Model		0.0085
Intercept	-0.14814	
A-Substrate loading	+4.86942E-003	0.0159
B-Acid concentration	+0.43274	0.5975
C-Reaction time	+3.00715E-003	0.0501
AB	-3.42708E-003	0.2681
AC	-8.09780E-005	0.0133
BC	-2.50219E-003	0.0115
A^2	+2.98688E-006	0.9706
B^2	-0.21390	0.0031
C^2	-5.36215E-006	0.3654

Statistical analysis of lignin of OPEFB pretreatment with H_2SO_4

Table A-5 Fit summary analysis of variance for independent variables on lignin of OPEFB pretreatment with H_2SO_4

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	0.1212	0.1890	0.3307	0.1873	
2FI	0.3075	0.1588	0.4277	0.1156	
Quadratic	0.0019	0.8699	0.9171	0.8234	Suggested
Cubic	0.0743	0.5465	0.9381	0.7369	Aliased

Table A- 6 Regression coefficients on lignin of OPEFB pretreatment with H_2SO_4

Source	Lignin (g/g OPEFB)	
	Coefficients	P-value
Model		0.0019
Intercept	+0.26	
A-Substrate loading	+4.742E-003	0.0236
B-Acid concentration	+5.414E-003	0.0086
C-Reaction time	-5.841E-003	0.0089
AB	-2.625E-003	0.2695
AC	+5.855E-003	0.0294
BC	+2.148E-003	0.3600
A^2	+6.098E-003	0.0098
B^2	+8.629E-003	0.0002
C^2	+1.912E-003	0.3221

Statistical analysis of glucose concentration of OPEFB pretreatment with H_2SO_4

Table A- 7 Fit summary analysis of variance for independent variables on glucose of OPEFB pretreatment with H_2SO_4

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	0.0562	0.0036	0.4068	0.2797	
2FI	0.0075	0.0083	0.7495	0.6129	
Quadratic	0.0010	0.0284	0.9298	0.8509	Suggested
Cubic	0.0037	0.0519	0.9873	0.9462	Aliased

Table A- 8 Regression coefficients on glucose of OPEFB pretreatment with H_2SO_4

Source	Glucose concentration (g/g OPEFB)	
	Coefficients	P-value
Model		0.0010
Intercept	+0.19982	
A-Substrate loading	-5.95065E-003	0.0001
B-Acid concentration	-0.26568	0.4469
C-Reaction time	-2.49843E-003	0.6342
AB	+3.30026E-003	0.1332
AC	+1.45797E-005	0.1352
BC	+3.04938E-003	0.0004
A^2	+8.38521E-005	0.0125
B^2	+0.063337	0.5204
C^2	+7.00107E-006	0.0055

Statistical analysis of xylose concentration of OPEFB pretreatment with H_2SO_4

Table A- 9 Fit summary analysis of variance for independent variables on xylose of OPEFB pretreatment with H_2SO_4

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	<0.0001	0.0205	0.8958	0.8735	
2FI	<0.0001	0.0508	0.9588	0.9364	
Quadratic	<0.0001	0.1167	0.9851	0.9683	Suggested
Cubic	<0.0001	0.6667	0.9982	0.9923	Aliased

Table A- 10 Regression coefficients on xylose of OPEFB pretreatment with H_2SO_4

Source	Xylose concentration (g/g OPEFB)	
	Coefficients	P-value
Model		<0.0001
Intercept	0.82430	
A-Substrate loading	-0.030299	< 0.0001
B-Acid concentration	-0.72236	0.9816
C-Reaction time	-3.71240E-003	0.1617
AB	0.019444	0.0474
AC	-6.67255E-005	0.1084
BC	0.011093	0.0010
A^2	3.39162E-004	0.0151
B^2	-0.15080	0.7136
C^2	-1.04282E-005	0.2197

Statistical analysis of cellulose of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

Table A-11 Fit summary analysis of variance for independent variables on cellulose of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	0.1152	0.1255	0.3362	0.1939	
2FI	0.2571	0.1099	0.4542	0.1565	
Quadratic	0.0586	0.2165	0.7819	0.5365	Suggested
Cubic	0.0321	0.9566	0.9610	0.8342	Aliased

Table A- 12 Regression coefficients on cellulose of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

Source	Cellulose (g/g OPEFB)	
	Coefficients	P-value
Model		0.0586
Intercept	+0.77379	
A-Substrate loading	-0.010253	0.0238
B-Acid concentration	-0.25799	0.0663
C-Reaction time	+3.22848E-003	0.7362
AB	+6.52792E-003	0.3762
AC	+1.09490E-004	0.1150
BC	-1.05995E-003	0.5839
A^2	-7.97080E-005	0.6860
B^2	+0.12742	0.3335
C^2	-3.93835E-005	0.0195

Statistical analysis of hemicellulose of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

Table A- 13 Fit summary analysis of variance for independent variables on hemicellulose of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	0.9426	0.0227	0.0265	-0.1822	
2FI	0.9968	0.0150	0.0437	-0.4779	
Quadratic	0.3834	0.0269	0.5837	0.1153	Suggested
Cubic	0.0525	0.0912	0.9490	0.7833	Aliased

Table A- 14 Regression coefficients on hemicellulose of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

Source	Hemicellulose (g/g OPEFB)	
	Coefficients	P-value
Model		0.3834
Intercept	-0.089857	
A-Substrate loading	+4.30379E-003	0.6496
B-Acid concentration	+0.34834	0.7762
C-Reaction time	+1.18441E-003	0.6587
AB	-2.69321E-003	0.5833
AC	+5.14697E-007	0.9905
BC	+8.49424E-005	0.9477
A^2	-1.01884E-004	0.4508
B^2	-0.26523	0.0132
C^2	-1.26823E-005	0.2028

Statistical analysis of lignin of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

Table A- 15 Fit summary analysis of variance for independent variables on lignin of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	0.0560	0.1496	0.4072	0.2801	
2FI	0.1420	0.1377	0.5288	0.2718	
Quadratic	0.1981	0.1301	0.6761	0.3118	Suggested
Cubic	0.1205	0.1726	0.9182	0.6522	Aliased

Table A- 16 Regression coefficients on lignin of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

Source	Lignin (g/g OPEFB)	
	Coefficients	P-value
Model		0.1981
Intercept	+0.061912	
A-Substrate loading	+2.22955E-003	0.3314
B-Acid concentration	+0.061443	0.0184
C-Reaction time	+1.65371E-003	0.5824
AB	-0.014447	0.2019
AC	-9.37497E-005	0.3398
BC	-5.74339E-004	0.8410
A^2	+4.33720E-004	0.1646
B^2	+0.26292	0.1923
C^2	+3.24894E-006	0.8760

Statistical analysis of glucose concentration of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

Table A- 17 Fit summary analysis of variance for independent variables on glucose of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	<0.0001	0.0028	0.7800	0.7328	
2FI	0.0035	0.0018	0.7847	0.6672	
Quadratic	0.0032	0.0032	0.9041	0.7962	Suggested
Cubic	0.0123	0.0034	0.9765	0.9003	Aliased

Table A- 18 Regression coefficients on glucose of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

Source	Glucose concentration (g/g OPEFB)	
	Coefficients	P-value
Model		0.0032
Intercept	-7.07128E-003	
A-Substrate loading	-3.76182E-003	0.0009
B-Acid concentration	0.11945	0.0009
C-Reaction time	5.25355E-004	0.0073
AB	6.77910E-004	0.8118
AC	-5.83480E-006	0.6464
BC	2.37213E-004	0.7550
A^2	8.85064E-005	0.0416
B^2	-0.077121	0.5736
C^2	-3.38572E-006	0.2285

Statistical analysis of xylose concentration of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

Table A- 19 Fit summary analysis of variance for independent variables on xylose of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	0.0027	0.0104	0.6238	0.5431	
2FI	0.0362	0.0075	0.6530	0.4637	
Quadratic	0.0257	0.0112	0.8293	0.6372	Suggested
Cubic	0.0275	0.0153	0.9641	0.8474	Aliased

Table A- 20 Regression coefficients on xylose of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

Source	Xylose concentration (g/g OPEFB)	
	Coefficients	P-value
Model		0.0257
Intercept	0.26984	
A-Substrate loading	-0.043391	0.0009
B-Acid concentration	0.60926	0.1207
C-Reaction time	5.14017E-003	0.9127
AB	0.025142	0.3463
AC	3.66108E-005	0.7514
BC	-3.40829E-003	0.6247
A^2	6.35175E-004	0.0929
B^2	-0.60709	0.6263
C^2	-3.85069E-005	0.1426

Statistical analysis of enzymatic digestibility of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

Table A- 21 Fit summary analysis of variance for independent variables on enzymatic digestibility of OPEFB pretreatment with H_2SO_4 and 5 % (w/v) NaOH

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	0.0171	0.0469	0.5056	0.3657	Suggested
2FI	0.0780	0.0399	0.5896	0.3526	
Quadratic	0.1336	0.0367	0.7168	0.3983	
Cubic	0.0172	0.0367	0.9721	0.8812	Aliased

Table A- 22 Regression coefficients on furfural of OPEFB pretreatment with H2SO4 and 5 % (w/v) NaOH

Source	Enzymatic digestibility (%)	
	Coefficients	P-value
Model		0.0171
Intercept	+68.94900	
A-Substrate loading	-5.53279E-003	0.9360
B-Acid concentration	-0.35438	0.8524
C-Reaction time	+0.068199	0.0020

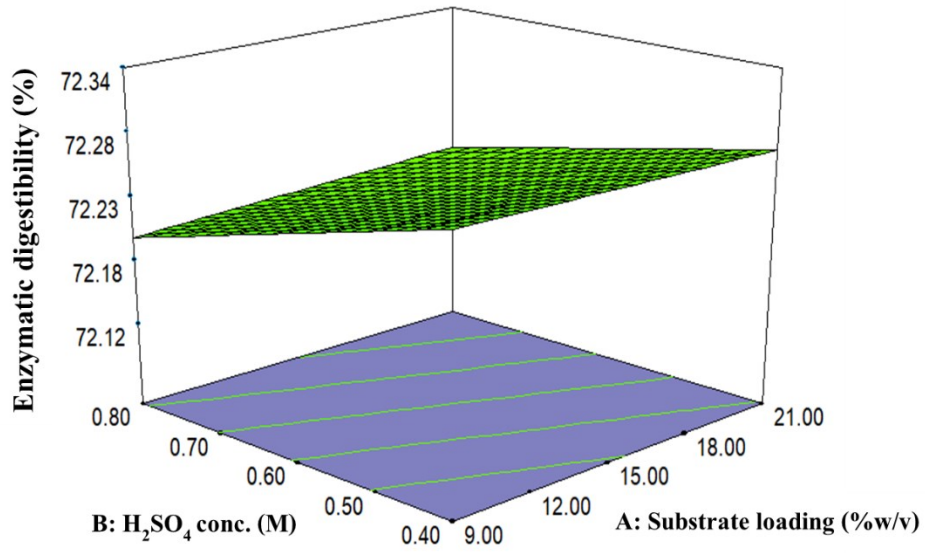


Figure A- 1 Contour and 3D of response surface plots showing the interactions between substrate loading vs. acid concentration affecting enzymatic digestibility

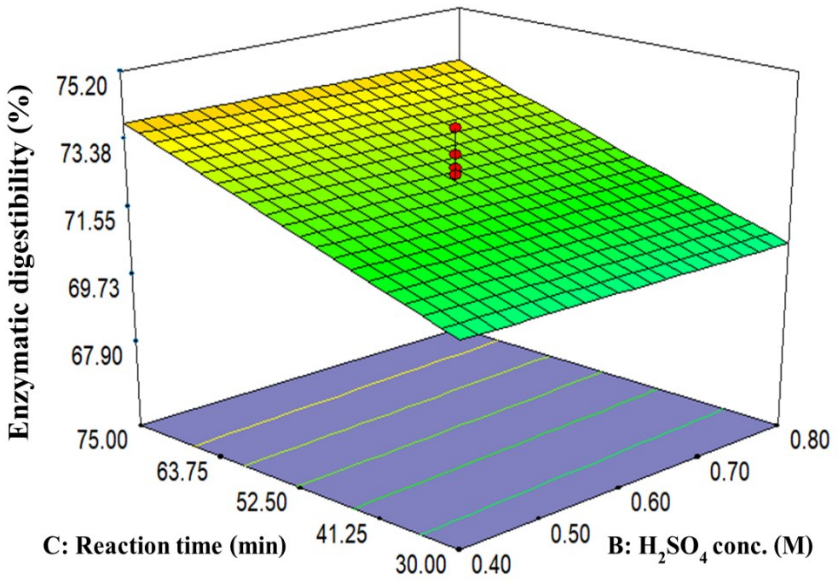


Figure A- 2 Contour and 3D of response surface plots showing the interactions between reaction time vs. acid concentration affecting enzymatic digestibility

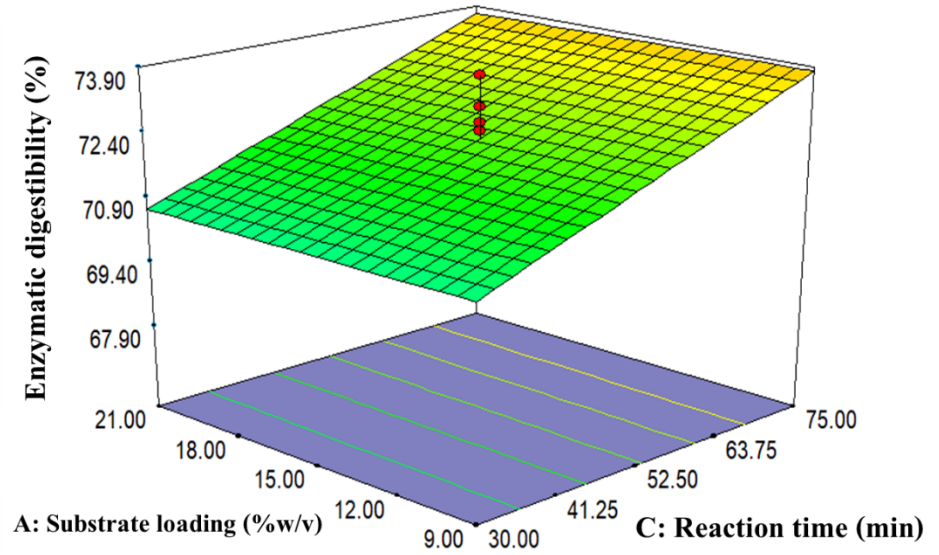


Figure A- 3 Contour and 3D of response surface plots showing the interactions between substrate loading vs. reaction time affecting enzymatic digestibility

Table A-23 Characterization of OPEFB after pretreatment with H_2SO_4

Conditions			Result liquid		Result solid		
Substrate loading (% w/v)	H_2SO_4 conc. (M)	Reaction time (min)	Xylose (g/g)	Glucose (g/g)	Hemicellulose (g/g)	Cellulose (g/g)	Lignin (g/g)
9	0.40	30	0.328	0.042	0.031	0.575	0.269
21	0.40	30	0.157	0.019	0.071	0.562	0.278
9	0.40	75	0.239	0.020	0.091	0.622	0.247
21	0.40	75	0.139	0.015	0.066	0.609	0.271
9	0.80	30	0.278	0.018	0.085	0.595	0.283
21	0.80	30	0.095	0.012	0.087	0.610	0.273
9	0.80	75	0.313	0.033	0.079	0.620	0.261
21	0.80	75	0.153	0.026	0.018	0.627	0.283
5	0.60	53	0.400	0.039	0.080	0.600	0.268
25	0.60	53	0.088	0.014	0.064	0.600	0.280
15	0.60	15	0.201	0.018	0.071	0.555	0.274
15	0.60	90	0.209	0.022	0.057	0.612	0.251
15	0.20	53	0.211	0.027	0.038	0.657	0.278

Table A-23 Characterization of OPEFB after pretreatment with H_2SO_4 (cont.)

Conditions			Result liquid		Result solid		
Substrate loading (% w/v)	H_2SO_4 conc. (M)	Reaction time (min)	Xylose (g/g)	Glucose (g/g)	Hemicellulose (g/g)	Cellulose (g/g)	Lignin (g/g)
15	1.00	53	0.177	0.029	0.041	0.635	0.304
15	0.60	53	0.211	0.018	0.077	0.611	0.260
15	0.60	53	0.217	0.015	0.078	0.605	0.260
15	0.60	53	0.205	0.017	0.079	0.619	0.243
15	0.60	53	0.199	0.017	0.076	0.607	0.258
Untreated			No analysis		0.300	0.411	0.164

Table A-24 Characterization of OPEFB after pretreatment with H_2SO_4 and NaOH

Conditions				Result liquid		Result solid			
Substrate loading (% w/v)	H_2SO_4 conc. (M)	Reaction time (min)	NaOH conc. (% w/v) at 20 min	Xylose (g/g)	Glucose (g/g)	Hemicellulose (g/g)	Lignin (g/g)	Cellulose (g/g)	Enzymatic digestibility (%)
9	0.40	30	5	0.227	0.014	0.064±0.002	0.162±0.002	0.711±0.002	77.51±0.59
21	0.40	30	5	0.118	0.009	0.034±0.003	0.274±0.013	0.610±0.014	76.20±0.16
9	0.40	75	5	0.250	0.027	0.046±0.004	0.183±0.015	0.662±0.004	82.20±0.33
21	0.40	75	5	0.198	0.019	0.056±0.001	0.224±0.004	0.640±0.012	80.88±1.12
9	0.80	30	5	0.222	0.024	0.045±0.006	0.261±0.003	0.679±0.008	79.50±0.47
21	0.80	30	5	0.129	0.012	0.042±0.006	0.282±0.006	0.630±0.011	80.98±0.19
9	0.80	75	5	0.210	0.035	0.069±0.005	0.250±0.009	0.632±0.007	82.00±0.89
21	0.80	75	5	0.181	0.029	0.025±0.004	0.243±0.006	0.621±0.003	82.65±0.29
5	0.60	53	5	0.443	0.050	0.054±0.004	0.240±0.004	0.674±0.006	80.00±0.20
25	0.60	53	5	0.132	0.016	0.077±0.002	0.219±0.004	0.640±0.009	80.00±0.98
15	0.60	15	5	0.167	0.011	0.069±0.003	0.144±0.013	0.578±0.019	75.50±1.04
15	0.60	90	5	0.245	0.033	0.047±0.002	0.237±0.012	0.641±0.006	81.19±0.49
15	0.20	53	5	0.158	0.010	0.032±0.002	0.176±0.007	0.721±0.002	83.50±0.75

Table A-24 Characterization of OPEFB after pretreatment with H_2SO_4 and NaOH (cont.)

Conditions				Result liquid		Result solid			
Substrate loading (% w/v)	H_2SO_4 conc. (M)	Reaction time (min)	NaOH conc. (% w/v) at 20 min	Xylose (g/g)	Glucose (g/g)	Hemicellulose (g/g)	Lignin (g/g)	Cellulose (g/g)	Enzymatic digestibility (%)
15	1.00	53	5	0.178	0.028	0.032±0.001	0.288±0.014	0.650±0.033	78.70±1.66
15	0.60	53	5	0.209	0.022	0.075±0.004	0.193±0.014	0.688±0.016	80.70±0.27
15	0.60	53	5	0.200	0.022	0.077±0.003	0.172±0.010	0.651±0.003	80.50±0.50
15	0.60	53	5	0.217	0.022	0.069±0.005	0.218±0.007	0.667±0.020	81.09±0.10
15	0.60	53	5	0.194	0.021	0.065±0.004	0.209±0.017	0.656±0.002	81.88±1.08
Untreated				No analysis		0.300±0.017	0.264±0.039	0.411±0.004	40.76±0.47

Statistical analysis of reducing sugar from OPEFB with SSF by *K.marxianus*

Table A- 25 Fit summary analysis of variance for independent variables on reducing sugar of OPEFB fermentation with SSF by *K. marxianus* at 48 h

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	0.0589	<0.0001	0.3158	0.1968	
2FI	0.5511	<0.0001	0.3468	-0.0375	
Quadratic	<0.0001	0.0041	0.9804	0.9594	Suggested
Cubic	0.0002	0.0044	0.9950	0.9729	Aliased

Table A- 26 Regression coefficients on reducing sugar of OPEFB fermentation with SSF by *K.*marxianus at 48 h

Source	Reducing sugar (g/g OPEFB)	
	Coefficients	P-value
Model		<0.0001
Intercept	+2.90960	
A-Temperature	-0.22300	< 0.0001
B-Substrate loading	+0.045900	0.1871
C-pH	+0.32542	0.1687
D-Yeast concentration	+0.044375	0.1225
AB	-9.33333E-004	0.0157
AC	+3.33333E-004	0.8459
AD	+1.86667E-003	0.0447
BC	+3.10000E-003	0.2406
BD	-2.90000E-003	0.0386
CD	-5.75000E-003	0.3782
A^2	+3.13037E-003	< 0.0001
B^2	-9.56667E-004	0.0370
C^2	-0.034417	0.0053
D^2	-8.72917E-003	0.0048

Table A-27 Reducing sugar from OPEFB with SSF by *K.marxianus*

Condition				Reducing sugar (g/g OPEFB)					
Temp. (°C)	Substrate loading (% w/v)	pH	Yeast conc. (% v/v)	At 0 h	At 12 h	At 24 h	At 48 h	At 72 h	At 96 h
SSF									
33.75	7.50	4.5	2.00	No	0.0320	0.0243	0.0172	0.0413	0.0283
41.25	7.50	4.5	2.00	No	0.0828	0.0959	0.0769	0.0301	0.0191
33.75	12.50	4.5	2.00	No	0.0306	0.0226	0.0191	0.0129	0.0187
41.25	12.50	4.5	2.00	No	0.0566	0.0597	0.0593	0.0638	0.0602
33.75	7.50	5.5	2.00	No	0.0369	0.0240	0.0225	0.0163	0.0463
41.25	7.50	5.5	2.00	No	0.0799	0.0881	0.0833	0.0709	0.0717
33.75	12.50	5.5	2.00	No	0.0255	0.0290	0.0380	0.0125	0.0140
41.25	12.50	5.5	2.00	No	0.1106	0.0807	0.0879	0.0698	0.0378
33.75	7.50	4.5	4.00	No	0.0331	0.0153	0.0276	0.0373	0.0077
41.25	7.50	4.5	4.00	No	0.0723	0.0856	0.1260	0.0713	0.0679
33.75	12.50	4.5	4.00	No	0.0332	0.0231	0.0109	0.0095	0.0051
41.25	12.50	4.5	4.00	No	0.0670	0.0294	0.0739	0.0377	0.0340
33.75	7.50	5.5	4.00	No	0.0543	0.0268	0.0152	0.0527	0.0632
41.25	7.50	5.5	4.00	No	0.0477	0.0184	0.1327	0.0248	0.0243
33.75	12.50	5.5	4.00	No	0.0260	0.0231	0.0310	0.0162	0.0063
41.25	12.50	5.5	4.00	No	0.0774	0.0951	0.0742	0.0441	0.0704
30.00	10.00	5.0	3.00	No	0.0732	0.1223	0.1340	0.1449	0.0944
45.00	10.00	5.0	3.00	No	0.2899	0.2801	0.3046	0.3086	0.3121
37.50	5.00	5.0	3.00	No	0.0300	0.0204	0.0138	0.0184	0.0122
37.50	15.00	5.0	3.00	No	0.0698	0.0423	0.0253	0.0149	0.0159
37.50	10.00	4.0	3.00	No	0.0349	0.0212	0.0052	0.0066	0.0073
37.50	10.00	6.0	3.00	No	0.0193	0.0117	0.0134	0.0156	0.0097
37.50	10.00	5.0	1.00	No	0.0301	0.0125	0.0047	0.0054	0.0078
37.50	10.00	5.0	5.00	No	0.0283	0.0175	0.0122	0.0189	0.0062
37.50	10.00	5.0	3.00	No	0.0343	0.0274	0.0354	0.0246	0.0297
37.50	10.00	5.0	3.00	No	0.0376	0.0416	0.0402	0.0208	0.0265
37.50	10.00	5.0	3.00	No	0.0309	0.0322	0.0377	0.0185	0.0176
37.50	10.00	5.0	3.00	No	0.0305	0.0329	0.0386	0.0164	0.0250

Table A-28 Reducing sugar from OPEFB with SHF by *K.marxianus*

Condition				Reducing sugar (g/g OPEFB)					
Temp. (°C)	Substrate loading (% w/v)	pH	Yeast conc. (% v/v)	At 0 h	At 12 h	At 24 h	At 48 h	At 72 h	At 96 h
SHF									
37.50	10.00	5.0	1.00	0.5842	0.3784	0.2642	0.1011	0.0518	0.0424

Statistical analysis of ethanol concentration from OPEFB with SSF by *K. marxianus*

Table A- 29 Fit summary analysis of variance for independent variables on ethanol yield of OPEFB fermentation with SSF by *K. marxianus* at 48 h

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	0.3655	0.0005	0.1647	0.0194	
2FI	0.4284	0.0005	0.3880	0.0281	
Quadratic	<0.0001	0.0163	0.9578	0.9123	Suggested
Cubic	0.0045	0.0075	0.9833	0.9098	Aliased

Table A- 30 Regression coefficients on ethanol yield of OPEFB fermentation with SSF by *K. marxianus* at 48 h

Source	Ethanol yield (g/g OPEFB)	
	Coefficients	P-value
Model		<0.0001
Intercept	-4.3268	
A-Temperature	0.1917	<0.0001
B-Substrate loading	0.1302	0.0055
C-pH	0.2054	0.5225
D-Yeast concentration	-0.0759	0.0177
AB	-1.180E-003	0.0029
AC	-7.900E-003	0.0003
AD	3.500E-004	0.6718
BC	-0.0132	0.0001
BD	1.425E-003	0.2605
CD	-3.750E-004	0.9516
A^2	-1.936E-003	<0.0001
B^2	-1.035E-003	0.0213
C^2	0.0221	0.0434
D^2	7.281E-003	0.0114

Table A-31 Ethanol yield from OPEFB with SSF by *K.marxinus*

Condition				Ethanol yield (g/g OPEFB)					
Temp. (°C)	Substrate loading (% w/v)	pH	Yeast conc. (%v/v)	At 0 h	At 12 h	At 24 h	At 48 h	At 72 h	At 96 h
SSF									
33.75	7.50	4.50	2.00	No	0.106^{d}	0.171^{c}	0.206^{b}	0.212^{ab}	0.222^{a}
41.25	7.50	4.50	2.00	No	0.132^{d}	0.199^{c}	0.228^{b}	0.236^{b}	0.250^{a}
33.75	12.50	4.50	2.00	No	0.158^{d}	0.240^{c}	0.259^{b}	0.277^{a}	0.290^{a}
41.25	12.50	4.50	2.00	No	0.150^{d}	0.232^{c}	0.249^{b}	0.264^{a}	0.259^{a}
33.75	7.50	5.50	2.00	No	0.128^{c}	0.211^{b}	0.267^{a}	0.266^{a}	0.267^{a}
41.25	7.50	5.50	2.00	No	0.105^{c}	0.208^{b}	0.219^{a}	0.219^{a}	0.223^{a}
33.75	12.50	5.50	2.00	No	0.153^{d}	0.253^{c}	0.263^{b}	0.267^{b}	0.273^{a}
41.25	12.50	5.50	2.00	No	0.108^{e}	0.177^{d}	0.189^{c}	0.185^{b}	0.207^{a}
33.75	7.50	4.50	4.00	No	0.137^{e}	0.147^{d}	0.173^{c}	0.185^{b}	0.204^{a}
41.25	7.50	4.50	4.00	No	0.133^{e}	0.153^{d}	0.213^{c}	0.222^{b}	0.235^{a}
33.75	12.50	4.50	4.00	No	0.191^{c}	0.251^{b}	0.273^{a}	0.277^{a}	0.281^{a}
41.25	12.50	4.50	4.00	No	0.099^{c}	0.232^{b}	0.240^{a}	0.242^{a}	0.250^{a}
33.75	7.50	5.50	4.00	No	0.139^{c}	0.191^{b}	0.253^{a}	0.226^{a}	0.245^{a}
41.25	7.50	5.50	4.00	No	0.124^{d}	0.177^{c}	0.218^{b}	0.228^{a}	0.227^{a}
33.75	12.50	5.50	4.00	No	0.212^{d}	0.233^{c}	0.241^{b}	0.250^{ab}	0.256^{a}
41.25	12.50	5.50	4.00	No	0.088^{d}	0.150^{c}	0.180^{b}	0.189^{ab}	0.200^{a}
30.00	10.00	5.00	3.00	No	0.053^{d}	0.144^{c}	0.169^{b}	0.189^{ab}	0.196^{a}
45.00	10.00	5.00	3.00	No	0.013^{d}	0.051^{c}	0.091^{b}	0.100^{a}	0.103^{a}
37.50	5.00	5.00	3.00	No	0.070^{c}	0.135^{b}	0.193^{a}	0.194^{a}	0.195^{a}
37.50	15.00	5.00	3.00	No	0.064^{d}	0.213^{c}	0.233^{b}	0.240^{ab}	0.247^{a}
37.50	10.00	4.00	3.00	No	0.055^{d}	0.250^{c}	0.263^{b}	0.268^{ab}	0.273^{a}
37.50	10.00	6.00	3.00	No	0.107^{d}	0.225^{c}	0.259^{b}	0.267^{ab}	0.273^{a}
37.50	10.00	5.00	1.00	No	0.098^{c}	0.255^{b}	0.281^{a}	0.290^{a}	0.292^{a}
37.50	10.00	5.00	5.00	No	0.080^{d}	0.189^{c}	0.255^{b}	0.267^{a}	0.273^{a}
37.50	10.00	5.00	3.00	No	0.100^{c}	0.210^{b}	0.248^{a}	0.251^{a}	0.254^{a}
37.50	10.00	5.00	3.00	No	0.107^{c}	0.225^{b}	0.250^{a}	0.256^{a}	0.255^{a}
37.50	10.00	5.00	3.00	No	0.100^{d}	0.220^{c}	0.243^{b}	0.251^{a}	0.253^{a}
37.50	10.00	5.00	3.00	No	0.104^{c}	0.229^{b}	0.245^{a}	0.254^{a}	0.258^{a}

Table A-32 Ethanol yield from OPEFB with SHF by *K. marxinus*

Condition				Ethanol yield (g/g OPEFB)					
Temp. (°C)	Substrate loading (% w/v)	pH	Yeast conc. (%v/v)	At 0 h	At 12 h	At 24 h	At 48 h	At 72 h	At 96 h
SHF									
37.50	10.00	5.00	1.00	0	0.085^{d}	0.158^{c}	0.258^{b}	0.264^{ab}	0.264^{a}

Statistical analysis of reducing sugar from OPEFB with SSF by *S. cerevisiae*

Table A- 33 Fit summary analysis of variance for independent variables on reducing sugar of OPEFB fermentation with SSF by *S. cerevisiae* at 48 h

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	<0.0001	<0.0001	0.6869	0.6324	
2FI	0.0004	<0.0001	0.7931	0.6714	
Quadratic	<0.0001	0.0003	0.9503	0.8968	Suggested
Cubic	0.0036	0.0002	0.9848	0.9177	Aliased

Table A- 34 Regression coefficients on reducing sugar of OPEFB fermentation with SSF by *S.cerevisiae* at 48 h

Source	Reducing sugar (g/g OPEFB)	
	Coefficients	P-value
Model		<0.0001
Intercept	7.3042	
A-Temperature	-0.2756	<0.0001
B-Substrate loading	-0.2729	0.0015
C-pH	-0.4506	0.1885
D-Yeast concentration	-0.1197	0.3309
AB	5.074E-003	0.0006
AC	-8.238E-003	0.1663
AD	1.538E-003	0.5933
BC	0.0157	0.0853
BD	-5.087E-003	0.2488
CD	-3.000E-004	0.9889
A^2	3.873E-003	<0.0001
B^2	1.661E-003	0.2490
C^2	0.0580	0.1158
D^2	0.0205	0.0330

Table A-35 Reducing sugar from OPEFB with SSF by *S.cerevisiae*

Condition				Reducing sugar (g/g OPEFB)					
Temp. (°C)	Substrate loading (% w/v)	pH	Yeast conc. (% v/v)	At 0 h	At 12 h	At 24 h	At 48 h	At 72 h	At 96 h
SSF									
33.75	7.50	4.50	2.00	No	0.0391	0.0213	0.0337	0.0293	0.0049
41.25	7.50	4.50	2.00	No	0.3184	0.4835	0.1877	0.0855	0.0973
33.75	12.50	4.50	2.00	No	0.0445	0.0192	0.0074	0.0330	0.0170
41.25	12.50	4.50	2.00	No	0.3184	0.3656	0.3634	0.2136	0.1834
33.75	7.50	5.50	2.00	No	0.0275	0.0233	0.0255	0.0295	0.0075
41.25	7.50	5.50	2.00	No	0.2997	0.6151	0.0591	0.0605	0.0567
33.75	12.50	5.50	2.00	No	0.0350	0.0357	0.0240	0.0381	0.0156
41.25	12.50	5.50	2.00	No	0.2808	0.3883	0.3691	0.1001	0.1037
33.75	7.50	4.50	4.00	No	0.0235	0.0192	0.0395	0.0100	0.0113
41.25	7.50	4.50	4.00	No	0.2967	0.3052	0.2797	0.1565	0.2093
33.75	12.50	4.50	4.00	No	0.0326	0.0235	0.0305	0.0347	0.0138
41.25	12.50	4.50	4.00	No	0.3115	0.2377	0.3386	0.2075	0.1770
33.75	7.50	5.50	4.00	No	0.0363	0.0232	0.0285	0.0355	0.0160
41.25	7.50	5.50	4.00	No	0.2676	0.3752	0.1549	0.0883	0.0919
33.75	12.50	5.50	4.00	No	0.0422	0.0198	0.0409	0.0146	0.0319
41.25	12.50	5.50	4.00	No	0.2974	0.3070	0.3470	0.1293	0.1733
30.00	10.00	5.00	3.00	No	0.0011	0.0376	0.0409	0.0849	0.0660
45.00	10.00	5.00	3.00	No	0.3815	0.4890	0.4136	0.3614	0.4065
37.50	5.00	5.00	3.00	No	0.1512	0.2268	0.0214	0.0142	0.0192
37.50	15.00	5.00	3.00	No	0.1715	0.1073	0.0804	0.0585	0.0782
37.50	10.00	4.00	3.00	No	0.1755	0.1397	0.0811	0.0530	0.0393
37.50	10.00	6.00	3.00	No	0.1576	0.1519	0.0536	0.0041	0.0030
37.50	10.00	5.00	1.00	No	0.1068	0.1845	0.0868	0.0357	0.0314
37.50	10.00	5.00	5.00	No	0.1061	0.0320	0.0961	0.0261	0.0255
37.50	10.00	5.00	3.00	No	0.1058	0.0425	0.0351	0.0321	0.0263
37.50	10.00	5.00	3.00	No	0.1044	0.0434	0.0298	0.0274	0.0323
37.50	10.00	5.00	3.00	No	0.1023	0.0379	0.0338	0.0324	0.0303
37.50	10.00	5.00	3.00	No	0.1087	0.0277	0.0358	0.0326	0.0309

Table A-36 Reducing sugar from OPEFB with SHF by *S.cerevisiae*

Condition				Reducing sugar (g/g OPEFB)					
Temp. (°C)	Substrate loading (% w/v)	pH	Yeast conc. (% v/v)	At 0 h	At 12 h	At 24 h	At 48 h	At 72 h	At 96 h
SHF									
37.50	10.00	5.00	3.00	0.5800	0.3190	0.1815	0.0262	0.0012	0.0010

Statistical analysis of ethanol concentration from OPEFB with SSF by *S. cerevisiae*

Table A- 37 Fit summary analysis of variance for independent variables on ethanol yield of OPEFB fermentation with SSF by *S.cerevisiae* at 48 h

	Sequential P-value	Lack of Fit P-value	R-Squared	Adjusted R-Squared	selection
Linear	<0.0001	<0.0001	0.7307	0.6838	
2FI	0.0021	<0.0001	0.7425	0.5911	
Quadratic	<0.0001	0.0002	0.9381	0.8713	Suggested
Cubic	0.0123	<0.0001	0.9745	0.8624	Aliased

Table A- 38 Regression coefficients on ethanol yield of OPEFB fermentation with SSF by *S.cerevisiae* at 48 h

Source	Ethanol yield (g/g OPEFB)	
	Coefficients	P-value
Model		<0.0001
Intercept	-6.7377	
A-Temperature	0.2254	<0.0001
B-Substrate loading	0.1111	0.1934
C-pH	0.9641	0.5677
D-Yeast concentration	0.2046	0.7882
AB	-1.407E-003	0.1879
AC	1.900E-003	0.7134
AD	-1.517E-003	0.5592
BC	1.550E-003	0.8414
BD	3.750E-004	0.9228
CD	3.750E-004	0.9845
A^2	-3.223E-003	<0.0001
B^2	-3.572E-003	0.0129
C^2	-0.1043	0.0051
D^2	-0.0252	0.0063

Table A-39 Ethanol yield from OPEFB with SSF by *S.cerevisiae*

Condition				Ethanol yield (g/g OPEFB)					
Temp. (°C)	Substrate loading (% w/v)	pH	Yeast conc. (% v/v)	At 0 h	At 12 h	At 24 h	At 48 h	At 72 h	At 96 h
SSF									
33.75	7.50	4.50	2.00	No	0.155	0.236	0.237	0.267	0.271
41.25	7.50	4.50	2.00	No	0.030	0.035	0.081	0.193	0.200
33.75	12.50	4.50	2.00	No	0.146	0.237	0.257	0.294	0.399
41.25	12.50	4.50	2.00	No	0.005	0.007	0.011	0.022	0.058
33.75	7.50	5.50	2.00	No	0.122	0.236	0.268	0.271	0.278
41.25	7.50	5.50	2.00	No	0.041	0.055	0.075	0.084	0.087
33.75	12.50	5.50	2.00	No	0.144	0.244	0.262	0.276	0.279
41.25	12.50	5.50	2.00	No	0.031	0.036	0.042	0.048	0.052
33.75	7.50	4.50	4.00	No	0.144	0.259	0.279	0.287	0.289
41.25	7.50	4.50	4.00	No	0.060	0.045	0.057	0.076	0.080
33.75	12.50	4.50	4.00	No	0.156	0.257	0.277	0.286	0.283
41.25	12.50	4.50	4.00	No	0.011	0.011	0.012	0.038	0.042
33.75	7.50	5.50	4.00	No	0.109	0.256	0.271	0287	0.292
41.25	7.50	5.50	4.00	No	0.059	0.086	0.087	0.093	0.096
33.75	12.50	5.50	4.00	No	0.136	0.271	0.283	0.292	0.297
41.25	12.50	5.50	4.00	No	0.022	0.027	0.028	0.035	0.038
30.00	10.00	5.00	3.00	No	0.128	0.250	0.265	0.273	0.279
45.00	10.00	5.00	3.00	No	0.002	0.002	0.003	0.004	0.005
37.50	5.00	5.00	3.00	No	0.186	0.216	0.239	0.248	0.245
37.50	15.00	5.00	3.00	No	0.115	0.189	0.213	0.224	0.233
37.50	10.00	4.00	3.00	No	0.182	0.199	0.205	0.220	0.222
37.50	10.00	6.00	3.00	No	0.163	0.208	0.217	0.224	0.229
37.50	10.00	5.00	1.00	No	0.136	0.204	0.212	0.225	0.222
37.50	10.00	5.00	5.00	No	0.131	0.204	0.217	0.237	0.238
37.50	10.00	5.00	3.00	No	0.145	0.264	0.293	0.300	0.306
37.50	10.00	5.00	3.00	No	0.144	0.265	0.288	0.289	0.295
37.50	10.00	5.00	3.00	No	0.121	0.266	0.290	0.296	0.303
37.50	10.00	5.00	3.00	No	0.128	0.261	0.291	0.297	0297

Table A-40 Ethanol yield from OPEFB with SHF by *S.cerevisiae*

Condition				Ethanol yield (g/g OPEFB)					
Temp. (°C)	Substrate loading (% w/v)	pH	Yeast conc. (% v/v)	At 0 h	At 12 h	At 24 h	At 48 h	At 72 h	At 96 h
SHF									
37.50	10.00	5.00	3.00	0	0.117	0.230	0.272	0.280	0.290

APPENDIX B

Raw data for cost calculation

B-1 Electricity Tariff, chemical cost and power of equipment for cost calculation

Table B-1 Time of Use Tariff (TOU Tariff)

MONTHLY TARIFF

	Energy Charge (Baht/kWh)		Service Charge (Baht/Month)
	On Peak	Off Peak	
1.3.1: 12 - 24 kV.	4.5827	2.1492	312.24
1.3.2: Below 12 kV.	5.2674	2.1827	38.22

On Peak : Monday – Friday from 09.00 AM to 10.00 PM

Off Peak : Monday – Friday from 10.00 PM to 09.00 AM

: Saturday – Sunday , National Labor Day and normal public holiday (excluding substitution holiday and Royal Ploughing Day) from 00.00 AM to 12.00 PM

Table B-2 Chemical cost

Name	Quantity	Price(Baht)
Sodium hydroxide (AR grade)	1 Kg	450
Sulfuric acid 98 % (commercial grade)	30kg/gallon	450
Yeast Malt Broth (Difco™)	500 g	1,650
Cellulase enzyme powder (commercial grade)	25 g	1,310
Cellulase from *Tricoderma ressei* (ATCC) C8546-10KU	1 bottle	7,900
Cellobiase C6105-50ML	1 bottle	4,600
Citric acid	1 kg	1,800
Tri-Sodium citrate	500 g	2,000

Table B-3 Power of equipment

Equipment	Power (kW)
Oven	2.40
Autoclave	2.00
Shaking incubator	0.80
Centrifuge	0.16

APPENDIX C

Analysis methodology

C-1 Cellulose and lignin analysis

- **Apparatus**

1. Vacuum pump
2. Oven and muffle furnace
3. Desiccator
4. Analytical balance
5. Hot plate
6. Fume hood
7. pH meter

- **Reagents**

1. 72 % w/w sulfuric acid (H_2SO_4)
2. Cetyltriethylammonium bromide (CTAB), reagent grade
3. 99.9 % w/w Acetone, reagent grade
4. Decahydronaphthalene, reagent grade
5. Acid detergent solution
6. Distilled water

Acid detergent solution preparation

Add 1000 mL 1.00 N (Normality) sulfuric acid in 2000 mL beaker that containing with 20 g of cetyltriethylammonium bromide, then stir and put only until smooth.

- **Materials**

1. 30 mL fritted glass crucibles
2. 1000 mL suction flask
3. 600 mL beaker
4. Glass tray
5. Glass stirring rod
6. 100 mL Cylinder

- **Procedure**

1. Weigh 1.00 g of the prepared sample and place in a 600 mL beaker.
2. Add 100 mL acid detergent solution and 2 mL decahydronaphthalene , heat to boil 10 min, then gently boil for another 60 min within fume hood.
3. Treated sample was filtered by using vacuum filter pump with 30 mL crucible which known its exact weight, then rinse with hot water 3 - 5 times and acetone 2 - 3 times, respectively.
4. Take crucible that contained treated sample dried in oven at 105 °C for 3 h or until constant weight. Put dry crucible cool to room temperature in desiccator then weigh it.
5. Put dry crucible in glass tray, and add haft a glass of 72 % w/w H_2SO_4 every an hour for 3 h. Meanwhile, use a glass stirring rod to mix sample is thoroughly wetted.
6. Acid solution was filtered with vacuum filter pump follow by section 3 until pH to 7, then make to dry and weigh follow by section 4.
7. Bring dry crucible to calcine in muffle furnace at 550 °C for 2 h, cool down in desiccator, and weigh it.

Calculation

$$ADF = [(W_2 - W_1) \times 100] / S$$

$$L = [(W_3 - W_4) \times 100] / S$$

$$C = ADF - L$$

When ADF is acid detergent fiber (%), L is lignin content (%), C is cellulose content (%), W_1 is empty crucible weight (g), W_2 is weight of crucible with treated sample by acid detergent (g), W_3 is weight of

crucible with treated sample by 72% w/w H_2SO_4 (g), W_4 is crucible weight after burning in muffle furnace (g) and S is initial sample weight (g).

C-2 Hemicellulose analysis

- **Apparatus**

1. Vacuum pump
2. Oven and muffle furnace
3. Desiccator
4. Analytical balance
5. Hot plate
6. Fume hood
7. pH meter

- **Materials**

1. 30 mL fritted glass crucibles
2. 1000 mL suction flask
3. 600 and 1000 mL beaker
4. Glass stirring rod
5. 100 mL cylinder
6. 2000 mL volumetric flask

- **Chemicals**

1. Sodium lauryl sulphate, reagent grade
2. Disodium ethylenediaminetetraacetate (EDTA) dihydrate, crystal, reagent grade
3. Sodium borate decahydrate ($Na_2B_4O_7.10H_2O$, reagent grate)
4. Disodium hydrogen phosphate anhydrous (Na_2HPO_4), reagent grade
5. Triethylene glycol, reagent grade
6. Sodium sulphite anhydrous, reagent grade
7. Acetone, reagent grade
8. Distilled water

Neutral detergent solution preparation

1. Add 60.0 g sodium lauryl sulphate in 1000 mL beaker then dissolve with distilled water into homogeneous solution.
2. Add 20 mL triethylene glycol, 9.12 g disodium hydrogen phosphate anhydrous, 13.62 g Sodium borate decahydrate and 37.22 g disodium ethylenediaminetetraacetate dehydrate, respectively. Use a glass stirring rod or magnetic bar to dissolve total chemicals as mixed solution.
3. Pour solution in volumetric flask, and add to make up the total volume to 2000 mL by using distilled water.

Procedure

1. Take 30 mL crucible to dry in oven at 105 °C for 2 h then put in desiccator before weighing.
2. Weigh 1.00 g of the crushed dried sample to place in a 600 mL beaker.
2. Add 100 mL neutral detergent solution, 2 mL decahydronaphthalene and 0.5 g sodium sulphate then heat to boil 10 min. Next, adjust the heat to gently boil for another 60 min.
3. Filter treated sample by using vacuum filter pump with 30 mL crucible which known its exact weight from section 1, then wash with hot water 3 - 5 times and acetone 2 - 3 times, respectively.
4. Dry crucible that contained treated sample in hot air oven at 105 °C for 8 h or one night. Put the crucible cool in desiccator then weigh it.
5. Take the crucible in section 4 to burn in muffle furnace at 550 °C for 2 h, cool down in desiccator, and weigh it to find ash.

Calculation

$$NDF = \{ [(W_2 - W_1) \times 100] / S \} - \% \text{ nuetral insoluble ash}$$

$$\% \text{ nuetral insoluble ash} = [(W_3 - W_1) \times 100] / S$$

$$H = NDF - ADF$$

When NDF is neutral detergent fiber (%), H is hemicellulose content (%), W_1 is empty crucible weight (g), W_2 is weight of crucible with treated sample by neutral detergent (g), W_3 is crucible weight after burning in muffle furnace (g) and S is initial sample weight (g).

C-3 Moisture analysis

- **Apparatus**

1. Oven and muffle furnace
2. Desiccator
3. Analytical balance

- **Materials**

1. Moisture can

- **Procedure**

1. Take moisture can to clean by using distilled water, and dry in hot air oven at 105 ºC for 2 h. Keep dry moisture can in desiccator to weigh it.
2. Weigh 2.00 g of the crushed dried sample to place in dry moisture can with closed lid of section 1 then weigh before drying in hot air oven at 105 ºC for 2 h or until constant weight.
3. After drying, keep moisture can (section 2) in desiccator then weigh it again.

Calculation

$$\%\text{Moisture} = [(W_1 - W_2) \times 100] / S$$

When W_1 is weight of empty moisture can containing with sample before drying (g) W_2 is weight of empty moisture can containing with sample after drying (g).

C-4 Analysis of reducing sugar by DNS method (Miller, 1959)

- **Apparatus**

1. Hot plate
2. UV-Vis spectrophotometer
3. Digital temperature controller (WILHL, China)

- **Materials**

1. 10, 50 and 1000 mL volumetric flask
2. 1 mL pipette
3. Stirring rod
4. 30 mL test tube caps

- **Chemicals**

1. 3, 5-dinitrosalicylic acid
2. Sodium hydroxide
3. Sodium potassium tartrate
4. Phenol (99%, crystalized)

DNS (3, 5-dinitrosalicylic acid) reagent preparation

Dissolve 10 g sodium hydroxide, 100 g sodium potassium tartrate, 2 g phenol and 10 g 3, 5-dinitrosalicylic acid, respectively, in 600 mL beaker. Pour mixed solution in volumetric flask then adjust total volume to 1000 mL. Keep DNS solution in brown reagent bottle at room temperature.

Preparation of glucose standard solution

Prepare 1.0 g/L glucose solution; 0.1 g glucose was dissolved with 100 mL distilled water in 100 mL volumetric flask. Dilute 1 g/L glucose solution into 0.2, 0.4, 0.6 and 0.8 g/L.

- **Procedure**

1. Pipette 1.0 mL sample solution and 1.0 mL DNS reagent in 30 mL test tube caps.
2. Put test tube caps (section 1) in hot water at 80 ºC for 10 min.
3. Then soak sample test tube in cold water immediately for 5 min.
4. Add 10 mL distilled water in sample test tube (section 3) to analyze reducing sugar at 540 nm by using spectrophotometer.

Note: standard solution was analyzed follow by 1 – 4.

C-5 Glucose and xylose analysis

Glucose and xylose were analyzed by HPLC (Agilent 1200).

The sample was diluted with deionized water, and filtered through 0.22 μm, 13 mm Nylon membrane filler.

Column: HPX-87H 300 mm x 7.8 mm column

Column temperature: 65 °C.

Mobile phase: 50 mM sulfuric acid

Flow rate: 0.6 mL/min

Injection volume: 20 μL

Detector: refractive index

C-6 Ethanol analysis

Ethanol was analyzed by GC

Column: HP-FFAP

Max Temperature: 240 ℃

Nominal length: 25 m

Nominal diameter: 320 µm

Nominal film thickness: 0.50 µm

Oven

Equilibration time: 5 min

Maximum temp: 240 ℃

Initial temp: 150 ℃

Initial temp: 5 min

Inlet (Split/split less)

Initial temp: 150 ℃

Pressure: 7.4 psi

Split ratio: 20.1:1

Split flow: 40mL/min

Total flow: 44.6 mL/min

Detector: FID

Temperature: 250 ℃

Hydrogen flow: 30 mL/min

Air flow: 300 mL/min

Nitrogen flow: 25 mL/min

C-7 Enzyme activity calculation

C-7.1 Measurement of cellulase activities by filter paper assay (Ghose, 1987)

- **Apparatus**

1. Hot plate
2. UV-Vis spectrophotometer
3. 4 digit analytical balance
4. Shaking incubator
5. Centrifuge

- **Materials**

1. Whatman qualitative filter paper No. 1, 10 cm × 60 cm
2. 25 and 600 mL beaker
3. Spatula
4. 30 mL test tube caps
5. 100 – 1000 μL micropipette

- **Chemicals**

1. 1.5 mg/L enzyme solution (1.5 mg cellulase in 1 mL buffer)
2. 50 mM citrate buffer at pH 4.8
3. DNS reagent

- **Procedures**

1. For enzyme solution, add 50 mg prepared filter paper, 1 mL of 50 mM citrate buffer (pH = 4.8) and 0.5 mL of enzyme solution in 30 mL test tube cap. The mixture was incubated in shaking incubator at 50 ºC and 150 rpm for an hour. Next, the tube was boiled in boiling water immediately to denature an enzyme for 5 min, and then it was centrifuged to separate liquid and solid. Take liquid sample to reducing sugar analysis by using DNS method
2. For blanks solution, add 50 mg prepared filter paper, 1.5 mL of 50 mM citrate buffer in the tube for analysis follow by section 1.

One unit of filter paper (FPU) activity was defined as the enzyme amount, which liberated 1 μmole of reducing sugar from Whatman no.1 filter paper in 1 minute.

C-7.2 Measurement of ß-glucosidase activities by cellubias assay (Ghose, 1987)

- **Apparatus**

1. Hot plate
2. UV-Vis spectrophotometer
3. Shaking incubator
4. Centrifuge

- **Materials**

1. 600 mL beaker
2. 10 mL volumetric flask
3. 30 mL test tube caps
4. 100 – 1000 μL micropipette

- **Chemicals**

1. Enzyme solution (1 mL enzyme in 10 mL buffer)
2. 50 mM citrate buffer at pH 4.8
3. DNS reagent
4. 15 mM cellobiose (dissolve in buffer)

- **Procedures**

1. Add 1.0 mL enzyme solution in 30 mL test tube cap; incubate at 50 ºC for 10 min. At least two dilutions must be made of each enzyme sample investigated.
2. Add 1.0 mL cellobiose solution, and then incubate for another 30 min.
3. Stop enzymatic reaction in boiling water for 5 minute before put the tube in cold water immediately.
4. If it has sediment, it will be centrifuged and kept liquid part to measure reducing sugar by DNS method.
5. For cellobiose blank, Add 1.0 mL citrate buffer in test tube cap to incubate at 50 ºC for 10 min. Make experiments follow by article 2 – 4.

One unit of cellobiase is defined as the amount of enzyme converts 1 μmol of cellobiose to 2 μmol of glucose in 1 minute under the assay conditions.

C-8 Microbial population count by spread plate technique

- **Apparatus**

1. Laminar flow clean bench
2. Autoclave
3. Shaking incubator
4. Incubator
5. Refrigerator

- **Materials**

1. 250 mL Erlenmeyer flask with airlock
2. 10 mL Pipette
3. 30 mL test tube caps
4. 100 – 1000 μL micropipette
5. Inoculating loop
6. Alcohol burner
7. Petri dishes
8. Micropipette tips
9. Permanent marker
10. Tri-shaped cell spreader
11. Plastic bag and plastic band

- **Chemicals**

1. 1.5 %w/v NaCl
2. 70% w/v Ethanol
3. YM agar (3.0 g/L yeast extract, 3.0 g/L malt extract, 5.0 g/L peptone, 10.0 g/L dextrose, 20.0 g/L agar)
4. YM broth (5.0 g/L animal tissue, 3.0 g/L yeast extract, 3.0 g/L malt extract, 10.0 g/L dextrose)
5. Yeast solution (*Saccharomyces cerevisiae* TISTR 5606) and *Kluyveromyces marxianus* (TISTR5116)

YM broth preparation

Add 5.25 g yeast malt powder in 250 mL distilled water, and heat to dissolve the medium completely. Take the solution to autoclave at 121 ºC for 15 min. It was placed to cold down in Laminar flow clean bench at room temperature before filling yeast cell.

YM Agar plate preparation

Suspend 41.0 g of powder in 1000 mL distilled water. Mix thoroughly, heat with frequent agitation and boil for 1 minute to completely dissolve the power. The YM solution was autoclaved at 121 ºC for 15 min. Put it within Laminar flow clean bench until the solution begins to warm. Pour the YM solution in haft of petri dishes then wait until it into agar. Keep the agar plates in plastic bag at 4 ºC.

Inoculum preparation

A single colony of *S. cerevisiae* and *K. marxianus* yeast on agar plate was added into 250 mL Erlenmeyer flasks containing 100 mL of YM broth by using inoculating loop, and was incubated in shaking incubator at 30 °C, 150 rpm for 24 h.

Comment; 1.5% w/v NaCl, Petri dishes, Micropipette tips, the tubes and pipette should be put in the plastic bag that cover with rubber band to sterilize in autoclave at 121 ºC for 15 min. For Tri-shaped cell spreader and inoculating loop, they should dip 70% w/v ethanol and then burn with the fire every time before using.

- **Procedures**

1. Pipette 1.0 mL yeast solution into a test tube cap containing 9.0 mL sterile 1.5% NaCl, mix thoroughly.
2. Pipette 1.0 mL from the tube (article 1) into another tube containing 9.0 mL sterile 1.5% NaCl, mix thoroughly.
3. Pipette follow by article 1 and 2 until the desired degree of dilution is reached as show in Figure C-1.

4. Pipette 0.1 mL from the dilution series onto the agar plates and then spread the liquid all over the surface by using tri-shaped cell spreader. Suggestion; rinse spreader with 70% ethanol and sterilize the rod by flaming from alcohol burner.
5. Petri dishes were incubated in the incubator at 30 ºC for 24 h.
6. Count the number of discrete colonies, which should choose petri dishes with grown yeast about 30 – 300 colonies.
7. Average the numbers of colonies and calculate the CFU value per 1 mL of yeast solution on an agar plate.

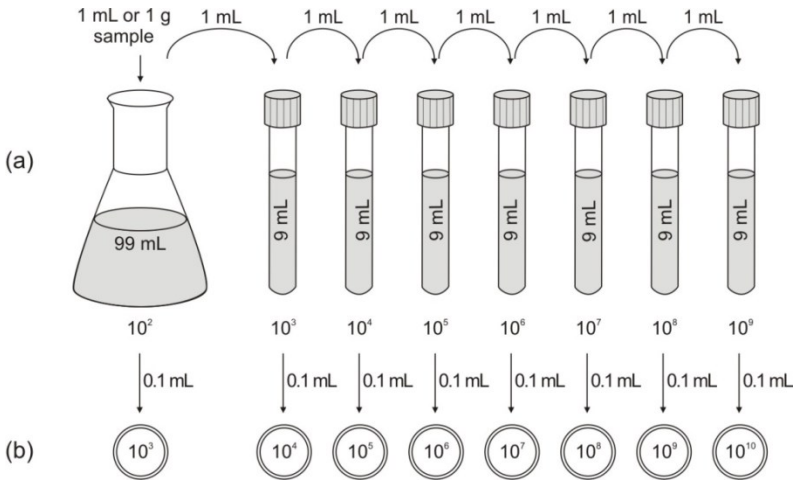


Figure C-1 the sample is diluted in sterile 1.5% NaCl with a 10-fold dilution series.

Source: Practical Microbiology, page 30.

Calculation

Colony forming unit per milliliter (CFU/mL) = 10 × Number of counted yeasts × Dilution

APPENDIX D

Chemical preparations

D-1 0.2, 0.4, 0.6, 0.8 and 1.0 mole H_2SO_4

Example: 1.0 mole H_2SO_4 1000mL

Mw = 98.08 g/mol, Basicity = 2, Density = 1.84 g/cm^3

So H_2SO_4 Mole = 98.08 g/mol

Sulfuric acid (H_2SO_4), reagent grade = 98.08 g

Then % assay from used bottle = 95 – 98 %, so average = 96.5%

Calculation, H_2SO_4 assay 96.5 % used acid 98.08 g

If H_2SO_4 assay 100 % used acid $(100 \times 98.08)/96.5 = 101.64$ g

Covert g to mL by divided density 101.64/1.84 = 55.24 mL (in 1000 mL distilled water)

Example: 0.2 mole H_2SO_4 1000mL from 1.0 mole

Calculation, $M_1V_1 = M_2V_2$

1.0 mole x V_1 = 0.2 mole x 1000 mL

V_1 = 200 mL

Add 200 mL of 1.0 mole H_2SO_4 in 1000 mL volumetric flask then adjust total volume to 1000 mL by distilled water.

D-2 5 % (w/v) NaOH

Example: 5 % w/v NaOH 1000 mL

Weigh 50 g NaOH to dissolve with distilled water in 500 mL beaker. Pour the solution in 1000 mL volumetric flask then adjust total volume to 1000 mL by distilled water.

D-3 1.0 N H_2SO_4 1000 mL

Mw = 98.08 g/mol, Basicity = 2, Density = 1.84 g/cm^3

So H_2SO_4 Normality = 98.08/2 = 49.04 g/mol

Sulfuric acid (H_2SO_4), reagent grade = 49.04 g

Then % assay from used bottle = 95 – 98 %, so average = 96.5%

Calculation, H_2SO_4 assay 96.5% used acid 49.04 g

If H_2SO_4 assay 100% used acid $(100 \times 49.04)/96.5 = 50.82$ g

Covert g to mL by divided density $50.82/1.84 = 27.62$ mL (in 1000 mL distilled water)

D-4 72% (w/w) H_2SO_4 1000 mL from 96.5% (w/w) H_2SO_4

Add 746.11 mL of 96.5% (w/w) H_2SO_4 in 1000 mL volumetric flask then adjust total volume to 1000 mL by distilled water.

D-5 50 mM citrate buffer at pH 4-6

Example: pH 4.8

4.1. Prepare 50 mM citric acid; dissolve 2.627 g citric acid monohydrate and then make to 250 mL total volume by distilled water.

4.2. Prepare 50 mM tri-sodium citrate; dissolve 7.353 g tri-sodium citrate in distilled water, and then adjust volume until 500 mL.

4.3. Pour a little of 50 mM tri-sodium citrate in 50 mM citric acid, and adjust pH until 4.8 then keep in brown reagent glass bottle.

D-6 1.5 % (w/v) NaCl

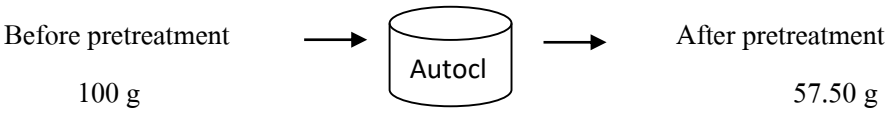
Dissolve 1.5 g NaCl in distilled water, add and adjust in 100 mL volumetric flask.

APPENDIX E

Calculations

E-1 The amount of remaining oil palm wastes after pretreatment.

Example: **Step I**, 15 % (w/v) OPEFB was treated with 0.2 M H_2SO_4 at 121 ℃ for 53 min.



Calculations

Mass of cellulose = [% w/w cellulose in solid fraction × mass of fraction (g)]/100

= (65.70 % × 57.50)/100

= 37.78 g

Mass of hemicellulose = [% w/w hemicellulose in solid fraction × mass of fraction (g)]/100

= (3.77 % × 57.50)/100

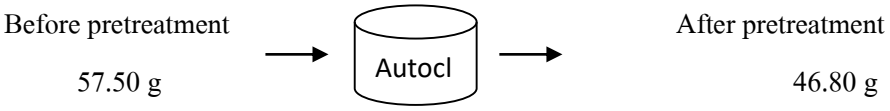
= 1.16 g

Mass of linin = [% w/w lignin in solid fraction × mass of fraction (g)]/100

= (27.82 % × 57.50)/100

= 16 g

Step II, 10 % (w/v) OPEFB was treated with 5 %(w/v) NaOH at 121 ℃ for 15 min.



Calculations

Mass of cellulose = [% w/w cellulose in solid fraction × mass of fraction (g)]/100

= (72.10 % × 46.80)/100

= 33.74 g

Mass of hemicellulose = [% w/w hemicellulose in solid fraction × mass of fraction (g)]/100

= (3.24 % × 46.80)/100

= 1.52 g

Mass of linin = [% w/w lignin in solid fraction × mass of fraction (g)]/100

= (17.60 % × 46.80)/100

= 8.24 g

E-2 Calculation of enzyme unit

E-2.1 Determination of cellulase enzyme activity

- reducing sugar from hydrolysis was 3.2500 g/L

- hydrolysis time was 60 min

- enzyme concentration was 1.5 mg/mL

Solution 1000 mL gave sugar 3.2500 g

If solution 1.5 mL gave sugar $(3.2500 \times 1.5)/1000 = 4.875 \times 10^{-3}$ g

Glucose sugar 180 g was thought to be 1 mole

If glucose sugar 4.875×10^{-3} g was thought to be $(1 \times 4.875 \times 10^{-3})/180 = 27.0833$ micromole

Hydrolysis time 60 min gave sugar 27.0833 micromole

If hydrolysis time 1 minute gave sugar 27.0833/60 = 0.4514 micromole

Sugar 1 micromole/min was thought to be 1 FPU

If sugar 0.4514 micromole/min was thought to be (1 x 0.4514)/1 = 0.4514 FPU

Enzyme solution 0.5 mL was contained with activity 0.4514 FPU

If enzyme solution 1 mL was contained with activity (0.4514 x 1)/0.5 = 0.9028 FPU

So, cellulase activity was 0.9028 /1.5 = 0.6019 FPU/mg enzyme

E-2.2 Determination of ß-glucosidase enzyme activity

- reducing sugar from hydrolysis was 17.954 g/L

- hydrolysis time was 30 min

- enzyme concentration was 0.118 mg/mL (1mL /10 mL buffer, density = 1.18 g/mL)

Solution 1000 mL gave sugar 17.954 g

If solution 2.0 mL gave sugar (17.954 x 2.0)/1000 = 0.0359 g

Glucose sugar 180 g was thought to be 1 mole

If glucose sugar 0.0359 g was thought to be (1 x 0.0359)/180 = 199.4889 micromole

Hydrolysis time 30 min gave sugar 199.4889 micromole

If hydrolysis time 1 minute gave sugar 199.4889/30 = 6.6496 micromole

Sugar 1 micromole/min was thought to be 1 U

If sugar 0.7454 micromole/min was thought to be (1 x 6.6496)/2 = 3.3248 U

So, enzyme 1 mL was contained with activity 3.3248 U

ß-glucosidase activity was 3.3248/0.118 = 28.1763 U/mg enzyme

APPENDIX F

Standard curve

F-1 Reducing sugar standard curve by using DNS method

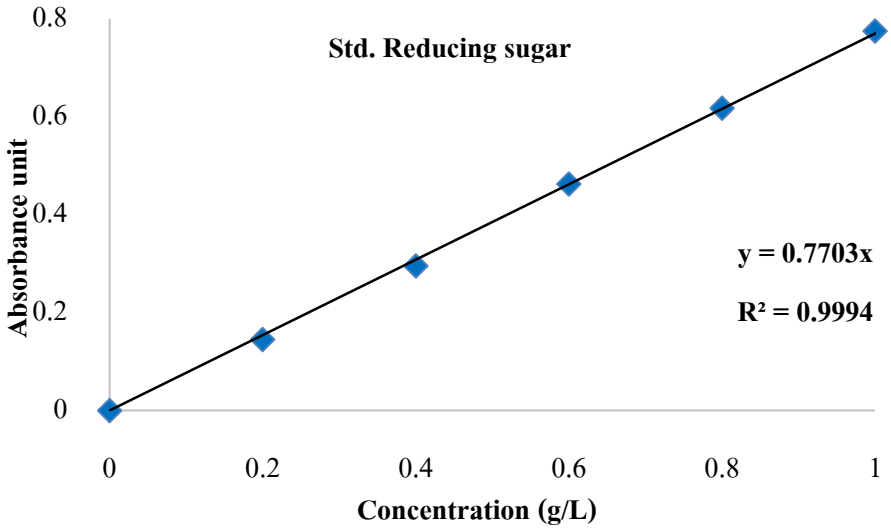


Figure F-1 Reducing sugar standard calibration curve with UV spectrophotometer, HP8453

F-2 Glucose standard curve by using HPLC

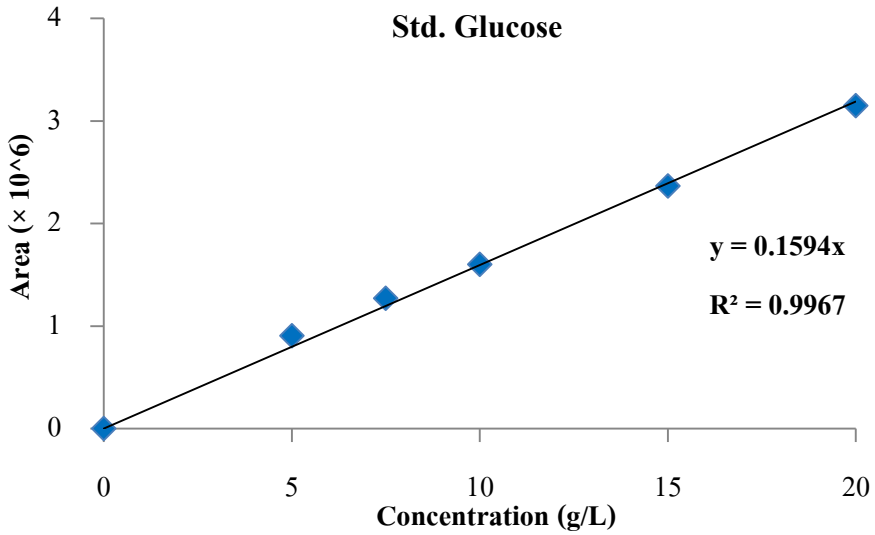


Figure F-2 Glucose standard calibration curve with HPLC, Agilent 12000

F-3 Xylose standard curve by using HPLC

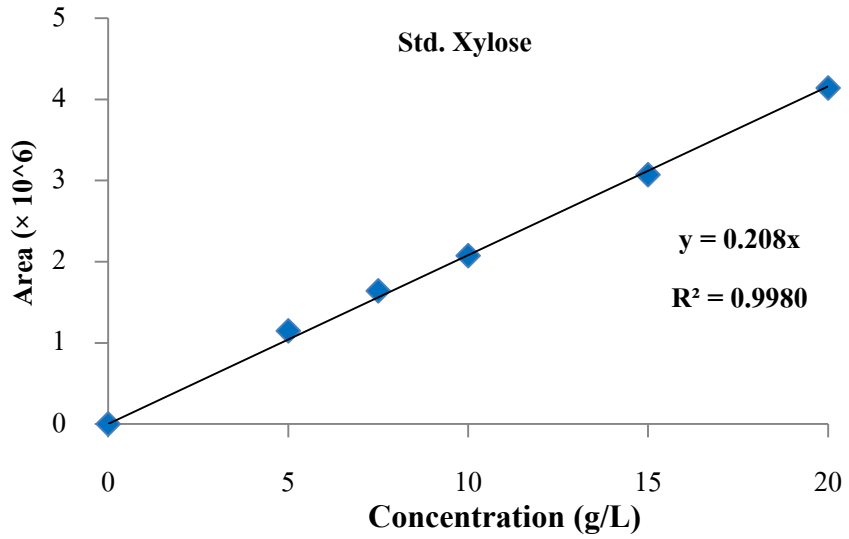


Figure F-3Xylose standard calibration curve with HPLC, Agilent 12000

F-4 Ethanol standard curve by using GC

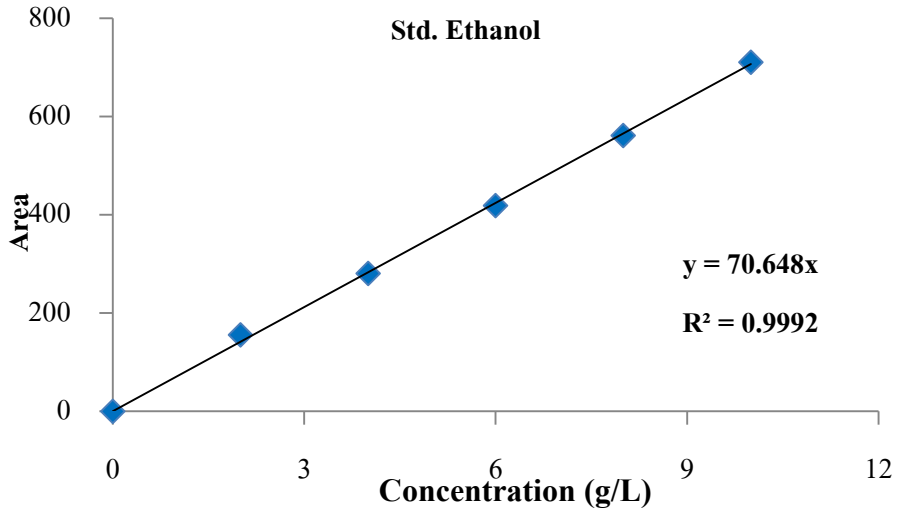


Figure F-4 Ethanol standard calibration curve with GC, 6890

VITAE

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