BIOETHANOL PRODUCTION FROM AGRICULTURAL WASTES BY SEPARATE HYDROLYSIS AND FERMENTATION METHOD

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MASTER OF ENGINEERING IN RENEWABLE ENERGY ENGINEERING

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
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ชื่อเรื่อง การผลิตไบโอเอทานอลจากวัสดุเหลือทิ้งทางการเกษตรโดยวิธีแยกการย่อยสลายและการหมัก

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บทคัดย่อ

ชีวมวลลิกโนเซลลูโลสเป็นแหล่งพลังงานทดแทนที่อุดมสมบูรณ์ที่สุดอย่างหนึ่งสำหรับการผลิตเชื้อเพลิงชีวภาพ และไบโอเอทานอลเป็นหนึ่งในพลังงานทดแทนที่มีข้อได้เปรียบทางด้านสิ่งแวดล้อม และในปัจจุบันมีการสนับสนุนให้ใช้ร่วมกับแก๊สโซลีน ซึ่งไบโอเอทานอลนี้สามารถผลิตได้จากวัตถุดิบหลายชนิด โดยเฉพาะอย่างยิ่งแหล่งวัตถุดิบทดแทนที่มีปริมาณมากอย่างลิกโนเซลลูโลส การผลิตไบโอเอทานอลจากลิกโนเซลลูโลสจึงได้รับความสนใจกันอย่างแพร่หลายทั่วโลก ในการศึกษาครั้งนี้ได้ใช้วิธีการปรับสภาพวัตถุดิบที่แตกต่างกัน การปรับสภาพชีวมวลของเสียทางการเกษตรมีแบ่งออกเป็นวิธีการทำหญ้าหมัก (silage) วิธีทางเคมี และวิธีทางชีวภาพ จากผลการศึกษาพบว่าการปรับสภาพด้วยวิธีทางเคมีเหมาะสำหรับวัตถุดิบทั้ง 4 ประเภท ได้แก่ ต้นทานตะวัน ต้นข้าวฟ่าง ใบอ้อย และต้นข้าวโพด เพื่อผลิตเอทานอลในขั้นตอนต่อไป ดังนั้นจึงเลือกวิธีทางเคมีในการปรับสภาพวัตถุดิบต้นทานตะวัน ต้นข้าวฟ่าง ใบอ้อย และต้นข้าวโพดซึ่งเป็นวิธีที่เหมาะสมต่อการย่อยสลายน้ำตาลในขั้นตอนต่อไป นอกจากนี้ ในการทดลองยังได้ใช้สถิติพื้นผิวตอบสนอง (Response Surface Methodology, RSM) แบบเซ็นทรัลคอมโพสิท (Central Composite Design, CCD) เพื่อประเมินและศึกษาสภาวะที่เหมาะสมของอุณหภูมิ (30, 35 และ 40 องศาเซลเซียส) ความเข้มข้นของโซเดียมไฮดรอกไซด์ (1, 1.5 และ 2 เปอร์เซ็นต์) และระยะเวลาการปรับสภาพ 1, 2 และ 3 วัน ซึ่งเป็นตัวแปรอิสระต่อผลผลิตน้ำตาลทั้งหมดและน้ำตาลรีดิวซ์ที่ได้ตามการตอบสนองของฟังก์ชัน และทำการศึกษาปฏิสัมพันธ์ของผลกระทบและตัวแปร โดยใช้ซอฟต์แวร์ Design-Expert 11 กำหนดการทดสอบด้วยค่า p น้อยกว่า 0.05 ผลการทดลอง พบว่า สภาวะที่เหมาะสมของการปรับสภาพและการย่อยน้ำตาลที่เหมาะสมของพืชทั้ง 4 ชนิด ได้แก่ การปรับสภาพด้วยโซเดียมไฮดรอกไซด์ความเข้มข้น 2 เปอร์เซ็นต์ ที่อุณหภูมิ 40 องศาเซลเซียส เป็นระยะเวลา 3 วัน มีผลทำให้ได้ปริมาณน้ำตาลทั้งหมดและน้ำตาลรีดิวซ์มากที่สุด ดังนั้นกราฟ 3 มิติที่ได้จึงแสดงความสัมพันธ์ระหว่างเวลาและความเข้มข้นของโซเดียมไฮดรอกไซด์อย่างชัดเจน ซึ่งแสดงให้เห็นว่าทั้งสองปัจจัยมีผลกระทบอย่างมากต่อการย่อยสลายน้ำตาลจากวัสดุลิกโนเซลลูโลส ในการผลิตเอทานอลจากเศษวัสดุเหลือทางการเกษตรครั้งนี้ได้ใช้

ยีสต์ *Saccharomyces cerevisiae* TISTR 5020 โดยทำการปรับสภาพวัตถุดิบด้วยโซเดียมไฮดรอกไซด์ น้ำ และ *Trichoderma* spp. ที่อุณหภูมิห้อง เป็นเวลา 3 วัน จากนั้นทำการย่อยน้ำตาลด้วยเอนไซม์เซลลูเลส 2 เปอร์เซ็นต์ เป็นระยะเวลา 24 ชั่วโมง ประสิทธิภาพในการย่อยสลายน้ำตาลดีที่สุดเมื่อวัตถุดิบได้รับการปรับสภาพด้วยโซเดียมไฮดรอกไซน์ 2 เปอร์เซ็นต์ ทำการหมักเอทานอลด้วยยีสต์เป็นเวลา 5 วัน พบว่าผลผลิตเอทานอลสูงที่สุดในวันที่ 3 จากการหมักสารละลายที่ได้จากการปรับสภาพด้วยโซเดียมไฮดรอกไซด์ 2 เปอร์เซ็นต์ ดังนั้นการปรับสภาพด้วยโซเดียมไฮดรอกไซด์ 2 เปอร์เซ็นต์จึงถูกนำมาใช้ในการหมักเอทานอลในระดับที่ใหญ่ขึ้น ผลการทดลองพบว่าวัตถุดิบที่ย่อยสลายด้วยเอนไซม์เซลลูเลส 2 เปอร์เซ็นต์ มีค่าน้ำตาลสำหรับการหมักโดยวัดด้วยวิธี DNS เท่ากับ 218.286 กรัมต่อลิตร จากการหมักด้วยยีสต์ *S. cerevisiae* TISTR 5020 ที่ความเข้มข้น 10 เปอร์เซ็นต์เป็นระยะเวลา 3 วัน สามารผลิตไบโอเอทานอลได้สูงถึง 7.3 เปอร์เซ็นต์ และหลังจากการกลั่นทำให้ความเข้มข้นของเอทานอลเพิ่มสูงขึ้นถึง 12.5 เปอร์เซ็นต์ จากการวัดค่าความร้อนได้ค่าความร้อนสูงสุด เท่ากับ 1.838 เมกกะจูลต่อกิโลกรัม จากผลงานวิจัยนี้ กระบวนการการผลิตไบโอเอทานอลจากวัตถุดิบลิกโนเซลลูโลสมีความเป็นไปได้ในเชิงเศรษฐศาสตร์ และสามารถประยุกต์ใช้กับกระบวนการผลิตขนาดใหญ่ได้

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Author	Mr.Numchok Manmai
Degree	Master of Engineering in Renewable Energy Engineering
Advisor Committee Chairperson	Dr. Rameshprabu Ramaraj

ABSTRACT

Lignocellulosic biomass is one of the most abundant renewable resources for biofuel production. Bioethanol is a renewable energy with major environmental advantages. It represents biofuel which is mostly used in combination with gasoline. It can be produced from different types of renewable feedstocks. One of the most abundant renewable resources for bioethanol production is lignocellulosic biomass. The production of bioethanol from lignocellulosic biomass has attracted worldwide interest. In the present study the slurry, obtained after different pretreatment methods were applied on agricultural waste biomass using silage, chemical and biological pretreatments. The study results revealed that chemical pretreatment is suitable for sunflower stalk, sorghum stalk, sugarcane leaf and corn stalk bioethanol production. Accordingly, the chemical pretreatment was verified for the feasibility of the sugar production process from sunflower stalk, sorghum stalk, sugarcane leaf and corn stalk. Furthermore, the Response Surface Methodology (RSM) was used based on Central Composite Design (CCD) to evaluate and optimize the effect of temperature (30, 35 and 40 °C), NaOH concentration (1, 1.5 and 2%) and time (1, 2 and 3 days) as an independent variable on the total sugar and reducing sugar concentrations were used the response function. The interaction effects and optimal parameters were obtained using Design-Expert 11 software. The significance of the independent variables and their interactions were tested by p-value less than 0.05. The results showed that using 4 plants pretreated at 40 °C, 2% NaOH for 3 days released the highest total sugar and

reducing sugar. Hence, 3D graphs expressed a significant association between time and NaOH concentrations, it shows that both functions were affected sugar extraction from lignocellulosic materials. The present work is apportioned with production of ethanol from agricultural wastes biomass by *Saccharomyces cerevisiae* TISTR 5020. The powdered biomass was treated with sodium hydroxide (NaOH), water, silage and *Trichoderma* spp. to enzymatic hydrolysis by a cellulase enzyme. All of pretreatments were performed at room temperature for 3 days. The pretreatments resulted in enhancing the following enzymatic hydrolysis to 2% of the theoretical yield overnight. The best hydrolysis performance was obtained after pretreatment by 2% NaOH. The yeast showed promising results in fermentation in 3 to 5 days. The best results occurred with the hydrolysate using 2% NaOH as pretreatment. Consequently, the pretreatment with 2% NaOH was applied in a large scale. Results showed that hydrolysis with 2% cellulase enzyme containing fermentable sugar and carried out by DNS method is 218.286 g/L. After fermentation with 10% *S. cerevisiae* TISTR 5020 for 3 days bioethanol production reached 7.3 %, and after distillation bioethanol increased to 12.5%. High Heating Value (HHV) was 1.838 MJ/kg. In this research, bioethanol production process from lignocellulosic materials can be economically feasible and production can be applied large scale.

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ABBREVIATION

ANOVA	Analysis of variance
ATP	Adenosine tri phosphate
CBP	Consolidated bio processing
CCD	Central composite design
DMC	Direct microbial conversion
DNS	3,5-Dinitrosalicylic acid
DP	Degree of polymerization
EU	The European Union
FPU	Filter- paper units
GDP	Gross domestic product
HHV	Higher heating value
LPG	Liquefied petroleum gas
NADH	Nicotinamide adenine dinucleotide hydride
NAD^+	Nicotinamide adenine dinucleotide
NGV	Natural gas vehicle
PEG	Polyethylene glycol
PGI	Phosphoglucose isomerase
rpm	Revolutions per minute
RSM	Response surface methodology
SHF	Separate hydrolysis and fermentation
SPSS	Statistical Package for the Social Sciences
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
U	Unit
UK	The United Kingdom
US	The United States of America

Chapter 1
Introduction

Fuel consumption

Fuel is a factor in driving the world economy is highly depend on diverse fossil energy sources such as natural gas, petroleum and coal. All the energy sources there are being used for the production of electricity and other materials (Sarkar et al., 2012; Gupta and Verma, 2015). Immoderate consumption of the fuel especially in large suburb areas. Due to speedy growth in citizenry and industrialization. Worldwide fuel demand a lot of quantity and tendency has been increasing every year (Petrou and Pappis, 2014).

Thailand energy report 2015, Energy production in Thailand decreased, resulting in imports met more domestic demand. The final energy consumption increased by 4.0 percent because Thai economy started to recover (GDP grew by 2.8 percent) while the energy prices are in a downtrend due to the oversupply of oil, natural gas and coal in the world market. The prices of Diesel, Gasoline and Gasohol increased from the low level. The jet fuel consumption increased by the number of foreign tourists. The foreign tourists were 29.9 million increases about 5 million people compare to previous year. The electricity consumption increased because the longer period of hot weather occurred and the expansion of the business sector is another key factor that affected the increasing electricity consumption in 2015 (Energy Policy and Planning Office, 2015).

Furthermore, crude oil supply is 1,028 thousand barrels per day by 85 percent of imports. The 8.8 percent increase in imports, mainly from Middle East countries. The rest is domestic production rose 10.0%, the refining capacity of the country stood at 1,252 thousand barrels per day. Crude was used in refining for 90 percent of the refining capacity. Petroleum products consumption is at 132 million liters per day, up 4.3 percent**.** The diesel consumption is at 60.1 million liters per day accounted for 46 percent of all petroleum products. It is increased 4.1 percent by the prices reduction. The consumption of gasoline and diesel fuel was at 26.4 million liters per day.

Accounted for 20 percent of all petroleum products consumption. The demand rose to 13.2 percent due to the low oil prices that encourage the auto LPG and NGV users turning to use more oil because it is cheaper and more convenient evenly over the service station. Jet fuel consumption was at 16.5 million liters per day, up 9.4 percent from the expansion in tourism sector. In 2015, the foreign tourists come to visit at 29.9 million people, up from about 5 million from the years ago (Energy Policy and Planning Office, 2015).

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Petrochemical industry accounted for most of the 32 percent decrease of 20.6 percent from the slowdown of downstream industries and the export sector is still shrinking. Households sector accounted for 31 percent, down 4.3 percent, it was a result from the adjusting retail LPG prices structure to reflect actual costs, that effected the prices in household sector higher than the last year prices so there was no motive to smuggle LPG. Automobile consumption fell 12.3 percent due to lower oil prices resulting that some users turn to oil instead of LPG. Industry consume 3.0 percent up compared to the previous year by adjusting the price to equal the household and transportation sector price (Energy Policy and Planning Office, 2015).

With the reason the energy consumption demand has been increasing every year. In consequence researchers find the alternative sources for the energy. The alternative sources of energy are being used in many countries. Biomass from agricultural waste is the most profusion biomass on the earth. Using the biomass from agricultural waste is the potential promising natural renewable is inexpensive, cost

effective and sustainable sources used for considerable and commercial production of bio-energy as bio-ethanol. The renew- able fuels such as bio-diesel and bio-hydrogen, derived from sugarcane, corn, switchgrass, algae, etc., can be used as petroleum-based fuels in the future as fossil fuels are going to depleted soon due to higher energy consumption (Service, 2016).

The potential of ethanol producer countries

Many Countries in the world uppermost ethanol producer countries such as Brazil, US, China, India, France, Russia, South Africa, UK and Saudi Arabia as shown in Figure 1 (Gupta and Verma, 2015) The total ethanol production in 2008 was about 7266.8 Millions of gallon and the largest ethanol producer country in 2008 is United States, which produced nearly 9000 Millions of gallon and the least ethanol producer country in 2008 is Paraguay, which produced nearly 23.7 Millions of gallon. It has been found that US by corn is the first and Brazil by sugarcane is the second largest producer of bioethanol followed by China in the world. China produced the bioethanol using sugarcane, cassava and yams, while the European Union by wheat and sugar beet. In US, the cereals grains including wheat and maize are also used for ethanol production. The biofuel production of different countries about 23 countries by using different crops by the year 2004–2009 and it was seen that many countries use sugar and starchy crops for bioethanol production, where these crops impose problem of food insecurity presented in Table 1 (Gupta and Verma, 2015).

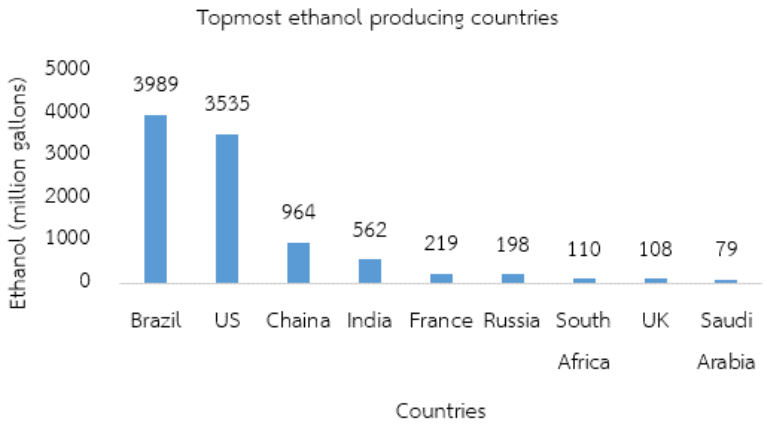


Figure 1 Topmost ethanol producing countries (Gupta and Verma, 2015)

Table 1 World's total production of fuel ethanol (billion liters) from year 2004 to 2013 adopted from (Gupta and Verma, 2015)

Countries	Major feedstock sugar and starchy crops	Ethanol production (billion liters) per year									
		2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
US	Corn/maize	13	15	18.3	24.6	34	41	49.5	54.2	50.4	50.3
Brazil	Sugarcane	15	15	17.5	19	27	26	27.6	21.0	21.6	25.5
Germany	Wheat	0.02	0.2	0.5	–	0.5	0.8	1.5	0.8	0.8	0.8
France	Sugar beet, wheat	0.1	0.15	–	–	1.2	0.9	1.1	1.1	1.0	1.0
China	Corn, sugarcane, maize, cassava	2	1	1	1.8	1.9	2.1	2.1	2.1	2.1	2.0
Argentina	Sugarcane	–	–	–	0.02	–	–	0.1	0.2	0.2	0.5
Italy	Cereals	–	–	0.13	–	0.13	0.1	0.1	0.0	–	–
Spain	Barley, wheat	0.2	0.3	0.4	–	0.4	0.4	0.6	0.5	0.4	0.4
India	Sugarcane, wheat	–	0.3	0.3	0.2	0.3	0.2	–	–	0.5	–
Canada	wheat/cereal	0.2	0.2	0.2	0.8	0.9	1.1	1.4	1.8	1.8	1.8
Poland	Rye	–	0.05	0.12	–	0.12	–	0.2	–	–	0.2
Czech Republic	Sugar beet	–	0.15	0.0	–	–	–	–	–	–	–
Colombia	Sugarcane	–	0.2	0.2	0.3	0.3	0.3	0.4	0.3	0.4	0.4
Sweden	Wheat	–	0.2	0.14	–	0.14	–	–	–	–	–
Malaysia	–	–	–	–	–	–	–	–	–	–	–
UK	–	–	–	–	–	–	0.2	0.3	–	–	–
Denmark	Wheat	–	0.1	–	–	–	–	–	–	–	–
Austria	Wheat	–	0.1	–	–	–	0.1	–	–	0.2	–
Slovakia	Corn	–	0.1	–	–	–	–	–	–		–
Thailand	Sugarcane, cassava	0.2	–	–	0.3	0.3	0.4	0.4	0.5	0.7	1.0
Australia	Sugarcane	0.07	–	–	0.1	–	–	–			0.3
Belgium	Wheat	–	–	–	–	–	0.2	0.3	0.4	0.4	0.4

Countries	Major feedstock sugar and starchy crops	Ethanol production (billion liters) per year									
		2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
EU	Various/cereal and sugar beet	–	–	–	2.16	–	–	4.5	4.3	4.2	4.5
World total		31	33	39	49.6	67	76	86	86.1	83.1	87.2

Thailand in 2017, fuel ethanol production is forecast to increase to 1.4 billion liters, up around 7 percent from 2016. Molasses-based ethanol still dominates Thailand's overall ethanol production, accounting for around 70 percent of fuel ethanol. The demand for molasses is expected to increase to 3.8 million metric tons, up 8 percent from 2016. Presently, there are 21 fuel ethanol plants with production capacity of 1.5 billion liters per year. Production capacity of molasses-based ethanol is currently around 0.9 billion liters per year. Other producers use cassava and sugarcane as inputs with production capacity of 0.5 and 0.1 billion liters, respectively (Service, 2016).

The production of nonfuel ethanol is controlled by the government. The Liquor Distillery Organization, which is under the authority of the Excise Department of the Ministry of Finance, has a monopoly on the production of industrial grade ethanol in Thailand with a production capacity of 20 million liters per year. Meanwhile, domestic demand for industrial grade ethanol, particularly for medical, pharmacy, paints and cosmetics uses, is around 18 million liters per year. The primary feedstock for industrial ethanol production is molasses and cassava (Service, 2016).

Presently, fuel ethanol production capacity is at 81 percent of full capacity. Production capacity is expected to reach 96 percent by 2017. Ethanol producers reportedly have received approval from the government to expand their production capacities. However, their investment has been delayed due to the concern about an economic instability (Service, 2016).

The objectives of research

1. To compare the pretreatment methods for agricultural waste biomass degradation.
2. To figure out the pretreatment methods effects on lignocellulosic components degradation and releasing more reducing sugar content.
3. To investigate the potential of producing bioethanol from agricultural materials including *Helianthus annuus* L. (Sunflower), *Sorghum bicolor* L. (Sorghum), *Zea mays* L. (Corn) and *Saccharum officinarum* L. (Sugarcane).

The scopes of research

1. Four raw materials, H. annuus L., S. bicolor L., Z. mays L. and S. officinarum L., will be explored the potential of bioethanol production.
2. Characterize the ability of yeast to produce ethanol by Separate Hydrolysis and Fermentation (SHF) method from the sugars present in *H. annuus* L., *S. bicolor* L., *Z. mays* L. and *S. officinarum* L.
3. Identify the proper pretreatments methods by Silage, NaOH, *Trichoderma spp*. and H2O before bioethanol production in the future applications.

Benefits of study

Biomass is a renewable source of energy with environment-friendly carbon neutral characteristics. World-wide a considerable amount of biomass is available in the form of wastes whose economy is primarily dependent on agricultural production. In the present work, an experimental investigation has been conducted using bioethanol production from *H. annuus* L., *S. bicolor* L., *Z. mays* L. and *S. officinarum* L. by fermentation process. These agricultural plant wastes are having been identified as the key industry for expansion to achieve economic advancement along with the development of greener production processes in Thailand, and also plenty of waste available from the industries. These wastes are called as biomass, which are value-added materials for fuel production. And the biomass appears to be one of the potential energy sources due to its abundance. In addition, the realization of waste biomass for producing value-added products and biochemicals increases the

economical and sustainable energy production opportunities for the biomass industry. Green development indicators are of the utmost importance in ensuring economic and sustainable development. In brief, the study will cover some basic experiments related to bioethanol production with the degradation effect of lignocellulosic biomass on ethanol yield. Also the study will be explaining the sustainable energy engineering aspects.

Chapter 2

Literatures review

Composition of lignocellulosic biomass

Biomass is the most logical carbon-based feedstock obtained from living organisms such as plants, animals, and microorganisms. Among biomasses, lignocellulose is the most common, which is composed of various polysaccharide celluloses, hemicelluloses, phenol-aldehyde polymer lignin, and soluble polar and non-polar substances. Because of its complex structure, the conversion technology of lignocelluloses materials to energy is costly and ineffective up to now. Besides, the compositions of various lignocelluloses are different, it is necessary to understand the structure of it to design suitable pretreatment, which can be improve the effectiveness of lignocellulose usage and reduce its costs (Chen et al., 2017).

Lignocelluloses there are composition of 40–50 percent cellulose, 25–30 percent hemicellulose, 15–20 percent lignin and traces of pectin, nitrogen compounds, and inorganic ingredients (Chen et al., 2017).

Cellulose, which is a homopolysaccharide composed of anhydroglucose units linked together by β-(1$\longrightarrow$4)-glycosidic bonds is the most profution polymer on the earth, has many advantageous properties such as biocompatibility, Its distinct polymer chains in orderly bundled arrangement and highly crystalline structure cause its stable properties, and its structure determines the framework of cell wall (Chen et al., 2017).

Hemicellulose is a mixture composed of different polysaccharides, including straight and branched chain ones, to connect different numbers of acetyl and methyl. This polysaccharide has a low degree of polymerization, and without crystalline regions, so it is relatively easily degraded into monosaccharides, such as arabinose, xylose, galactose, fructose, mannose, dextrose, or glucuronide (Chen et al., 2017).

Lignin is a complex hydrophobic, cross-linked aromatic polymer that interferes with the hydrolysis process. It has a three-dimensional heterogeneous polycrystalline reticulated polymer, which belongs to polyphenolic compounds. Such polymer is

formed by phenyl propane structural units via ether linkages and carbon–carbon bond connection attested in Figure 2 (Chen et al., 2017).

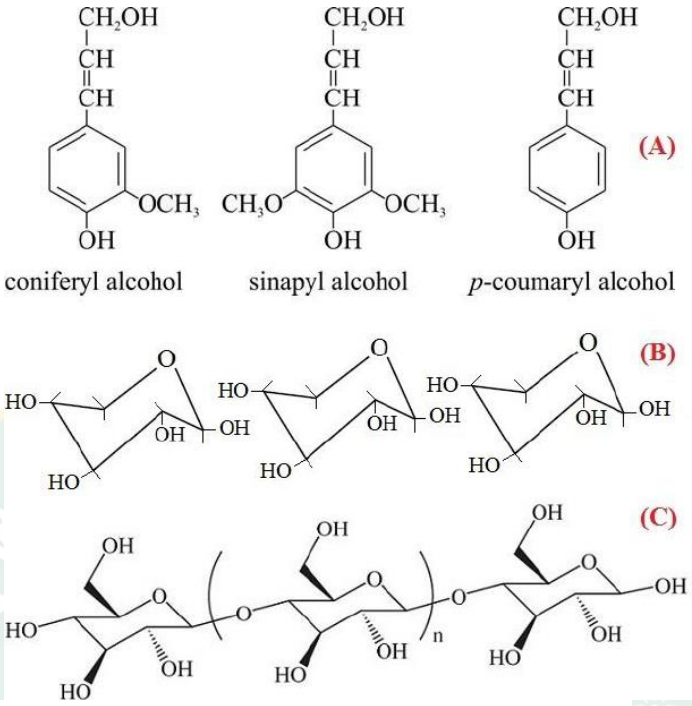


Figure 2 Structure unit of cellulose, hemicellulose, and lignin.(Chen et al., 2017)

The high crystallization zone; different binding forces between cellulose, hemicellulose, and lignin molecules are high degree of polymerization vesicle structure on the surface of cellulose; and overwrite protection effect by lignin and hemicellulose determine that the cell walls are stable and difficult to be degraded, as shown in Table 2. And Percent composition of lignocellulose components in various lignocellulosic materials, as presented in Table 3 (Chen et al., 2017).

Table 2 Structure and chemical composition of cellulose, hemicellulose, and lignin adopted from (Chen et al., 2017)

	Cellulose	Hemicellulose	Lignin
Structural unit	D-Glucopyranose	D-xylose, Mannose, Galactose, L-arabinose, Glucuronic acid	Syringyl, Guaiacyl, *Para*-hydroxy-phenyl
Bond join of structural unit	β-1.4-glycosidic linkage	β-1.4-Glycosidic linkage, β-1.2(or 3, 6)-Glycosidic linkage	C–C, R–O–R′
Polymeric level	1000–10,000	≤ 200	4000
Polymer	β-1.4-Glucan	Glucomannan, Galactoglucomannan, Xylan	G-,GS-, and GSH-type
Structure	Crystalline and amorphous area	Few crystalline area, Majority is amorphous area	Amorphous, non-uniform, nonlinear, 3D polymer
Binding forces	Hydrogen bond	Chemical bond	Chemical bond

Table 3 Percent composition of lignocellulose components in various lignocellulosic materials adopted from (Iqbal et al., 2013)

Lignocellulosic material	Lignin (%)	Hemicellulose (%)	Cellulose (%)	Reference
Sugar cane bagasse	20	25	42	(Kim and Day, 2011)
Sweet sorghum	21	27	45	(Kim and Day, 2011)
Corn cobs	15	35	45	(Prasad et al., 2007)
Corn stover	19	26	38	(YONGMING ZHU et al., 2005)
Rice straw	18	24	32.1	(Prasad et al., 2007)
Nut shells	30-40	25-30	25-30	(Abbasi and Abbasi, 2010)
Grasses	10-30	25-50	25-40	(Malherbe and Cloete, 2002)
Wheat straw	16-21	26-32	29-35	(McKendry, 2002)
Bagasse	23.33	16.52	54.87	(Guimarães et al., 2009)
Sponge gourd fibers	15.46	17.44	66.59	(Guimarães et al., 2009)

Lignocellulose pretreatment

Pretreatment is the importance step in the energy conversion from lignocelluloses, which provide the separation or solubilization of the complex components of lignocellulose. And the choice of pretreatment should consider the compatibility of raw materials, enzymes and organisms. This process generally can be classified into physical, chemical, physical-chemical, biological methods and their combinations (Jönsson and Martín, 2016; Chen et al., 2017).

Physical pretreatment methods

Commonly, physical pretreatment methods include mechanical crushing, microwave treatment, ultrasonic treatment, and high-energy electron radiation method. These methods cause less environmental pollution and the process is also relatively simple, but it requires relatively high energy and power, which increasing the cost of production (Jönsson and Martín, 2016; Chen et al., 2017).

Chemical pretreatment methods

In the chemical pretreatment, inorganic acids (sulfuric and hydrochloric acids) and organic acids (formic, acetic, and propionic acids) are used acid functions mainly depend on the separation and removal of lignin, and hydrolyzation of fibers also acids present a high pretreatment efficiency with wheat straw, and lesser amount of furfural is obtained than that in pretreatment with sulfuric acid (Jönsson and Martín, 2016; Chen et al., 2017).

The alkali pretreatment are used (NaOH and KOH) mainly depends on the solubility performance of lignin. In addition, this method exposes better productiveness on agricultural leftovers than on wood lignocellulose (Jönsson and Martín, 2016; Chen et al., 2017).

Biological pretreatment methods

The biological pretreatment in the main uses some microbes to decompose lignin. It can generate enzymes for lignin decomposing in the process. Fungal pretreatment with high lignin-decomposing and low cellulose-decomposing fungi of wheat straw for 10 days, which conducts to a reduction in acid loading for hydrolysis,

shows an augmentation in the release of fermentable sugars and a reduction in the concentration of fermentation inhibitors (Jönsson and Martín, 2016; Chen et al., 2017).

Combination pretreatment methods

A single procedure can have insecurities, for instance technological obstacle, environmental pollution, high-energy consumption, long reaction time, high requirement for reaction equipment corrosion resistance, and the absence of the requirement for industrial production, which cannot satisfy the intended effect. Combined pretreatment including mechanical crushing–chemical, physical or biological treatment and etc. This method integrates the advantages of several single pretreatment methods according to different lignocellulosic materials, which can significantly improve the efficiency of enzymatic hydrolysis (Jönsson and Martín, 2016; Chen et al., 2017).

Different types of pretreatment and respective yields

Different types of pretreatment and respective yields for sugarcane bagasse, wheat straw, rice straw, and corn straw are given in Table 4.

Table 4 Different pretreatments and respective yields for sugarcane bagasse, wheat straw, rice straw and corn straw

Substrate	Pretreatment	Hydrolysis	Yield of sugars	References
Sugarcane bagasse	Ball milling (4 h)	Enzymatic (Acremonium cellulase at 5 FPU/g substrate of cellulase and 20 U/g substrate of xylanase from Optimash BG at 45 °C, pH 5.0 for 72 h.	89.2 ± 0.7% (glucose), 77.2 ± 0.9% (xylose)	(Buaban et al., 2010)
	1% sulfuric acid (v/v) at 60 °C, 24 h (SLR 1:6)	In an autoclave at 121 °C for 40 min after removing the excess acid (1% (v/v) sulfuric acid).	Total sugar concentration of approximately 68.0 g/L.	(Takahashi et al., 2000)

Substrate	Pretreatment	Hydrolysis	Yield of sugars	References
Wheat Straw	Knife milling with 0.7–1.0 mm rejection screen, washed with water and dried.	At 90 °C with 1.85% (w:v) sulfuric acid for 18 h; liquid to solid ratio of 20:1. Suspension centrifuged and the residue is washed with hot water.	D-xylose: 12.80 ± 0.25 g/L, D-glucose: 1.70 ± 0.30 g/L	(Nigam, 2001)
Rice straw	Chopped to 5–6 mm size range.	4.4% sulfuric acid at 1:10 solid to liquid ratio in boiling water bath, 1 h, filtered and pH adjusted to 5.5.	Total sugar (20 g/L)	(Abbi et al., 1996)
		Soaked in water at 170 °C and 7.6 kg/cm^2, 30 min, finally cooled and pH adjusted to 5.5.	Total sugar (23 g/L)	
	Chopped, steam exploded (3.5 MPa, 275 °C, 2 min)	Enzymatic saccharification (*cytolase, novozyme*) (50 °C, 120 h)	Xylose yield (10–5 g/L)	(Moniruzzaman, 1995)
Corn straw	2% NaOH, 80 °C, 1 h.	Enzymatic hydrolysis by cellulase of *Trichoderma reesei* ZU-02 and cellobiose of *Aspergillus niger* ZU-07.	Xylose 23.6 g/L, glucose 56.7 g/L, arabinose 5.7 g/L	(Chen et al., 2008)

Enzymatic hydrolysis

Saccharification is the critical step for bioethanol production where complex carbohydrates are converted to simple monomers. Compared to acid hydrolysis, enzymatic hydrolysis requires less energy and mild environment conditions (Ferreira et al., 2009). The optimum conditions for cellulase have been reported as temperature of 40–50 °C and pH 4–5 (das Neves et al., 2007). Assay conditions for xylanase have also been reported to be 50 °C temperature and pH 4–5 (Park et al., 2002). Therefore, enzymatic hydrolysis is advantageous because of its low toxicity, low utility cost and low corrosion compared to acid or alkaline hydrolysis (Sun and Cheng, 2002; Taherzadeh and Karimi, 2007). Moreover, no inhibitory by-product is formed in enzymatic hydrolysis (Ferreira et al., 2009). However, enzymatic hydrolysis is carried

out by cellulase enzymes that are highly substrate specific (Taherzadeh and Karimi, 2007; Banerjee et al., 2010). Here cellulase and hemicellulase enzymes cleave the bonds of cellulose and hemicellulose respectively. Cellulose contains glucan and hemicellulose contains different sugar units such as mannan, xylan, glucan, galactan and arabinan. Cellulase enzymes involve endo and exoglucanase and β-glucosidases. Endoglucanase (endo 1,4-d glucanhydrolase or E.C. 3.2.1.4) attacks the low crystallinity regions of the cellulose fiber, exoglucanase (1,4-β-d glucan cellobiohydrolase or E.C. 3.2.1.91) removes the cellobiase units from the free chain ends and finally cellobiose units are hydrolysed to glucose by β-glucosidase (E.C. 3.2.1.21) (Taherzadeh and Karimi, 2007; Banerjee et al., 2010). Hemicellulolytic enzymes are more complex and are a mixture of at least eight enzymes such as endo-1,4-β-d-xylanases, exo-1,4-β-d xylocuronidases, α-l-arabinofuranosidases, endo-1,4-β-d mannanases, β-mannosidases, acetyl xylan esterases, α-glucoronidases and α-galactosidases (Jørgensen et al., 2003). Cellulose is hydrolysed to glucose whereas hemicellulose gives rise to several pentoses and hexoses. Several species of *Clostridium*, *Cellulomonas*, *Thermonospora*, *Bacillus*, *Bacteriodes*, *Ruminococcus*, *Erwinia*, *Acetovibrio, Microbispora*, *Streptomyces* are able to produce cellulase enzyme. Many fungi such as *Trichoderma*, *Penicillium*, *Fusarium*, *Phanerochaete*, *Humicola*, *Schizophillum* sp. also have been reported for cellulase production (Rabinovich et al., 2002; Sun and Cheng, 2002). Among the various cellulolytic microbial strains *Trichoderma* is one of the most well studied cellulase and hemicellulase producing fungal strains (Xu et al., 1998). *Trichoderma* is able to produce at least two cellobiohydrolases and five endoglucanases and three endoxylanases (Xu et al., 1998; Sandgren et al., 2001). However, *Trichoderma* lacks β-glucosidase activity that plays an efficient role in polymer conversion (Taherzadeh and Karimi, 2007; Kovács et al., 2009). On the other hand, *Aspergillus* is a very efficient β-glucosidase producer (Taherzadeh and Karimi, 2007). *Trichoderma* cellulase supplemented with extra β-glucosidase has been studied several times. Combination of *Trichoderma reesei* ZU-02 cellulase and cellobiase from *Aspergillus niger* ZU-07 improved the hydrolysis yield to 81.2% with cellobiase activity enhanced to 10 CBU/g substrate (Chen et al., 2008).

Various factors influence yields of monomer sugars from lignocellulose. Temperature, pH and mixing rate are the main factors of enzymatic hydrolysis of lignocellulosic material (Olsson and Hahn-Hägerdal, 1996; Taherzadeh and Karimi, 2007). Other factors that affect yield are substrate concentration, cellulase enzyme loading, and surfactant addition (Sun and Cheng, 2002; Alkasrawi et al., 2003; Börjesson et al., 2007). High substrate concentration may lead to substrate inhibition. Cellulase contributes to the major cost of the lignocellulosic ethanol technology (Banerjee et al., 2010). Therefore, an efficient pretreatment is to be selected to decrease cellulose crystallinity and to remove lignin to the maximum extent, so that hydrolysis time as well as cellulase loading will be minimized (Eggeman and Elander, 2005). Surfactants modify the cellulose surface by adsorbing lignin onto surfactant and thus the surfactant prevents the enzyme from unproductive binding with lignin and lowers enzyme loading (Eriksson et al., 2002).

Several studies have been reported on the conversion of cellulosic biomass to sugars by enzymatic hydrolysis. Belkacemi and Hamoudi (2003) studied enzymatic hydrolysis of corn stalk hemicellulose at 30 °C and pH 5. Saccharification was 90% and sugar was released after 10 h. Chen et al. (2008) studied enzymatic hydrolysis of maize straw using cellulase from *T. reesei* ZU-02 and cellobiase from *A. niger* ZU-07. Addition of 5 g/L Tween 80 improved hydrolysis yield by 7.5%. Borjesson et al. (2007) reported that PEG addition increased the enzymatic conversion of soft lignocellulose from 42% to 78% at 16 h where optimum hydrolysis temperature was 50 °C. Xu et al. (1998) presented that *T. reesei* decomposed 68.21% of alkali pretreated rice straw whereas 73.96% conversion was obtained from alkali assisted photocatalysis of rice straw after enzymatic hydrolysis. Alkaline peroxide pretreated wheat straw showed 96.75% yield after enzymatic hydrolysis whereas atmospheric autocatalytic or ganosolv illustrated wet wheat straw gave above 75% yield (Saha and Cotta, 2006).

Bioethanol fermentation

Yeast diversity and metabolism

Yeast, as other heterotrophic organisms, have the anabolism coupled with catabolism. In one hand, the oxidation of organic molecules, as sugars, yields adenosine 5-triphosphate (ATP) that, in turn, is used as an energy resource for the cell. On the other hand, such organic molecules can also be used as building blocks or to generate intermediary compounds for the synthesis of other compounds, some of which with high commercial value (Faria-Oliveira et al., 2015).

Following uptake by the hexose transporters, glucose enters the glycolytic pathway in order to be metabolized to pyruvate exhibited in Figure 3. steps from glucose to pyruvate where by the production of energy in the form of ATP is coupled to the generation of intermediates and reducing power in the form of NADH for biosynthetic pathways. The phosphorylation of glucose to glucose-6-phosphate, requiring ATP, is the initial step of glycolysis, by the action of the hexokinases and the glucokinase, which are linked to high-affinity glucose uptake. The glucose-6-phosphate is then isomerized to fructose-6-phosphate by the phosphoglucose isomerase, encoded by *PGI* 1 gene. The next step, done by the phosphofructokinase, also requires energy. The fructose-6-phosphate molecule is converted into fructose 1,6-biphosphate through the transfer of inorganic phosphate from ATP. In turn, yeast aldolase (fructose 1,6-bisphosphate) is responsible for the reversible cleavage of fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Faria-Oliveira et al., 2015).

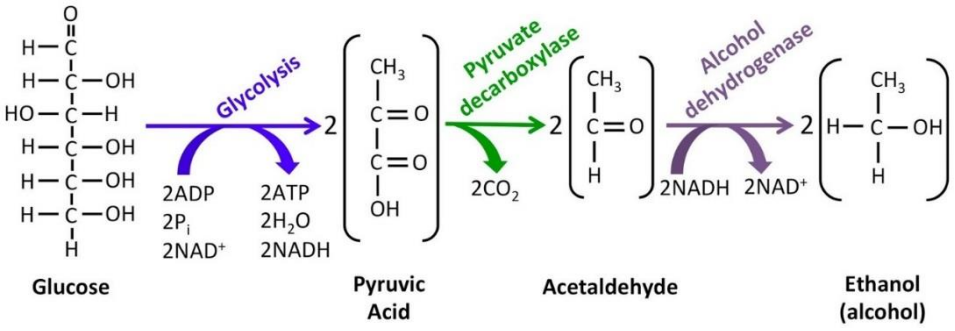


Figure 3 Glycolysis and alcoholic fermentation steps on *saccharomyces cerevisiae* (Faria-Oliveira et al., 2015)

These two resulting compounds can be interconverted, in a reversible way, by the action of the triosephosphate isomerase. Glyceraldehyde 3-phosphate is further metabolized to ultimately yield pyruvate, while some of the dihydroxyacetone phosphate follows gluconeogenesis. This step is fundamental for the osmotic and redox homoeostasis, as the dihydroxyacetone can be converted to glycerol yielding NAD^+. Glyceraldehyde 3-phosphate is first oxidized by NAD^+ and then phosphorylated under the catalysis of the 3-phosphate dehydrogenase. The resulting 1,3-diphosphoglycerate is, in turn, converted to 3-phosphoglycerate by the action of phosphoglycerate kinase, yielding 1 molecule of ATP. The enzyme phosphoglycerate mutase promotes the relocation of the phosphate group from C_3 to C_2, allowing the dehydration by the enolase, resulting in the phosphoenolpyruvate. Then the pyruvate kinase converts this highly energetic molecule to pyruvate, yielding a second molecule of ATP (Faria-Oliveira et al., 2015).

Process configurations for ethanol production

Bioethanol fermentation is carried out to convert these monomeric sugars into alcohols using yeast or bacteria. Four process configurations for ethanol production are possible based on the degree to which the above mentioned steps are consolidated as manifested in Figure 4 (Devarapalli and Atiyeh, 2015).

(i) In Separate hydrolysis and fermentation (SHF) configuration, the enzyme production, hydrolysis of biomass, hexose and pentose fermentation are carried out in separate reactors. In SHF, hydrolysis and fermentation can occur at their optimum conditions. However, the accumulation of glucose and cellobiose during hydrolysis inhibit the cellulases and reduce (Devarapalli and Atiyeh, 2015).

(ii) The annoyance of SHF led to the advancement of Simultaneous saccharification and fermentation (SSF) process. In SSF, both cellulose hydrolysis and hexose fermentation occur in the same reactor. However, SSF process has some limitations. In SSF, the rate of enzyme production limits the rate of alcohol production. In addition, cellulases used for hydrolysis and the fermenting microorganisms usually have different optimum pH and temperature conditions. It is important to have compatible conditions for both the enzyme and the microorganism. Another issue with

SSF is that most microorganisms used for fermentation of glucose cannot utilize xylose, a hemicellulose hydrolysis product (Devarapalli and Atiyeh, 2015).

(iii) In simultaneous saccharification and co-fermentation (SSCF) process, glucose and xylose are co-fermented in the same reactor. Strains of *Saccharomyces cerevisiae* and *Zymomonas mobilis* are genetically engineered to co-ferment both glucose and xylose (Devarapalli and Atiyeh, 2015).

(iv) Another method of process integration is the consolidated bioprocessing (CBP), in which one single microorganism is used for hydrolysis and fermentation steps. This potentially reduces the capital costs and increases process efficiency. However, microorganisms which can both produce enzymes for hydrolysis of biomass and then ferment released sugars are still in the early development stage (Devarapalli and Atiyeh, 2015).

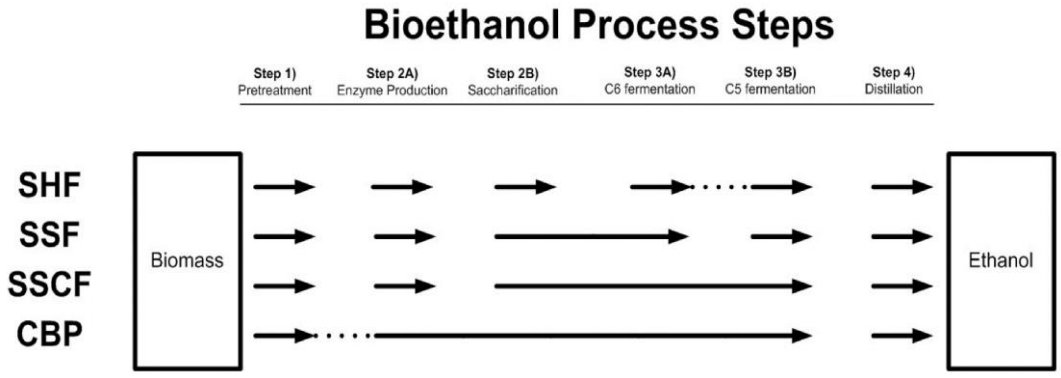


Figure 4 Bioethanol lignocellulosic biomass process configurations (i) separate hydrolysis & fermentation (SHF) (ii) simultaneous saccharification & fermentation (SSF) (iii) simultaneous saccharification & co-fermentation (SSCF) (iv) consolidated bioprocessing (CBP) (Devarapalli and Atiyeh, 2015)

The ethanol production after the pretreatment hinges on percentage of sugar concentration. Table 5 explained the effect of some treatments on the ethanol recovery from the dissimilar substrates. Obvious that same types of pretreatment have valuable difference on diverse types of crops e.g., using H_2SO_4 on sugarcane leaf litter 3.35 g/L and Wheat straw 19 g/L. The ethanol recovery mainly depends on type of crops rather than pretreatment used. It also makes worth to notice that every crop need to have more optimum pretreatment method in order to have maximum ethanol

recovery e.g., sugarcane leaf litter gives lower ethanol recovery if H_2O_2 is used in place of H_2SO_4 revealed in Table 5 (Singh et al., 2014).

Table 5 Ethanol potential after different pre-treatment adopted from (Singh et al., 2014)

Substrate	Treatment	Ethanol recovery (g/L)	References
Rice straw	Dilute H_2SO_4	6.5–11.35	(Karimi et al., 2006)
Sugarcane leaf litter	H_2O_2	1.30	(Dawson and Boopathy, 2007)
Sugarcane leaf litter	H_2SO_4	3.35	(Dawson and Boopathy, 2007)
Waste cotton	H_2SO_4	14.2	(Yu and Zhang, 2003)
Wheat straw	H_2SO_4	19	(Saha BC et al., 2005a,b)
Agave	HCl	7.4	(Hernandez-Salas et al., 2009)
Sugarcane bagasse	HCl	4.7	(Hernandez-Salas et al., 2009)
Agave	Alkaline-enzymatic	6.6	(Hernandez-Salas et al., 2009)
Sugarcane bagasse	Alkaline-enzymatic	12.9	(Hernandez-Salas et al., 2009)

Comparison between the two main fermentation techniques.

The processes usually employed in the fermentation of lignocellulosic hydrolysate are simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF). Conventionally or traditionally the SHF process has been employed but SSF is superior for ethanol production since it can improve ethanol yields by removing end product inhibition and eliminate the need for separate reactors. It is also cost effective but difference in optimum temperature conditions of enzyme

for hydrolysis and fermentation poses some limitations (Bjerre et al., 1996; Hamelinck et al., 2005; Das Neves et al., 2007). The higher ethanol yield coefficient from SSF would be partially due to more conversion of xylose to xylitol under the SSF conditions (Buaban et al., 2010). A comparative study between the two processes (SHF and SSF) is attested in Table 6.

Table 6.Comparison between the two main fermentation techniques

Fermentation process	Features and advantages	Limitations	References
Simultaneous saccharification and fermentation	Low costs Higher ethanol yields due to removal of end product inhibition of saccharification step. Reduces the number of reactors required.	Difference in optimum temperature conditions of enzyme for hydrolysis and fermentation.	(Bjerre et al., 1996; Hamelinck et al., 2005; Das Neves et al., 2007; Balat et al., 2008)
Separate hydrolysis and fermentation	Each step can be processed at its optimal operating conditions. Separate steps minimize interaction between the steps.	End product inhibition minimizes the yield of ethanol. Chance of contamination due to long period process.	(das Neves et al., 2007; Balat et al., 2008; Sanchez and Cardona, 2008)

Studies have appeared that SSF is a better alternative to SHF (Bjerre et al., 1996; Balat et al., 2008). Xylose consumption during fermentation in SHF may be due to the inhabitance of toxic compounds which inhibit the growth and the microorganism fermentation activity (Buaban et al., 2010). The hindrance of SSF can be removed by using thermo-tolerant microorganisms like *Kluyveromyces marxianus* which has been developed to withstand the higher temperatures needed for enzymatic hydrolysis (Bjerre et al., 1996).

A part from SSF or SHF, the available alternatives are consolidated bioprocessing (CBP) and simultaneous saccharification and co-fermentation (SSCF) (Cardona et al., 2010). In CBP, cellulase production, biomass hydrolysis and bioethanol fermentation are all together carried out in a single reactor (Bjerre et al., 1996). The process is also known as direct microbial conversion (DMC). Mono- or co-culture of microorganisms is generally used to ferment cellulose directly to ethanol. Application of CBP requires no capital investment for purchasing enzyme or its production (Hamelinck et al., 2005; Lynd et al., 2005). Bacteria such as *Clostridium thermocellum* and some fungi including *Neurospora crassa*, *Fusarium oxysporum* and *Paecilomyces* sp. have demonstrated this type of activity. However, CBP is not an efficient process because of poor ethanol yields and long fermentation periods 3 to12 days (Szczodrak and Fiedurek, 1996). In SSCF the co-fermenting microorganisms need to be cooperative in terms of operating pH and temperature (Das Neves et al., 2007). A association of *Candida shehatae* and *S. cerevisiae* were described as suitable for the SSCF process (Das Neves et al., 2007). Sequential fermentation with two different microorganisms in different time periods of the fermentation process for better utilization of sugar has also been employed using *S. cerevisiae* in the first phase for hexose utilization and *C. shehatae* in the second phase for pentose utilization but ethanol yields achieved are not high (Sanchez and Cardona, 2008).

Charpter 3

Materials and methodesexperimental design of the study

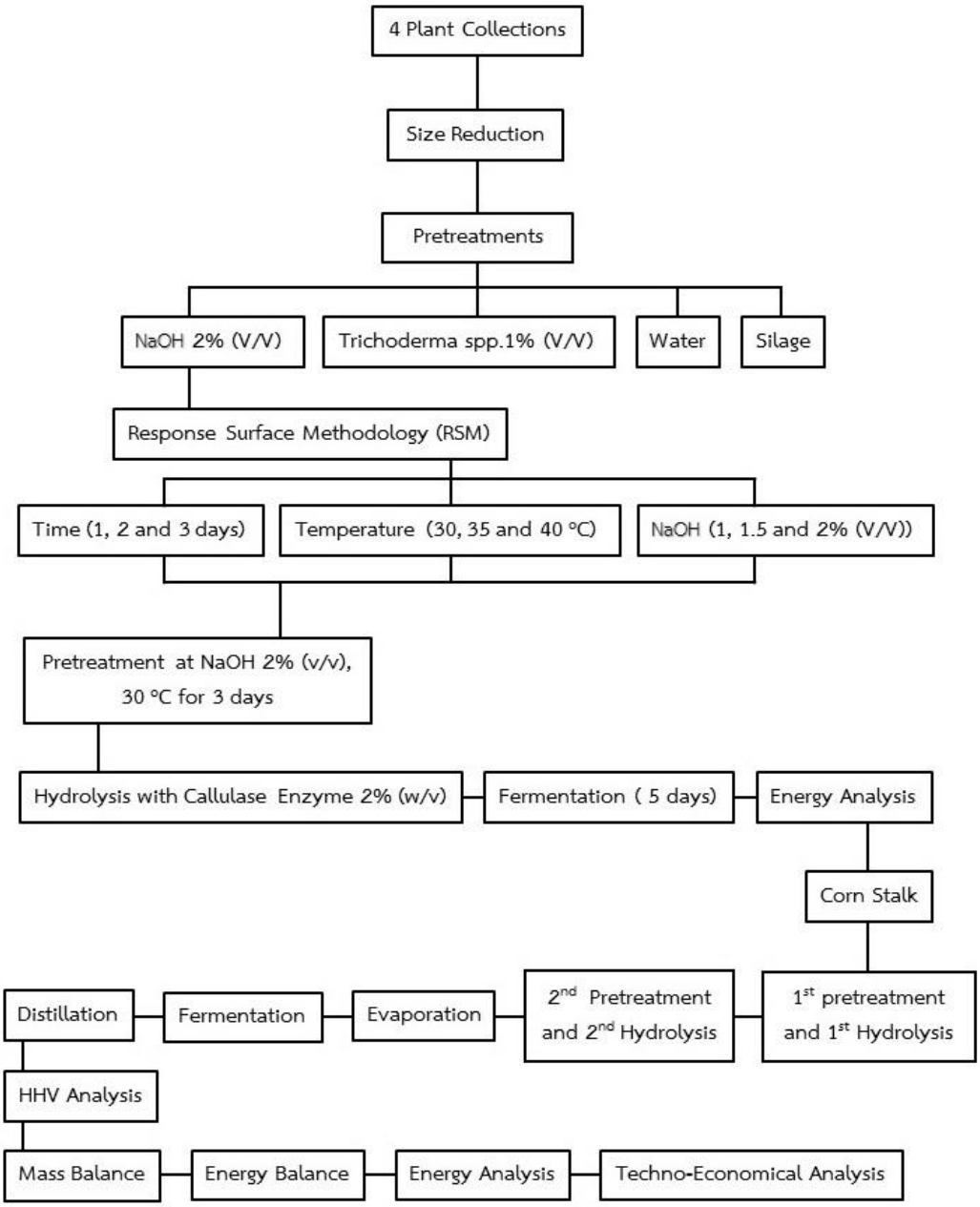


Figure 5 Experimental design of the study

Material preparation

Helianthus annuus L. (Sunflower), *Sorghum bicolor* L. (Sorghum), *Zea mays* L. (Corn) and *Saccharum officinarum* L. (Sugarcane) interpreted in Figure 7 and Figure 8 were used in this study were gathered stalk and leaf during harvest from the farm of

Program in Agronomy, Faculty of Agricultural Production, Maejo University, Chiang Mai, Thailand (18° 8’ 98” N 99°0’ 13” E) disclosed in Figure 6.

Figure 6 The farm of program in agronomy, faculty of agricultural production, Maejo university, Chiang Mai

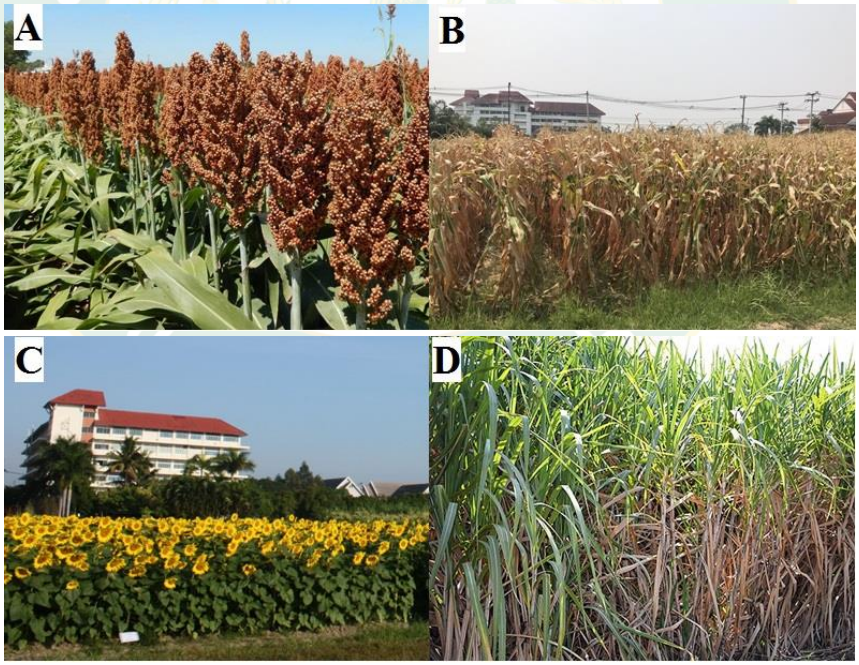


Figure 7 Row materials

It was originally dried by sunlight then grinded to a size of less than 1-4 cm by a rolling machine and blended up to a size of less than 1 mm diameter using a house blender. The final product was collected as powder. Finally, it was dried then at 50 ºC in a hot air oven before being used for the experiments followed Figure 9.

Figure 8 Material collections

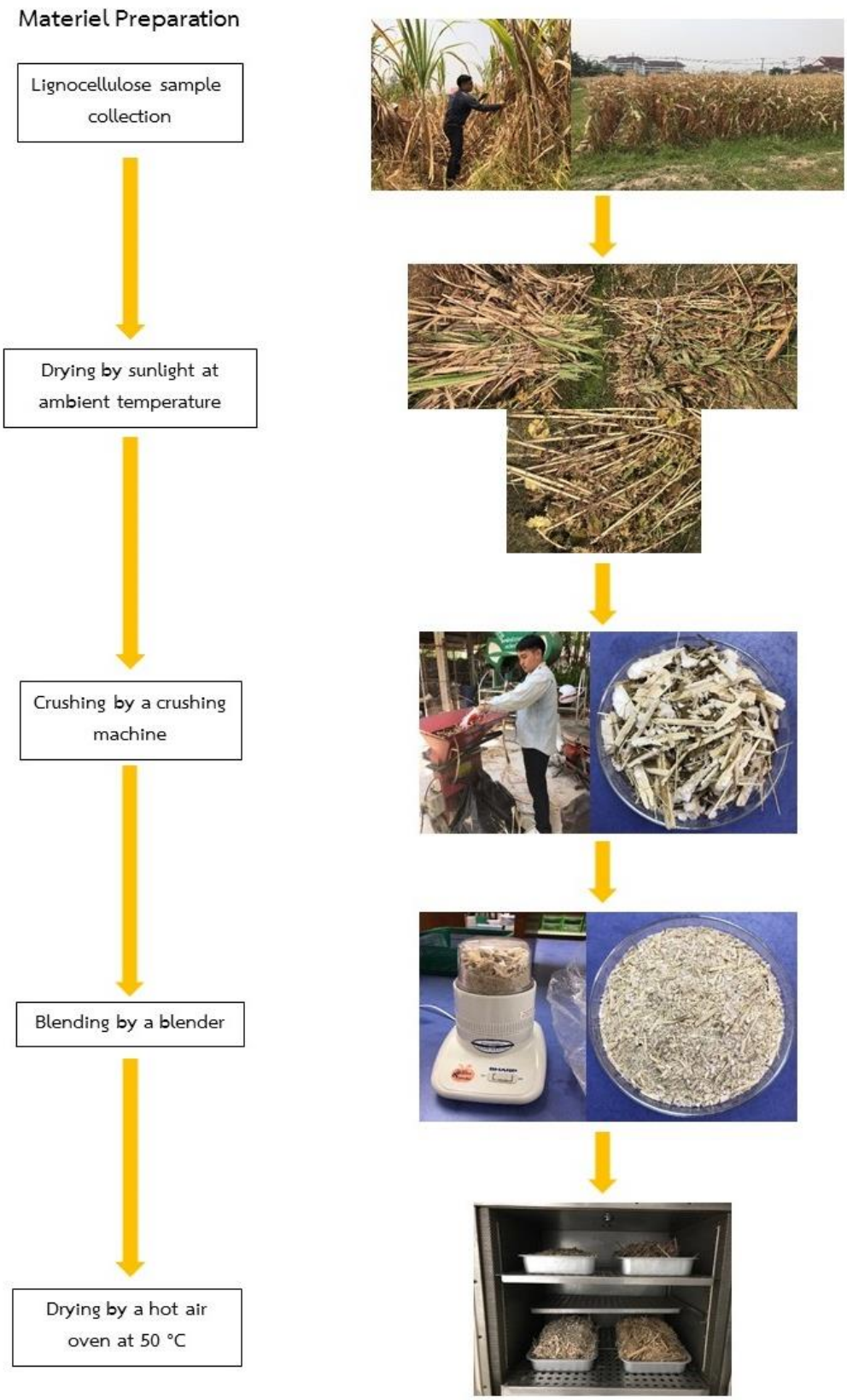


Figure 9 Material preparation (same procedure for 4 plants)

Pretreatment methods

The pretreatment was done by using distilled water (control), silage, 2% NaOH, and 1% of *Trichoderma* spp. from the Institute of Product Quality and Standardization, Maejo University exposed in Figure 10 and with some chemical and biological addition solid to liquid ratio of 1:3 were conducted at room temperature for 3 days, measured for comparison total sugar and reducing sugar analyzed by phenol – sulfuric procedure and DNS method. Before analyzed to take distilled water solid to liquid ratio of 1:4 to the condition sample.

Figure 10 Water, sodium hydroxide and *Trichoderma*

Hydrolysis for fermentable sugar method

The enzymatic hydrolysis of agricultural waste powder from the best condition pretreatment sample is 2% NaOH. Mixture of water and solid substrate. Two percent (2%) of cellulose enzyme was utilized for enzymatic hydrolysis without detoxification before hydrolysis emerged in Figure 11. The pH were adjusted to 5.0 by adding diluted HCl and temperature were adjusted to room temperature overnight. And assayed total sugar and reducing sugar. Then filtered and evaporated on the hot plate to fermentable sugar and checked sugar concentration.

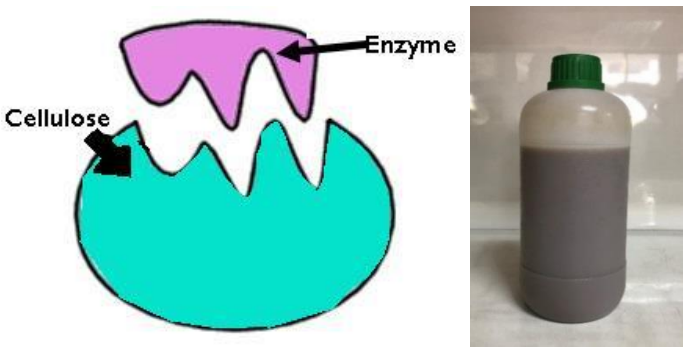


Figure 11 Cellulase enzyme

Medium and microorganism (Yeast) preparation

The microorganism used in this study was *Saccharomyces cerevisiae* TISTR5020 that obtained from Thailand Institute of Scientific and Technology Research (TISTR). *S. cerevisiae* TISTR5020 was maintained on YDP agar containing yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L and agar 15 g/L pH 5.6 by diluted NaOH for the microbial preservation in the aseptic refrigerator at 4 ºC and YDP broth containing and preparing as YDP agar but without agar. The media was sterilized at 121 ºC, 15 psi for 15 min in an autoclave expressed in Figure 12 and Figure 13.

For the inoculum preparation, yeast was inoculated to YDP broth and been put to a shaker with 150 rpm for 24 hat 35ºC. The yeast biomass was harvested by centrifugation at 7,000 rpm for 10 mins at 4 ºC, without centrifuge and used as inoculum excerpted in Figure 14.

Figure 12 Yeast extract, peptone, glucose and agar

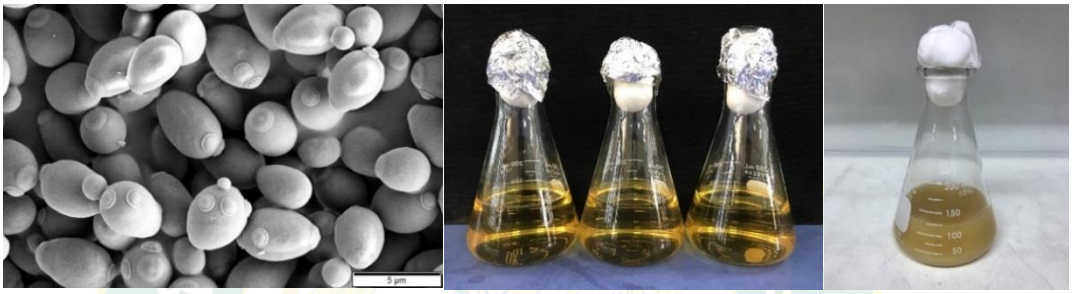


Figure 13 Yeast and yeast medium

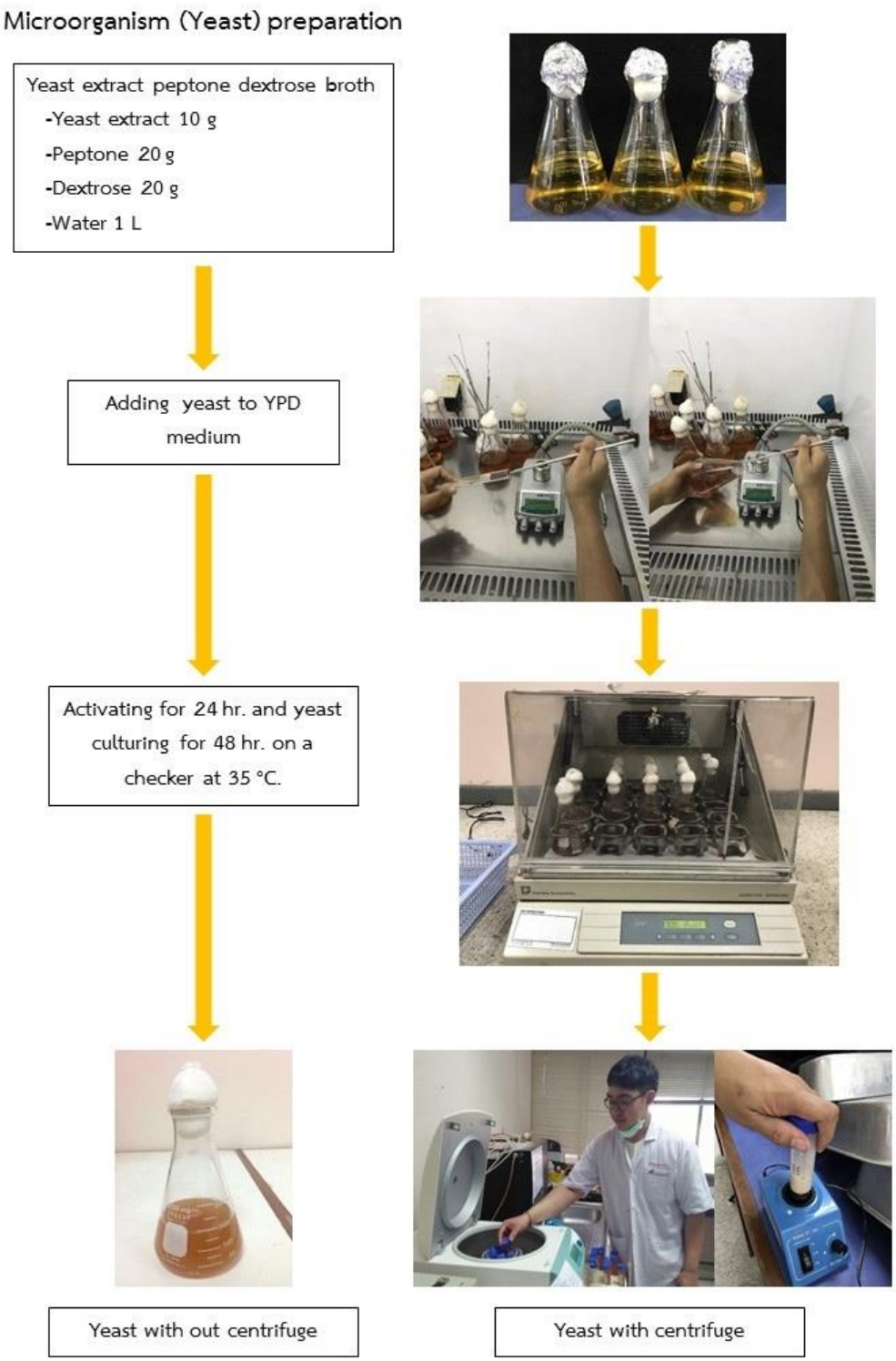


Figure 14 Yeast culturing

Fermentation

Cellulosic hydrolysate, obtained from batch hydrolysis, was utilized as a fermentation medium. The fermentation medium was not sterilized and ethanol fermentations were carried out with 1% of *S. cerevisiae* was centrifuged and 10% of the yeast without centrifuged in reactor at 33-35 ºC with a pH adjusted to 5.6 under anaerobic conditions. It was then incubated for five (5 days) and corrected the samples 1, 3, 5 days. The bioethanol content of each samples were measured using an ebulliometer shown in Figure 15.

Figure 15 Fermenter

Ethanol distillation

After fermentation, it is necessary to separate ethanol from the mixture of samples, water and yeasts, so-call distillation. The principle of this process is simply based on the different volatilities of ethanol from water. With the lower boiling point (78.3 ºC), ethanol evaporates sooner than water and recaptures again via condensation.

Analytical method

Total sugar and reducing sugar will be analyzed before and after the pretreatment process using phenol-sulfuric acid and DNS standard method.

The ethanol content will be using the ebulliometer for measuring in triplicates. The ebulliometer used the different boiling point of distilled water compare to alcohol solutions. A calculating dial will be used to calculate the percentage of ethanol in the solution by comparing two different boiling points from distilled water and the solution.

Statistical analysis

The data will be presented in mean ± sd (standard deviation) done in triplicates. The differences between means will be considered significant when $p<0.05$. All statistical analyses will be performed using the SPSS program version 23.0.

Response surface methodology (RSM) – Central composite design (CCD)

Response surface methodology (RSM) has been widely applied for the optimization of ethanol production from various substrates. RSM explores the relationships between several explanatory operating variables and one or more response variables. Central composite design (CCD) was applied to study process variables. The experimental runs were carried out according to a 29 full factorial designs for the three identified design independent variables, namely, initial NaOH concentration % (A), temperature °C (B), time (days) (C). The behavior of the plant pretreatment process is explained by the following empirical second order polynomial model in Equation 1 (Mäkelä, 2017).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_{ii}^2 + \varepsilon$$

...(Equation 1)

Where Y is the response variable; β_0 is the intercept; β_i, β_{ij} and β_{ii} are coefficients of the linear effect, double interactions; x_i, x_j are the independent variables or factors and ε is error.

Mass balance

Mass balance is intrinsically the law application of mass conservation. The mass of a solitary system remains constant irrespective of the changes occurring within the system. It forms a basis for mass balance calculations. The following Equation 2 describes in words the principle of general material balance applicable to processes both with and without chemical reactions (Lueking and Cole, 2017).

(Acculation) = (Input) - (Output) **...(Equation 2)**

The equation reduces further when there is no accumulation within the system, i.e., steady state. In that case one can write Equation 3 (Lueking and Cole, 2017):

(input) =.(Output) **...(Equation 3)**

This is applicable to a batch process which involves treatment of a given mass of materials in a process after which the products are taken out. If a process is operated such that, over long periods, continuous streams of materials enter into the processing unit and continuous streams leave the same then it is called a continuous process. In such a process, one is concerned with the rate of input and rate of output of materials. If the continuous process runs at steady state, then the chemical compositions of the input materials and output materials remain unchanged and there can be no accumulation within the system either. In such a situation, the material balance equation is written as:

(Rate of output of materials from the system) **...(Equation 4)**

Energy balances

Energy takes many forms, such as heat, kinetic energy, chemical energy, potential energy but because of interconversions it is not always easy to isolate separate constituents of energy balances. However, under some circumstances certain aspects predominate. In many heat balances in which other forms of energy are insignificant; in some chemical situations mechanical energy is insignificant and in some mechanical energy situations, as in the flow of fluids in pipes, the frictional losses appear as heat but the details of the heating need not be considered. We are seldom concerned with internal energies. Therefore practical applications of energy balances tend to focus on particular dominant aspects and so a heat balance, for example, can be a useful description of important cost and quality aspects of process situation. When unfamiliar with the relative magnitudes of the various forms of energy entering into a particular processing situation, it is wise to put them all down. Then after some preliminary calculations, the important ones emerge and other minor ones can be lumped together or even ignored without introducing substantial errors. With

experience, the obviously minor ones can perhaps be left out completely though this always raises the possibility of error. Energy balances can be calculated on the basis of external energy used per kilogram of product, or raw material processed, or on dry solids or some key component. The energy consumed in food production includes direct energy which is fuel and electricity used on the farm, and in transport and in factories, and in storage, selling, etc.; and indirect energy which is used to actually build the machines, to make the packaging, to produce the electricity and the oil and so on. Food itself is a major energy source, and energy balances can be determined for animal or human feeding; food energy input can be balanced against outputs in heat and mechanical energy and chemical synthesis. In the SI system there is only one energy unit, the joule. However, kilocalories are still used by some nutritionists and British thermal units (Btu) in some heat-balance work. The two applications used in this chapter are heat balances, which are the basis for heat transfer, and the energy balances used in analysis fluid flow (Lueking and Cole, 2017).

Techno-economical comparison of different pretreatment techniques for bioethanol production

This techno-economical study will compare the different technologies for bioethanol production from lignocellulosic materials. This study will be focuses in the technologies used in the experiment part. According to (Chovau et al., 2013), a techno-economic model assess the potential of research developments to reduce the production cost by process designs. Also, it can be used to estimate absolute production cost of bioethanol production from lignocellulosic materials by defined process and plant design assumptions.

Experimental procedure

Experimental I: chemical and biological pretreatment

The chemical and biological pretreatment was carried out in a plastic bag, each bag containing 5 g of dry substrate including sunflower, sorghum and corn stalks and sugar cane leaf. Some includable chemical and biological pretreating were water (control), silage, 2% NaOH, and 1% of *T.* spp. addition solid to liquid ratio of 1:3 was

15 ml. All conditions were conducted at room temperature for 3 days at room temperature (28-30 °C) after pretreatment took the samples to the beakers and added water 20 ml, mixed and corrected extract from dry sample for measuring sugar and selecting the best condition for scale up to ferment bioethanol Figure 16.

Figure 16 Pretreatment

Experimental II: optimization of parameters, experimental range and level of independent variables on temperature, NaOH and time pretreatments

Response surface methodology (RSM) is a combination of statistical and mathematical methods used to select the best experimental conditions requiring the lowest number of experiments in order to get appropriate results .A Central composite design (CCD) with three independent variables was applied to investigate the effect of temperature (ºC), NaOH concentration (%) and time (days) on all 4 plants pretreatment process. A total of 29 experiments were found to be sufficient to calculate the coefficients of the second-order polynomial regression model for three variables. Each variable was investigated at three levels: −1, 0 and +1 as shown in Table 7 and Table 8.

Table 7 Optimization of parameters, experimental range and level of independent variables on temperature, NaOH and time pretreatments

	Range and Level		
	−1	0	+1
Temperature (ºC)	30	35	40
NaOH concentration (%)	1	1.5	2
Time (days)	1	2	3

Table 8 Reducing sugar and total sugar design table

Run	Factor 1	Factor 2	Factor 3
	A: Temperature (°C)	B:NaOH (%)	C: Time (Days)
1	40	1	1
2	40	2	3
3	40	2	1
4	40	1.5	2
5	35	1.5	1
6	35	1.5	3
7	30	1	1
8	35	1.5	2
9	35	1.5	1
10	35	1	2
11	35	1.5	1
12	40	1.5	2
13	30	1.5	2
14	30	1	3
15	35	1.5	3
16	35	2	2
17	35	1.5	2
18	35	2	2
19	35	1.5	2
20	40	1.5	2
21	30	1.5	2
22	35	1.5	3
23	30	1.5	2
24	35	2	2
25	30	2	1
26	30	2	3
27	35	1	2
28	35	1	2
29	40	1	3

Experimental III: bioethanol fermentation from pretreatment with 2% NaOH and hydrolysis with 2% cellulase enzyme.

All 4 plants released highest total sugar and reducing sugar from 2% NaOH pretreatment, using 50 g of dry materials mixed with 2% NaOH 150 ml in a plastic bag presented in Figure 17 followed condition from Experiment II after pretreatment added water 200 ml, corrected extract from dry sample for measuring sugar.

Figure 17 NaOH pretreatment

The enzymatic hydrolysis of all 4 plant powder from the best condition pretreatment sample is 2% NaOH. Mixture of water and solid substrate. Two percent (2%) of cellulose enzyme was utilized for enzymatic hydrolysis without detoxification before hydrolysis exhibited in Figure 18. The pH were adjusted to 5.0 by adding diluted HCl and temperature were adjusted to room temperature (28-30 °C) overnight. And assayed total sugar and reducing sugar. Then filtered and evaporated until 100 ml on the hot plate manifested in Figure 19 to fermentable sugar and checked sugar concentration.

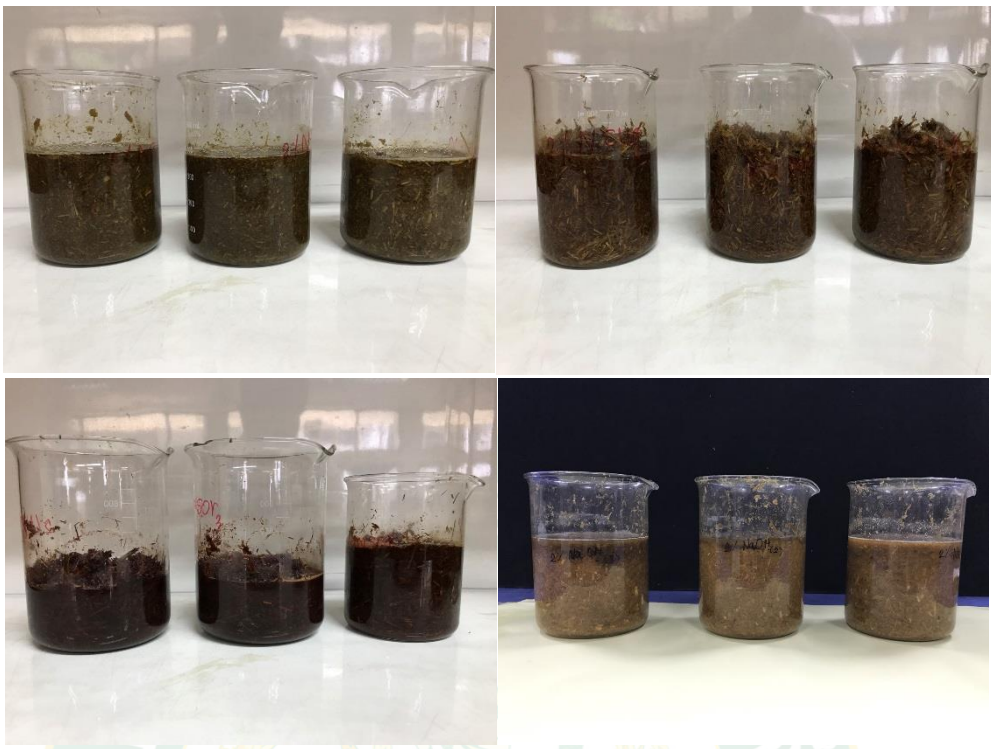


Figure 18 Cellulase enzyme hydrolysis

Figure 19 Filtering and evaporation

Cellulosic hydrolysate, obtained from batch hydrolysis, was utilized as a fermentation medium. The fermentation medium was not sterilized and ethanol fermentations were carried out with 1% of *S.* cerevisiae was centrifuged at 33-35 ºC with a pH adjusted to 5.6 under anaerobic conditions. It was then incubated for five (5 days) and corrected the samples 1, 3, 5 days. The bioethanol content of each samples were measured using an ebulliometer for selecting the beat plant can produce bioethanol.

Experimental IV: scale up on bioethanol production and from corn stalk for distillation

Corn stalk is plant released highest total sugar, reducing sugar and bioethanol from 2% NaOH pretreatment, using 1 kg of dry material mixed with 2% NaOH 3 L in a plastic bag followed condition from Experiment II after the first pretreatment filtered for collecting sugar extract, added water 4 L and adjusted pH until 5.0 by diluted HCl, Added 2% cellulose enzyme followed Experiment III, corrected extract from the first hydrolysis, then filtered and did the second pretreatment and hydrolysis likes the first step but used material from the first step, then finished to filter, mixed every steps to evaporate on the hot plate to 2 L and checked reducing sugar all of steps reveled in Figure 20, Figure 21 and Figure 22.

Figure 20 The first pretreatment and hydrolysis

Figure 21 The second pretreatment and hydrolysis

Figure 22 Evaporation

All of steps without detoxification before fermentation, obtained from batch hydrolysis, was utilized as a fermentation medium. The fermentation medium was not sterilized and ethanol fermentations were carried out with 10% of *S.* cerevisiae without centrifuged at 33-35 ºC with a pH adjusted to 5.6 under anaerobic conditions. It was then incubated for three days and corrected the sample. The bioethanol content of each samples were measured using an ebulliometer attested in Figure 23 before and after the distillation by a distillator and after distillation bioethanol was checked high heat value (HHV) by a bomb calorimeter.

Figure 23 Distillation

Chapter 4

Results and discussion

Characteristics and composition of the raw materials

Lignocelluloses are three-dimensional nanocomposites and a dynamic mixture of multifunctional components. Compositional analysis is not enough to investigate the effects of a pretreatment on a lignocellulose. For instance, it is not enough to know how much lignin has a biomass; it is also important to know where the lignin is located and how it interacts with the other components, e.g., celluloses and hemicelluloses. On the other hand, lignin re-localization and cell wall delamination by pretreatments are likely to be as important as lignin removal in the improvement of lignocelluloses hydrolysis.

The composition of the sunflower, sorghum and corn stalks and sugarcane leaf used in the present study is presented in Table 9. Sunflower stalk consisted of 22.3% lignin, 32.0% cellulose, 18.7% hemicellulose and extractives 8.1%. Sorghum stalk consisted of 9.9% lignin, 38.2% cellulose, 33.0% hemicellulose, extractives 15.8% and 3.1% ash. Sugarcane leaf consisted of 9.39% lignin, 44.78% cellulose and 27.38% hemicellulose. Corn stalk consisted of 28.0% lignin, 30.0% cellulose, 26.1% hemicellulose, extractives 28.0% and 4.9% ash.

After pretreatment in theory lignin will decrease and a cellulose was increasing. The increasing of cellulose after chemical pretreatments were reported by (Kang et al., 2013; Kim et al., 2016). A 16.6% increase in cellulose was recorded by (Sindhu et al., 2011) after the acidic pretreatment of sugarcane tops. The increase in lignin and cellulose after pretreatment is a phenomenon linked to the high solubilization of the hemicellulose fraction. Inorganic salts play a key role in the breakage of ether bonds between xylan polymers thus resulting in substantial hemicellulose solubilization (Kamireddy et al., 2013).

Table 9 The main components of the raw materials (on a dry basis).

Plants	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Extractives (%)	Ash (%)	Reference
Sunflower Stalk	32.000±0.200	18.700±2.400	22.300±0.300	8.100±0.100	-	(Antonopoulou et al., 2016)
Sorghum Stalk	38.200±0.200	33.000±0.100	9.900±0.100	15.800±0.100	3.100±0.200	(Xu et al., 2017)
Sugarcane leaf	44.780±0.000	27.380±0.000	9.390±0.000	-	-	(Moodley and Kana, 2017)
Corn Stalk	30.000±0.100	26.100±0.100	11.000±0.100	28.000±0.300	4.900±0.200	(Xu et al., 2017)

Bioethanol production from sunflower stalk

The products from chemical and biological pretreatment

This results in huge accumulation of sunflower stalks annually which do not find any suitable end use and are generally burnt in the fields causing environmental pollution. Therefore, sunflower stalk is lignocellulos afford a renewable and low cost raw material for bioethanol production. In Figure 24 appeared concentration of total sugar and reducing sugar from three different pretreatments comparing with control (without any pretreatment) of four materials. Sunflower stalk: the lowest amount of total sugar and reducing sugar were 8.860±1.373 and 2.707±0.167 g/L observed from control while the highest amount was 35.544±0.818 and 4.213±0.717 g/L from pretreatment with NaOH 2%. This means that sodium hydroxide affected adequately the structure of material and released more sugar. Total sugar and reducing sugar from pretreatment by silage and 1% Trichoderma spp. were 13.965±3.117, 2.293±0.122 and 20.544±1.701, 3.693±0.482 g/L.

Ruiz et al. (2013) reported Influence of acid pretreatment on sugar production the results in terms of solid, glucose and xylose recovery in the solids and in the liquid fractions obtained after pretreatment. Sugars recovery was calculated as a percentage of sugars present Solid recovery values (g of pretreated solids/100 g starting, dry

material) ranged from 20 to 62% depending on the operational conditions. The experiments performed in the center of the domain resulted in an average solid recovery of 39.2 ± 0.49 g/100 g.in the raw material that remains after pretreatment in solids and prehydrolysates. Experiments have been ordered as a function of increasing combined severity.

The highest value of xylose recovery in prehydrolysates, 79.3%, was found in the experiment performed at 150 °C and 2% sulfuric acid concentration. This recovery compares favorably with other results reported using hydrothermal pretreatments of sunflower stalks. For example, Díaz et al. (2011) found that the highest xylose recovery by LHW pretreatment was 73% and was attained from materials pretreated at 190 °C and the same operational time (5 min) as that employed in this work. Pretreated sunflower stalks by steam explosion resulted in only 27% of xylose recovery in prehydrolysates, as a highest value, obtained from operation at 210 °C, Ruiz et al. (2008). Compared to dilute acid pretreatment reports, Akpinar et al. (2011) attained 50% as a highest recovery of hemicellulosic sugars, corresponding to pretreatment at optimal conditions of 100 °C for 30 min and 4% sulfuric acid concentration.

Sunflower stalk pretreatment with 2% NaOH in this study Figure 24 was similar with research of Yıldız et al. (2016) reported the effect of alkali concentration on the content of sunflower stalk. Hemicellulose and lignin removal from sunflower stalks increased by increasing alkali concentration from 0.5 to 4%. Maximum cellulose recovery was obtained with 2% NaOH solution (91.41%) After the pretreatments by 2% NaOH, enzymatic hydrolysis was applied on recovered solids. It was observed that the saccharifications were increased by the more alkaline concentration and the highest yield of cellulose digestion (98,34%) and glucose recovery (70.20%).

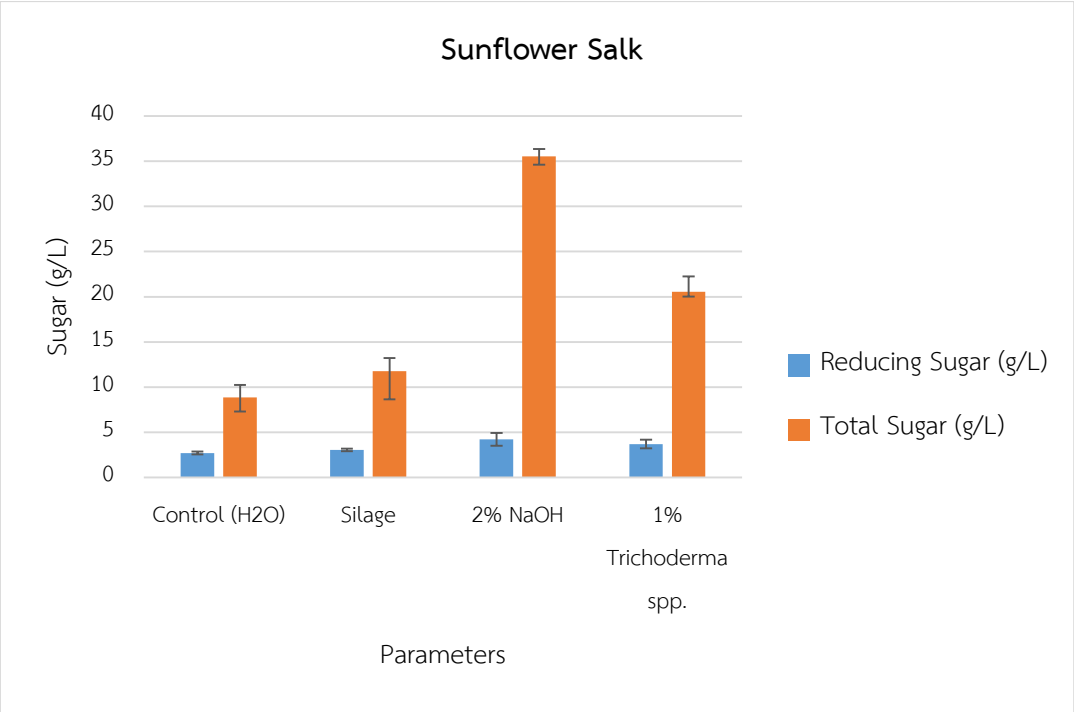


Figure 24 Sugar concentration of sunflower stalk pretreatments

Reponses surface methodology of pretreatments

In this study, the effect of three factors on reducing sugar production from sunflower stalk including temperature, NaOH concentration and time.

RSM development of reducing sugar from sunflower stalk

Model (sunflower stalk : reducing sugar)

All factors were selected as factors in the central composite design. As a response, the reducing sugar production rate was chosen, a total number of 29 experiments were employed for the response surface modeling Table 10, and the order of experiments was arranged randomly. The observed and predicted results for the reducing sugar production from sunflower stalk are also depicted in Table 10.

Table 10 Experimental designs of reducing sugar and predictive values from sunflower stalk

Run	Factor 1	Factor 2	Factor 3	Reducing sugar (g/L)	
	A: Temperature (°C)	B:NaOH (%)	C: Time (Days)	Observed	Predicted
1	40	1	1	1.420	1.410
2	40	2	3	6.040	6.030
3	40	2	1	2.320	2.300
4	40	1.5	2	3.120	3.170
5	35	1.5	1	1.620	1.750
6	35	1.5	3	3.930	3.650
7	30	1	1	1.000	0.987
8	35	1.5	2	3.120	3.000
9	35	1.5	1	1.620	1.750
10	35	1	2	2.380	2.330
11	35	1.5	1	1.930	1.750
12	40	1.5	2	3.230	3.170
13	30	1.5	2	2.960	2.960
14	30	1	3	2.310	2.300
15	35	1.5	3	3.470	3.650
16	35	2	2	3.620	3.700
17	35	1.5	2	3.100	3.000
18	35	2	2	3.710	3.700
19	35	1.5	2	3.020	3.000
20	40	1.5	2	3.120	3.170
21	30	1.5	2	2.840	2.960
22	35	1.5	3	3.500	3.650
23	30	1.5	2	3.010	2.960
24	35	2	2	3.710	3.700
25	30	2	1	2.000	1.980
26	30	2	3	4.210	4.200
27	35	1	2	2.380	2.330
28	35	1	2	2.160	2.330
29	40	1	3	3.040	3.030

The Design-Expert 11 software was used to calculate the coefficients of the second-order fitting equation and the model suitability was tested using the ANOVA test. Therefore, the second-order polynomial equation should be expressed by Equation 5 (conf. Equation 1):

$$\text{Reducing Sugar} = +3.00+0.1072A+0.6885B+0.9538C+0.1244AB+0.2276AC+0.3759BC +0.0666A^2+0.0132B^2-0.3014C^2+0.1494ABC+0.1604A^2B+0.1548A^2C +0.3060AB^2 \quad \textbf{...(Equation 5)}$$

Y= Reducing sugar (g/L)

A= Temperature (°C)

B= NaOH (%)

C= Time (days)

Statistical analysis (sunflower stalk : reducing sugar)

CCD was applied for the optimization of reducing sugar production conditions. The Model F-value of 108.74 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case B, C, AB, AC, BC, C^2, ABC, AB^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 1.49 implies the Lack of Fit is not significant relative to the pure error. There is a 24.20% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good for the model to fit interpreted in Table 11.

The R^2 of 0.9895 in Figure 25 is as close to the Adjusted R^2 of 0.9804. A negative Predicted R^2 implies that the overall mean may be a better predictor of your response than the current model. In some cases, a higher order model may also predict better.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 51.713 indicates an adequate signal. This model can be used to navigate the design space exposed in Table 12.

Table 11 ANOVA for quadratic model of reducing sugar from sunflower stalk

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Remark
					<	
Model	27.82	13	2.14	108.74	0.0001	significant
A-Temperature	0.0689	1	0.0689	3.5	0.0809	
B-NaOH	2.84	1	2.84	144.55	< 0.0001	significant
C-Time	5.46	1	5.46	277.43	< 0.0001	significant
AB	0.1238	1	0.1238	6.29	0.0241	significant
AC	0.4145	1	0.4145	21.07	0.0004	significant
BC	1.13	1	1.13	57.44	< 0.0001	significant
A^2	0.0305	1	0.0305	1.55	0.2323	not significant
B^2	0.0012	1	0.0012	0.0613	0.8078	not significant
C^2	0.6249	1	0.6249	31.76	< 0.0001	significant
ABC	0.1785	1	0.1785	9.07	0.0088	significant
A^2B	0.0882	1	0.0882	4.48	0.0514	not significant
A^2C	0.0822	1	0.0822	4.18	0.059	not significant
AB^2	0.321	1	0.321	16.31	0.0011	significant
Residual	0.2951	15	0.0197			
Lack of Fit	0.0284	1	0.0284	1.49	0.242	not significant
Pure Error	0.2667	14	0.0191			
Cor Total	28.11	28				

Table 12 Fit statistics of reducing sugar from sunflower stalk

Std. Dev.	0.1403	**R^2**	0.9895
Mean	2.89	**Adjusted R^2**	0.9804
C.V. %	4.85	**Predicted R^2**	-0.0358
		Adeq Precision	51.7128

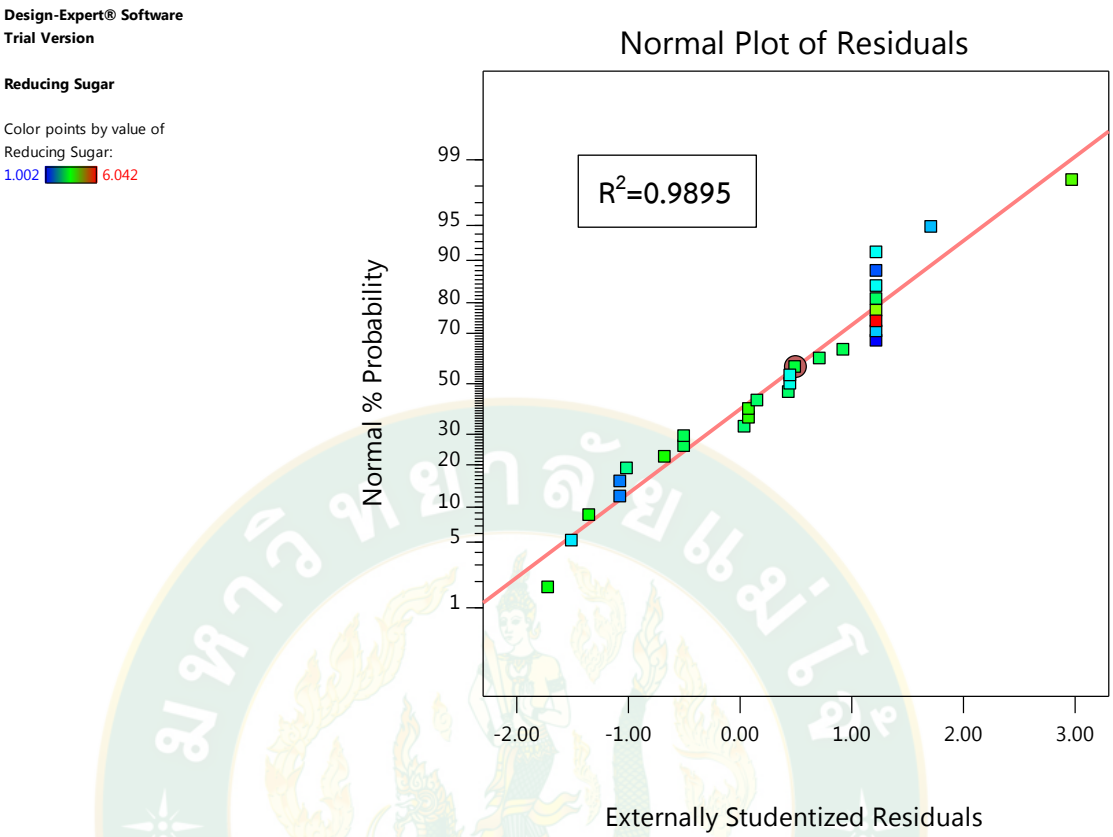


Figure 25 Comparison of predicted and actual value of reducing sugar from sunflower stalk

The effects of model parameters and their Interactions

The Design-Expert 11 software was used to produce three-dimensional (3D) response surfaces and two-dimensional (2D) contour plots. The 3D surfaces and 2D contour plots are graphical representations of the regression equation for the optimization of reaction conditions and are the most useful approach in revealing the conditions of the reaction system. In such plots, the response functions of two factors are presented while all other factors are at the fixed levels. The results of the interactions between three independent variables and the dependent variable are expressed in Figure 26, Figure 27 and Figure 28.

As in Figure26, Figure 27 and Figure 28 depending on the reaction, the temperature, NaOH concentration and time may have a positive or negative effect on the reducing sugar.

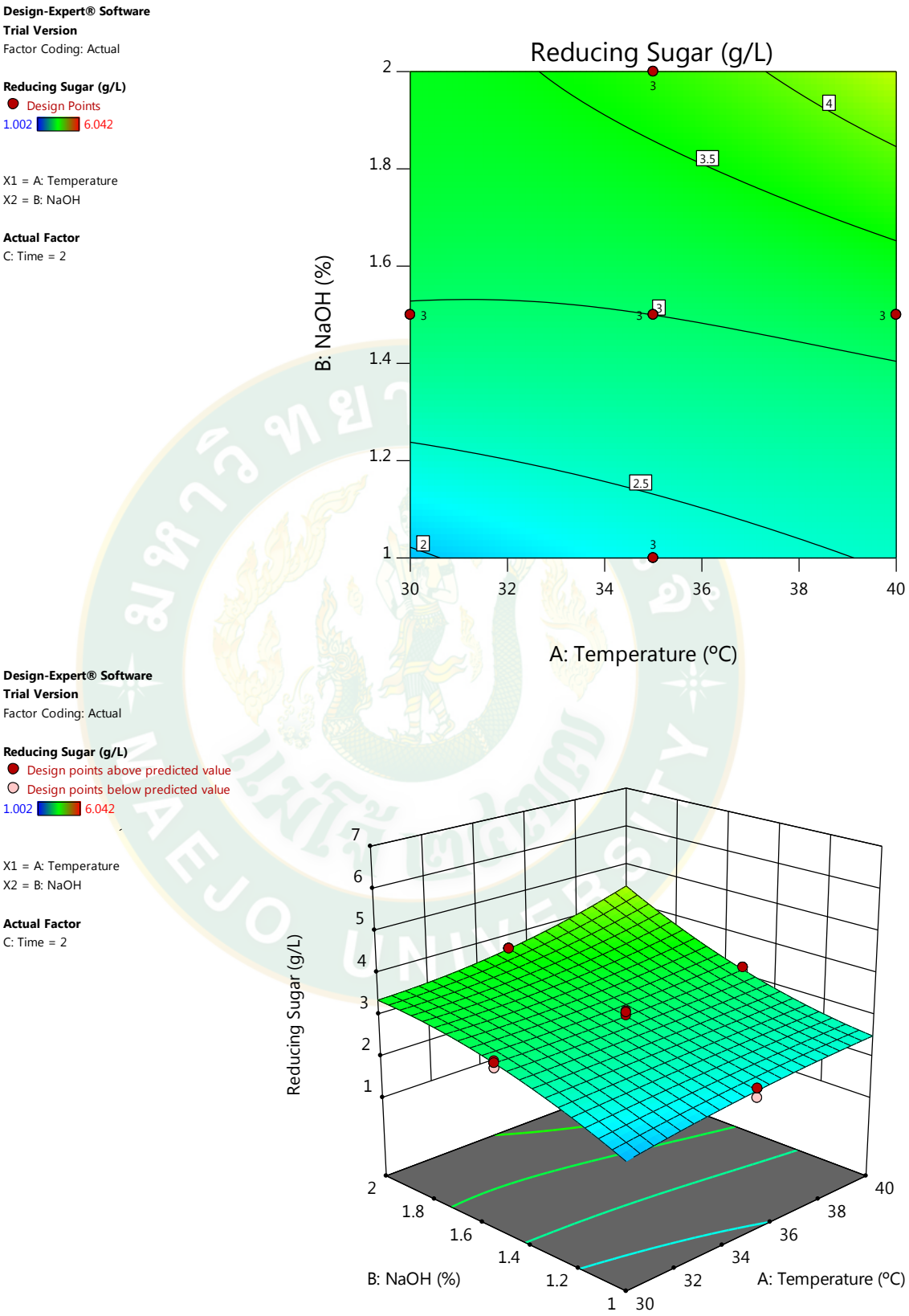


Figure 26 Reducing sugar yield from sunflower stalk (design points above/below predicted value), actual factor (time)

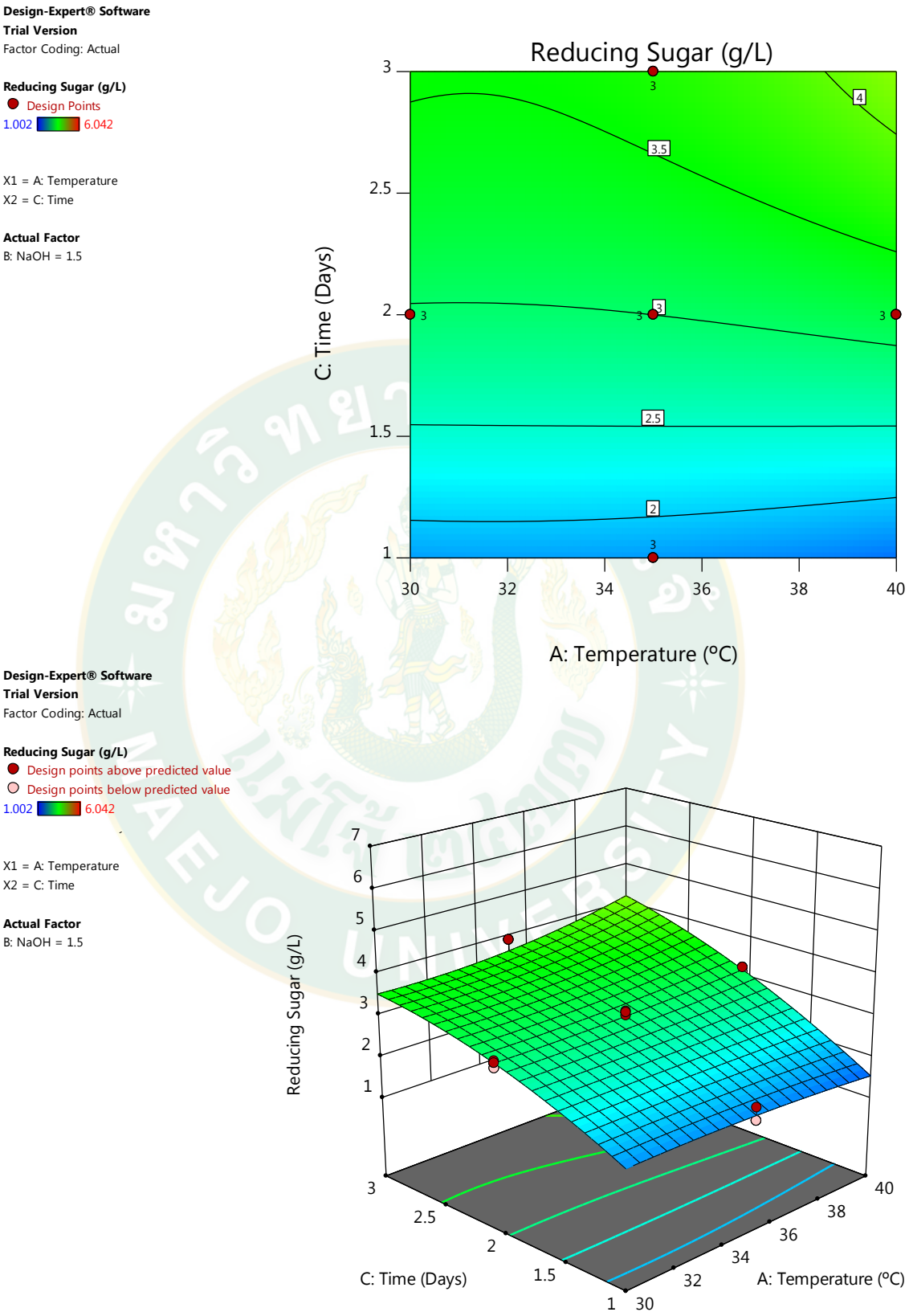


Figure 27 Reducing sugar yield from sunflower stalk (design points above/below predicted value), actual factor (NaOH)

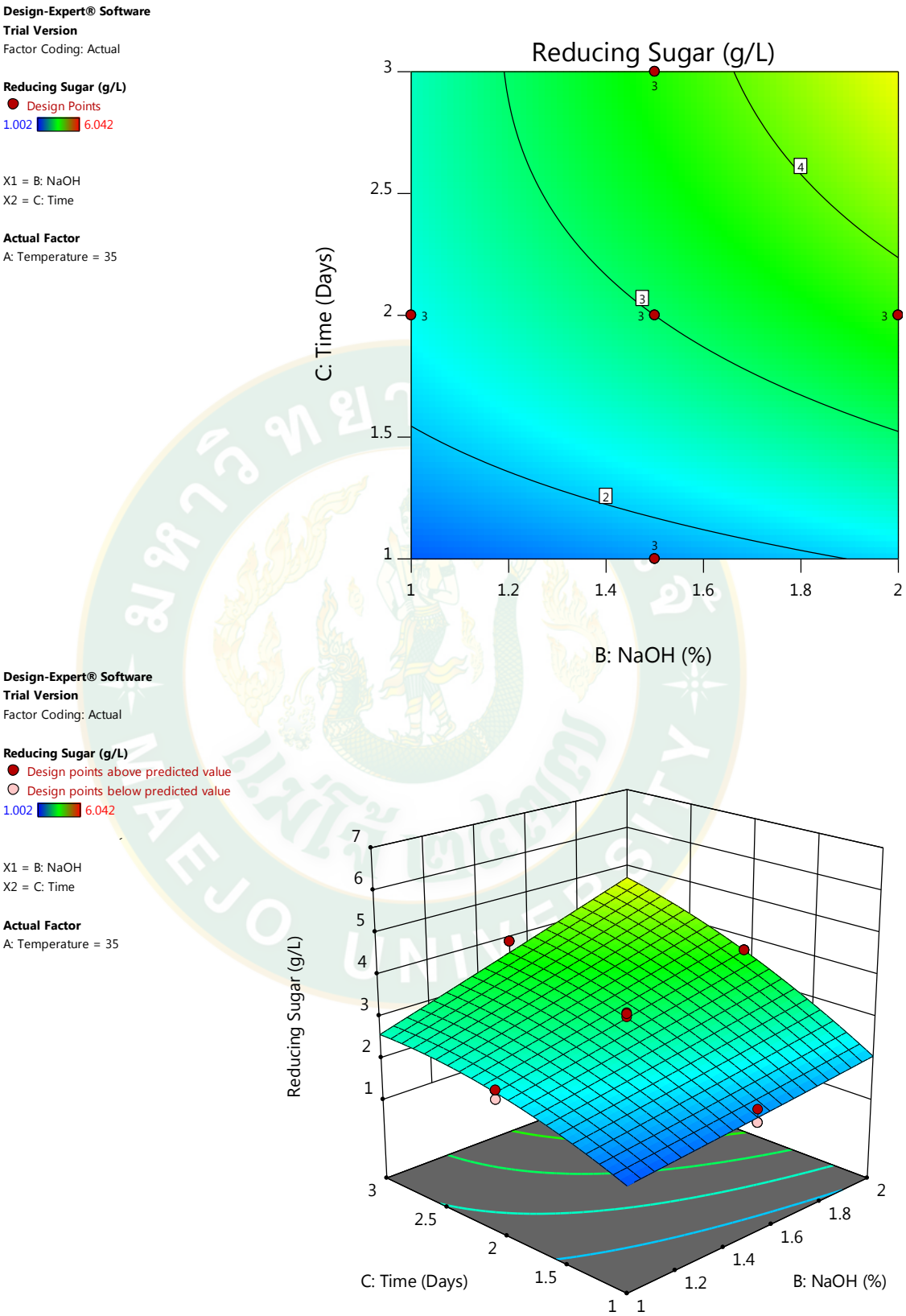


Figure 28 Reducing sugar yield from sunflower stalk (design points above/below predicted value), actual factor (temperature)

Figure 26 exhibited the interaction effect of NaOH and temperature on the reducing sugar production from sunflower stalk. As it can be seen in the plots, there is an increase in the reducing sugar rate with an increase of NaOH concentration, with the maximum rate in the 2% NaOH. On the other hand, the effect of temperature on reducing sugar production from sunflower stalk has not similar trends, regardless of the NaOH concentration. The reducing sugar rate increased slightly with the increase of temperature. It can be concluded from the contour plots that the optimum region of the reducing sugar production from sunflower stalk is in the 2% NaOH.

Figure 27 demonstrated the interaction effect of the time and temperature on the reducing sugar production from sunflower stalk. As can be seen in the plots, the increase of the time leads to an increase in the reducing sugar rate. The time has been increasing degradation rate. We can seen from the contour plots Figure 27 (2D) that the reducing sugar concentration is more than 3 g/L to 4 g/L in the time range of 2–3 days either at a low or high level of temperature. Therefore, it can be concluded that the increasing time does not affect lignocellulose degradation.

Figure 28 described the interaction effect of the time and NaOH concentration on reducing sugar production from sunflower stalk. The contour plots shown that the optimum region for the reducing sugar production rate is in the time range of 3 days and the NaOH concentration is in the range of 2%, respectively.

RSM development of total sugar from sunflower stalk

Model (sunflower stalk : total sugar)

All factors were selected as factors in the central composite design. As a response, the total sugar production rate was chosen, a total number of 29 experiments were employed for the response surface modeling disclosed in Table 13, and the order of experiments was arranged randomly. The observed and predicted results for the total sugar production from sunflower stalk are also depicted in Table 13.

Table 13 Experimental designs of total sugar and predictive values from sunflower stalk

Run	Factor 1	Factor 2	Factor 3	Total sugar (g/L)	
	A: Temperature (°C)	B:NaOH (%)	C: Time (Days)	Observed	Predicted
1	40	1	1	19.340	19.280
2	40	2	3	40.800	40.740
3	40	2	1	29.370	29.320
4	40	1.5	2	30.690	31.070
5	35	1.5	1	25.530	25.840
6	35	1.5	3	37.460	37.090
7	30	1	1	15.660	15.610
8	35	1.5	2	31.490	32.060
9	35	1.5	1	25.230	25.840
10	35	1	2	27.880	28.150
11	35	1.5	1	26.530	25.840
12	40	1.5	2	31.180	31.070
13	30	1.5	2	29.960	30.030
14	30	1	3	31.440	31.390
15	35	1.5	3	37.460	37.090
16	35	2	2	33.790	33.720
17	35	1.5	2	31.130	32.060
18	35	2	2	32.990	33.720
19	35	1.5	2	34.450	32.060
20	40	1.5	2	31.120	31.070
21	30	1.5	2	30.670	30.030
22	35	1.5	3	36.120	37.090
23	30	1.5	2	29.220	30.030
24	35	2	2	34.160	33.720
25	30	2	1	24.880	24.830
26	30	2	3	35.420	35.370
27	35	1	2	28.340	28.150
28	35	1	2	28.000	28.150
29	40	1	3	34.120	34.070

The Design-Expert 11 software was used to calculate the coefficients of the second-order fitting equation and the model suitability was tested using the ANOVA test. Therefore, the second-order polynomial equation should be expressed by Equation 6 (conf. Equation 1):

$$\text{Total Sugar} = +32.06 + 0.5237A + 2.79B + 5.62C + 0.4390AB - 0.0132AC - 1.08BC - 1.51A^2 - 1.13B^2 - 0.5994C^2 + 0.2355ABC + 0.9519A^2B + 0.9428A^2C + 1.50AB^2$$

...(Equation 6)

Y= Total sugar (g/L)

A= Temperature (°C)

B= NaOH (%)

C= Time (days)

Statistical analysis (sunflower stalk : total sugar)

CCD was applied for the optimization of total sugar production conditions. The Model F-value of 80.03 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case B, C, BC, A^2, B^2, AB^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 0.50 implies the Lack of Fit is not significant relative to the pure error. There is a 48.99% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good for the model to fit emerged in Table 14.

The R^2 of 0.9895 in Figure 29 is as close to the Adjusted R^2 of 0.9804. A negative Predicted R^2 implies that the overall mean may be a better predictor of your response than the current model. In some cases, a higher order model may also predict better.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 51.713 indicates an adequate signal. This model can be used to navigate the design space exemplified in Table 15.

Table 14 ANOVA for quadratic model of total sugar from sunflower stalk

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Remark
Model	775.51	13	59.65	80.03	< 0.0001	significant
A-Temperature	1.65	1	1.65	2.21	0.1581	not significant
B-NaOH	46.62	1	46.62	62.54	< 0.0001	significant
C-Time	189.79	1	189.79	254.6	< 0.0001	significant
AB	1.54	1	1.54	2.07	0.1709	not significant
AC	0.0014	1	0.0014	0.0019	0.9659	not significant
BC	9.25	1	9.25	12.41	0.0031	significant
A^2	15.73	1	15.73	21.1	0.0004	significant
B^2	8.71	1	8.71	11.69	0.0038	significant
C^2	2.47	1	2.47	3.31	0.0887	not significant
ABC	0.4437	1	0.4437	0.5952	0.4524	not significant
A^2B	3.11	1	3.11	4.17	0.0592	not significant
A^2C	3.05	1	3.05	4.09	0.0614	not significant
AB^2	7.75	1	7.75	10.4	0.0057	significant
Residual	11.18	15	0.7454			
Lack of Fit	0.3877	1	0.3877	0.5029	0.4899	not significant
Pure Error	10.79	14	0.771			
Cor Total	786.69	28				

Table 15 Fit statistics of total sugar from sunflower stalk

Std. Dev.	0.8634	**R^2**	0.9858
Mean	30.50	**Adjusted R^2**	0.9735
C.V. %	2.83	**Predicted R^2**	0.4832
		Adeq Precision	41.9048

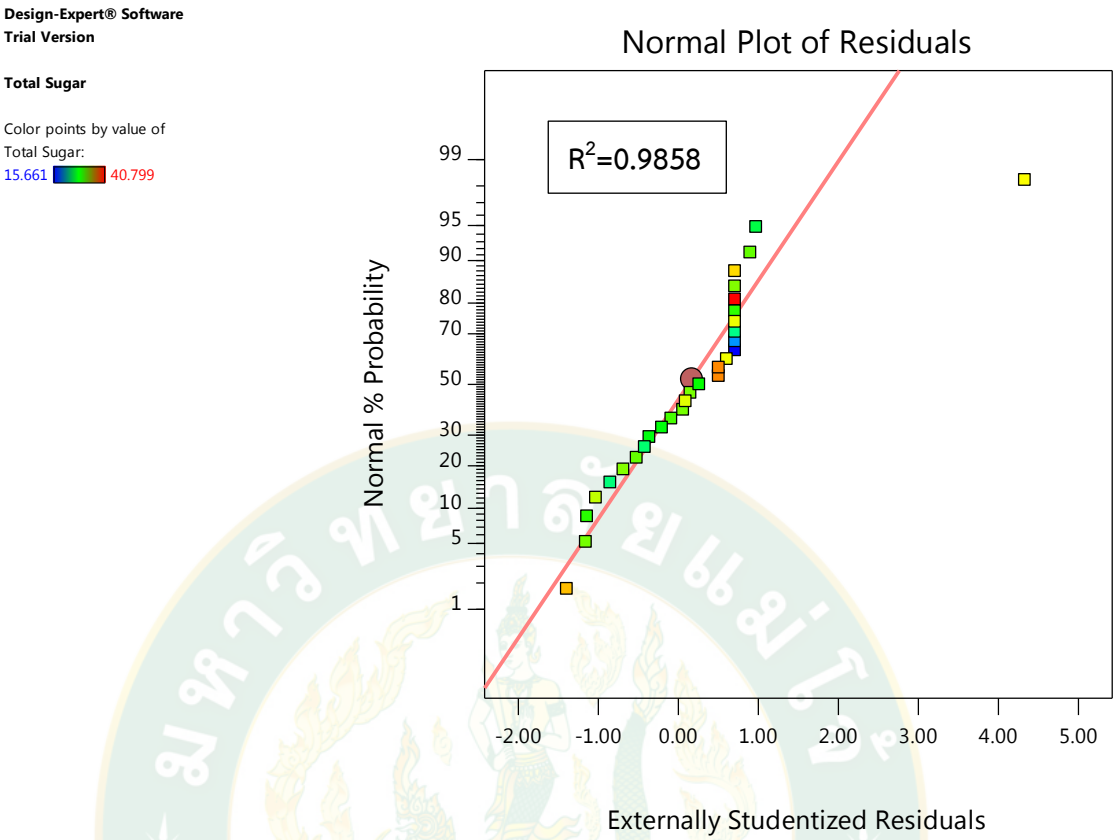


Figure 29 Comparison of predicted and actual value of total sugar from sunflower stalk

The effects of model parameters and their Interactions

The Design-Expert 11 software was used to produce three-dimensional (3D) response surfaces and two-dimensional (2D) contour plots. The 3D surfaces and 2D contour plots are graphical representations of the regression equation for the optimization of reaction conditions and are the most useful approach in revealing the conditions of the reaction system. In such plots, the response functions of two factors are presented while all other factors are at the fixed levels. The results of the interactions between three independent variables and the dependent variable are interpreted in Figure 30, Figure 31 and Figure 32.

As in Figure 30, Figure 31 and Figure 32 depending on the reaction, the temperature, NaOH concentration and time may have a positive or negative effect on the total sugar.

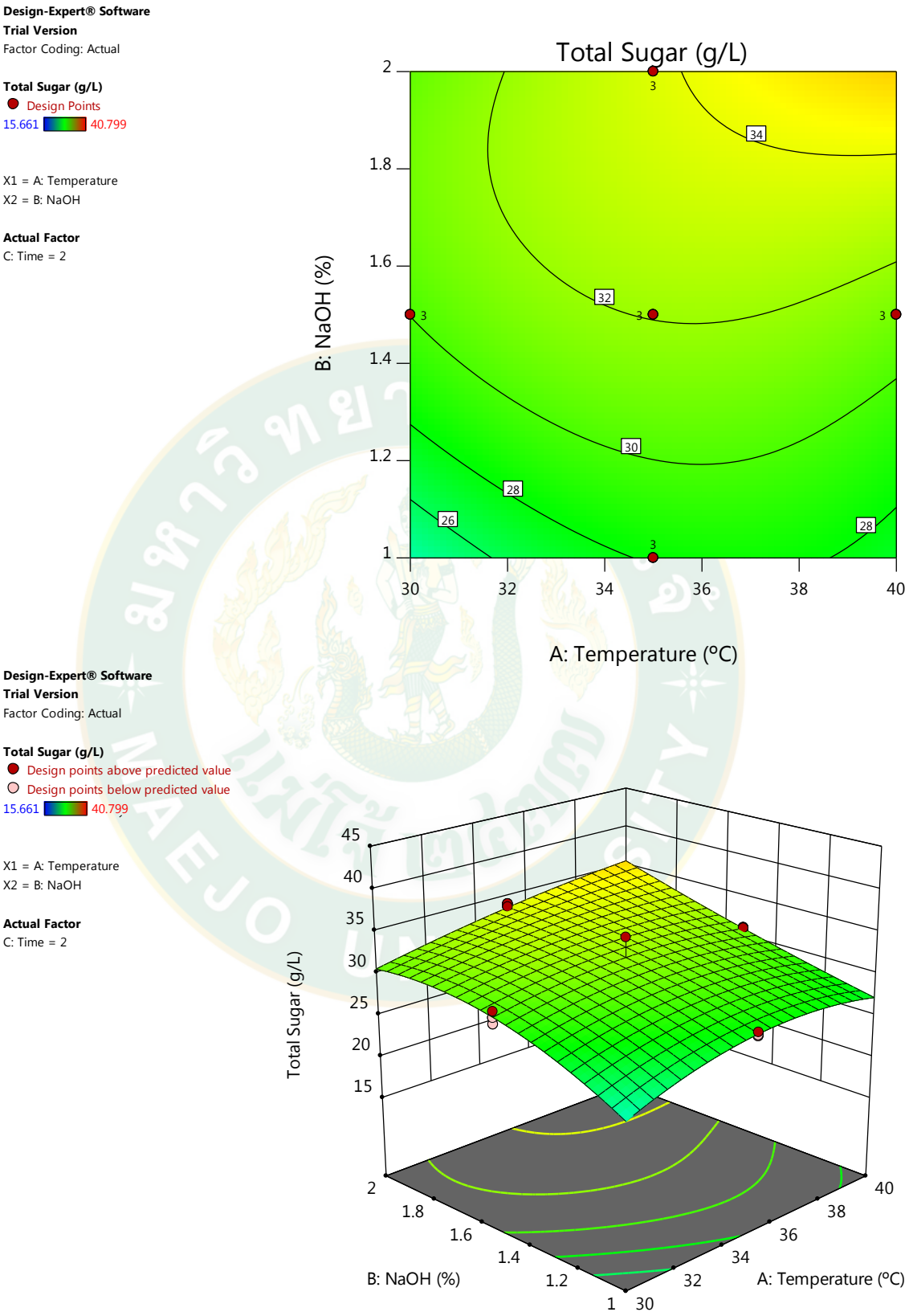


Figure 30 Total sugar yield from sunflower stalk (design points above/below predicted value), actual factor (time)

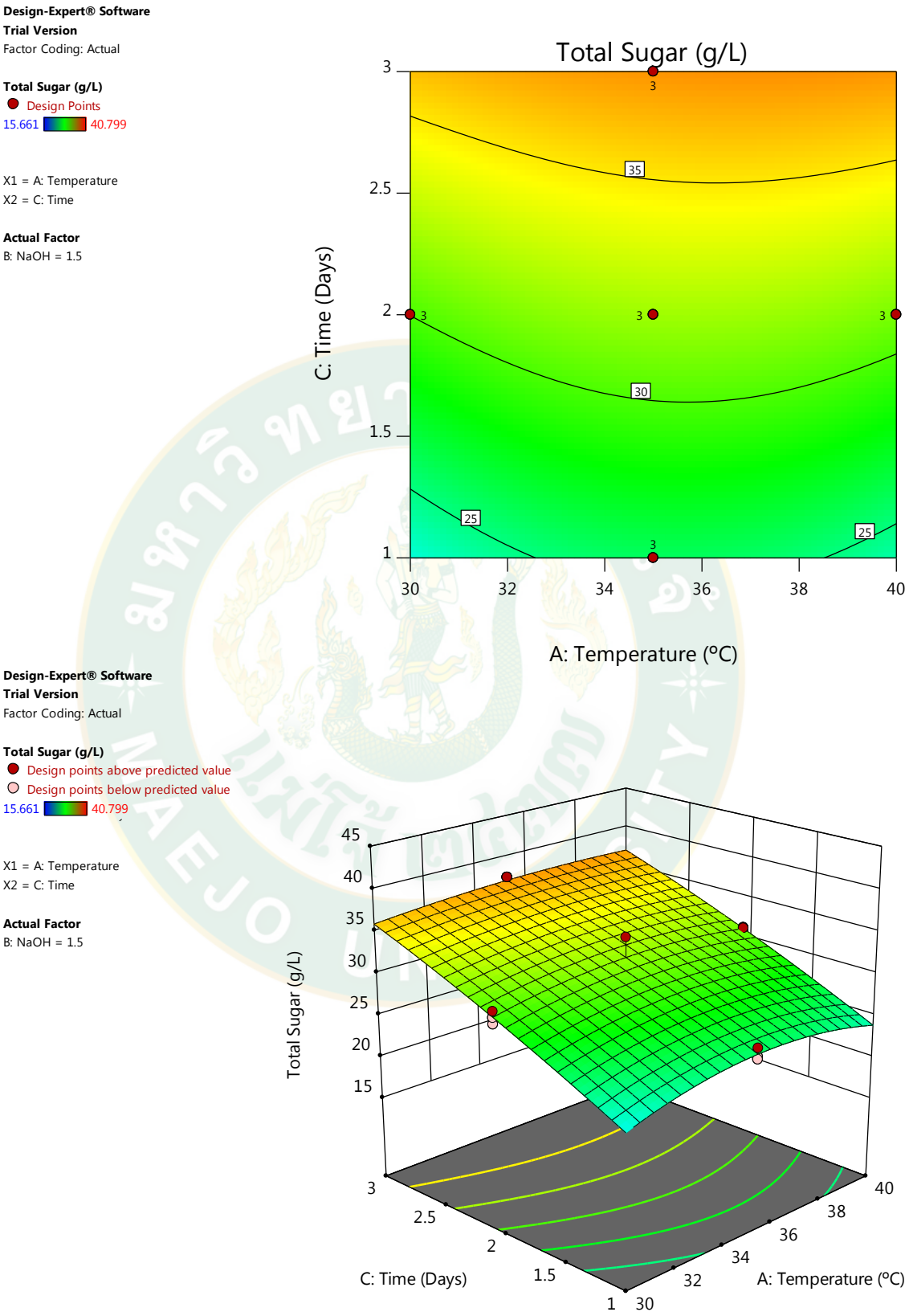


Figure 31 Total sugar yield from sunflower stalk (design points above/below predicted value), actual factor (NaOH)

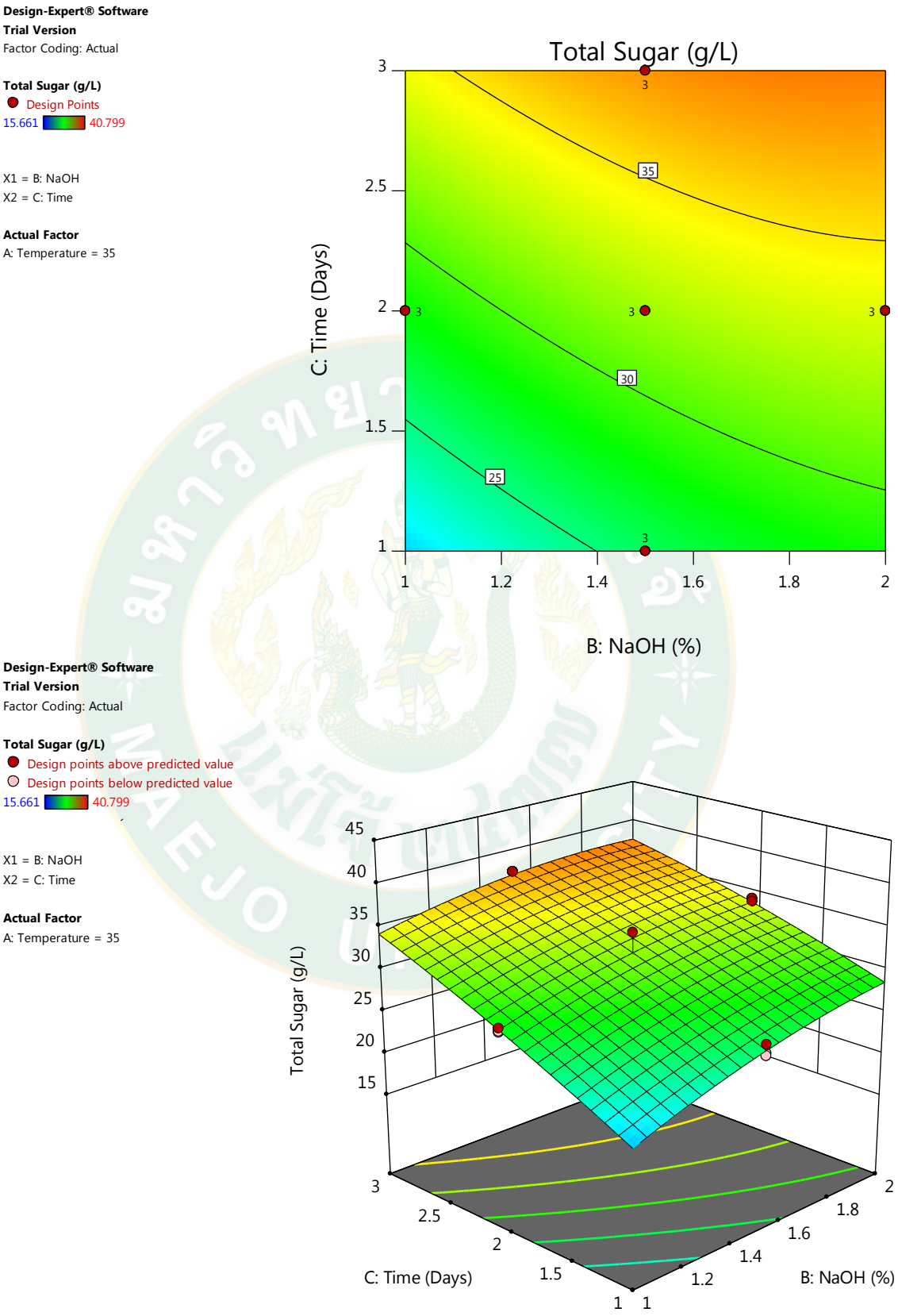


Figure 32 Total sugar yield from sunflower stalk (design points above/below predicted value), actual factor (temperature)

Figure 30 reported the interaction effect of NaOH and temperature on the total sugar production from sunflower stalk. As it can be seen in the plots, there is an increase in the total sugar rate with an increase of NaOH concentration, with the maximum rate in the 2% NaOH. On the other hand, the effect of temperature on total sugar production from sunflower stalk has similar trends, regardless of the NaOH concentration. The total sugar rate increased with the increase of time. It can be concluded from the contour plots that the optimum region of the total sugar production from sunflower stalk is the highest in the 2% NaOH for 3 days.

Figure 31 exposed the interaction effect of the time and temperature on the total sugar production from sunflower stalk. As can be seen in the plots, the increase of the time leads to an increase in the total sugar rate. The time has been increasing degradation rate. We can seen from the contour plots Figure 31 (2D) that the total sugar concentration is more than 35 g/L in the time range of 2.5 to 3 days either at a low or high level of temperature. Therefore, it can be concluded that the increasing time does not affect lignocellulose degradation.

Figure 32 shown the interaction effect of the time and NaOH concentration on total sugar production from sunflower stalk. The contour plots shown that the optimum region for the total sugar production rate is in the time range of 3 days and the NaOH concentration is in the range of 2%, respectively.

Sugar concentration on scale up from pretreatment and enzyme hydrolysis and evaporation

Sugar concentration of sunflower stalk from scaling up ; Pretreatment with 2% NaOH there were reducing sugar and total sugar 6.333±0.820 and 25.544±0.936 g/L Hydrolysis with 2% Cellulase enzyme there were reducing sugar and total sugar 20.267±6.058 and 143.860±39.517 g/L and after evaporation there were reducing sugar and total sugar 49.067±6.466 and 206.316±6.574 g/L attested in Table 16.

Table 16 Fermentable sugar from sunflower stalk

Plants	Parameter	Reducing Sugar (g/L)	Total Sugar (g/L)	Degree of Polymerisation (DP)	pH
Sunflower Stalk	2% NaOH	6.333±0.820	25.544±0.936	4.033	7.431±0.273
	2% Cellulase Enzyme	20.267±6.058	143.860±39.517	7.098	4.927±0.047
	Evaporation	49.067±6.466	206.316±6.574	4.205	5.600±0

Bioethanol yields from sunflower stalk

Ethanol yields were presented in Figure 33 at the 1st day there is highest ethanol concentration is 0.000 g/L, the 3th day there is the highest ethanol concentration is 12.562±0.000 g/L.

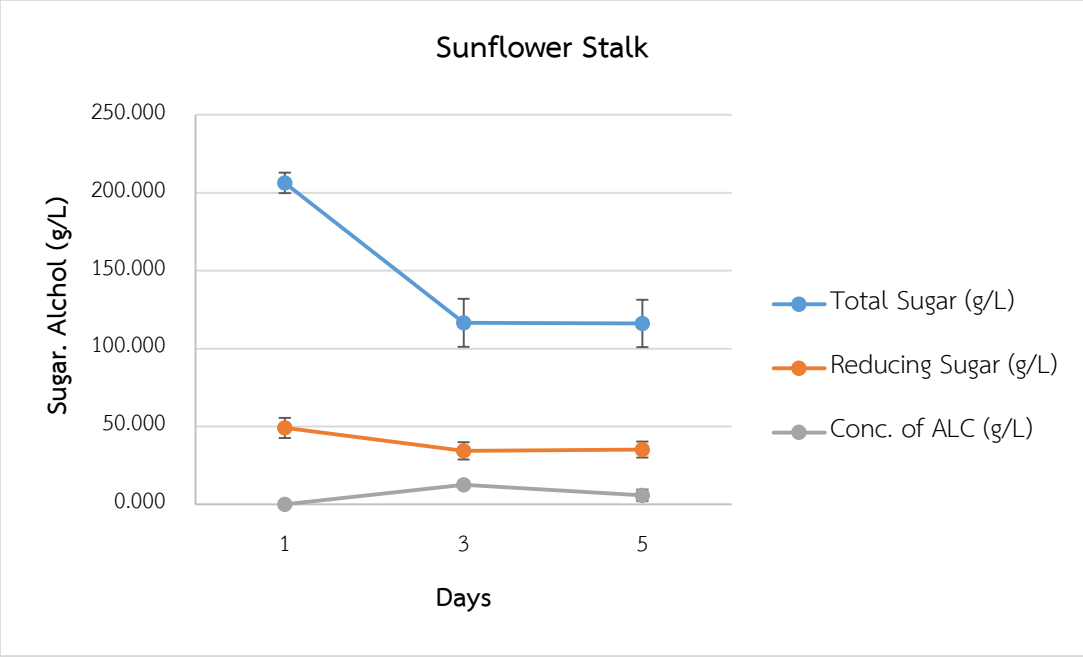


Figure 33 Ethanol concentration, total Sugar, and reducing sugar throughout fermentation (sunflower stalk)

Bioethanol production from sorghum stalk

The products from chemical and biological pretreatment

Sorghum stalk: Total sugar and reducing sugar from silage were lowest as 13.965±3.117, 2.293±0.12 g/L while pretreatment with 2% NaOH were the highest 27.158±0.913, 6.053±1.166 g/L. Total sugar and reducing sugar from pretreatment by water and 1% Trichoderma spp. were 17.123±1.574, 2.867±0.546 g/L and 24.667±0.540, 3.960±0.616 g/L exhibited in Figure 34.

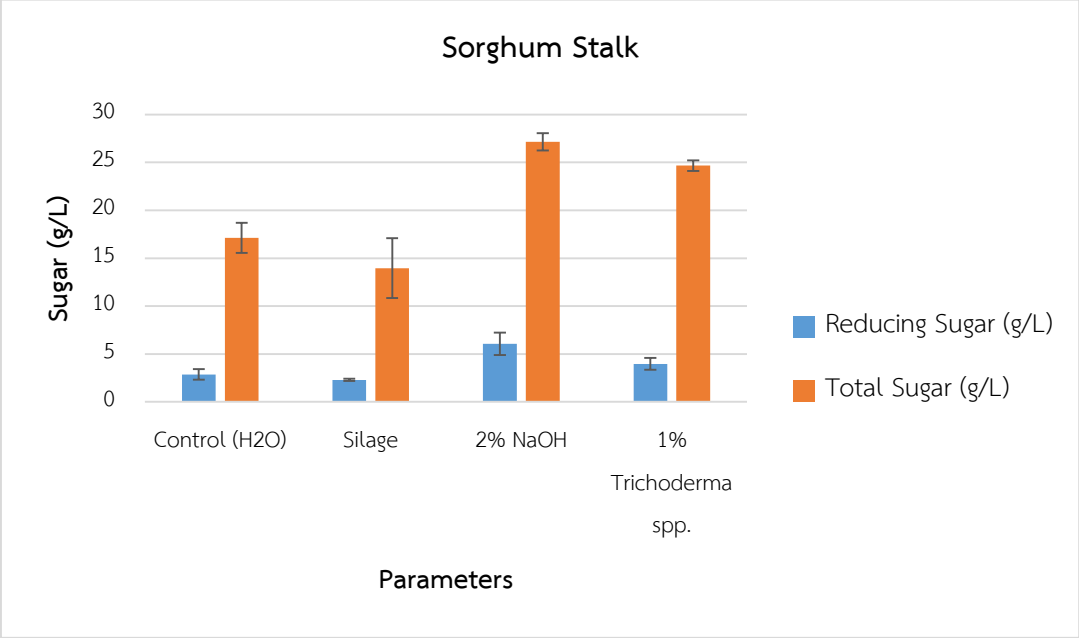


Figure 34 Sugar concentration of sorghum stalk pretreatments

Reponses surface methodology of pretreatments

In this study, the effect of three factors on reducing sugar production from sunflower stalk including temperature, NaOH concentration and time.

RSM development of reducing sugar from sorghum stalk

Model (sorghum stalk : reducing sugar)

All factors were selected as factors in the central composite design. As a response, the reducing sugar production rate was chosen, a total number of 29 experiments were employed for the response surface modeling manifested in Table 17, and the order of experiments was arranged randomly. The observed and predicted

results for the reducing sugar production from sorghum stalk are also depicted in Table 17.

Table 17 Experimental designs of reducing sugar and predictive values from sorghum stalk

Run	Factor 1	Factor 2	Factor 3	Reducing sugar (g/L)	
	A: Temperature (°C)	B:NaOH (%)	C: Time (Days)	Observed	Predicted
1	40	1	1	3.720	3.700
2	40	2	3	9.360	9.330
3	40	2	1	4.521	4.498
4	40	1.5	2	5.510	5.560
5	35	1.5	1	3.820	3.860
6	35	1.5	3	6.330	6.060
7	30	1	1	2.300	2.280
8	35	1.5	2	5.520	5.300
9	35	1.5	1	3.860	3.860
10	35	1	2	4.770	4.650
11	35	1.5	1	3.790	3.860
12	40	1.5	2	5.630	5.560
13	30	1.5	2	5.210	5.160
14	30	1	3	4.410	4.380
15	35	1.5	3	5.740	6.060
16	35	2	2	5.830	5.960
17	35	1.5	2	5.400	5.300
18	35	2	2	5.910	5.960
19	35	1.5	2	5.410	5.300
20	40	1.5	2	5.420	5.560
21	30	1.5	2	4.840	5.160
22	35	1.5	3	6.010	6.060
23	30	1.5	2	5.310	5.160
24	35	2	2	6.030	5.960
25	30	2	1	3.400	3.370
26	30	2	3	6.530	6.510
27	35	1	2	4.610	4.650
28	35	1	2	4.460	4.650
29	40	1	3	5.640	5.620

The Design-Expert 11 software was used to calculate the coefficients of the second-order fitting equation and the model suitability was tested using the ANOVA test. Therefore, the second-order polynomial equation should be expressed by Equation 7 (conf. Equation 1):

$$\text{Reducing Sugar} = +5.32+0.2007A+0.6550B+1.10C+0.1614AB+0.1891AC+0.4921BC +0.0306A^2-0.0248B^2-0.3659C^2+0.2359ABC+0.3106A^2B +0.3977A^2C+0.6245AB^2 \quad \textbf{...(Equation 7)}$$

Y= Reducing sugar (g/L)

A= Temperature (°C)

B= NaOH (%)

C= Time (days)

Statistical analysis (sorghum stalk : reducing sugar)

CCD was applied for the optimization of reducing sugar production conditions. The Model F-value of 111.97 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BC, C^2, ABC, A^2B, A^2C, AB^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 2.27 implies the Lack of Fit is not significant relative to the pure error. There is a 15.41% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good for the model to fit explained in Table 18.

The R^2 of 0.9898 in Figure 35 and A negative Predicted R^2 implies that the overall mean may be a better predictor of your response than the current model. In some cases, a higher order model may also predict better.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 57.825 indicates an adequate signal. This model can be used to navigate the design space revealed in Table 19.

Table 18 ANOVA for quadratic model of reducing sugar from sorghum stalk

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Remark
Model	44.86	13	3.45	111.97	< 0.0001	significant
A-Temperature	0.2416	1	0.2416	7.84	0.0135	significant
B-NaOH	2.57	1	2.57	83.53	< 0.0001	significant
C-Time	7.29	1	7.29	236.52	< 0.0001	significant
AB	0.2083	1	0.2083	6.76	0.0201	significant
AC	0.2861	1	0.2861	9.29	0.0081	significant
BC	1.94	1	1.94	62.87	< 0.0001	significant
A^2	0.0064	1	0.0064	0.2084	0.6546	not significant
B^2	0.0042	1	0.0042	0.137	0.7164	not significant
C^2	0.9211	1	0.9211	29.89	< 0.0001	significant
ABC	0.4451	1	0.4451	14.44	0.0017	significant
A^2B	0.3308	1	0.3308	10.73	0.0051	significant
A^2C	0.5423	1	0.5423	17.6	0.0008	significant
AB^2	1.34	1	1.34	43.38	< 0.0001	significant
Residual	0.4622	15	0.0308			
Lack of Fit	0.0645	1	0.0645	2.27	0.1541	not significant
Pure Error	0.3978	14	0.0284			
Cor Total	45.32	28				

Table 19 Fit statistics of reducing sugar from sorghum stalk

Std. Dev.	0.1755	**R^2**	0.9898
Mean	5.15	**Adjusted R^2**	0.9810
C.V. %	3.41	**Predicted R^2**	-0.4475
		Adeq Precision	57.8251

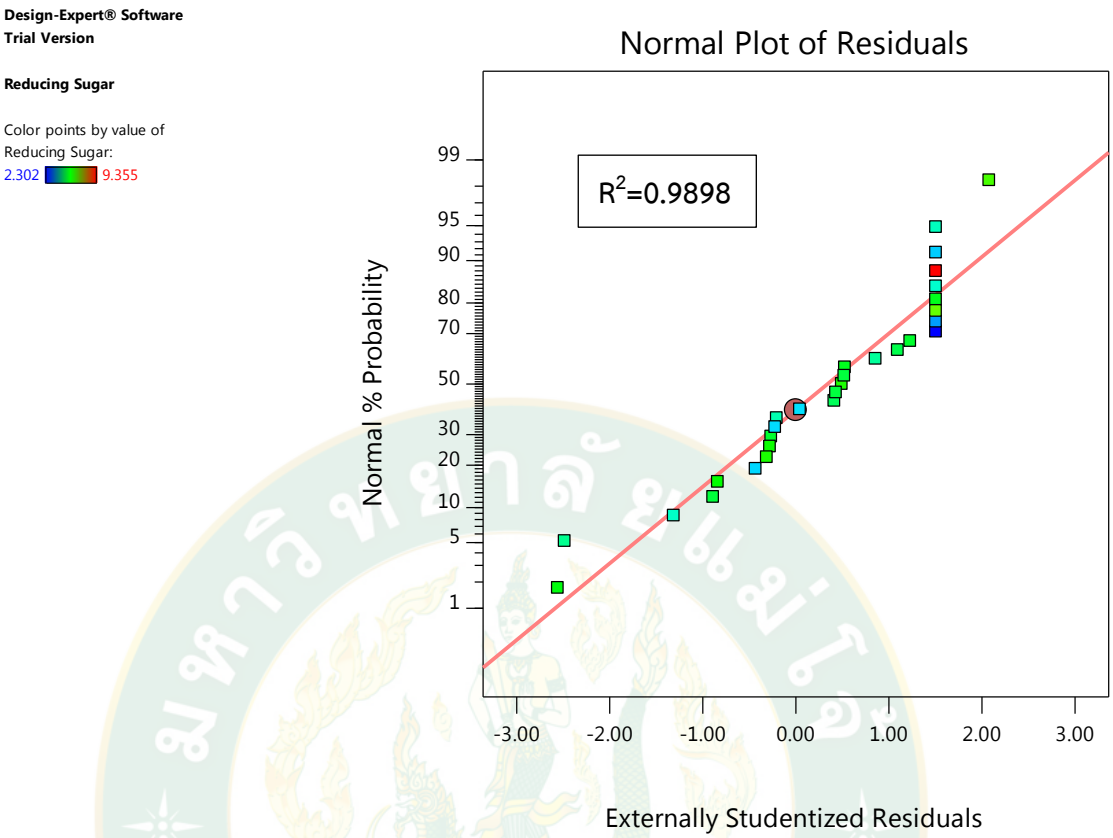


Figure 35 Comparison of predicted and actual value of reducing sugar from sorghum stalk

The effects of model parameters and their Interactions

The Design-Expert 11 software was used to produce three-dimensional (3D) response surfaces and two-dimensional (2D) contour plots. The 3D surfaces and 2D contour plots are graphical representations of the regression equation for the optimization of reaction conditions and are the most useful approach in revealing the conditions of the reaction system. In such plots, the response functions of two factors are presented while all other factors are at the fixed levels. The results of the interactions between three independent variables and the dependent variable are attested in Figure 36, Figure 37 and Figure 38.

As in Figure 36, Figure 37 and Figure 38 depending on the reaction, the temperature, NaOH concentration and time may have a positive or negative effect on the reducing sugar.

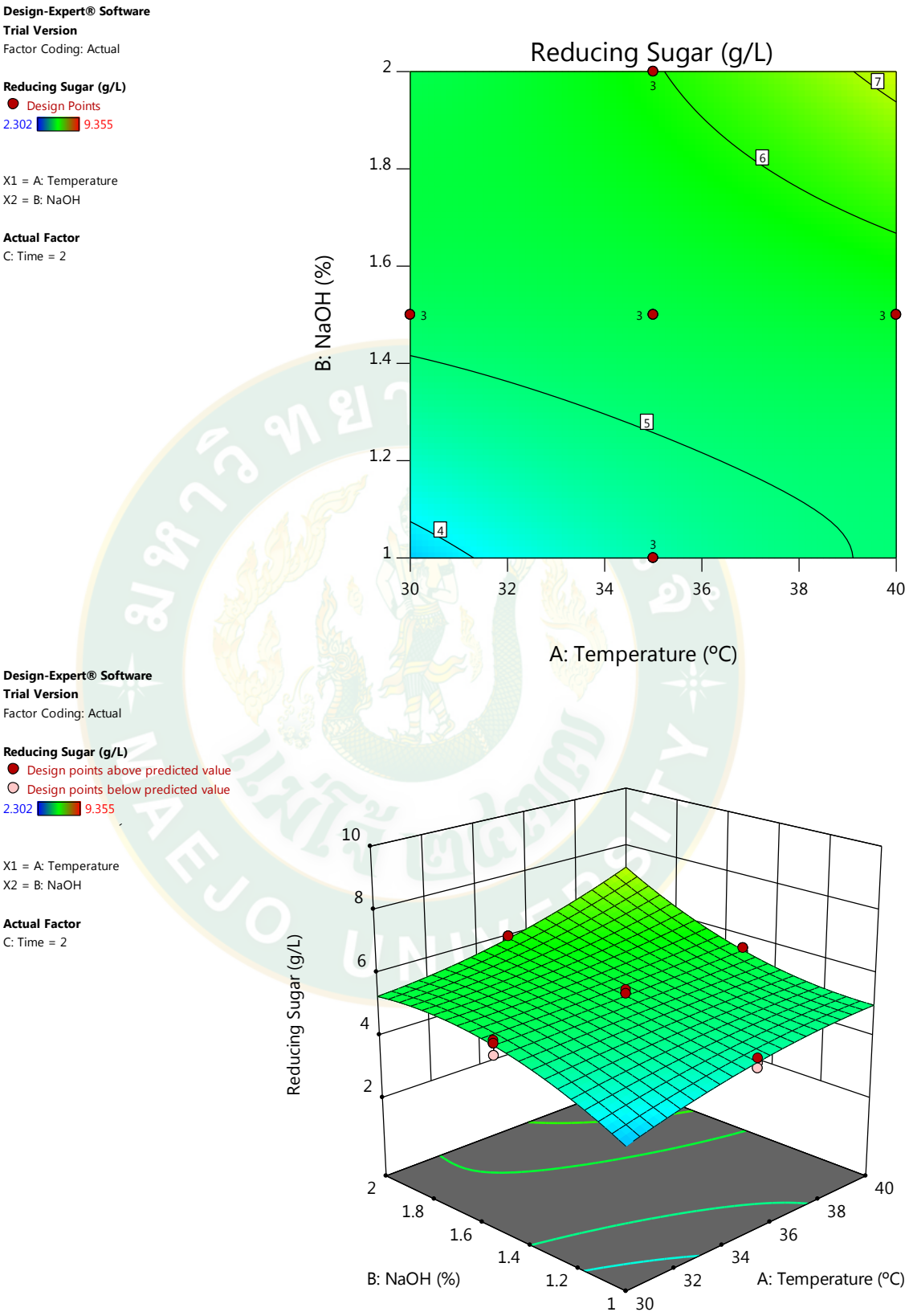


Figure 36 Reducing sugar yield from sorghum stalk (design points above/below predicted value), actual factor (time)

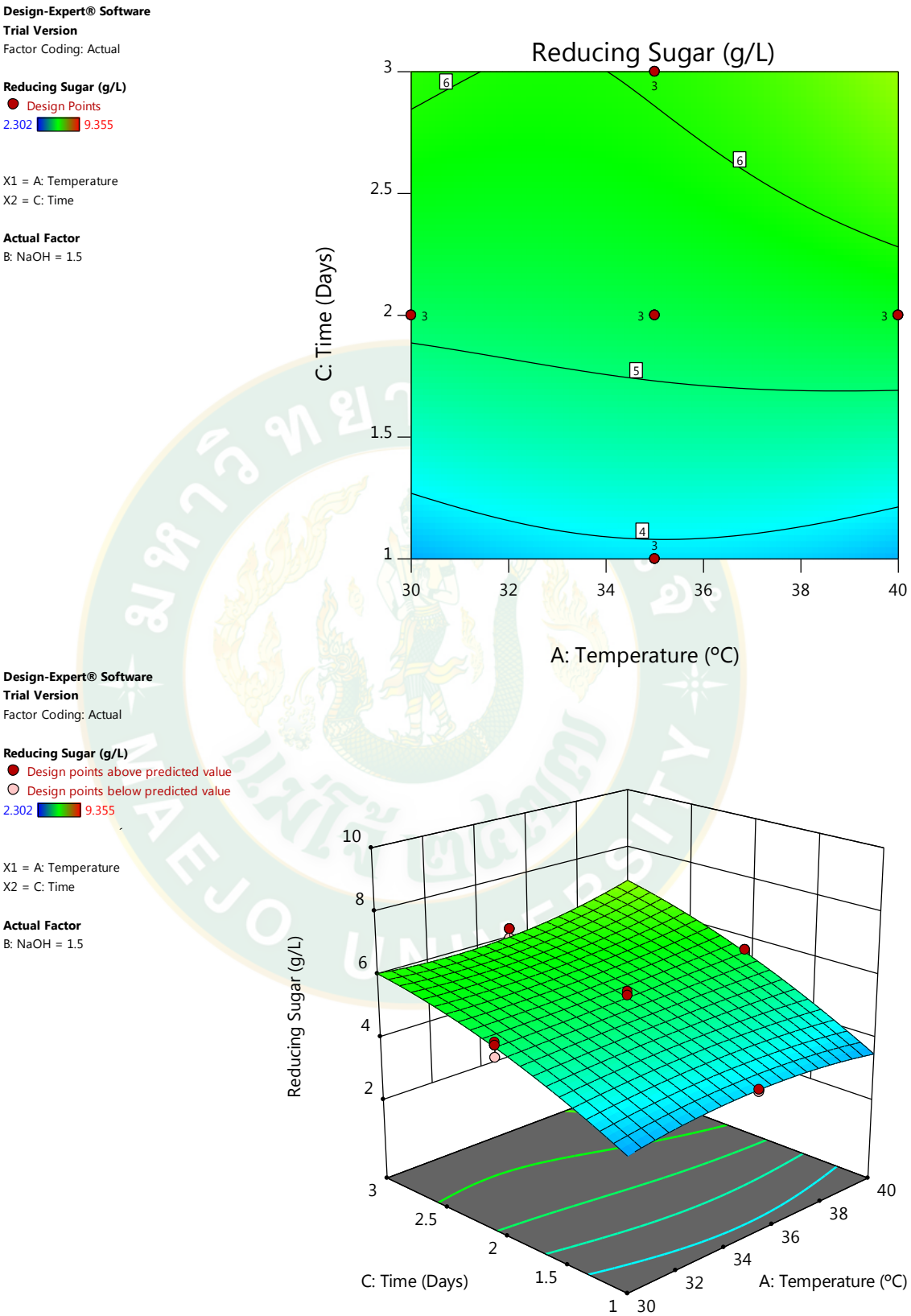


Figure 37 Reducing sugar yield from sorghum stalk (design points above/below predicted value), actual factor (NaOH)

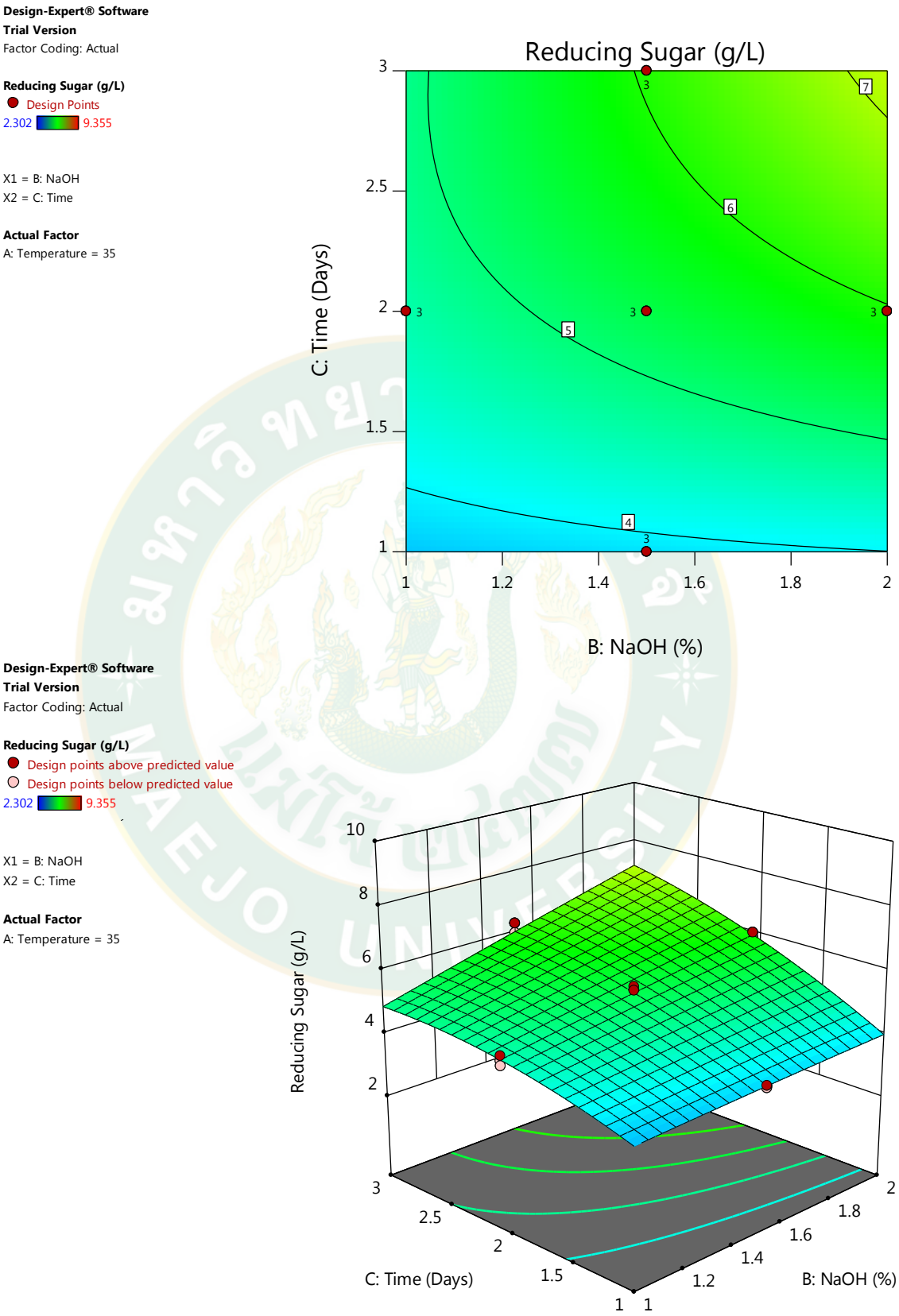


Figure 38 Reducing sugar yield from sorghum stalk (design points above/below predicted value), actual factor (temperature)

Figure 36 appeared the interaction effect of NaOH and temperature on the reducing sugar production from sorghum stalk. As it can be seen in the plots, there is an increase in the reducing sugar rate with an increase of NaOH concentration, with the maximum rate in the 2% NaOH. On the other hand, the effect of temperature on reducing sugar production from sorghum stalk has not similar trends, regardless of the NaOH concentration. The reducing sugar rate increased slightly with the increase of temperature. It can be concluded from the contour plots that the optimum region of the reducing sugar production from sorghum stalk is in the 2% NaOH.

Figure 37 demonstrated the interaction effect of the time and temperature on the reducing sugar production from sorghum stalk. As can be seen in the plots, the increase of the time leads to an increase in the reducing sugar rate. The time has been increasing degradation rate. We can seen from the contour plots Figure 37 (2D) that the reducing sugar concentration is more than 6 g/L in the time range of 2.5–3 days either at a low or high level of temperature. Therefore, it can be concluded that the increasing time does not affect lignocellulose degradation.

Figure 38 proved the interaction effect of the time and NaOH concentration on reducing sugar production from sorghum stalk. The contour plots shown that the optimum region for the reducing sugar production rate is in the time range of 3 days and the NaOH concentration is in the range of 2%, respectively.

RSM development of total sugar from sorghum stalk

Model (sorghum stalk : total sugar)

All factors were selected as factors in the central composite design. As a response, the total sugar production rate was chosen, a total number of 29 experiments were employed for the response surface modeling confirmed in Table 20, and the order of experiments was arranged randomly. The observed and predicted results for the total sugar production from sorghum stalk are also depicted in Table 20.

Table 20 Experimental designs of total sugar and predictive values from sorghum stalk

Run	Factor 1	Factor 2	Factor 3	Total sugar (g/L)	
	A: Temperature (°C)	B:NaOH (%)	C: Time (Days)	Observed	Predicted
1	40	1	1	17.450	17.360
2	40	2	3	38.980	38.880
3	40	2	1	27.360	27.260
4	40	1.5	2	28.700	29.160
5	35	1.5	1	23.430	23.890
6	35	1.5	3	35.460	34.510
7	30	1	1	15.760	15.660
8	35	1.5	2	29.490	29.530
9	35	1.5	1	23.430	23.890
10	35	1	2	25.540	26.020
11	35	1.5	1	24.430	23.890
12	40	1.5	2	29.270	29.160
13	30	1.5	2	27.860	27.810
14	30	1	3	29.230	29.140
15	35	1.5	3	35.560	34.510
16	35	2	2	31.700	31.620
17	35	1.5	2	29.190	29.530
18	35	2	2	30.900	31.620
19	35	1.5	2	31.450	29.530
20	40	1.5	2	29.120	29.160
21	30	1.5	2	27.970	27.810
22	35	1.5	3	34.123	35.148
23	30	1.5	2	27.220	27.810
24	35	2	2	31.860	31.620
25	30	2	1	25.680	25.590
26	30	2	3	33.520	33.420
27	35	1	2	26.340	26.020
28	35	1	2	25.800	26.020
29	40	1	3	32.410	32.310

The Design-Expert 11 software was used to calculate the coefficients of the second-order fitting equation and the model suitability was tested using the ANOVA test. Therefore, the second-order polynomial equation should be expressed by Equation 8 (conf. Equation 1):

$$\text{Total Sugar} = +29.64+0.6737A+2.80B+5.64C+0.2821AB+0.6586AC-1.12BC-1.18A^2 -0.8507B^2-0.1347C^2+0.2871ABC+1.04A^2B+0.3451A^2C+0.8260AB^2$$

...(Equation 8)

Y= Total sugar (g/L)

A= Temperature (°C)

B= NaOH (%)

C= Time (days)

Statistical analysis (sorghum stalk : total sugar)

CCD was applied for the optimization of total sugar production conditions. The Model F-value of 113.70 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, AC, BC, A^2, B^2, A^2B, AB^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 1.59 implies the Lack of Fit is not significant relative to the pure error. There is a 22.77% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good for the model to fit described in Table 21.

The R^2 of 0.9900 in Figure 39 a negative Predicted R^2 of -0.0452 implies that the overall mean may be a better predictor of your response than the current model. In some cases, a higher order model may also predict better.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 48.677 indicates an adequate signal. This model can be used to navigate the design space illustrated in Table 22.

Table 21 ANOVA for quadratic model of total sugar from sorghum stalk

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Remark
Model	775.51	13	59.65	80.03	< 0.0001	significant
A-Temperature	1.65	1	1.65	2.21	0.1581	not significant
B-NaOH	46.62	1	46.62	62.54	< 0.0001	significant
C-Time	189.79	1	189.79	254.6	< 0.0001	significant
AB	1.54	1	1.54	2.07	0.1709	not significant
AC	0.0014	1	0.0014	0.0019	0.9659	not significant
BC	9.25	1	9.25	12.41	0.0031	significant
A^2	15.73	1	15.73	21.1	0.0004	significant
B^2	8.71	1	8.71	11.69	0.0038	significant
C^2	2.47	1	2.47	3.31	0.0887	not significant
ABC	0.4437	1	0.4437	0.5952	0.4524	not significant
A^2B	3.11	1	3.11	4.17	0.0592	not significant
A^2C	3.05	1	3.05	4.09	0.0614	not significant
AB^2	7.75	1	7.75	10.4	0.0057	significant
Residual	11.18	15	0.7454			
Lack of Fit	0.3877	1	0.3877	0.5029	0.4899	not significant
Pure Error	10.79	14	0.771			
Cor Total	786.69	28				

Table 22 Fit statistics of total sugar from sorghum stalk

Std. Dev.	0.6864	**R^2**	0.9900
Mean	29.58	**Adjusted R^2**	0.9812
C.V. %	2.40	**Predicted R^2**	-0.0452
		Adeq Precision	48.6769

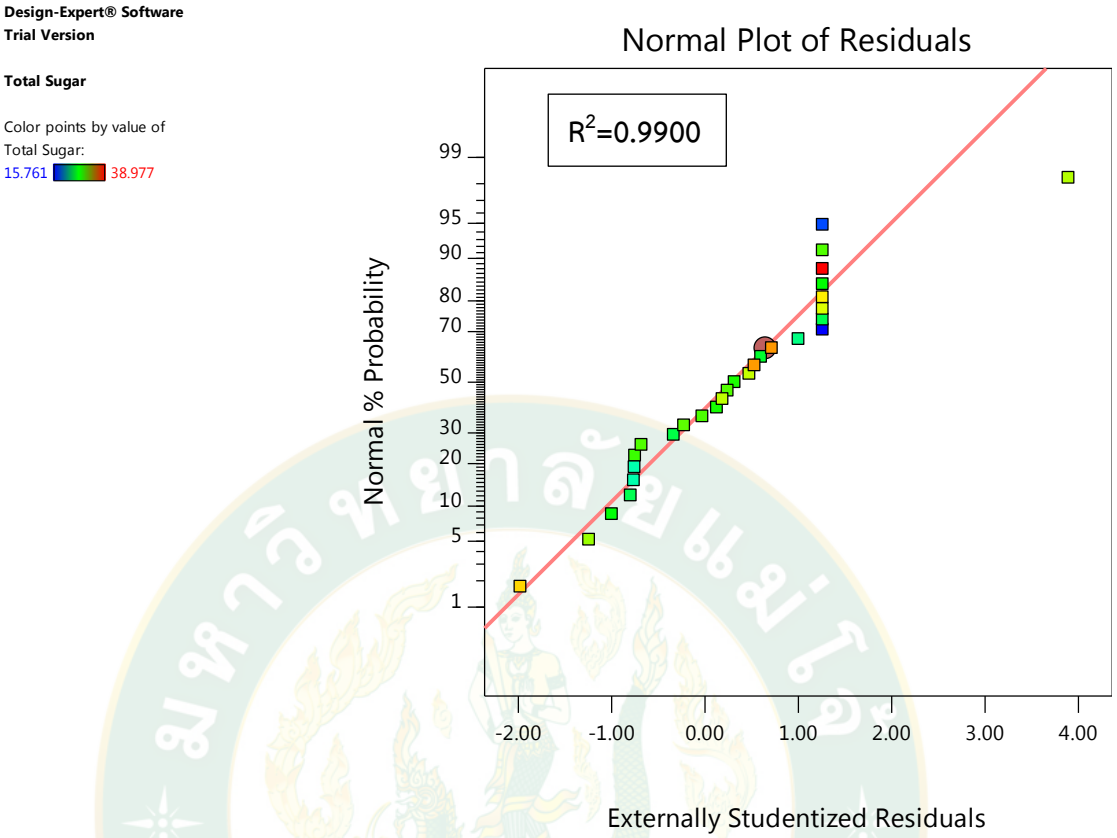


Figure 39 Comparison of predicted and actual value of total sugar from sorghum stalk

The effects of model parameters and their Interactions

The Design-Expert 11 software was used to produce three-dimensional (3D) response surfaces and two-dimensional (2D) contour plots. The 3D surfaces and 2D contour plots are graphical representations of the regression equation for the optimization of reaction conditions and are the most useful approach in revealing the conditions of the reaction system. In such plots, the response functions of two factors are presented while all other factors are at the fixed levels. The results of the interactions between three independent variables and the dependent variable are interpreted in Figure 40, Figure 41 and Figure 42.

As in Figure 40, Figure 41 and Figure 42 depending on the reaction, the temperature, NaOH concentration and time may have a positive or negative effect on the total sugar.

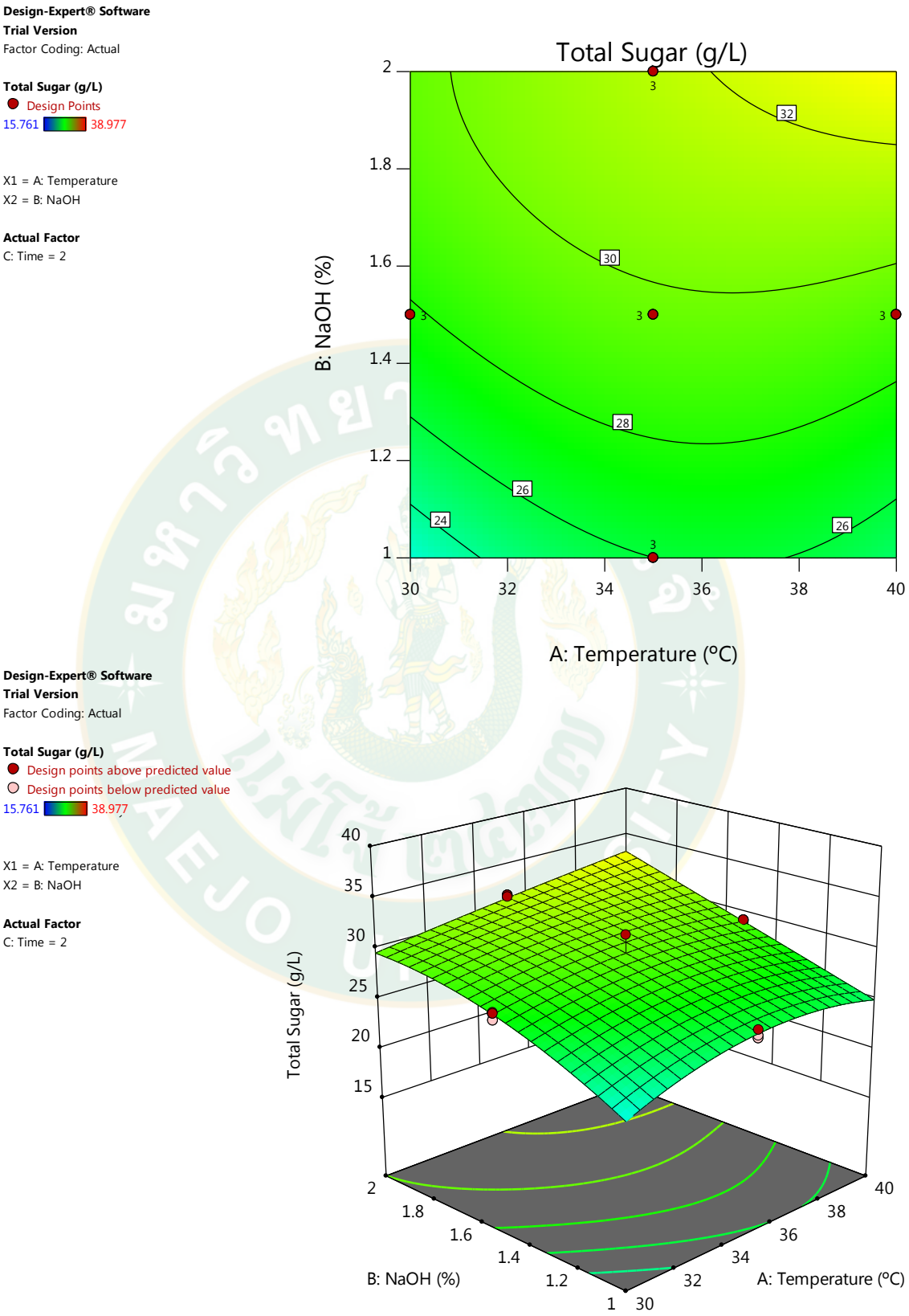


Figure 40 Total sugar yield from sorghum stalk (design points above/below predicted value), actual factor (time)

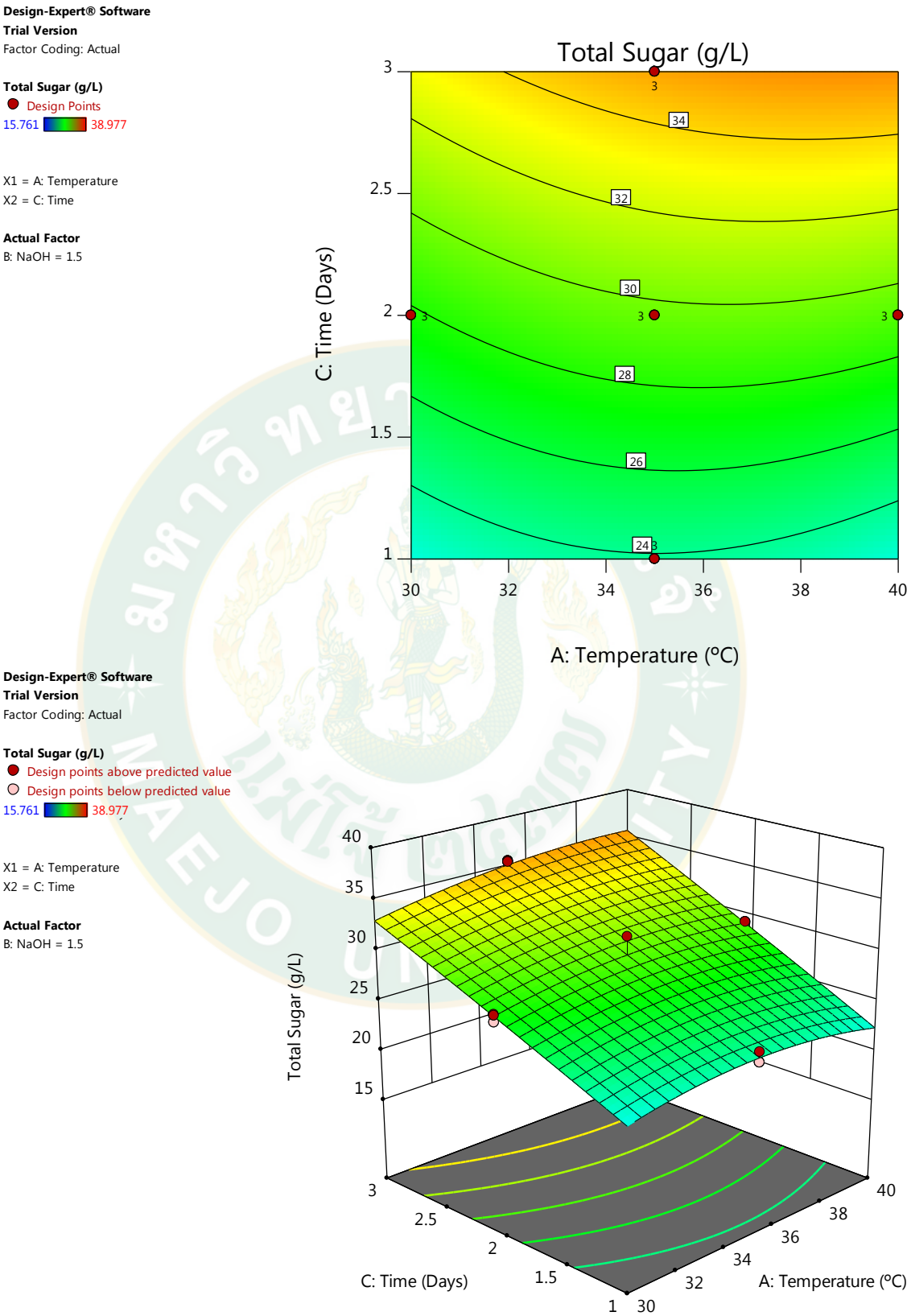


Figure 41 Total sugar yield from sorghum stalk (design points above/below predicted value), actual factor (NaOH)

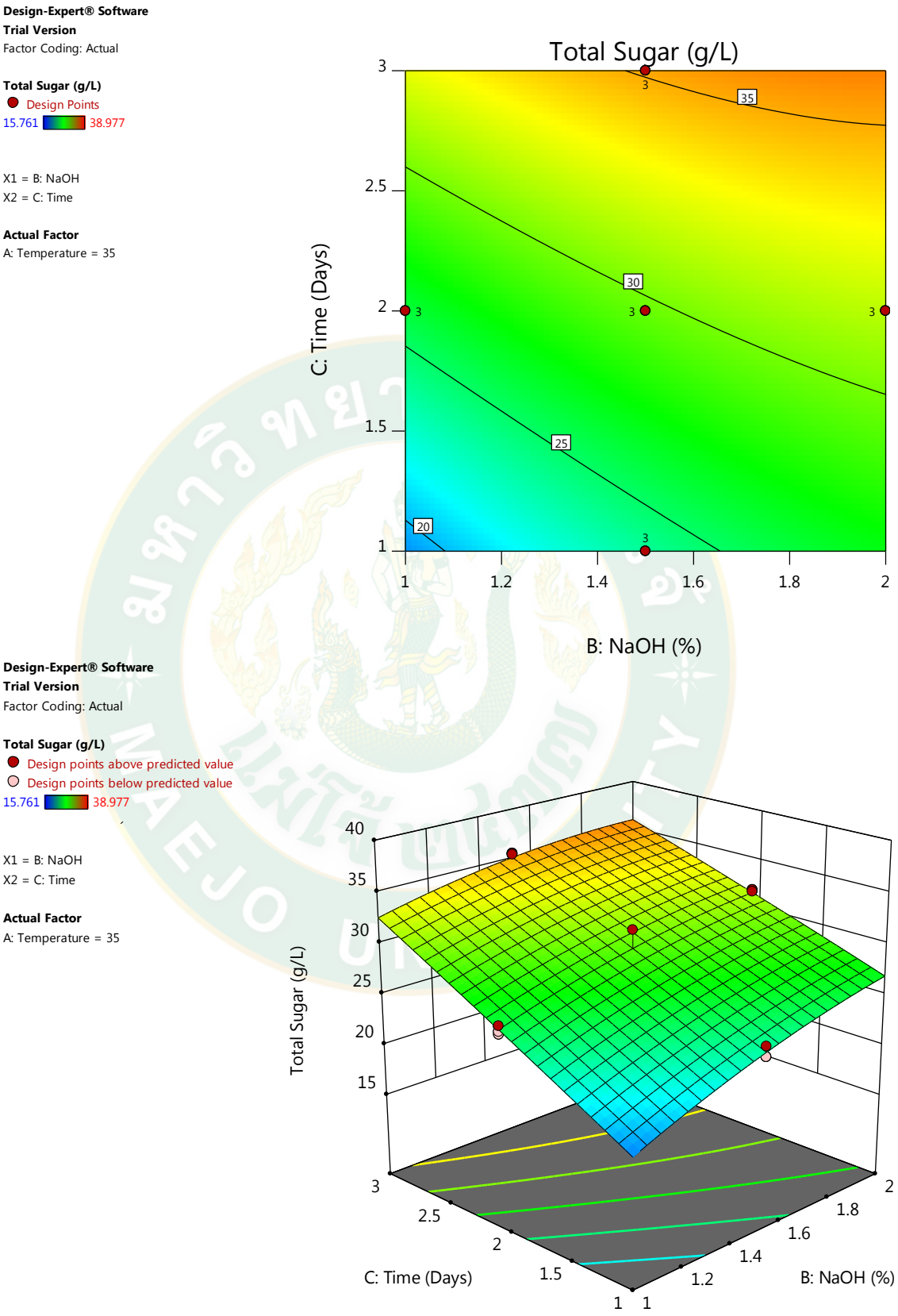


Figure 42 Total sugar yield from sorghum stalk (design points above/below predicted value), actual factor (temperature)

Figure 40 disclosed the interaction effect of NaOH and temperature on the total sugar production from sorghum stalk. As it can be seen in the plots, there is an increase in the total sugar rate with an increase of NaOH concentration, with the maximum rate in the 2% NaOH. On the other hand, the effect of temperature on total sugar production from sorghum stalk has not similar trends, regardless of the NaOH concentration. The total sugar rate is not increased with the increase of temperature. It can be concluded from the contour plots that the optimum region of the total sugar production from sorghum stalk is the highest in the 2% NaOH.

Figure 41 exposed the interaction effect of the time and temperature on the total sugar production from sorghum stalk. As can be seen in the plots, the increase of the time leads to an increase in the total sugar rate. The time has been increasing degradation rate. We can see from the contour plots Figure 41 (2D) that the total sugar concentration is more than 34 g/L in the time range of 2.75 to 3 days either at a low or high level of temperature. Therefore, it can be concluded that the increasing time does not affect lignocellulose degradation.

Figure 42 emerged the interaction effect of the time and NaOH concentration on total sugar production from sorghum stalk. The contour plots shown that the optimum region for the total sugar production rate is in the time range of 3 days and the NaOH concentration is in the range of 2%, respectively.

Sugar concentration on scale up from pretreatment and enzyme hydrolysis and evaporation

Sugar concentration of sorghum stalk from scaling up ; Pretreatment with 2% NaOH there were reducing sugar and total sugar 6.480±0.538 and 28.263±2.263 g/L. Hydrolysis with 2% Cellulase Enzyme there were reducing sugar and total sugar 28.800±7.632 and 151.228±12.470 g/L and after evaporation there were reducing sugar and total sugar 62.667±16.518 and 276.842±6.403g/L expressed in Table 23.

Table 23 Fermentable sugar from sorghum stalk

Plants	Parameter	Reducing Sugar (g/L)	Total Sugar (g/L)	Degree of Polymerisation (DP)	pH
Sorghum Stalk	2% NaOH	6.480±0.538	28.263±2.263	4.362	6.791±0.162
	2% Cellulase Enzyme	28.800±7.632	151.228±12.470	5.251	4.250±0.195
	Evaporation	62.667±16.518	276.842±6.403	4.418	5.600±0

Bioethanol yields from sorghum stalk

Ethanol yields were recited in Figure 43 at the 1st day there is highest ethanol concentration is 0.000 g/L, the 3th day there is the highest ethanol concentration is 7.328± 1.813 g/L.

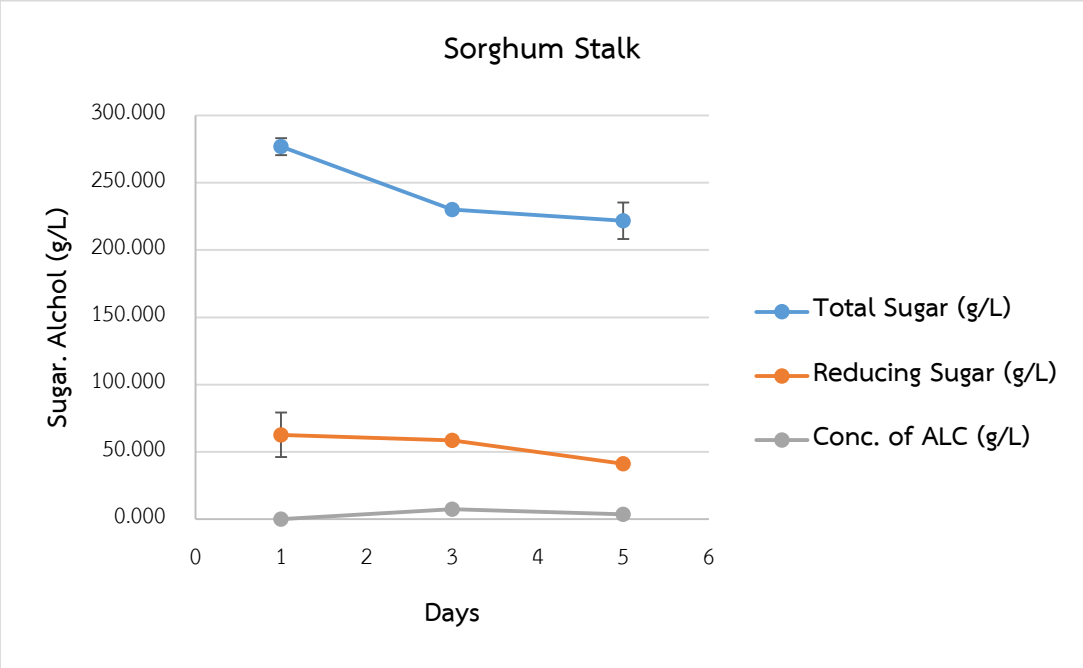


Figure 43 Ethanol concentration, total sugar, and reducing sugar throughout fermentation (sorghum stalk)

Bioethanol production from sugarcane leaf

The products from chemical and biological pretreatment

Sugarcane leaf: total sugar and reducing sugar were 14.789±1.891, 3.360±0.212; 15.053±2.346, 12.035±0.373 and 2.493±0.623 which observed from control (water), silage and 1% Trichoderma spp. were similar while the highest amount was obtained from pretreatment with NaOH 2% as 17.912±0.500, 4.147±0.266 g/L reported in Figure 44.

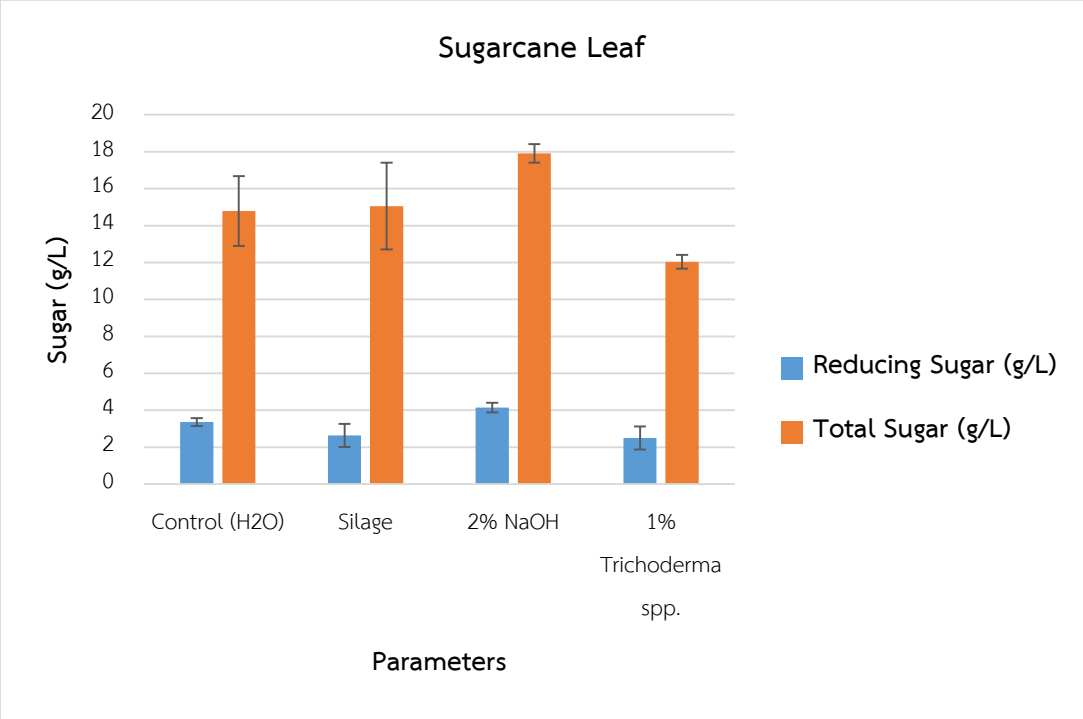


Figure 44 Sugar concentration of sugarcane leaf pretreatments

Reponses surface methodology of pretreatments

In this study, the effect of three factors on reducing sugar production from sunflower stalk including temperature, NaOH concentration and time.

RSM development of reducing sugar from sugarcane leaf

Model (sugarcane leaf : reducing sugar)

All factors were selected as factors in the central composite design. As a response, the reducing sugar production rate was chosen, a total number of 29

experiments were employed for the response surface modeling quoted in Table 24, and the order of experiments was arranged randomly. The observed and predicted results for the reducing sugar production from sugarcane leaf are also depicted in Table 24.

Table 24 Experimental designs of reducing sugar and predictive values from sugarcane leaf

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Remark
Model	29.08	13	2.24	91.71	< 0.0001	significant
A-Temperature	0.1104	1	0.1104	4.53	0.0503	not significant
B-NaOH	2.91	1	2.91	119.45	< 0.0001	significant
C-Time	5.39	1	5.39	221.07	< 0.0001	significant
AB	0.0336	1	0.0336	1.38	0.2591	not significant
AC	1.66	1	1.66	67.91	< 0.0001	significant
BC	1.33	1	1.33	54.47	< 0.0001	significant
A^2	0.0216	1	0.0216	0.8857	0.3616	not significant
B^2	0.0519	1	0.0519	2.13	0.1652	not significant
C^2	0.8047	1	0.8047	32.99	< 0.0001	significant
ABC	0.1058	1	0.1058	4.34	0.0548	not significant
A^2B	0.1503	1	0.1503	6.16	0.0254	significant
A^2C	0.002	1	0.002	0.0811	0.7798	not significant
AB^2	0.8067	1	0.8067	33.07	< 0.0001	significant
Residual	0.3659	15	0.0244			
Lack of Fit	0.0017	1	0.0017	0.0662	0.8007	not significant
Pure Error	0.3641	14	0.026			
Cor Total	29.45	28				

The Design-Expert 11 software was used to calculate the coefficients of the second-order fitting equation and the model suitability was tested using the ANOVA test. Therefore, the second-order polynomial equation should be expressed by Equation 9 (conf. Equation 1):

Reducing Sugar = +2.63+0.1357A+0.6968B+0.9480C+0.0648AB+0.4550AC+0.4075BC -0.0560A^2-0.0869B^2-0.3420C^2+0.1150ABC+0.2094A^2B-0.0240A^2C +0.4851AB^2 ...**(Equation 9)**

Y= Reducing sugar (g/L)

A= Temperature (°C)

B= NaOH (%)

C= Time (days)

Statistical analysis (sugarcane leaf : reducing sugar)

CCD was applied for the optimization of reducing sugar production conditions. The Model F-value of 91.71 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case B, C, AC, BC, C^2, A^2B, AB^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 0.07 implies the Lack of Fit is not significant relative to the pure error. There is a 80.07% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good for the model to fit proved in Table 25.

The R^2 of 0.9876 in Figure 45 and The Predicted R^2 of 0.9155 is in reasonable agreement with the Adjusted R^2 of 0.9768; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 47.293 indicates an adequate signal. This model can be used to navigate the design space shown in Table 26.

Table 25 ANOVA for quadratic model of reducing sugar from sugarcane leaf

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Remark
Model	29.080	13	2.24	91.71	< 0.0001	significant
A-Temperature	0.1104	1	0.1104	4.53	0.0503	not significant
B-NaOH	2.910	1	2.91	119.45	< 0.0001	significant
C-Time	5.390	1	5.39	221.07	< 0.0001	significant
AB	0.0336	1	0.0336	1.38	0.2591	not significant
AC	1.660	1	1.66	67.91	< 0.0001	significant
BC	1.330	1	1.33	54.47	< 0.0001	significant
A^2	0.0216	1	0.0216	0.8857	0.3616	not significant
B^2	0.0519	1	0.0519	2.13	0.1652	not significant
C^2	0.8047	1	0.8047	32.99	< 0.0001	significant
ABC	0.1058	1	0.1058	4.34	0.0548	not significant
A^2B	0.1503	1	0.1503	6.16	0.0254	significant
A^2C	0.002	1	0.002	0.0811	0.7798	not significant
AB^2	0.8067	1	0.8067	33.07	< 0.0001	significant
Residual	0.3659	15	0.0244			
Lack of Fit	0.0017	1	0.0017	0.0662	0.8007	not significant
Pure Error	0.3641	14	0.026			
Cor Total	29.45	28				

Table 26 Fit statistics of reducing sugar from sugarcane leaf

Std. Dev.	0.1562	**R^2**	0.9876
Mean	2.39	**Adjusted R^2**	0.9768
C.V. %	6.53	**Predicted R^2**	0.9155
		Adeq Precision	47.2930

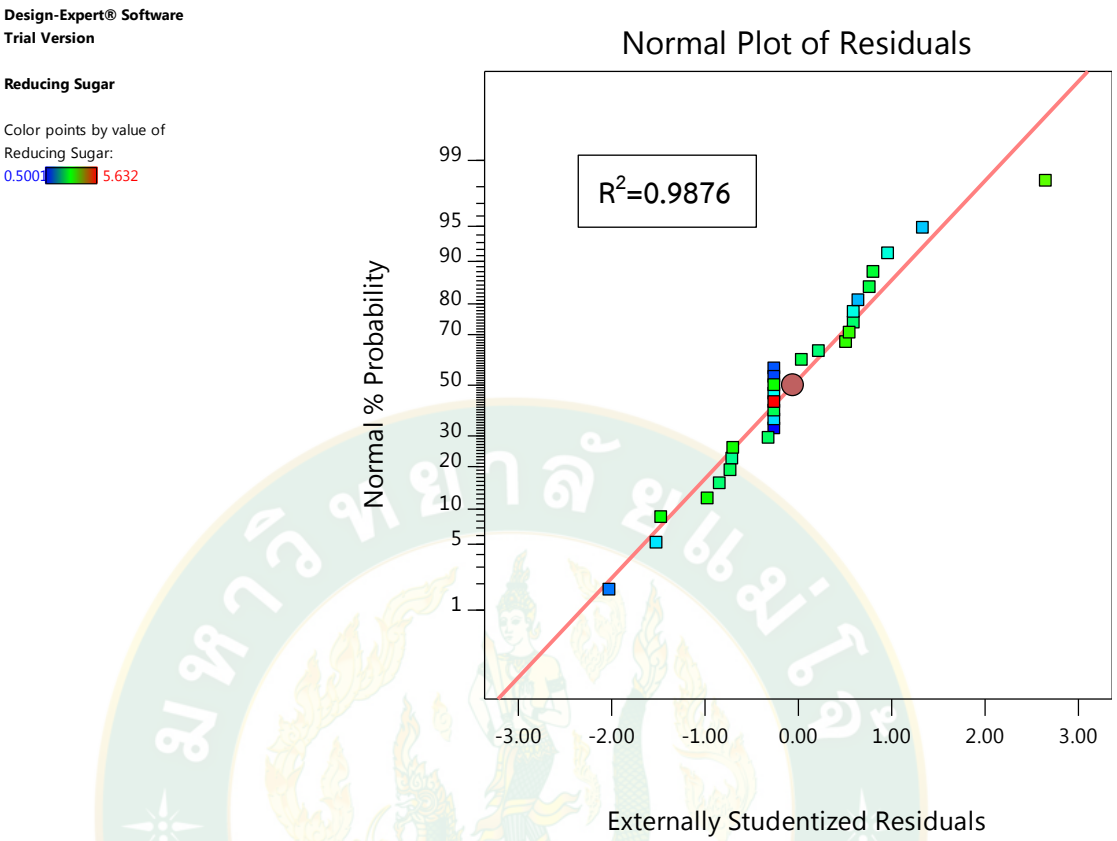


Figure 45 Comparison of predicted and actual value of reducing sugar from sugarcane leaf

The effects of model parameters and their Interactions

The Design-Expert 11 software was used to produce three-dimensional (3D) response surfaces and two-dimensional (2D) contour plots. The 3D surfaces and 2D contour plots are graphical representations of the regression equation for the optimization of reaction conditions and are the most useful approach in revealing the conditions of the reaction system. In such plots, the response functions of two factors are presented while all other factors are at the fixed levels. The results of the interactions between three independent variables and the dependent variable are presented in Figure 46, Figure 47 and Figure 48.

As in Figure 46, Figure 47 and Figure 48 depending on the reaction, the temperature, NaOH concentration and time may have a positive or negative effect on the reducing sugar.

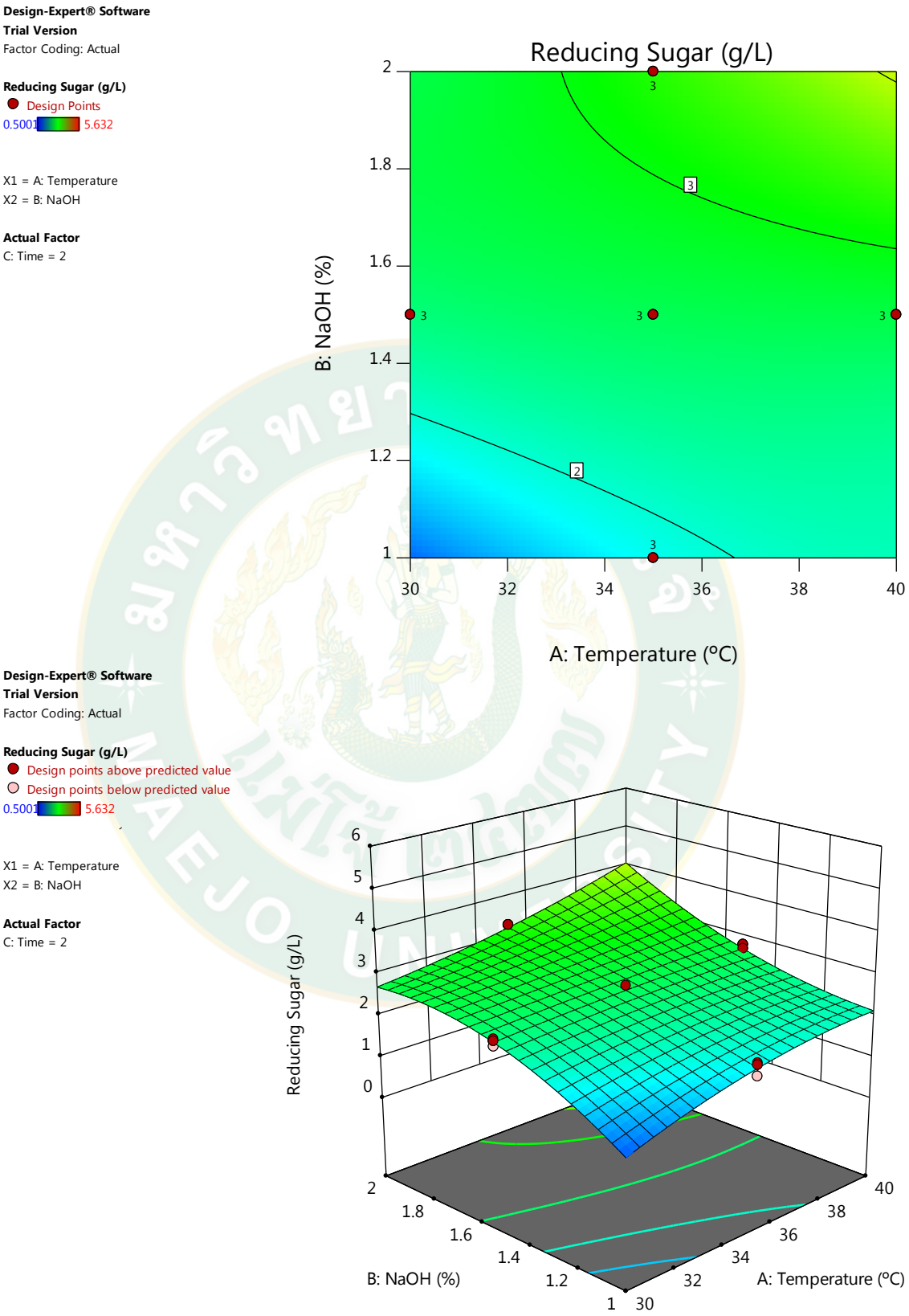


Figure 46 Reducing sugar yield from sugarcane leaf (design points above/below predicted value), actual factor (time)

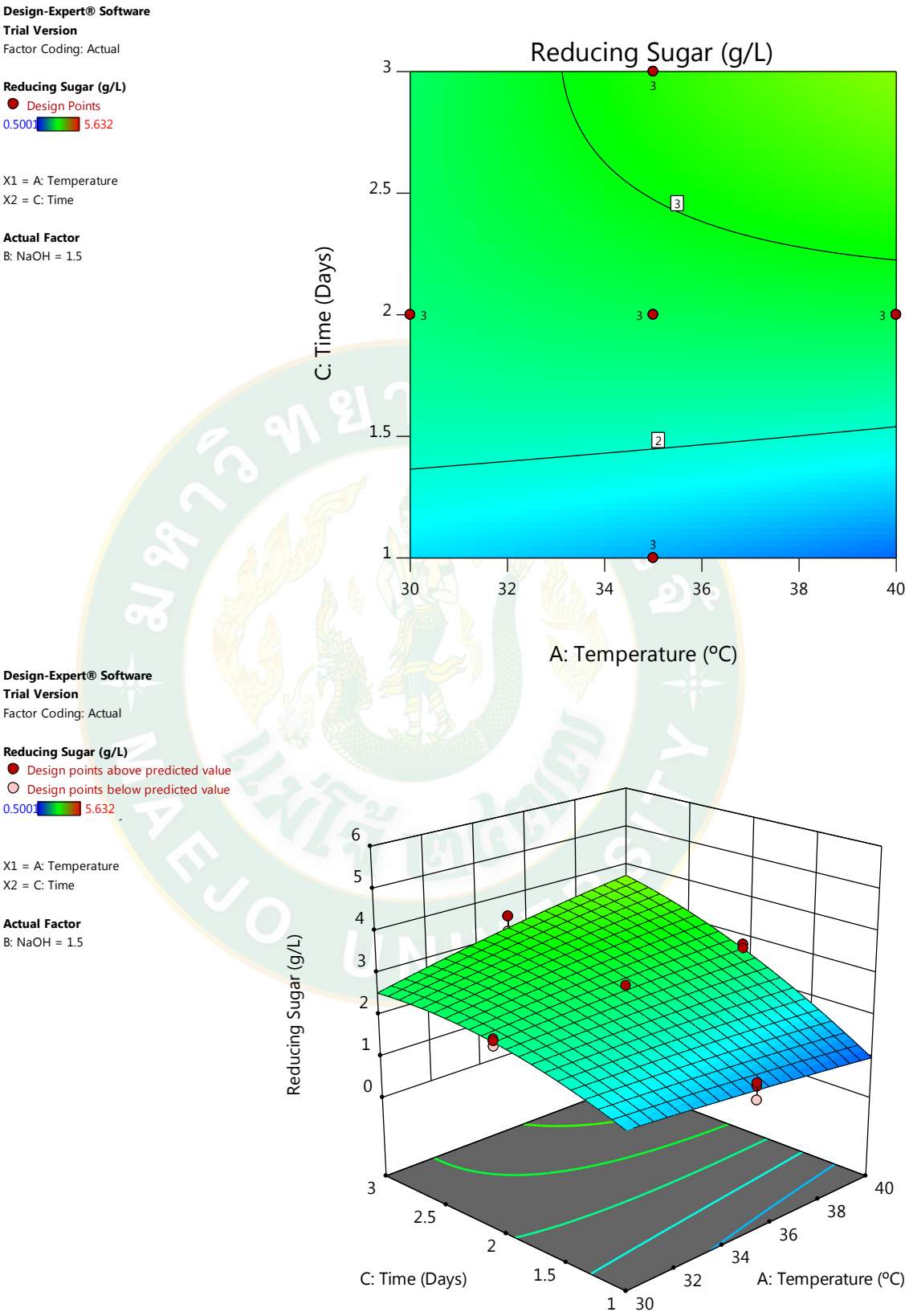


Figure 47 Reducing sugar yield from sugarcane leaf (design points above/below predicted value), actual factor (NaOH)

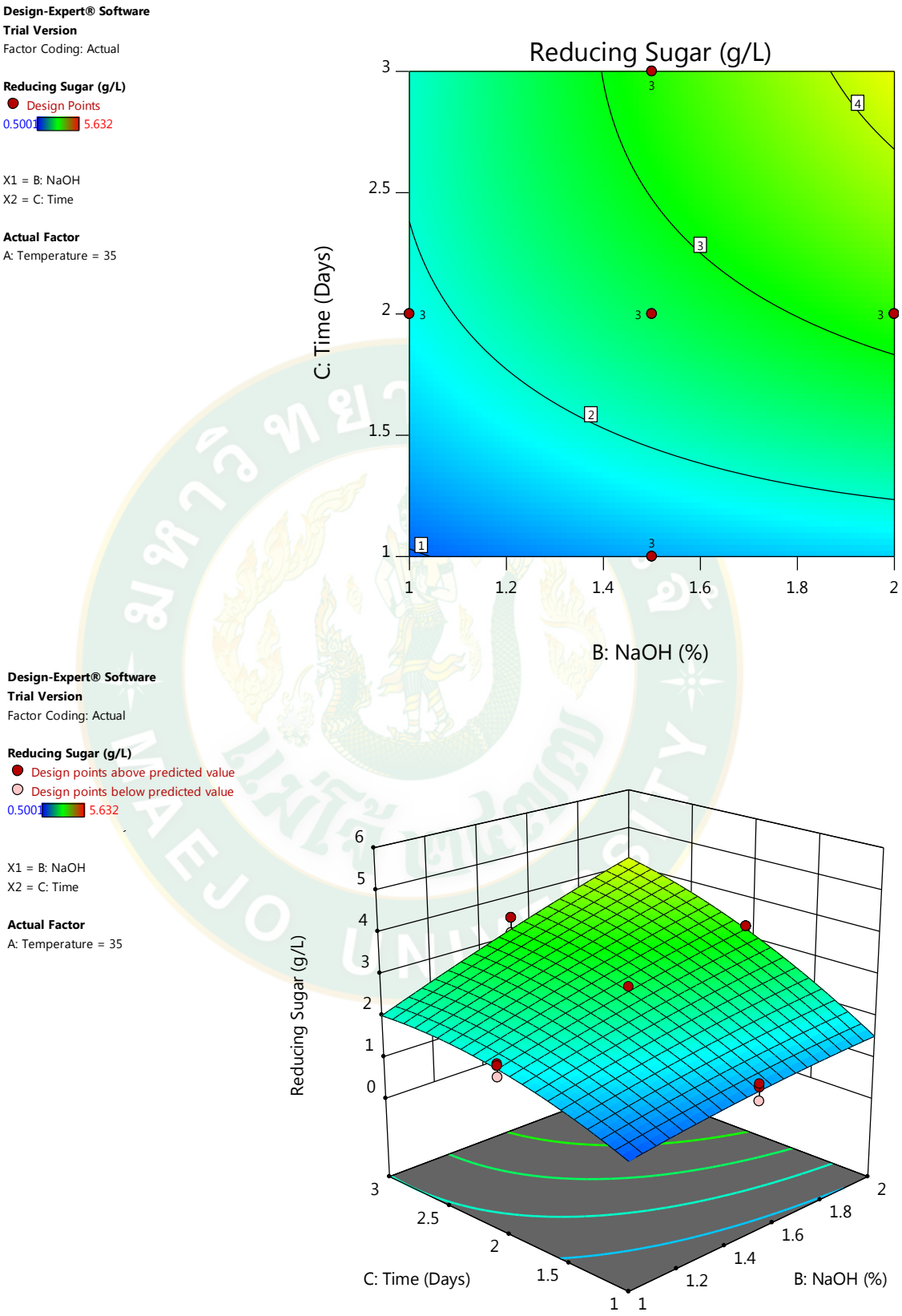


Figure 48 Reducing sugar yield from sugarcane leaf (design points above/below predicted value), actual factor (temperature)

Figure 46 exhibited the interaction effect of NaOH and temperature on the reducing sugar production from sugarcane leaf. As it can be seen in the plots, there is an increase in the reducing sugar rate with an increase of NaOH concentration, with the maximum rate in the 2% NaOH. On the other hand, the effect of temperature on reducing sugar production from sugarcane leaf has not similar trends, regardless of the NaOH concentration. The reducing sugar rate increased slightly with the increase of temperature. It can be concluded from the contour plots that the optimum region of the reducing sugar production from sugarcane leaf is in the 2% NaOH.

Figure 47 manafested the interaction effect of the time and temperature on the reducing sugar production from sugarcane leaf. As can be seen in the plots, the increase of the time leads to an increase in the reducing sugar rate. The time has been increasing degradation rate. We can see from the contour plots Figure 47 (2D) that the reducing sugar concentration is more than 3 g/L in the time range of 2.5–3 days either at a low or high level of temperature. Therefore, it can be concluded that the increasing time does not affect lignocellulose degradation.

Figure 48 explained the interaction effect of the time and NaOH concentration on reducing sugar production from sugarcane leaf. The contour plots shown that the optimum region for the reducing sugar production rate is in the time range of 3 days and the NaOH concentration is in the range of 2%, respectively.

RSM development of total sugar from sugarcane leaf

Model (sugarcane leaf: total sugar)

All factors were selected as factors in the central composite design. As a response, the total sugar production rate was chosen, a total number of 29 experiments were employed for the response surface modeling revealed in Table 27, and the order of experiments was arranged randomly. The observed and predicted results for the total sugar production from sugarcane leaf is also depicted in Table 27.

Table 27 Experimental designs of total sugar and predictive values from sugarcane leaf

Run	Factor 1	Factor 2	Factor 3	Total sugar (g/L)	
	A: Temperature (°C)	B:NaOH (%)	C: Time (Days)	Observed	Predicted
1	40	1	1	7.235	7.159
2	40	2	3	32.612	32.536
3	40	2	1	25.471	25.395
4	40	1.5	2	22.693	23.166
5	35	1.5	1	17.431	17.097
6	35	1.5	3	26.556	26.479
7	30	1	1	7.001	6.925
8	35	1.5	2	22.291	23.198
9	35	1.5	1	15.127	17.097
10	35	1	2	17.783	18.738
11	35	1.5	1	18.432	17.097
12	40	1.5	2	23.085	23.166
13	30	1.5	2	20.761	21.668
14	30	1	3	15.343	15.267
15	35	1.5	3	26.256	26.479
16	35	2	2	25.579	25.312
17	35	1.5	2	23.221	23.198
18	35	2	2	24.892	25.312
19	35	1.5	2	25.289	23.198
20	40	1.5	2	23.417	23.166
21	30	1.5	2	22.671	21.668
22	35	1.5	3	26.323	26.479
23	30	1.5	2	21.271	21.668
24	35	2	2	25.164	25.312
25	30	2	1	18.883	18.807
26	30	2	3	26.422	26.346
27	35	1	2	18.341	18.738
28	35	1	2	19.788	18.738
29	40	1	3	26.319	26.243

The Design-Expert 11 software was used to calculate the coefficients of the second-order fitting equation and the model suitability was tested using the ANOVA test. Therefore, the second-order polynomial equation should be expressed by Equation 10 (conf. Equation 1):

$$\text{Total Sugar} = +23.20+0.7487A+3.29B+4.69C+0.1960AB+1.29AC-1.59BC-0.7806A^2-1.17B^2 -1.41C^2-1.39ABC+2.65A^2B+0.5724A^2C+2.25AB^2 \quad \textbf{...(Equation 10)}$$

Y= Total sugar (g/L)

A= Temperature (°C)

B= NaOH (%)

C= Time (days)

Statistical analysis (sugarcane leaf : total sugar)

CCD was applied for the optimization of total sugar production conditions. The Model F-value of 62.95 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case B, C, AC, BC, B^2, C^2, ABC, A^2B, AB^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 0.66 implies the Lack of Fit is not significant relative to the pure error. There is a 42.93% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good for the model to fit attested in Table 28.

The R^2 of 0.9820 in Figure 49 and The Predicted R^2 of 0.1512 is not as close to the Adjusted R^2 of 0.9664 as one might normally expect; i.e. the difference is more than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 35.894 indicates an adequate signal. This model can be used to navigate the design space appeared in Table 29.

Table 28 ANOVA for quadratic model of total sugar from sugarcane leaf

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Remark
Model	863.03	13	66.39	62.95	< 0.0001	significant
A-Temperature	3.36	1	3.36	3.19	0.0944	not significant
B-NaOH	64.83	1	64.83	61.48	< 0.0001	significant
C-Time	132.02	1	132.02	125.19	< 0.0001	significant
AB	0.3073	1	0.3073	0.2914	0.5972	not significant
AC	13.37	1	13.37	12.68	0.0028	significant
BC	20.31	1	20.31	19.26	0.0005	significant
A^2	4.19	1	4.19	3.97	0.0647	not significant
B^2	9.45	1	9.45	8.96	0.0091	significant
C^2	13.66	1	13.66	12.96	0.0026	significant
ABC	15.51	1	15.51	14.71	0.0016	significant
A^2B	24.06	1	24.06	22.82	0.0002	significant
A^2C	1.12	1	1.12	1.07	0.3184	not significant
AB^2	17.35	1	17.35	16.46	0.001	significant
Residual	15.82	15	1.05			
Lack of Fit	0.7147	1	0.7147	0.6625	0.4293	not significant
Pure Error	15.1	14	1.08			
Cor Total	878.85	28				

Table 29 Fit statistics of total sugar from sugarcane leaf

Std. Dev.	1.03	**R^2**	0.9820
Mean	21.57	**Adjusted R^2**	0.9664
C.V. %	4.76	**Predicted R^2**	0.1512
		Adeq Precision	35.8942

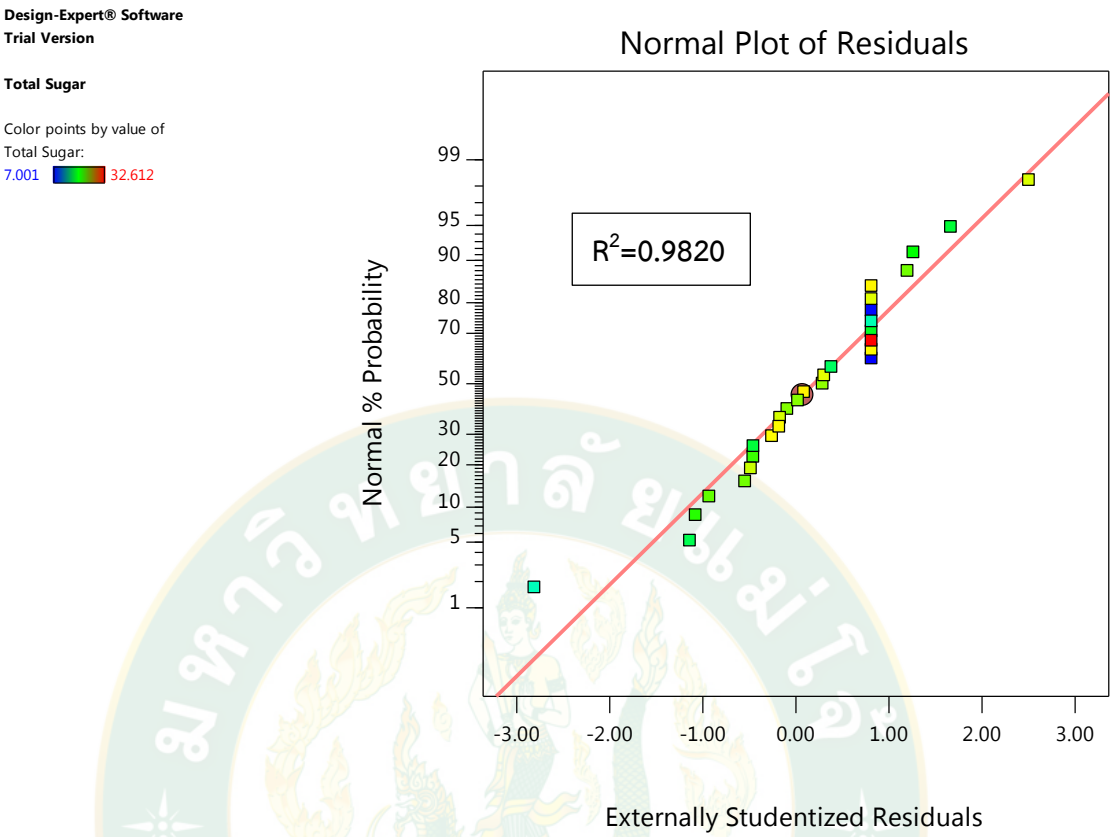


Figure 49 Comparison of predicted and actual value of total sugar from sugarcane leaf

The effects of model parameters and their Interactions

The Design-Expert 11 software was used to produce three-dimensional (3D) response surfaces and two-dimensional (2D) contour plots. The 3D surfaces and 2D contour plots are graphical representations of the regression equation for the optimization of reaction conditions and are the most useful approach in revealing the conditions of the reaction system. In such plots, the response functions of two factors are presented while all other factors are at the fixed levels. The results of the interactions between three independent variables and the dependent variable are demonstrated in Figure 50, Figure 51 and Figure 52.

As in Figure 50, Figure 51 and Figure 52 depending on the reaction, the temperature, NaOH concentration and time may have a positive or negative effect on the total sugar.

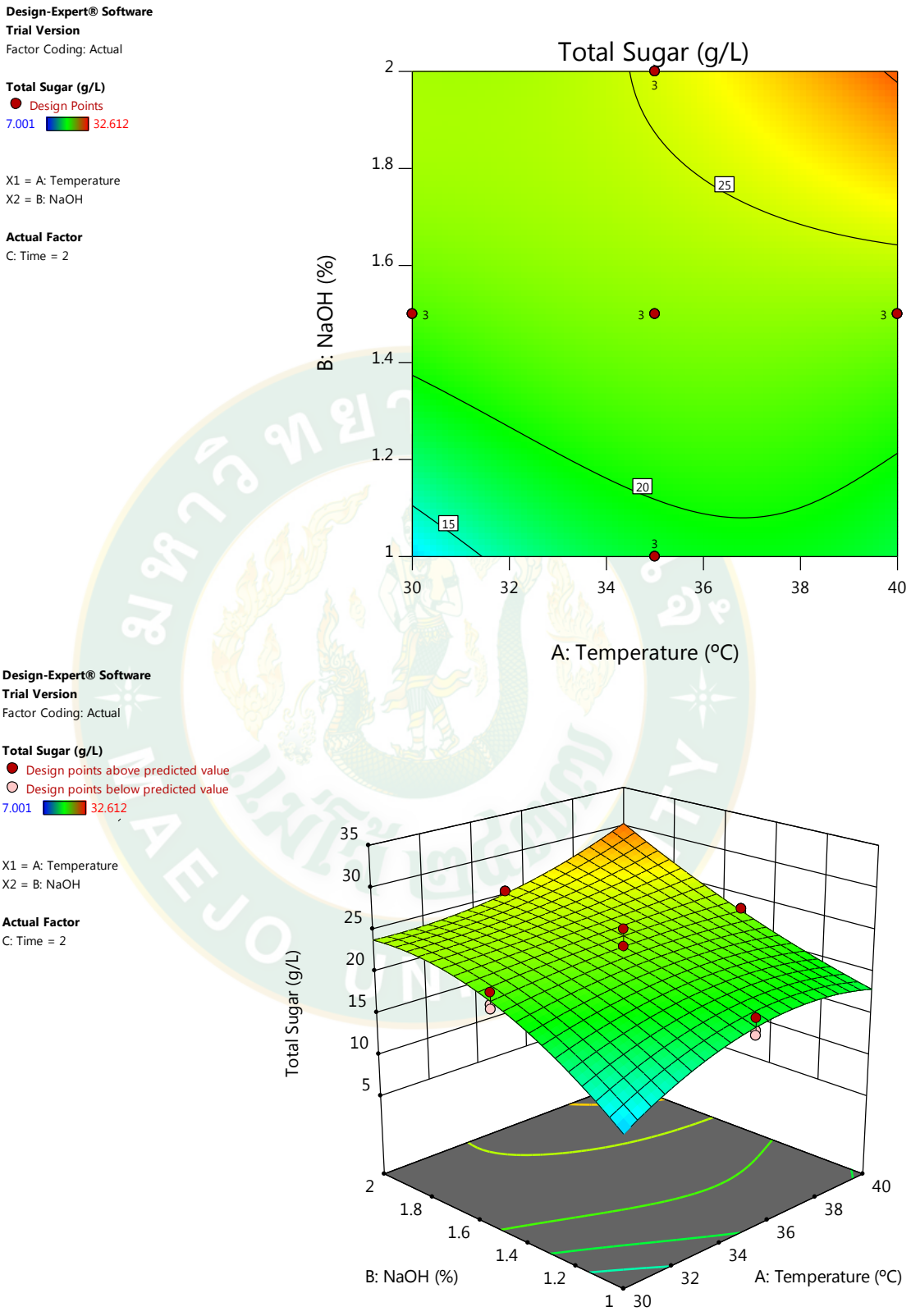


Figure 50 Total sugar yield from sugarcane leaf (design points above/below predicted value), actual factor (time)

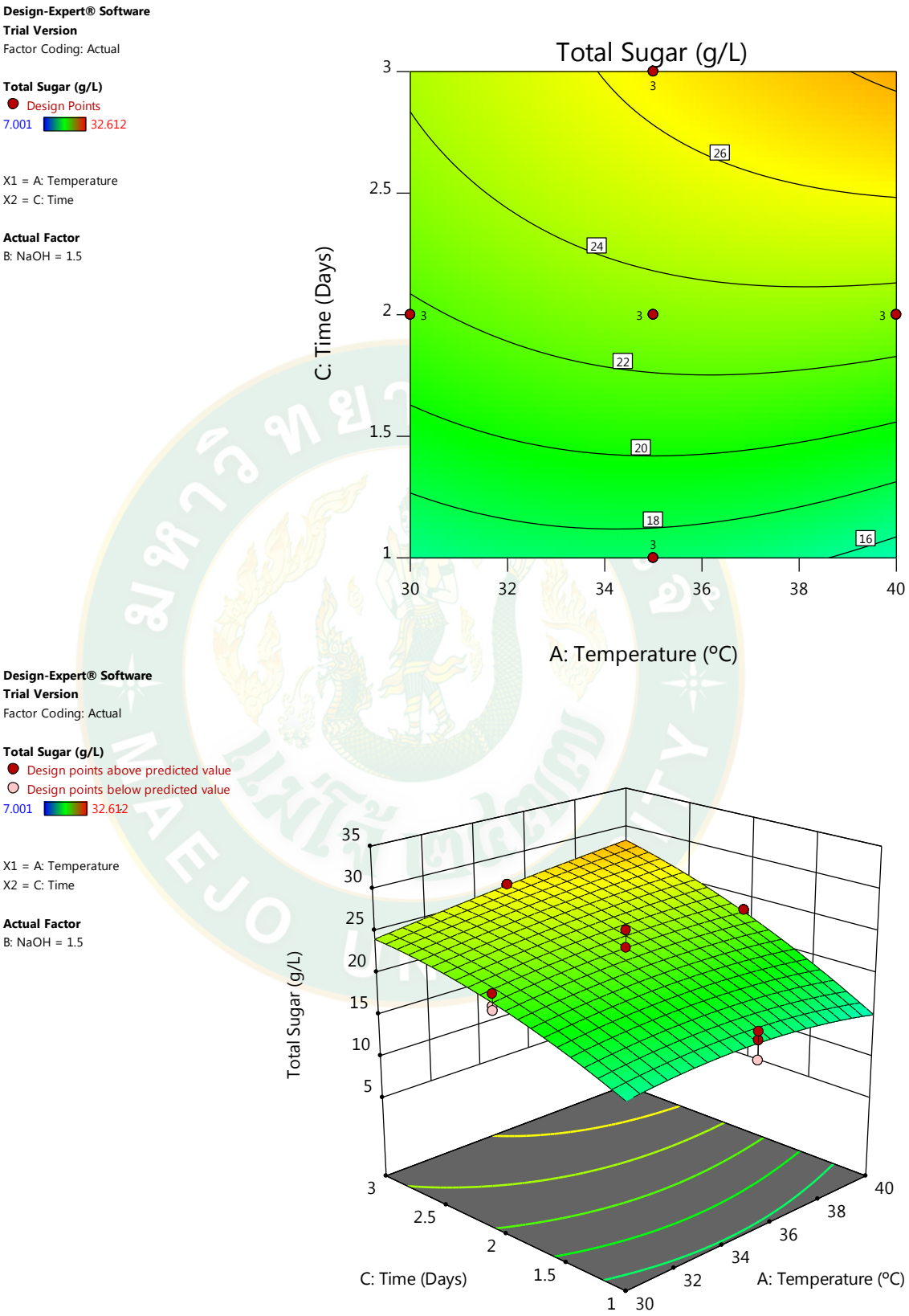


Figure 51 Total sugar yield from sugarcane leaf (design points above/below predicted value), actual factor (NaOH)

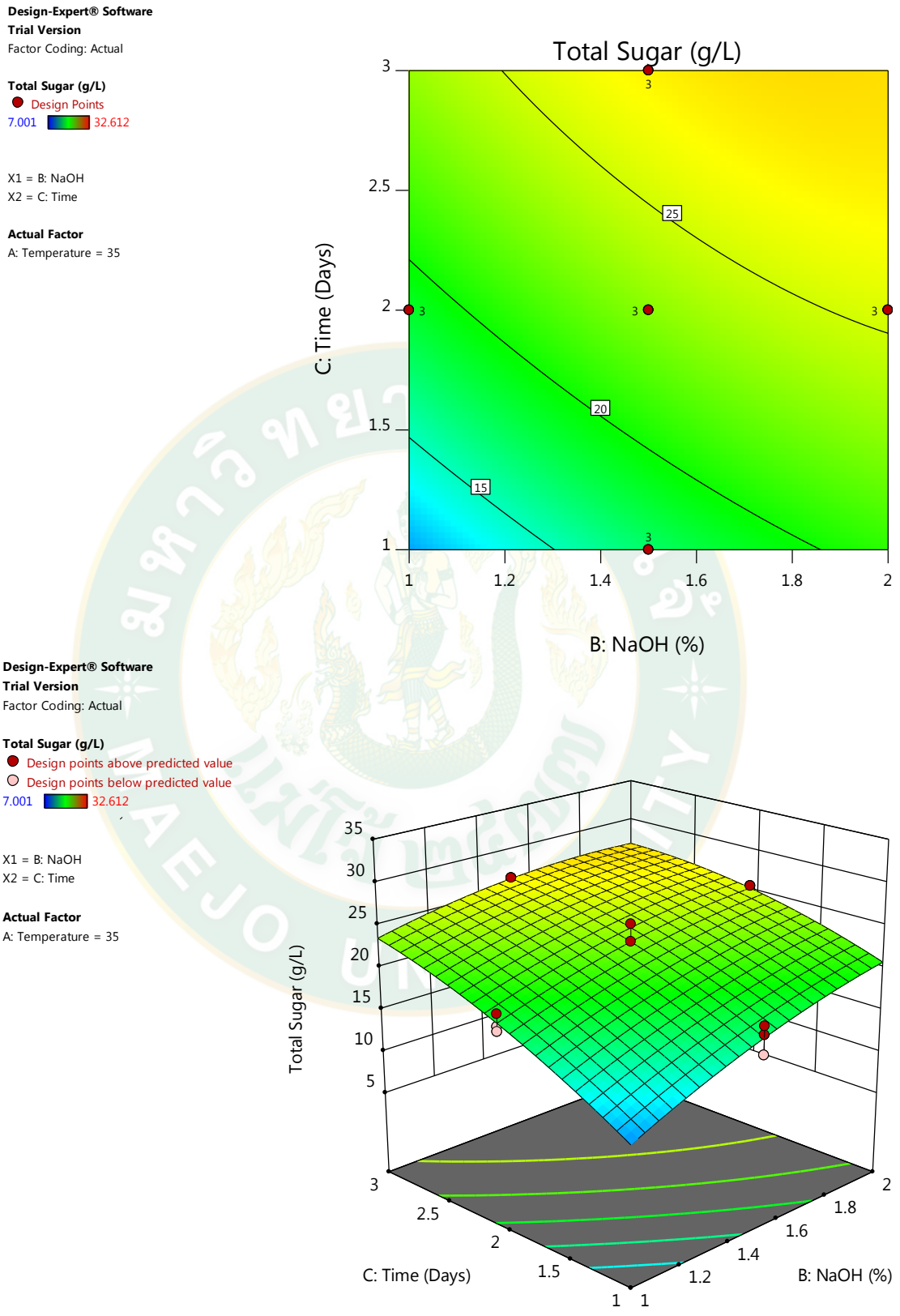


Figure 52 Total sugar yield from sugarcane leaf (design points above/below predicted value), actual factor (temperature)

Figure 50 described the interaction effect of NaOH and temperature on the total sugar production from sugarcane leaf. As it can be seen in the plots, there is an increase in the total sugar rate with an increase of NaOH concentration, with the maximum rate in the 2% NaOH. On the other hand, the effect of temperature on total sugar production from sugarcane leaf has not similar trends, regardless of the NaOH concentration. The total sugar rate is not increased with the increase of temperature. It can be concluded from the contour plots that the optimum region of the total sugar production from sugarcane leaf is the highest in the 2% NaOH.

Figure 51 illustrated the interaction effect of the time and temperature on the total sugar production from sugarcane leaf. As can be seen in the plots, the increase of the time leads to an increase in the total sugar rate. The time has been increasing degradation rate. We can seen from the contour plots Figure 51 (2D) that the total sugar concentration is more than 23 g/L in the time range of 2.75 to 3 days either at a low or high level of temperature. Therefore, it can be concluded that the increasing time does not affect lignocellulose degradation.

Figure 52 interpreted the interaction effect of the time and NaOH concentration on total sugar production from sorghum stalk. The contour plots shown that the optimum region for the total sugar production rate is in the time range of 3 days and the NaOH concentration is in the range of 2%, respectively.

Sugar concentration on scale up from pretreatment and enzyme hydrolysis and evaporation

Sugar concentration of sorghum stalk from scaling up ; Pretreatment with 2% NaOH there were reducing sugar and total sugar 6.480±0.538 and 28.263±2.263 g/L. Hydrolysis with 2% Cellulase Enzyme there were reducing sugar and total sugar 28.800±7.632 and 151.228±12.470 g/L and after evaporation there were reducing sugar and total sugar 62.667±16.518 and 276.842±6.403g/L disclosed in Table 30.

Table 30 Fermentable sugar from sugarcane leaf

Plants	Parameter	Reducing Sugar (g/L)	Total Sugar (g/L)	Degree of Polymerisation (DP)	pH
Sugarcane Leave	2% NaOH	6.293±0.227	27.772±2.296	4.413	7.954±0.184
	2% Cellulase Enzyme	17.600±2.177	111.228±5.402	6.320	4.213±0.059
	Evaporation	37.067±2.810	205.614±9.493	5.547	5.600±0

Bioethanol yields from sugarcane leaf

Ethanol yields were exposed in Figure 53 at the 1st day there is highest ethanol concentration is 0.000 g/L, the 3th day there is the highest ethanol concentration is 5.234± 0.907 g/L.

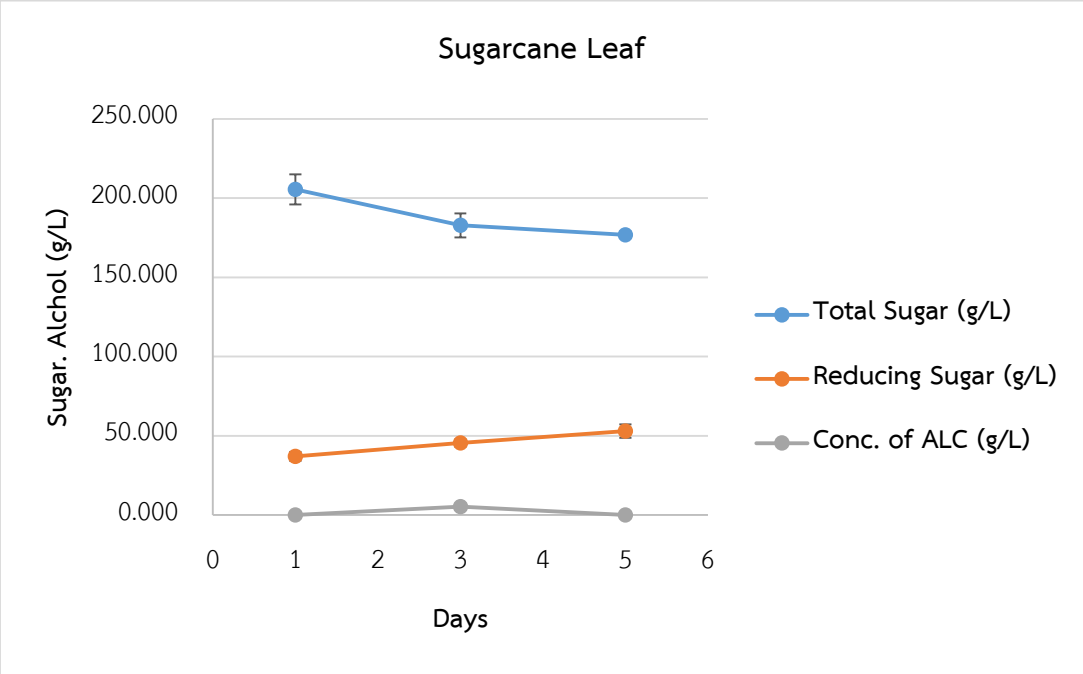


Figure 53 Ethanol concentration, total sugar, and reducing sugar throughout fermentation (sugarcane leaf)

Bioethanol production from corn stalk

The products from chemical and biological pretreatment

In this study corn stalk can produce total sugar and reducing sugar from silage were lowest as 14.754±0.747, 2.413±0.234 g/L while pretreatment with 2% NaOH were the highest 25.702±0.548, 2.560±0.069 g/L. Total sugar and reducing sugar from pretreatment by water and 1% *T.* spp. were 13.228±0.409, 2.520±0.040 g/L and 15.491±0.402, 2.227±0.151 g/L emerged in Table 54.

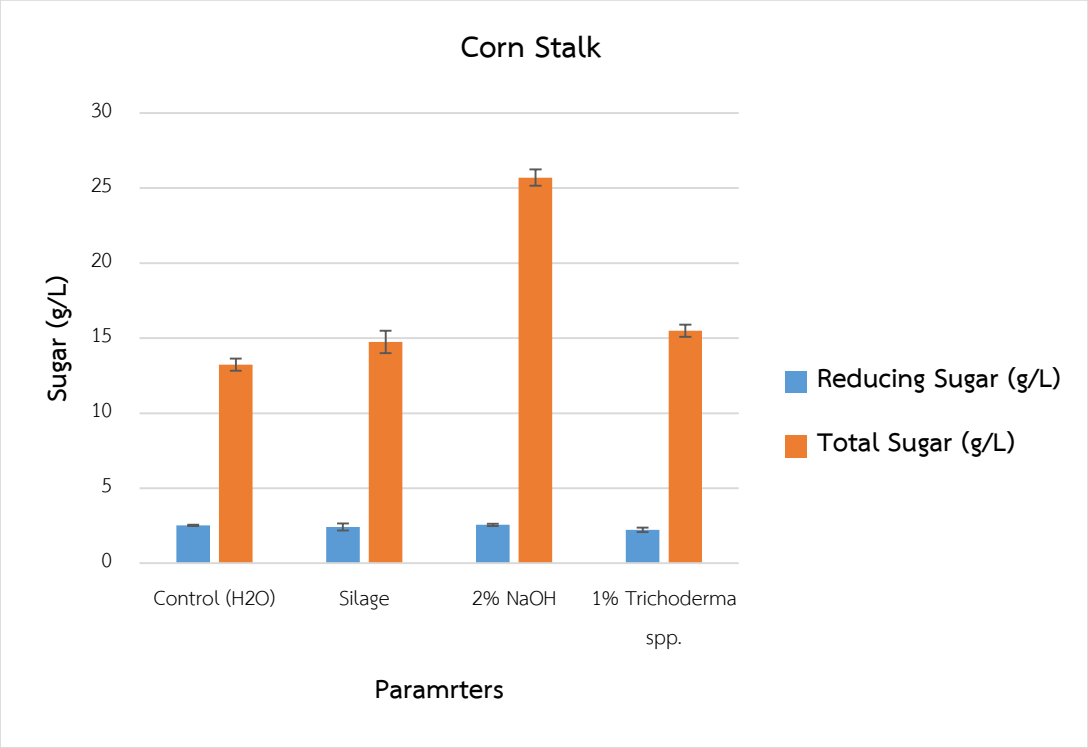


Figure 54 Sugar concentration of corn stalk pretreatment

Reponses surface methodology of pretreatments

In this study, the effect of three factors on reducing sugar production from sunflower stalk including temperature, NaOH concentration and time.

RSM development of reducing sugar from corn stalk

Model (corn stalk : reducing sugar)

All factors were selected as factors in the central composite design. As a response, the reducing sugar production rate was chosen, a total number of 29

experiments were employed for the response surface modeling expressed in Table 31, and the order of experiments was arranged randomly. The observed and predicted results for the reducing sugar production from corn stalk are also depicted in Table 31.

Table 31 Experimental designs of reducing sugar and predictive values from corn stalk

Run	Factor 1	Factor 2	Factor 3	Reducing sugar (g/L)	
	A: Temperature (°C)	B:NaOH (%)	C: Time (Days)	Observed	Predicted
1	40	1	1	1.720	1.720
2	40	2	3	6.250	6.250
3	40	2	1	2.660	2.670
4	40	1.5	2	3.400	3.680
5	35	1.5	1	1.940	2.120
6	35	1.5	3	4.360	4.260
7	30	1	1	1.360	1.370
8	35	1.5	2	3.630	3.690
9	35	1.5	1	2.010	2.120
10	35	1	2	2.850	3.040
11	35	1.5	1	2.460	2.120
12	40	1.5	2	3.790	3.680
13	30	1.5	2	3.470	3.570
14	30	1	3	1.880	1.890
15	35	1.5	3	4.100	4.260
16	35	2	2	4.180	4.380
17	35	1.5	2	3.670	3.690
18	35	2	2	4.430	4.380
19	35	1.5	2	3.650	3.690
20	40	1.5	2	3.880	3.680
21	30	1.5	2	3.530	3.570
22	35	1.5	3	4.360	4.260
23	30	1.5	2	3.750	3.570
24	35	2	2	4.570	4.380
25	30	2	1	2.880	2.890
26	30	2	3	4.430	4.440
27	35	1	2	3.300	3.040
28	35	1	2	3.010	3.040
29	40	1	3	4.020	4.030

The Design-Expert 11 software was used to calculate the coefficients of the second-order fitting equation and the model suitability was tested using the ANOVA test. Therefore, the second-order polynomial equation should be expressed by Equation 11 (conf. Equation 1):

$$\text{Reducing Sugar} = +3.69+0.0532A+0.6693B+1.07C-0.1131AB+0.4784AC+0.2884BC -0.0614A^2+0.0251B^2-0.4952C^2+0.0311ABC+0.2353A^2B -0.0742A^2C+0.4590AB^2 \quad \textbf{...(Equation 11)}$$

Y= Reducing sugar (g/L)

A= Temperature (°C)

B= NaOH (%)

C= Time (days)

Statistical analysis (corn stalk : reducing sugar)

CCD was applied for the optimization of reducing sugar production conditions. The Model F-value of 62.64 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case B, C, AC, BC, C^2, A^2B, AB^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 0.17 implies the Lack of Fit is not significant relative to the pure error. There is a 68.26% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good for the model to fit reported in Table 32.

The R^2 of 0.9818 in Figure 55 and The Predicted R^2 of 0.7381 is not as close to the Adjusted R^2 of 0.9662 as one might normally expect; i.e. the difference is more than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 36.285 indicates an adequate signal. This model can be used to navigate the design space proved in Table 33.

Table 32 ANOVA for quadratic model of reducing sugar from corn stalk

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Remark
Model	30.56	13	2.35	62.64	< 0.0001	significant
A-Temperature	0.017	1	0.017	0.4519	0.5116	not significant
B-NaOH	2.69	1	2.69	71.63	< 0.0001	significant
C-Time	6.85	1	6.85	182.47	< 0.0001	significant
AB	0.1024	1	0.1024	2.73	0.1194	not significant
AC	1.83	1	1.83	48.78	< 0.0001	significant
BC	0.6653	1	0.6653	17.73	0.0008	significant
A^2	0.0259	1	0.0259	0.6907	0.419	not significant
B^2	0.0043	1	0.0043	0.1156	0.7386	not significant
C^2	1.69	1	1.69	44.95	< 0.0001	significant
ABC	0.0078	1	0.0078	0.2065	0.656	not significant
A^2B	0.1898	1	0.1898	5.06	0.04	not significant
A^2C	0.0189	1	0.0189	0.5031	0.489	not significant
AB^2	0.7222	1	0.7222	19.24	0.0005	significant
Residual	0.5629	15	0.0375			
Lack of Fit	0.0069	1	0.0069	0.1744	0.6826	not significant
Pure Error	0.556	14	0.0397			
Cor Total	31.12	28				

Table 33 Fit statistics of reducing sugar from corn stalk

Std. Dev.	0.1937	**R^2**	0.9819
Mean	3.43	**Adjusted R^2**	0.9662
C.V. %	5.65	**Predicted R^2**	0.7381
		Adeq Precision	36.2851

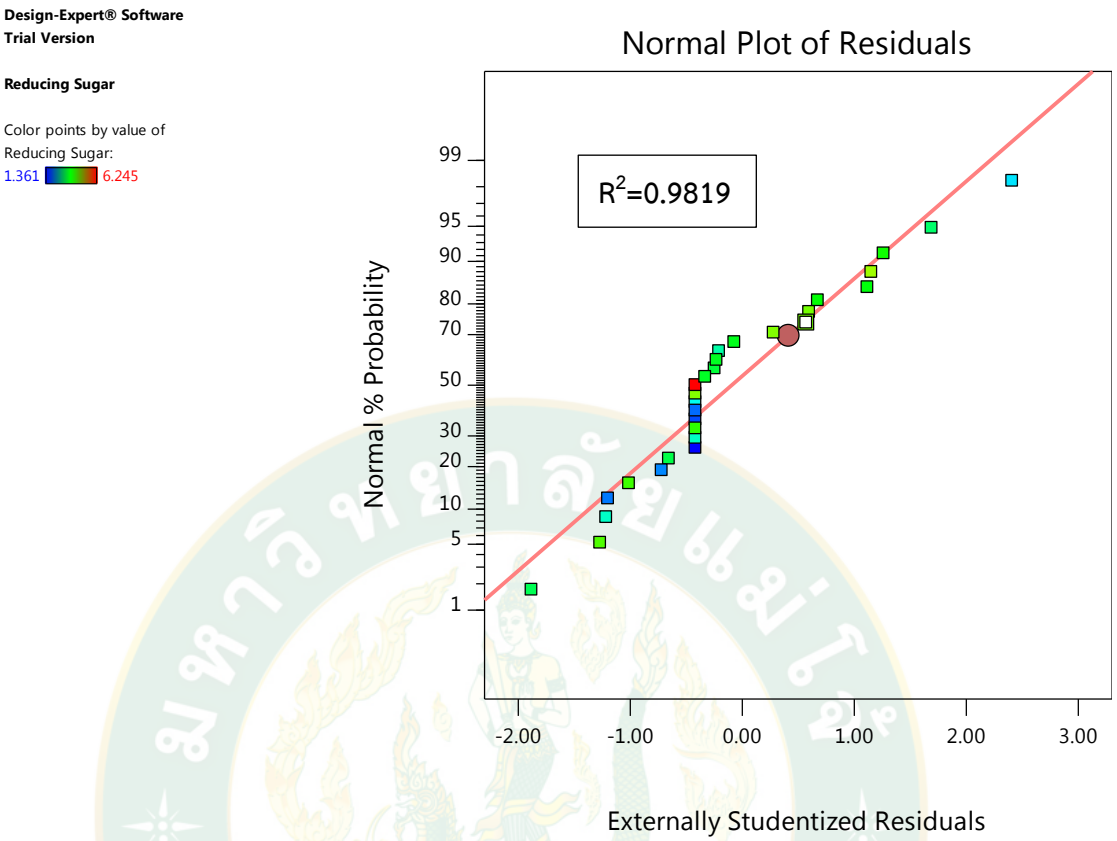


Figure 55 Comparison of predicted and actual value of reducing sugar from corn stalk

The effects of model parameters and their Interactions

The Design-Expert 11 software was used to produce three-dimensional (3D) response surfaces and two-dimensional (2D) contour plots. The 3D surfaces and 2D contour plots are graphical representations of the regression equation for the optimization of reaction conditions and are the most useful approach in revealing the conditions of the reaction system. In such plots, the response functions of two factors are presented while all other factors are at the fixed levels. The results of the interactions between three independent variables and the dependent variable are exemplified in Figure 56, Figure 57 and Figure 58.

As in Figure 56, Figure 57 and Figure 58 depending on the reaction, the temperature, NaOH concentration and time may have a positive or negative effect on the reducing sugar.

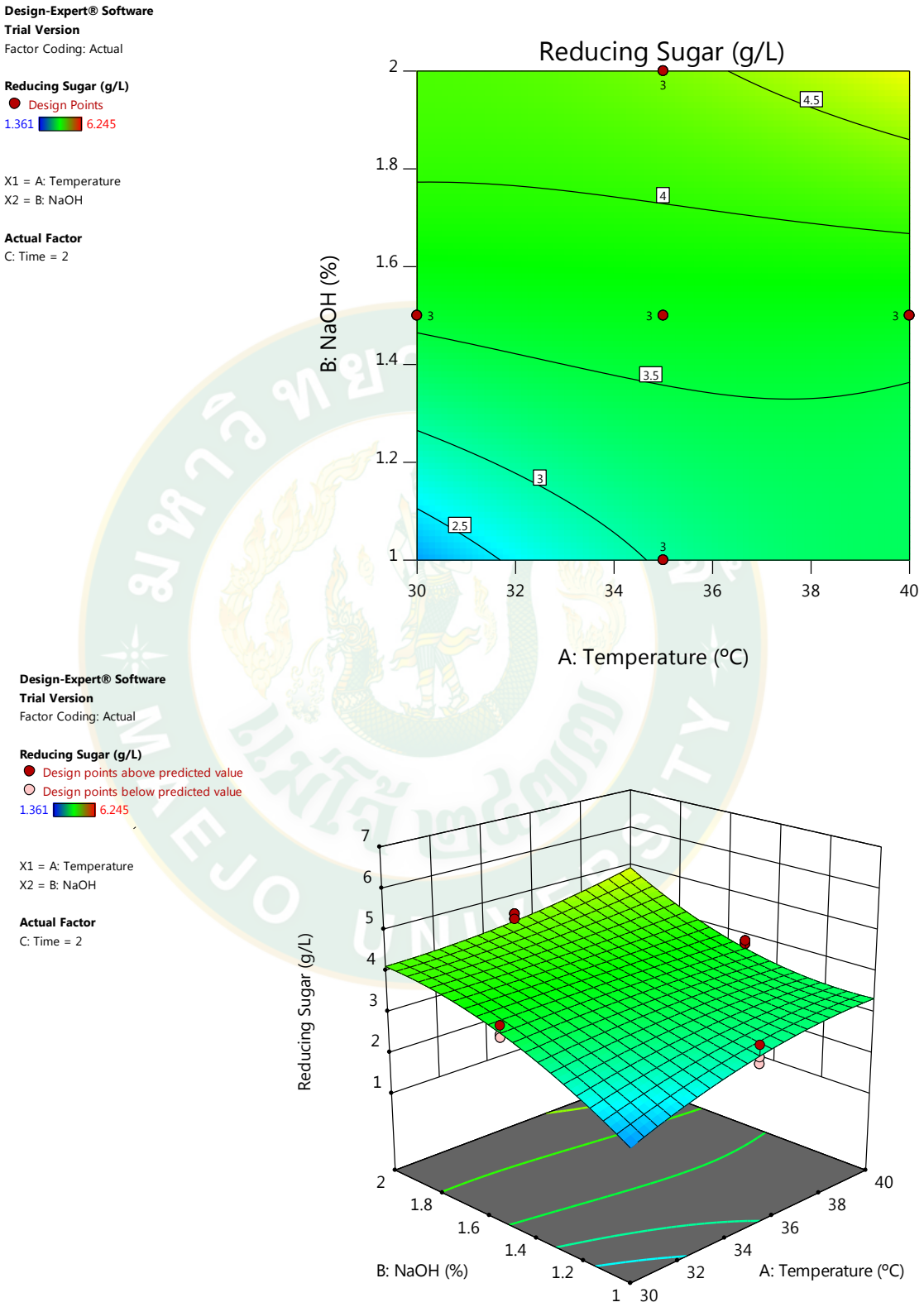


Figure 56 Reducing sugar yield from corn stalk (design points above/below predicted value), actual factor (time)

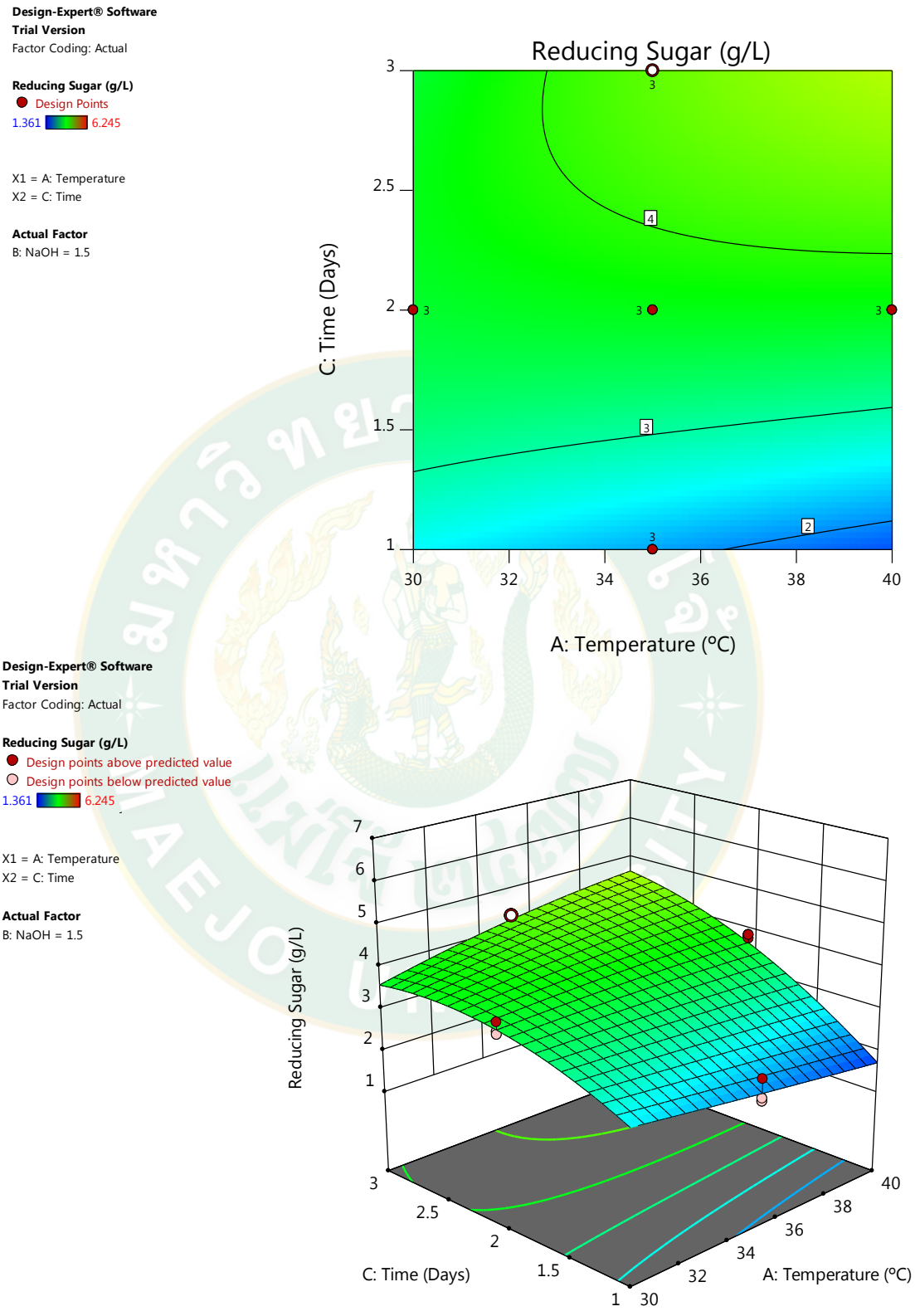


Figure 57 Reducing sugar yield from corn stalk (design points above/below predicted value), actual factor (NaOH)

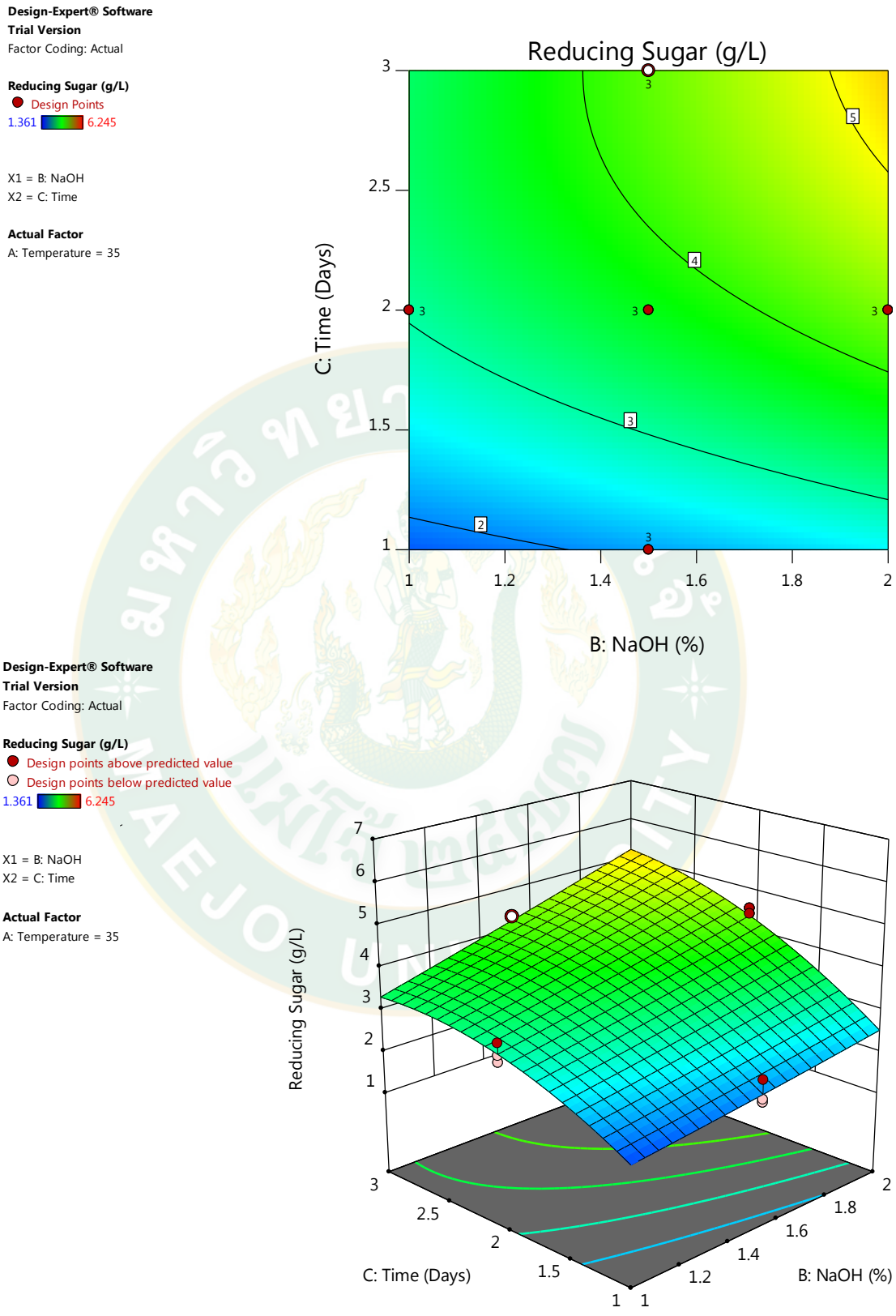


Figure 58 Reducing sugar yield from corn stalk (design points above/below predicted value), actual factor (temperature)

Figure 56 shown the interaction effect of NaOH and temperature on the reducing sugar production from corn stalk. As it can be seen in the plots, there is an increase in the reducing sugar rate with an increase of NaOH concentration, with the maximum rate in the 2% NaOH. On the other hand, the effect of temperature on reducing sugar production from corn stalk has not similar trends, regardless of the NaOH concentration. The reducing sugar rate increased slightly with the increase of temperature. It can be concluded from the contour plots that the optimum region of the reducing sugar production from corn stalk is in the 2% NaOH.

Figure 57 presented the interaction effect of the time and temperature on the reducing sugar production from corn stalk. As can be seen in the plots, the increase of the time leads to an increase in the reducing sugar rate. The time has been increasing degradation rate. We can see from the contour plots in Figure 57 (2D) that the reducing sugar concentration is more than 2 g/L in the time range of 2–3 days either at a low or high level of temperature. Therefore, it can be concluded that the increasing time does not affect lignocellulose degradation.

Figure 58 shown the interaction effect of the time and NaOH concentration on reducing sugar production from corn stalk. The contour plots shown that the optimum region for the reducing sugar production rate is in the time range of 3 days and the NaOH concentration is in the range of 2%, respectively.

RSM development of total sugar from corn stalk

Model (corn stalk : total sugar)

All factors were selected as factors in the central composite design. As a response, the total sugar production rate was chosen, a total number of 29 experiments were employed for the response surface modeling exhibited in Table 43, and the order of experiments was arranged randomly. The observed and predicted results for the total sugar production from sorghum stalk are also depicted in Table 34.

Table 34 Experimental designs of total sugar and predictive values from corn stalk

Run	Factor 1	Factor 2	Factor 3	Total sugar (g/L)	
	A: Temperature (°C)	B:NaOH (%)	C: Time (Days)	Observed	Predicted
1	40	1	1	14.910	14.850
2	40	2	3	37.510	37.440
3	40	2	1	24.820	24.750
4	40	1.5	2	26.230	26.680
5	35	1.5	1	20.890	21.360
6	35	1.5	3	32.990	32.700
7	30	1	1	13.280	13.210
8	35	1.5	2	27.010	27.260
9	35	1.5	1	20.950	21.360
10	35	1	2	23.070	23.580
11	35	1.5	1	21.970	21.360
12	40	1.5	2	26.820	26.680
13	30	1.5	2	25.410	25.360
14	30	1	3	26.790	26.730
15	35	1.5	3	33.120	32.700
16	35	2	2	29.270	29.160
17	35	1.5	2	26.770	27.260
18	35	2	2	28.480	29.160
19	35	1.5	2	29.040	27.260
20	40	1.5	2	26.710	26.680
21	30	1.5	2	25.570	25.360
22	35	1.5	3	31.730	32.700
23	30	1.5	2	24.830	25.360
24	35	2	2	29.480	29.160
25	30	2	1	23.310	23.240
26	30	2	3	29.150	29.090
27	35	1	2	23.980	23.580
28	35	1	2	23.440	23.580
29	40	1	3	30.060	29.990

The Design-Expert 11 software was used to calculate the coefficients of the second-order fitting equation and the model suitability was tested using the ANOVA test. Therefore, the second-order polynomial equation should be expressed by Equation 12 (conf. Equation 1):

$$\text{Total Sugar} = +27.26+0.6580A+2.79B+5.67C+0.6199AB+1.06AC-1.27BC-1.24A^2-0.8817B^2 -0.2247C^2+0.6519ABC+0.9275A^2B+0.2297A^2C+1.19AB^2 \quad \textbf{...(Equation 12)}$$

Y= Total sugar (g/L)

A= Temperature (°C)

B= NaOH (%)

C= Time (days)

Statistical analysis (corn stalk : total sugar)

CCD was applied for the optimization of total sugar production conditions. The Model F-value of 115.87 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BC, A^2, B^2, ABC, A^2B, AB^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 1.17 implies the Lack of Fit is not significant relative to the pure error. There is a 29.68% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good for the model to fit explained in Table 35.

The R^2 of 0.9900 in Figure 59 and the Predicted R^2 of 0.2181 is not as close to the Adjusted R^2 of 0.9816 as one might normally expect; i.e. the difference is more than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 50.877 indicates an adequate signal. This model can be used to navigate the design space manifested in Table 36.

Table 35 ANOVA for quadratic model of total sugar from corn stalk

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Remark
Model	707.72	13	54.44	115.87	< 0.0001	significant
A-Temperature	2.6	1	2.6	5.53	0.0328	significant
B-NaOH	46.7	1	46.7	99.4	< 0.0001	significant
C-Time	192.9	1	192.9	410.59	< 0.0001	significant
AB	3.07	1	3.07	6.54	0.0219	significant
AC	8.99	1	8.99	19.13	0.0005	significant
BC	12.81	1	12.81	27.27	0.0001	significant
A^2	10.53	1	10.53	22.41	0.0003	significant
B^2	5.35	1	5.35	11.38	0.0042	significant
C^2	0.3473	1	0.3473	0.7391	0.4035	not significant
ABC	3.4	1	3.4	7.24	0.0168	significant
A^2B	2.95	1	2.95	6.28	0.0242	significant
A^2C	0.1809	1	0.1809	0.3851	0.5442	not significant
AB^2	4.84	1	4.84	10.3	0.0059	significant
Residual	7.05	15	0.4698			
Lack of Fit	0.5455	1	0.5455	1.17	0.2968	not significant
Pure Error	6.5	14	0.4644			
Cor Total	714.77	28				

Table 36 Fit statistics of total sugar from corn stalk

Std. Dev.	0.6854	**R^2**	0.9901
Mean	26.12	**Adjusted R^2**	0.9816
C.V. %	2.62	**Predicted R^2**	0.2181
		Adeq Precision	50.8767

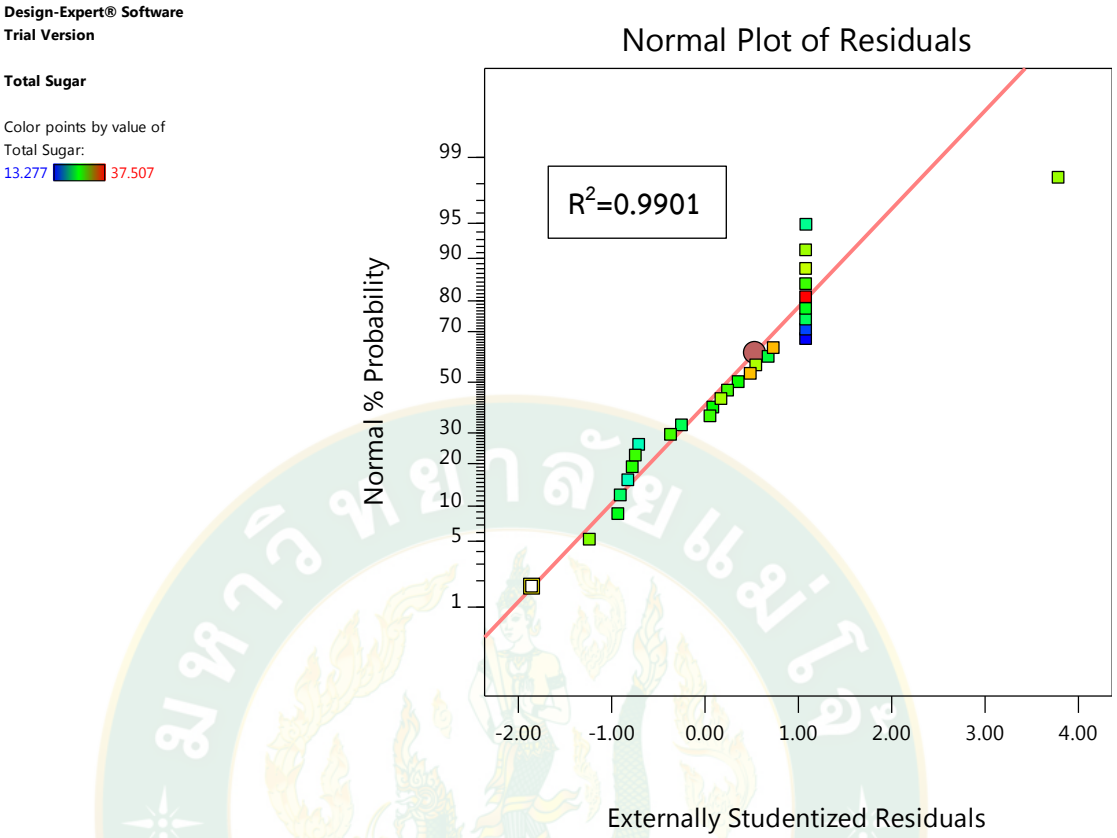


Figure 59 Comparison of predicted and actual value of total sugar from corn stalk

The effects of model parameters and their Interactions

The Design-Expert 11 software was used to produce three-dimensional (3D) response surfaces and two-dimensional (2D) contour plots. The 3D surfaces and 2D contour plots are graphical representations of the regression equation for the optimization of reaction conditions and are the most useful approach in revealing the conditions of the reaction system. In such plots, the response functions of two factors are presented while all other factors are at the fixed levels. The results of the interactions between three independent variables and the dependent variable are explained in Figure 60, Figure 61 and Figure 62.

As in Figure 60, Figure 61 and Figure 62 depending on the reaction, the temperature, NaOH concentration and time may have a positive or negative effect on the total sugar.

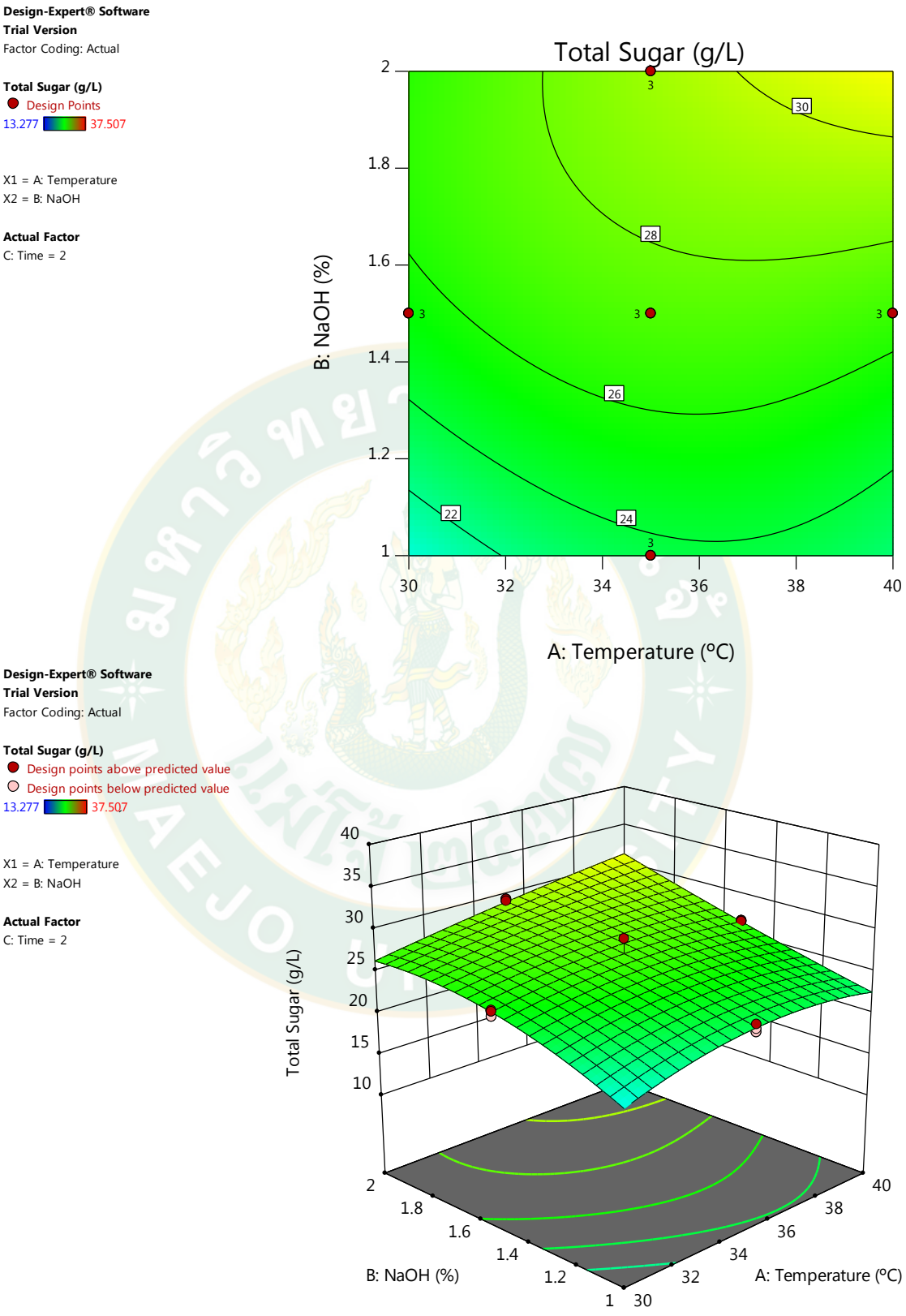


Figure 60 Total sugar yield from corn stalk (design points above/below predicted value), actual factor (time)

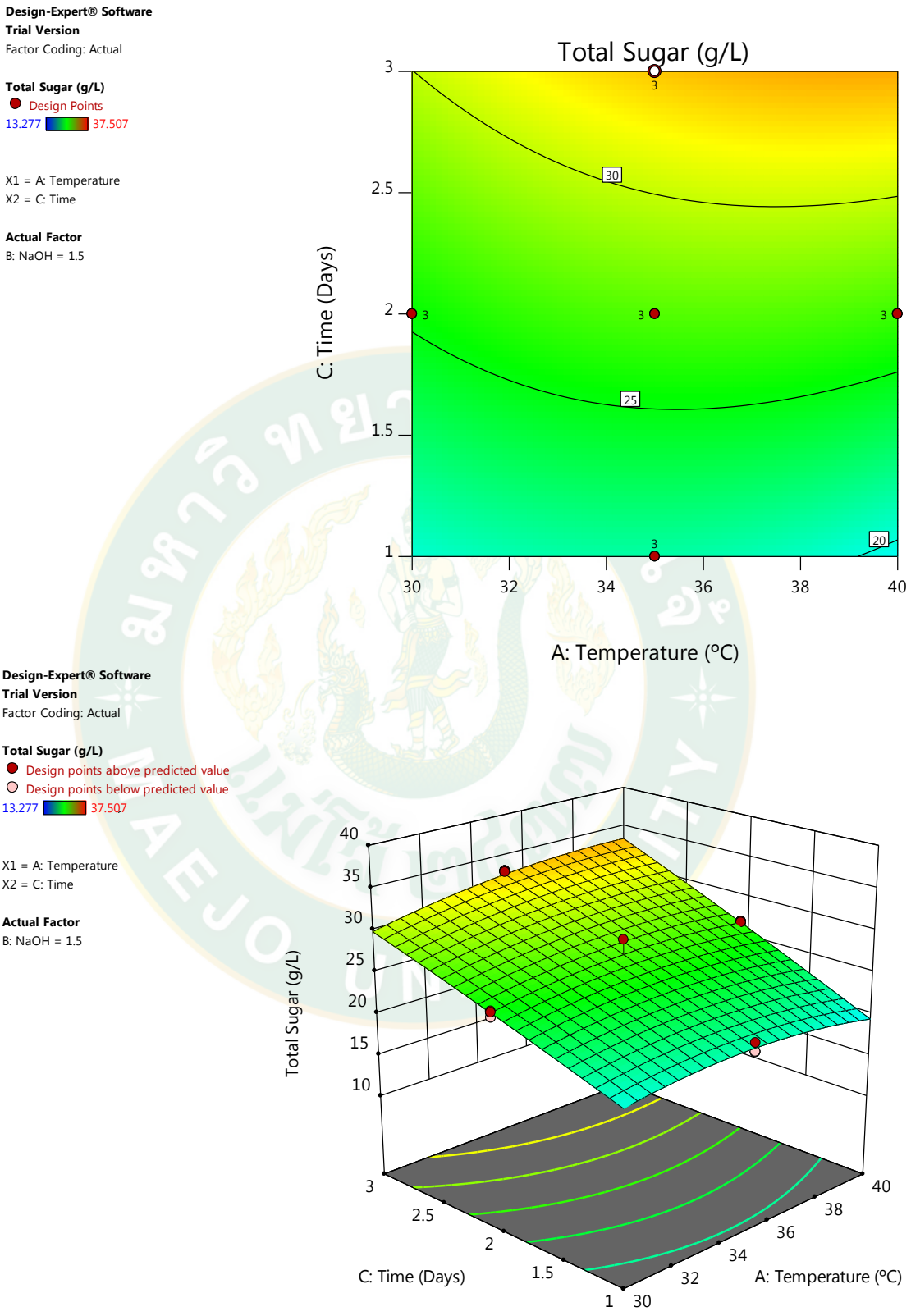


Figure 61 Total sugar yield from corn stalk (design points above/below predicted value), actual factor (NaOH)

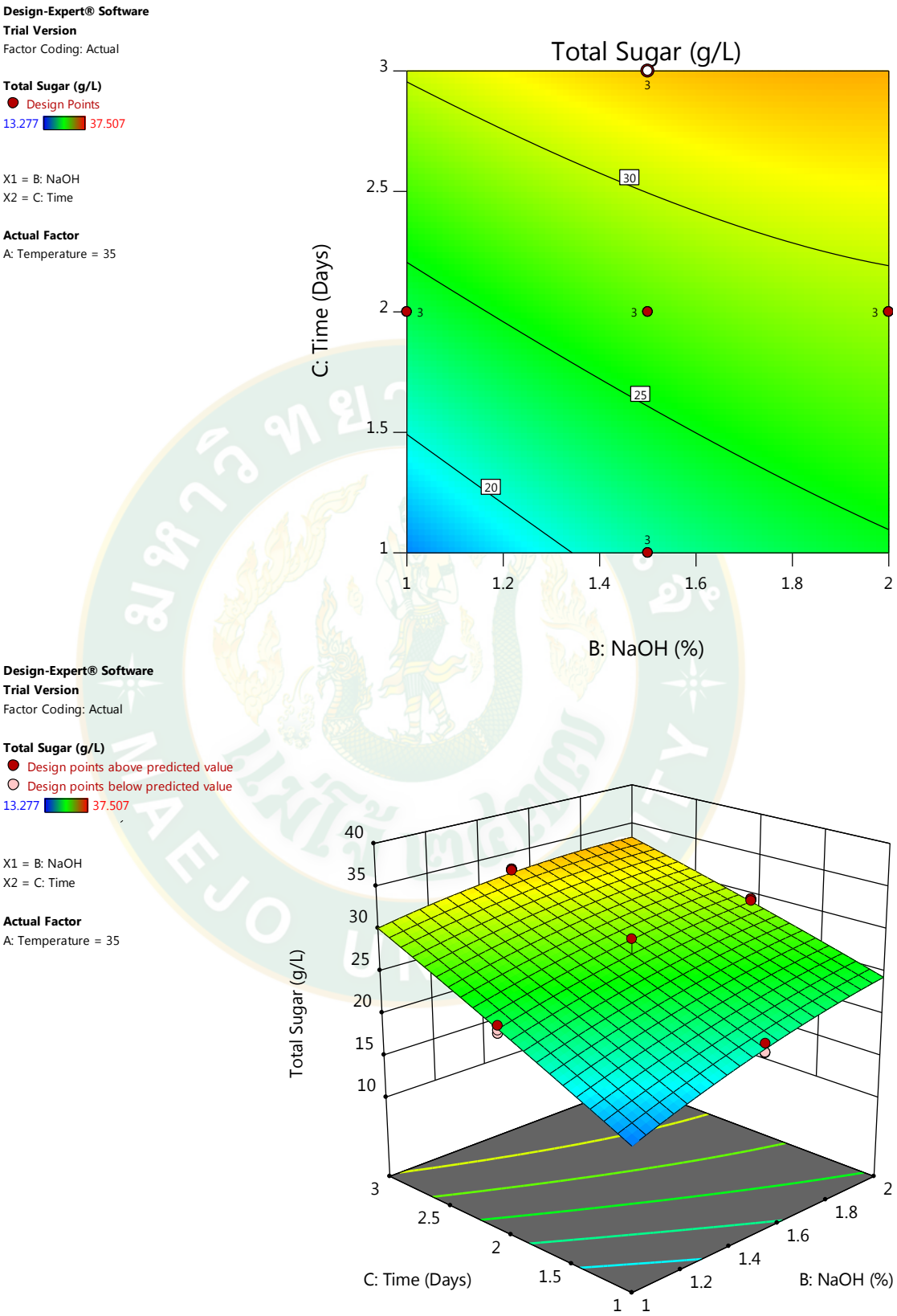


Figure 62 Total sugar yield from corn stalk (design points above/below predicted value), actual factor (temperature)

Figure 60 revealed the interaction effect of NaOH and temperature on the total sugar production from corn stalk. As it can be seen in the plots, there is an increase in the total sugar rate with an increase of NaOH concentration, with the maximum rate in the 2% NaOH. On the other hand, the effect of temperature on total sugar production from corn stalk has not similar trends, regardless of the NaOH concentration. The total sugar rate is not increased with the increase of temperature. It can be concluded from the contour plots that the optimum region of the total sugar production from corn stalk is the highest in the 2% NaOH.

Figure 61 attested the interaction effect of the time and temperature on the total sugar production from corn stalk. As can be seen in the plots, the increase of the time leads to an increase in the total sugar rate. The time has been increasing degradation rate. We can see from the contour plots in Figure 61 (2D) that the total sugar concentration is more than 30 g/L in the time range of 2.5 to 3 days either at a low or high level of temperature. Therefore, it can be concluded that the increasing time does not affect lignocellulose degradation.

Figure 62 demonstrated the interaction effect of the time and NaOH concentration on total sugar production from sorghum stalk. The contour plots shown that the optimum region for the total sugar production rate is in the time range of 3 days and the NaOH concentration is in the range of 2%, respectively.

Sugar concentration on scale up from pretreatment, enzyme hydrolysis and evaporation

Sugar concentration of corn stalk from scaling up ; Pretreatment with 2% NaOH there were reducing sugar and total sugar 6.480±0.538 and 28.263±2.263 g/L. Hydrolysis with 2% cellulase enzyme there were reducing sugar and total sugar 28.800±7.632 and 151.228±12.470 g/L and after evaporation there were reducing sugar and total sugar 62.667±16.518 and 276.842±6.403g/L described in Table 37.

Table 37 Fermentable sugar from corn stalk

Plants	Parameter	Reducing Sugar (g/L)	Total Sugar (g/L)	Degree of Polymerisation (DP)	pH
Sugarcane Leave	2% NaOH	6.560±0.069	28.649±2.283	4.367	8.627±0.216
	2% Cellulase Enzyme	47.200±2.884	185.965±11.225	3.940	4.953±0.060
	Evaporation	77.143±2.060	213.509±8.227	2.770	5.600±0

Bioethanol yields from corn stalk

Ethanol yields were illustrated in Figure 63 at the 1st day there is highest ethanol concentration is 0.000 g/L, the 3th day there is the highest ethanol concentration is 23.495±0.670 g/L

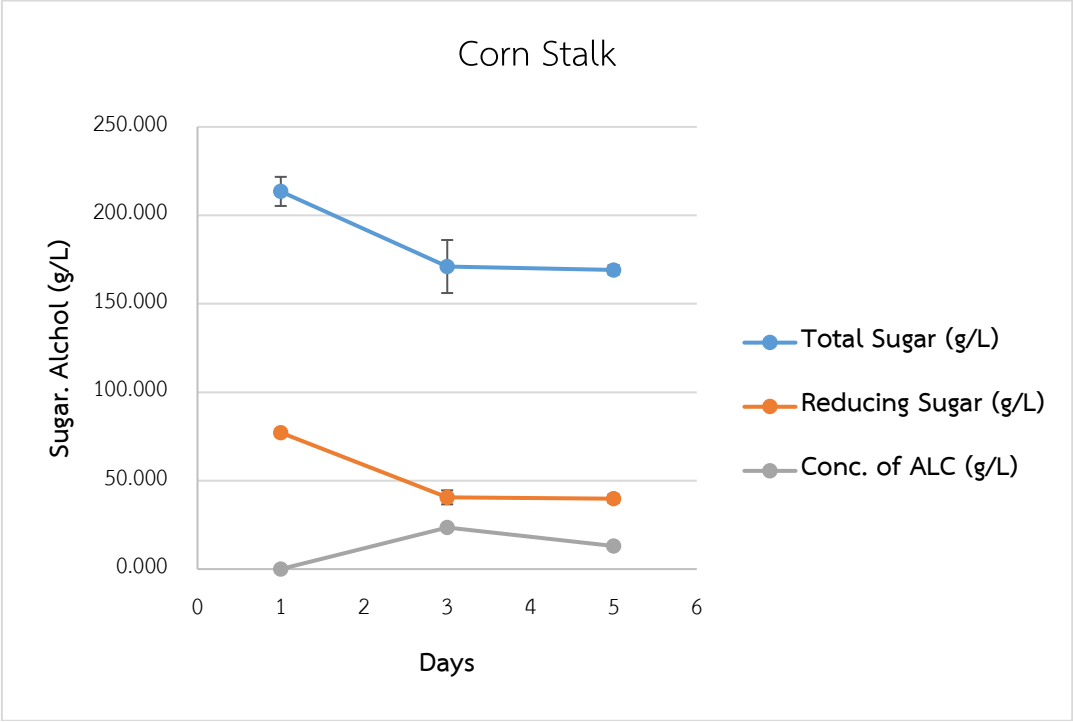


Figure 63 Ethanol concentration, total sugar, and reducing sugar throughout fermentation (corn stalk)

Comparison study between lab scale of sunflower stalk, sorghum stalk, sugarcane leaf and corn stalk for bioethanol production

In the present investigation, total sugar estimation was carried out by Phenol sulphuric acid method was pretreated with 2% NaOH sunflower stalk, sorghum stalk, sugarcane leaf and corn stalk substrates. The maximum total sugar sunflower stalk, sorghum stalk, sugarcane leaf and corn stalk substrates were found 35.544±0.818, 27.158±0.913, 17.912±0.500 and 25.702±0.548 g/L Table 38. From the Table 38 reported that sunflower stalk is maximum release of total sugar compared to other crops. The residual sugar estimation was carried out by DNS method. The Reducing sugar of 2% NaOH treated sorghum stalk, sunflower stalk, sugarcane leaf and corn stalk substrates were found 6.053±1.166, 4.213±0.717, 4.147±0.266 and 2.560±0.069 g/L respectively from Table 38.

Table 38 Sugar from four plants

Plants	Pretreatments		
	Parameters	Reducing Sugar (g/L)	Total Sugar (g/L)
Sunflower Stalk	Control	2.707±0.167^{de}	8.860±1.373^{i}
	Silage	3.040±0.144^{cde}	11.754±1.446^{h}
	2% NaOH	4.213±0.717^{a}	35.544±0.818^{a}
	1% *T.* spp.	3.693±0.482^{bc}	20.544±1.701^{d}
Sorghum Stalk	Control	2.867±0.546^{cde}	17.123±1.574^{ef}
	Silage	2.293±0.122^{e}	13.965±3.117^{gh}
	2% NaOH	6.053±1.166^{b}	27.158±0.913^{b}
	1% *T.* spp.	3.960±0.616^{b}	24.667±0.540^{c}
Sugarcane Leaf	Control	3.360±0.212^{bcd}	14.789±1.891^{fg}
	Silage	2.640±0.629^{de}	15.053±2.346^{fg}
	2% NaOH	4.147±0.266^{b}	17.912±0.500^{e}
	1% *T.* spp.	2.493±0.623^{de}	12.035±0.373^{h}
Corn Stalk	Control	2.520±0.040^{de}	13.228±0.409^{gh}
	Silage	2.413±0.234^{e}	14.754±0.747^{fg}
	2% NaOH	2.560±0.069^{de}	25.702±0.548^{bc}
	1% *T.* spp.	2.227±0.151^{e}	15.491±0.402^{efg}

Sugar bioethanol and energy productivity (lab scale)

The comparison of sugar production from sunflower, sorghum and corn stalks and sugarcane leaf was shown in Table 39. The highest sugar concentration and sugar productivity from corn stalk 77.143 g/L and 3.214 g/L/h were produced for 24 h. Corn stalk fermentation can produce ethanol and ethanol productivity 23.455 g/L and 0.326 g/L/h were produced for 72 h interpreted in Table 39. The comparison of energy

production disclosed in Table 40 corn stalk was produced the highest energy from sugar 1,234.286 kJ/L and energy from ethanol 704.854 kJ/L exposed in Table 40.

Table 39 Sugar and bioethanol productivity

Plants	Sugar	Bioethanol	Saccharification time	Fermentation time	Theoretical Ethanol	Ethanol Yield (Theoretical)	Ethanol yield coefficient	Bioethanol Productivity	Sugar Productivity
	(g/L)	(g/L)	(h)	(h)	(g/L)	(%)	(g/g Sugar)	(g/L/h)	(g/L/h)
Sunflower Stalk	49.067	12.562	24	72	25.024	50.198	0.256	0.174	2.044
Sorghum Stalk	62.667	7.328	24	72	31.96	22.927	0.117	0.102	2.611
Sugarcane Leaf	37.067	5.234	24	72	18.904	27.687	0.141	0.073	1.544
Corn Stalk	77.143	23.495	24	72	39.343	59.719	0.305	0.326	3.214

Table 40 Energy productivity

Plants	Energy from Sugar	Energy from Bioethanol	Bioethanol Productivity	Sugar Productivity	Energy Productivity	
	(kJ/L)	(kJ/L)	(g/L/h)	(g/L/h)	kJ/h	W
Sunflower Stalk	785.067	376.848	0.174	2.044	37.945	10.549
Sorghum Stalk	1,002.667	219.828	0.102	2.611	44.831	12.463
Sugarcane Leaf	593.067	157.020	0.073	1.544	26.892	7.476
Corn Stalk	1,234.286	704.854	0.326	3.214	61.218	17.019

Scale up on bioethanol production and from corn stalk for distillation

This is results from experiment IV, Since the pretreatment with 2% NaoH and hydrolysis with 2% cellulase enzyme contained fermentable sugar was carried out by DNS method is 218.286 g/L. After fermented for 3 days bioethanol contented 7.3 % and after distilled bioethanol increased to 12.5% and HHV 1.838 MJ/kg emerged in Table 41.

Table 41 Characteristic of sugar and bioethanol from corn stalk

Parameters	Results
Fermentable sugar (g/L)	218.286
Bioethanol (%)	7.3
Bioethanol after distillation (%)	12.5
HHV (MJ/kg)	1.838
Energy from fermentable sugar (kJ)	3,492.576
Energy from bioethanol after distillation (kJ)	2,944.125

Techno-economic analysis of scale up on bioethanol production from corn stalk

In the recent years, bioethanol production on a large scale has attained significant interest in the economic possibility, however always has been focused on high percent of bioethanol that used associated raw materials with high productivities so as to decrease the unit cost from bioethanol production on a commercial scale. The crude enzymes are amylase and cellulase were used for the enzymatic hydrolysis biomass. In the hydrolysis process, crude enzymes extraction in incubator at 37℃ for 3 hrs and reaction was arrested at 4℃ for 15 minutes. Purification of the crude enzymes and optimization parameters may give better result for degradation of starch or cellulose and hemicelluloses present in biomass (Hemalatha et al., 2015). In this research,1 L of 12.5% bioethanol has lose a unit cost of bioethanol production 47.629 Baht expressed in Table 42 as a result of cellulase enzyme and electricity of an incubator were used for scarification and temperature controlling in the fermentation process. If cost reduction in this part, bioethanol production from lignocellulosic agricultural waste It is more interesting to produce at the industrial level.

Table 42 The unit cost of bioethanol production from corn stalk

Vessel total		**1 liter**	
Items	**Units**	**Quantity**	**Baht**
Biomass	0 Baht/kg	1 kg	0
NaOH	20 Baht/kg	0.06 kg	1.200
Water	1.020 Baht/L	7 L	7.140
Enzyme cellulase	185.734 Baht/kg	0.070 kg	13.001
Peptone	30.956 Baht/kg	0.005 kg	0.155
Glucose	46.433 Baht/kg	0.01 kg	0.464
Yeast extract	18.573 Baht/kg	0.01 kg	0.186
Milling machine	3.2483 Baht/Unit	15 W	0.049
Blender	3.2483 Baht/Unit	400 W	1.299
Incubator	3.2483 Baht/Unit	1,680 W	5.457
Disstillator	3.2483 Baht/Unit	400 W	1.299
Centrifuge	3.2483 Baht/Unit	550 W	1.787
Hot pate	3.2483 Baht/Unit	4,000 W	15.592
Unit cost			**47.629**

Mass and energy balance of scale up on bioethanol production from corn stalk

A mass balance starting from 1 kg of dry corn stalk for our overall process for sugar yield is reported in Figure 64. In 2% NaOH pretreatment and the enzymatic hydrolysis, the data were converted according to the results obtained from pretreatment and the enzymatic hydrolysis It was found that 86.7 g sugar/kg dry corn stalk and after distillation was obtained, 218,298 g of fermentable sugar and 1 kg of dry corn stalk was produced ethanol 57.312 g from Figure 65. An energy balance in this process used total energy 25.183 kW_t but produced energy 4.123 kW_t.

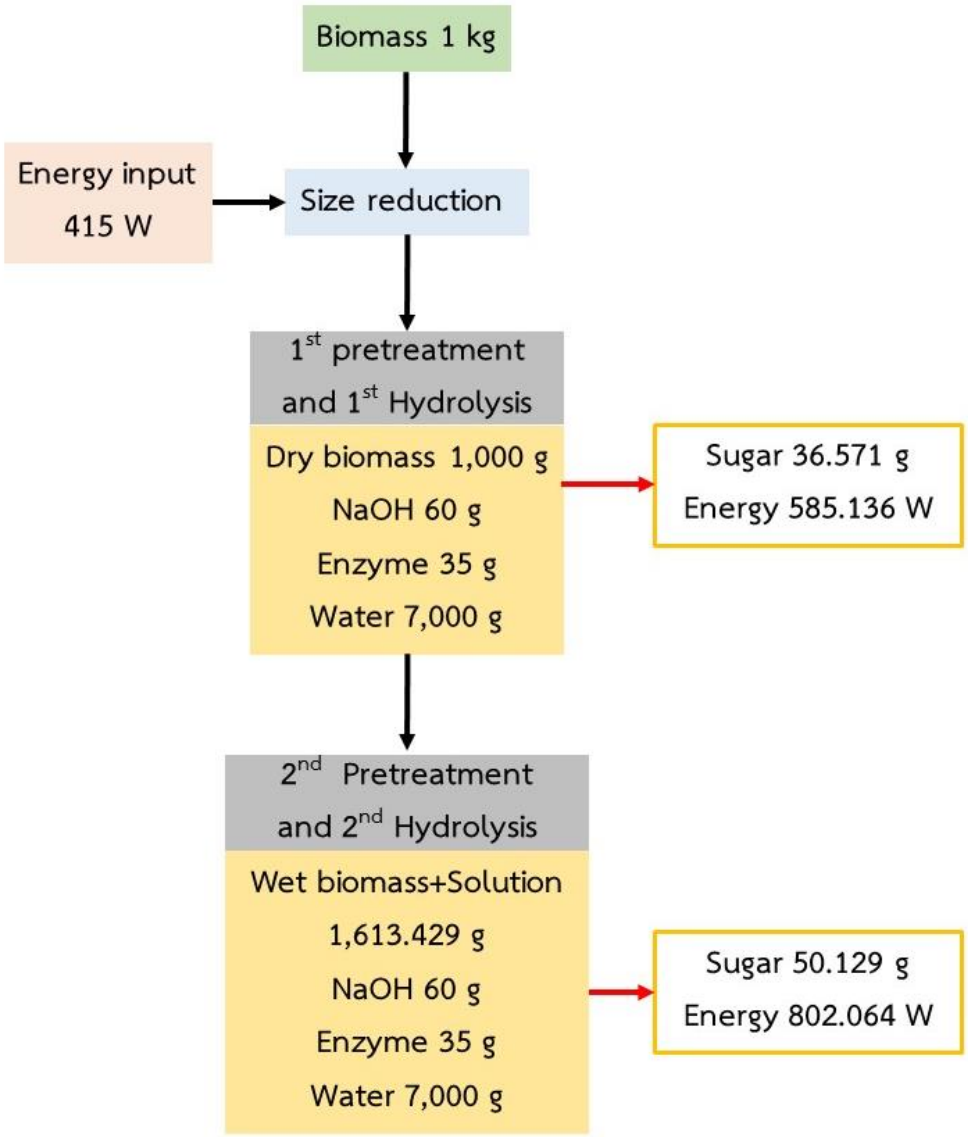


Figure 64 Mass and energy balances of sugar production from corn stalk

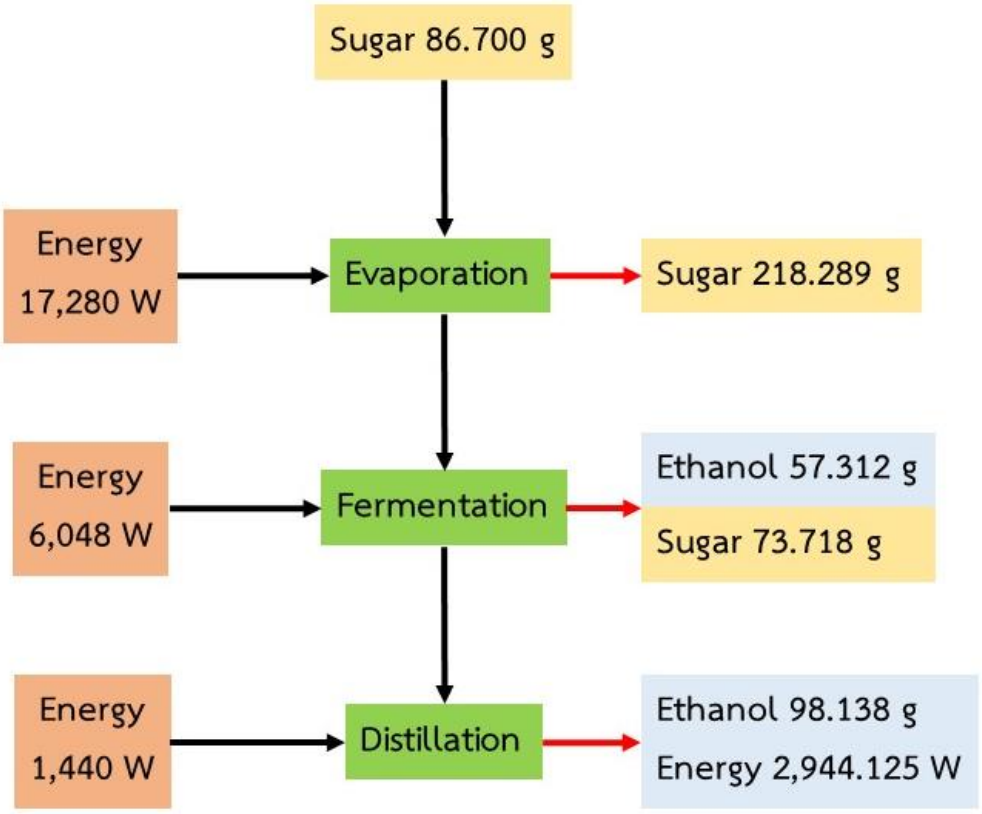


Figure 65 Mass and energy balances of ethanol production from corn stalk

Comparison between fermentable sugar and bioethanol from corn stalk and commercial scale of fresh sweet sorghum stalk

From Table 43 the comparison between bioethanol production from corn stalk and commercial scale of fresh sweet sorghum stalk. A results were calculated from a mass 1 kg to 10 kg of dry corn stalk (In this thesis) and the mass starting from 10 kg of fresh sweet sorghum stalks for our overall process for ethanol yield is shown in Figure 42 it is found that. In the research (corn stalk) used NaOH and callulase enzyme for sacharifrcation method produced fermentable sugar 2,182.860 g/L, *S. cerevisiae* TISTR 5020 and SHF method for fermentation produced bioethanol 98.138 g/L. But comparison with (Li et al., 2013) used *S. cerevisiae* TSH1 was used as the fermentation strain in the solid fermentation step. Then pretreatment and hydrolysis with NaOH and callulase enzyme after sacharifrcation used *Z. Mobilis* TSH-01 as the fermentation strain in the C5-C6 co-fermentation step. the enzymatic hydrolysis and C5-C6 co-fermentation stage, the data were converted according to the results obtained from

batch experiments performed in a shake flask instead of a large-scale instrument. It was found that 919 g/10 kg of sweet sorghum stalk or 91.9 kg ethanol/tonne fresh sweet sorghum stalk was obtained, 627 g/10 kg of sweet sorghum stalk or 62.7 kg ethanol/tonne from non-structural carbohydrates and 292 g/10 kg of sweet sorghum stalk or 29.2 kg/ tonne of ethanol from structural carbohydrates.

Table 43 Comparison between fermentable sugar and bioethanol from corn stalk and commercial scale of sorghum stalk

Parameters	Comparison with commercial scale	
	Corn stalk SHF method (Thesis)	Sorghum stalk
Fermentable sugar from juice (g/L)	-	1,403.000
Fermentable sugar from stalk (g/L)	2,182.860	656.000
Bioethanol production from juice (g/L)	-	627.000
Bioethanol production from stalk (g/L)	98.138	292.000
Total fermentable (g/L)	2,182.860	2,059.000
Total bioethanol (g/L)	98.138	919.000

Chapter 5

Summary

The potential of agricultural wastes (sorghum stalks, sugarcane leaves, corn stalks and sunflower stalks) as an alternative crop for ethanol production was investigated in this study were treated with changed pretreatments and 2%NaOH pretreatment showed the best result (the highest total sugar). By using enzyme at 50℃, acidic pH (5.0), those total sugar were breaking into fermentable sugars. The present study thus projects sunflower, sorghum and corn stalks and sugarcane leaf as alternative lignocellulosic agricultural wastes available in plenty in this country for bioethanol production on a commercial scale. RSM is an optimization method which collects a group of mathematical, engineering and statistical techniques to define the relationships between the response and the independent variables. It is divided into three major stages which are preliminary determination of independent parameters and levels, selection of experimental design, and graphical presentation of result analysis. RSM analysis was helpful to optimize the conditions and suggested suitable parameter for design the final experiments.

In this paper a techno-economic analysis for bioethanol production in Thailand agricultural biomass wastes (i.e. sorghum stalks, sugarcane leaves, corn stalks and sunflower stalks) was presented. Ethanol production costs for the evaluated from the crop biomass. The ethanol yield depended sugar content of the lignocellulosic residues, and the technology efficiency. Energy efficiency was tested with bomb colorimeter, which can have measured the accurate heating values. According to the healing values results showed that agricultural wastes (sorghum stalks, sugarcane leaves, corn stalks and sunflower stalks) has huge potential and ability to produce bioethanol as sustainable applications. Furthermore, the high sugar content, the low purchase price, and the low energy consumption make these agricultural waste biomasses the most promising residue to produce bioethanol in Thailand. The selling of the electricity surplus is the key aspect to reduce the fuel ethanol production cost.

REFERENCES

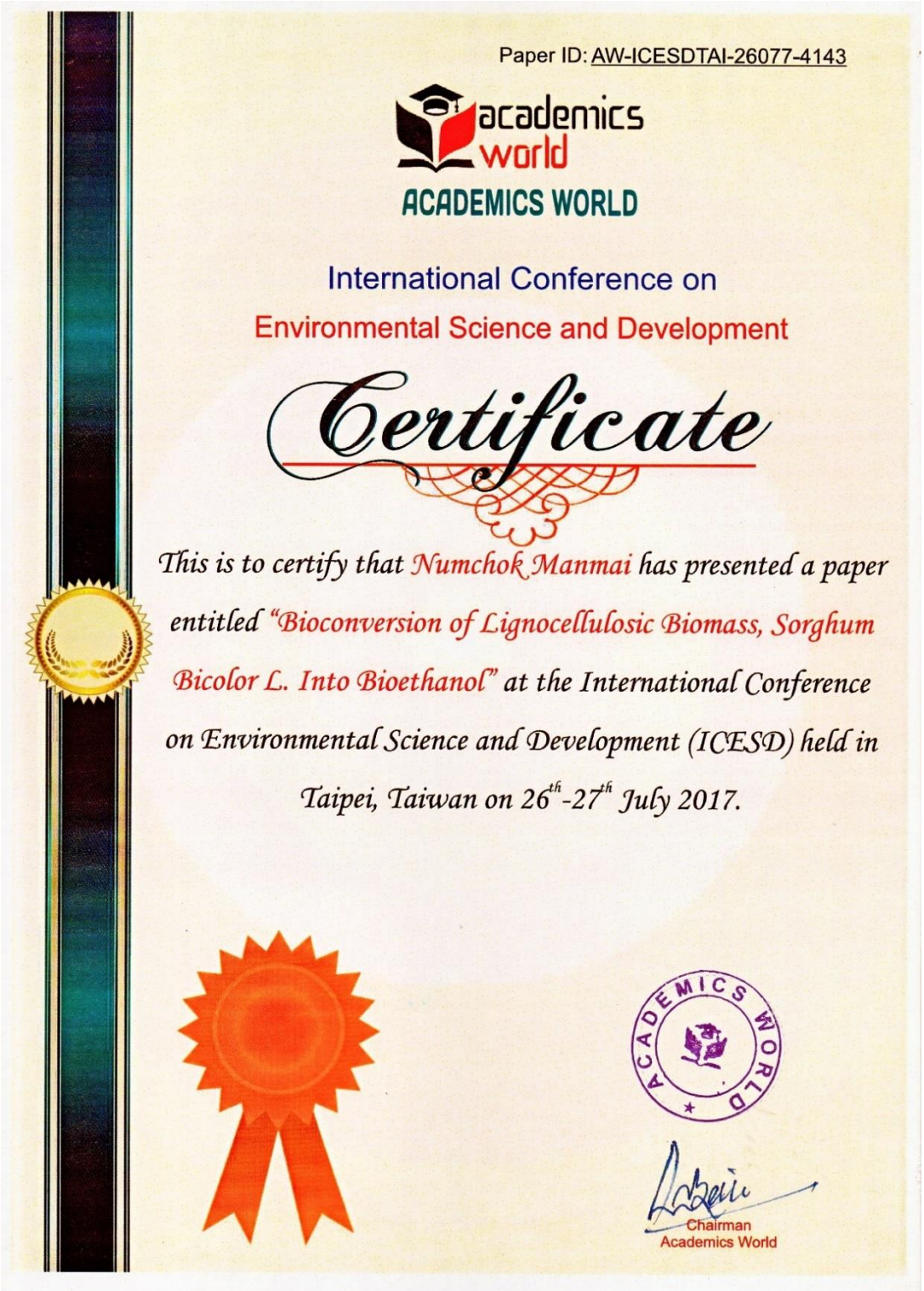
APPENDIX

APPENDIX I

Research publication

Appendix Table 1 List of publications

Title:	Bioconversion of lignocellulosic biomass, *Sorghum bicolor* L. into bioethanol
Type:	Proceeding: Oral presentation
Author:	Numchok Manmai, Thidarat Siriboon, Sawitree Tipnee Yuwalee Unpaprom and Rameshprabu Ramaraj
Conference:	Academics World 73rd International Conference, International Conference on Environmental Science and Development (ICESD), Taipei, Taiwan, 26th–27th July 2017
Pages:	41–45
Title:	Comparison of sugarcane leaves biomass pretreatments for bioethanol production
Type:	Proceeding: Oral presentation
Author:	Numchok Manmai, Thidarat Siriboon, Nigran Homdoung, Yuwalee Unpaprom and Rameshprabu Ramaraj
Conference:	1st National Graduate Research Conference and Creative Innovation Competition 17th–18th August 2017
Pages:	325–332



Paper ID: AW-ICESDTAI-26077-4143

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ACADEMICS WORLD

International Conference on

Environmental Science and Development

Certificate

This is to certify that Numchok Manmai has presented a paper entitled "Bioconversion of Lignocellulosic Biomass, Sorghum Bicolor L. Into Bioethanol" at the International Conference on Environmental Science and Development (ICESD) held in Taipei, Taiwan on 26th-27th July 2017.

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Appendix Figure 1 Certificate I

BIOCONVERSION OF LIGNOCELLULOSIC BIOMASS, SORGHUM BICOLOR L. INTO BIOETHANOL

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Abstract: Lignocellulosic biomass is one of the most abundant renewable resources for biofuel productions and value-added chemicals. The production of bioethanol from lignocellulosic biomass has attracted worldwide interest. The present work apportionments with production of ethanol from sorghum stalk powderby*Saccharomyces cerevisiae*TISTR 5020. The powder was treated with sodium hydroxide, water, silage and*Trichoderma spp.* to enzymatic hydrolysis by acellulaseenzyme. All of pretreatments were performed at room temperature for 3 days. The pretreatments resulted in ameliorati0ng the following enzymatic hydrolysis to 2% of the theoretical yield overnight. The best hydrolysis performance was obtained after pretreatment by 2% NaOH. The yeast showed promising results in fermentation for 3 days from 5 days. In the best case, the hydrolysate of 2% NaOH pretreated. Furthermore, the highest volumetric ethanol productivity was observed in the hydrolysatesof 2%NaOH pretreated powder.

Keywords: Bioethanol, Pretreatment, Hydrolysis, Sorghum stalks

I. INTRODUCTION

Fuel is a factor in driving the world economy is highly depending on diverse fossil energy sources such as natural gas, petroleum and coal. All the energy sources there are being used for the production of electricity and other materials [1]. Immoderate consumption of the fuel especially in large suburb areas due to speedy growth in citizenry and industrialization. Worldwide fuel demand a lot of quantity and tendency has been increasing every year [2].

Thailand Energy Report 2015, Energy production in Thailand decreased, resulting in imports met more domestic demand. The final energy consumption increased by 4.0 percent because Thai economy started to recover (GDP grew by 2.8 percent) while the energy prices are in a downtrend due to the oversupply of oil, natural gas and coal in the world market. The prices of Diesel, Gasoline and Gasohol increased from the low level. The jet fuel consumption increased by the number of foreign tourists. The foreign Tourists were 29.9 million increases about 5 million people compare to previous year. The electricity consumption increased because the longer period[3].

Furthermore, crude oil supply is 1,028 thousand barrels per day by 85 percent of imports. The 8.8 percent increase in imports, mainly from Middle East countries. The rest is domestic production rose 10.0%, the refining capacity of the country stood at 1,252 thousand barrels per day. Crude was used in refining for 90 percent of the refining capacity. Petroleum products consumption is at 132 million liters per day, up 4.3 percent. The diesel consumption is at 60.1 million liters per day accounted for 46 percent of all petroleum products. It is increased 4.1 percent by the prices reduction. The consumption of gasoline and diesel fuel was at 26.4 million liters per day. Accounted for 20 percent of all petroleum products consumption. The demand rose to 13.2 percent due to the low oil prices that encourage the auto LPG and NGV users turning to use more oil because it is cheaper and more convenient evenly over the service station. Jet fuel consumption was at 16.5 million liters per day, up 9.4 percent from the expansion in tourism sector. In 2015, the foreign tourists come to visit at 29.9 million people, up from about 5 million from the years ago [3].

Lignocellulosic biomass and waste are furnish renewable resources for the biofuel production. Given the possible viability in the medium term, innovative approaches for the production of bioethanol, especially by pretreatment and hydrolysis of cellulosic materials, have conducted to growing attractin energy crops. These comprise agricultural wastes and grasses for speedy growth and high biomass potency, such as sunflower (*Helianthus annuus*L.) is used as oil in Thailand,In Asia; rice (*Oryza sativa* L.), wheat (*Triticumaestivum* L.), corn (*Zea mays* L.) , and sugarcane (*Saccharumofficinarum*L.)lot of planting, straw and baggaseare a major field-based residue that is produced in large amounts 667.6, 145.2, 33.9 and 74.9 million tons[4],[5]. Another potential crop for the bioethanol productionis sorghum.

Sorghum (*Sorghum bicolor* L.) is a type ofagricultural yields in the world scenario, and among the considers that make it highly tendency, is its broad genetic variety and high durability to abiotic

Appendix Figure 2 Proceeding I-I

stresses such as rainlessness and heat. Furthermore, sorghum requires less fertilizer than other agronomy plants for high performance and tolerate for environmentof conditions [6]. This makes the sorghum crop suitable for growing under ideal conditions as well as on marginal lands where other crops do not thrive. Unlike sugar cane, sorghum is grown from seed and has a much shorter growing season of around 120 to 130 days. This cereal is tolerant to dry periods during its cycle and produces economically lucrative crops of grains and green mass even under low rainfall or unstable conditions [7],[8].

Sorghum is grown in Brazil predominantly for the production of grain and forage, although sweet sorghum is also used to produce alcohol [10]. With cutting when sorghum reaches the maturation stage, use of only the leaves and stems that constitute the straw used in this work, can produce approximately 7.81 t of dry matter/ha in around 110 days[9], [10]. Considering two crop harvests a year, sorghum can yield about 15.62 t/ha of biomass which can be exploited to produce second generation ethanol. In addition, 15.6 t/ha of panicles with a high value for silage or as direct feed is produced. Therefore, highly potential of sorghum stalk was performed as a raw materialforethanol fermentation with different pretreatment methods.

II. DETAILS EXPERIMENTAL

2.1. Feedstock

Sorghum was gathered postharvest the farm of Program in Agronomy, Faculty of Science, Maejo University, Chiang Mai, Thailand (18°8'98"N 99°0'13"E) Showed in **Fig.1.A**. It was originally air dried at ambient temperature Showed in **Fig.1.B,C**, rolled to a size of < 1-4 cm. long with a rolling machine Showed in **Fig.2.A,B** and chopped up again to a size of < 1 mm diameter with a house blender until to become powder. The final product was collected as powder. Finally, it was dried at ambient temperature before being used for the experiments. The components and other nutrients of SS were characterized. Sorghum Stalks contained 42.03 ± 3.38 % of cellulose and 24.53 ±4.45 % of hemicellulose and 9.89 ±2.35 % of lignin.

2.2. Pretreatments

Pretreatment was conducted at room temperature for 3 days, by the use of silage, distilled water, 2% NaOH and 1% *Trichoderma spp*. **Fig.3.A**. with some chemical and biological addition solid to liquid ratio of 1:3 were carried out, for comparison total sugar and reducing sugar analyzed by phenol – sulfuric procedure and DNS method. Before analyzed to take H_2O solid to liquid ratio of 1:4 to the condition sample.

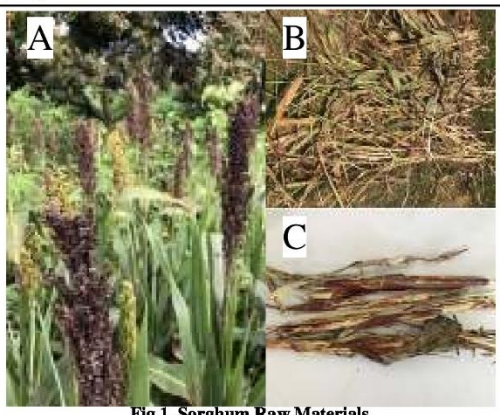

Fig.1. Sorghum Raw Materials

Fig.2. Material preparations

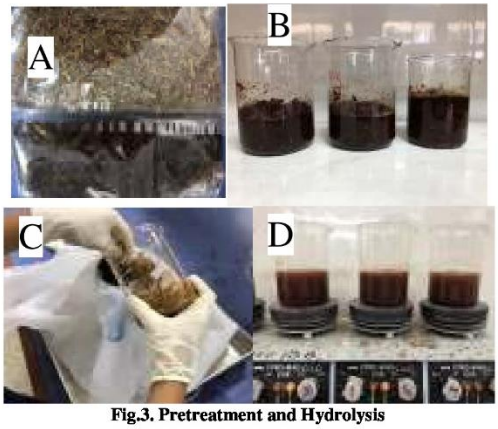

Fig.3. Pretreatment and Hydrolysis

2.3. Hydrolysis method

The enzymatic hydrolysis of sorghum stalk powder cellulosic residue from the best condition pretreatment sample was performed in 500 mL of Beaker, containing a 350 mL mixture of water and solid substrate. 2% cellulose enzyme was utilized for enzymatic hydrolysis **Fig.3.B**. The pH and temperature were adjusted to 5.0 by diluted HCl and room temperature overnight, respectively. And

assayed Total Sugar and Reducing Sugar. Then filtered and evaporated until 100 mL on the hot plate to fermentable sugar and checked sugar concentration again **Fig.3.C,D**.

2.4. Microorganism and media

The microorganism from Biotechnology program, Faculty of Science, Maejo University, Chiang Mai, Thailand. The organism was identified as *Saccharomyces cerevisiae* TISTR 5020 in our laboratory. *S. cerevisiae* was maintained on YM medium containing yeast extract 10 g/L, peptone 20 g/L and glucose 20 g/L pH 5.6 by dilutedNaOH and shake 150 rpm in the shaker for 24 h, at 37°C. When yeastgrow in the medium overnight was centrifuged 7,000 rpm for 10 min, at 4 °C.

2.5. Fermentation

Cellulosic hydrolysate obtained from batch hydrolysis, was utilized as a fermentation medium. Ethanol fermentations were carried out at 30 °C under anaerobic conditions, with 1% of *S. cerevisiae* was centrifuged inoculated into a 250 mL flask with a working volume of 100 mLfor 5-day **Fig.4**.

Fig.4. Fermentation

III. RESULTS AND DISCUSSION

Various crop residues rich in lignocellulosics like wheat straw, rice straw, corn stalks and cobs, groundnut shells etc. have been exploited for ethanol production. However little effort has been made to utilize sorghum wastes as a substrate for ethanol production. Sorghum was cultivated global production ofdry sorghumis about 53 Tg.In Africa and Asia, over60% of sorghum is used for human food [15].

The utilization ofwasted sorghum grain could provide1.4 GL ofbioethanol, replacing 1 GL ofgasoline.Sorghum dry milling could produce 1.2 dry kgofDDGS per kg ofethanol as a coproduct from waste sorghum. About 1.7 Tg ofsorghum would besaved by DDGS, thereby producing another 752 ML ofbioethanol. Therefore, the wasted sorghum graincould produce 2.1 GL ofbioethanol.For sorghum straw, 60% ground cover requiresat least 2:7 Mg of crop residues per hectare. Under these practices, 10.3 Tg ofsorghum strawwould be globally available and could produce 2.8 GLofbioethanol.Wasted sorghum grain and sorghum straw couldproduce 4.9 GL ofbioethanol globally, replacing 3.5 GL ofgasoline in an E85 midsize passenger vehicle,or about 0.3% ofthe global gasoline consumption.

There is no bioethanol available from sorghumstraw in Africa because the low yield requires that all straw be left in the field to conserve soil. This results in huge accumulation of sorghum stalks annually which do not find any suitable end use and are generally burnt in the fields causing environmental pollution [15].

Therefore, sorghum stalks as lignocellulosics afford a renewable and low cost raw material for bioethanol production. Fermentable sugars in sorghum are mainly sucrose, glucose, and fructose.

Pretreatment typically breaks down the macroscopicrigidity of the biomass and reduces the physical barriersto mass transport.Table 1 performed total sugar and reducing sugar from three different pretreatments comparing with control (without any pretreatment) of sorghum stalk. The lowest amount of total sugar was 6.980±1.560 g/L observed from control while the highest amount was 13.610±0.439g/L from pretreatment with NaOH 2%. This means that sodium hydroxide affected adequately the structure of material and released more sugar. Total sugar from pretreatment by H_2O and 1% *Trichoderma spp.* were 8.560±0.786 g/L and 12.333±0.268 g/L, respectively. As most of sugar exits as long chain polymers like cellulose, the amounts of reducing sugar of sorghum stalk were obtained as low contents which ranged from 1.147±0.061 g/L to 3.027±0.583 g/L. Reducing Sugar Total Sugar from Hydrolysis,ethanol yields were presented in Table 2, 3, 4 and Figure 5.

Sugar contents and profiles of the sorghum stalks were suitable for ethanol fermentation. Sincethegoalof pretreatment step is enhancing hydrolysis process by disturbing the structure of cell wall and making cellulose chain available to enzyme, pretreatment with 2% NaOH which resulted the highest amount of total sugar was applied and continued to the next step, saccharification.The observed mean of ethanol production from sorghum stalks was found to be7.328±1.813g/L, which was higher than leaves and stem.Consequently, the present study demonstrated that sorghum is apromising alternative energy crop for ethanol production that canmake large contributions to ethanol-producing nations due to itswide adaptability, high biomass productivity and short growthperiod.

Table1: Reducing Sugar and Total Sugar from Pretreatments

Plant	Pretreatments	Reducing Sugar (g/L)	Total Sugars (g/L)
Sorghum Stalks	Silage	1.147±0.061	6.980±1.560
	Control (H_2O)	1.433±0.273	8.560±0.786
	2% NaOH	3.027±0.583	13.610±0.439
	1% Trichoderma spp.	1.980±0.308	12.333±0.268

Table2: Reducing Sugar and Total Sugar from Hydrolysis

Plant	Hydrolysis	Reducing Sugar (g/L)	Total Sugars (g/L)
Sorghum Stalks	2% Cellulase Enzyme	7.200±1.908	37.807±3.117

Table3: Reducing Sugar and Total Sugar from Evaporated for Fermentable Sugar

Plant	Evaporating	Reducing Sugar (g/L)	Total Sugars (g/L)
Sorghum Stalks	After Pretreated By 2% Cellulase Enzyme	15.667±4.130	69.211±1.601

Table4: Utilization of Sorghum Stalks for Ethanol Production

Plant	Days	Reducing Sugar (g/L)	Total Sugar (g/L)	Conc. Of ALC (g/L)
Sorghum Stalks	0	16.000±3.811	69.211±1.601	0.000±0.000
	3	14.633±0.208	57.544±0.548	7.328±1.813
	5	10.300±0.265	55.439±3.384	2.094±0.907

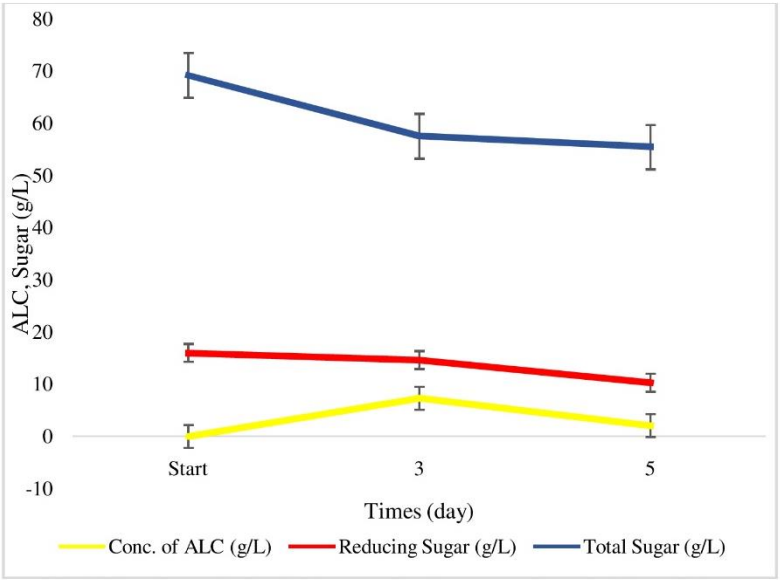

Fig.5. Ethanol concentration, total sugar, and reducing sugar throughout fermentation

Appendix Figure 5 Proceeding I-IV

Table5: Ethanol concentration of different biomass

Substrate	Pretretment and Hydrolysis	Ethanol ConC. (g/L)	References
Sugarcane leaf	H_2O_2	1.30	[13]
Sugarcane leaf	H_2SO_4	3.35	[13]
Sunflower straw	Thermal, 80 ◦C	2.90	[14]
Sunflower straw	Thermal, 120 ◦C	2.82	[14]
Sunflower straw	H_2SO_4 0.1%	2.95	[14]
Sunflower straw	H_3PO_4 0.1%	2.73	[14]
Sunflower straw	HCl 0.1%	2.52	[14]
Sunflower straw	NaOH 0. 1%	2.34	[14]
Sorghum stalk	NaOH-enzyme	7.33	This Study

CONCLUSION

Thepotential of sorghum stalk as an alternative crop for ethanol productionwas investigated in thisstudy. Sorghumstalk was treated with changed pretreatments and NaOH 2% pretreatment showed the best result (the highest total sugar). By using enzyme at 50°C, acidic pH (5.0), those total sugar were breaking into fermentable sugars. The present study thus projects sorghum stalks as an alternative lignocellulosic agricultural waste available in plenty in Thailand for bioethanol production on a commercial scale.

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Appendix Figure 7 Certificate II

การประชุมวิชาการและประกวดนวัตกรรมบัณฑิตศึกษาแห่งชาติ ครั้งที่ 1
1st National Graduate Research Conference and Creative Innovation Competition
วันที่ 17-18 สิงหาคม 2560 โรงแรมดิเอ็มเพรส จังหวัดเชียงใหม่

การเปรียบเทียบวิธีการปรับสภาพของใบอ้อยสำหรับการผลิตไบโอเอทานอล

Comparison of Sugarcane Leaves Biomass Pretreatments for Bioethanol Production

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บทคัดย่อ

ไบโอเอทานอลเป็นพลังงานทดแทนและพลังงานสะอาดที่เป็นผลดีต่อสิ่งแวดล้อม ซึ่งชีวมวลประเภทลิกโนเซลลูโลสจากวัสดุเหลือทิ้งทางการเกษตรนั้นมีปริมาณมากสามารถใช้ผลิตไบโอเทานอลได้ อีกทั้งการผลิตไบโอเอทานอลจากชีวมวลกำลังเป็นที่สนใจทั่วโลก งานวิจัยนี้จึงมุ่งเน้นการผลิตไบโอเอทานอลจากใบอ้อยด้วยวิธีแยกการย่อยสลายและการหมัก (SHF) โดยใบอ้อยถูกปรับสภาพด้วยน้ำ การทำหญ้าหมัก การใช้โซเดียมไฮดรอกไซด์ (NaOH) และการใช้เชื้อรา *Trichoderma* spp. ที่อุณหภูมิห้องเพื่อเลือกสภาวะในการปรับสภาพที่ดีที่สุดมาไฮโดรไลซิสโดยเอมไซม์เซลลูเลสต่อ เชื้อจุลินทรีย์ปกติทั่วไปที่ใช้ในหมักไบโอเอทานอลคือ ยีสต์ *Saccharomyces cerevisiae* ซึ่งในงานวิจัยนี้ได้ใช้ยีสต์ *Saccharomyces cerevisiae* TISTR 5020 สำหรับการหมักโดยใช้ระยะเวลาในการหมัก 5 วัน ผลการทดลองพบว่า การปรับสภาพด้วยโซเดียมไฮดรอกไซด์ให้ผลผลิตน้ำตาลมากกว่าการปรับสภาพด้วยวิธีอื่น นอกจากนี้ยังพบว่าการปรับสภาพด้วยวิธีดังกล่าว มีผลทำให้ผลผลิตเอทานอลเกิดขึ้นสูงที่สุดในวันที่ 3 ของการหมัก

คำสำคัญ: ไบโอเอทานอล ใบอ้อย ไฮโดรไลซิสด้วยเอนไซม์ การหมัก

Abstract

Bioethanol is a renewable and clean energy with its major environmental benefits. One way to produce bioethanol is by lignocellulosic biomass from agricultural waste products. Conveniently, agricultural waste products were abundantly available in Thailand. Since bioethanol production from agricultural waste biomass has attracted a worldwide interest, this study was done to evaluate the potential of sugarcane (*Saccharum officinarum* L.) leaves for bioethanol production by separation hydrolysis and fermentation (SHF) method. The leaves were then pretreated with water, silage, sodium hydroxide (NaOH) and *Trichoderma* spp. at room temperature to select the best condition for enzymatic hydrolysis by a cellulase enzyme. Yeast *(Saccharomyces cerevisiae* TISTR 5020) was used in the fermentation assay which lasted for five (5) days. *S. cerevisiae* was the most common microorganism used to produce ethanol commercially. The results showed that the pretreated samples with NaOH produced more sugar than the other samples. Additionally, NaOH pretreatments articulated on the third day of fermentation achieved highest ethanol concentration.

Keywords: Bioethanol, sugarcane leaves, enzymatic hydrolysis, fermentation

INTRODUCTION

The production of biofuels, such as biogas, biodiesel, bioethanol, have been encouraged as a sustainable option to tackle the problems associated with rising crude oil prices, global warming and diminishing petroleum reserves. Biofuels have emerged as a best choice to meet these requirements in a sustainable

Appendix Figure 8 Proceeding II-I

manner (Saini et al., 2015a). Agricultural wastes, which are abundant and inexpensive, have been suggested to be the most promising alternative for the renewable feedstock (Binod et al., 2010). In agriculture, after the agriculture products have been harvested and processed it generates a huge amount of agricultural by-products such as stalks and bagasse. These agricultural residues were generally used for fertilizer or for animal feed. For the time being, some areas eradicated these wastes by burning to save time and energy but can caused another problem—air pollution. Nowadays, agricultural residues have been introduced into the bioethanol production due to its lignocellulose component (Boochapun et al., 2014). Bioethanol produced from lignocellulosic biomass have been expected to be one of the major alternatives to petroleum based fuels due to its availability and ubiquity in Thailand.

Bioethanol is the most common and one of the practically important liquid biofuel and can be produced from a variety of low-priced substrates. The varied raw materials used in manufacturing bioethanol are conveniently classified into three main types: sugars, starches, and cellulose materials. Bioethanol production from agricultural biomass involves four major steps: biomass pretreatment, enzymatic hydrolysis, fermentation and distillation (Saini et al., 2015b).

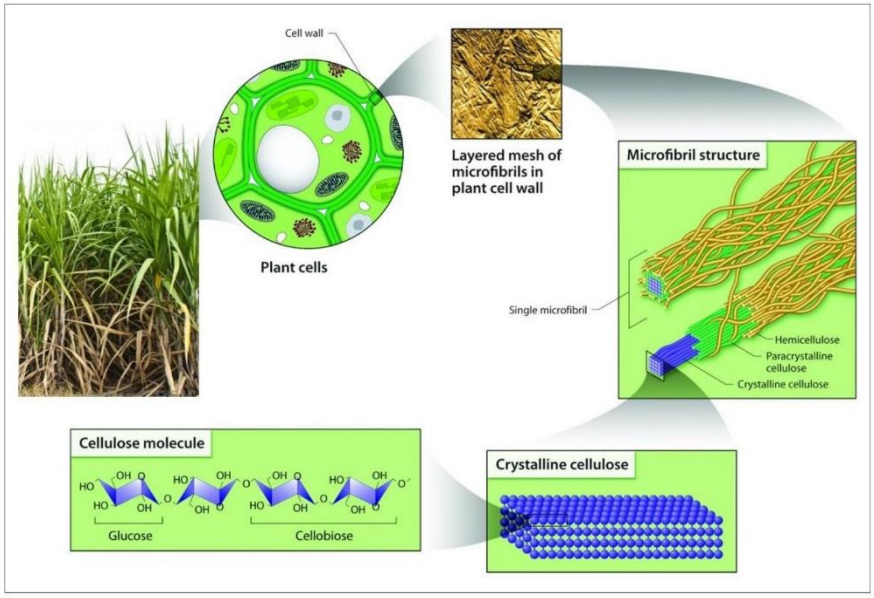

Figure 1 Structural organization of the plant cell wall adapted from (Quiroz-Castañeda and Folch-Mallol, 2013)

The main challenge in ethanol production from lignocellulosic biomass is the feedstock pretreatment. (Ingram and Doran, 1995). Lignocellulose plant biomass consists of three major components: cellulose (40–50 %), hemicellulose (20–40 %) and lignin (20–30 %) showed in Figure 1. Cellulose is surrounded by hemicellulose and lignin functioning as matrix and encrusting materials. During pretreatment, the matrix of cellulose and lignin bound by hemicellulose will be broken. This reduce the crystallinity of cellulose and increase the fraction of amorphous cellulose, the most suitable form for enzymatic attack (Saini et al., 2015b).

Appendix Figure 9 Proceeding II-II

การประชุมวิชาการและประกวดนวัตกรรมบัณฑิตศึกษาแห่งชาติ ครั้งที่ 1
1st National Graduate Research Conference and Creative Innovation Competition
วันที่ 17-18 สิงหาคม 2560 โรงแรมดิเอ็มเพรส จังหวัดเชียงใหม่

In countries like Thailand, agricultural production of various crops such as cotton, mustard, chilli, sugarcane, rice, corn, sorghum, sweet sorghum, pulses, oilseeds, etc. generated massive amount of wastes. Hence, this could be a solution to the growing problem of agricultural wastes management, to used it yo generate biofuels such as bioethanol, in an environmentally friendly manner. Sugarcane is one of the crops in Thailand that is cultivated abundantly due to its economic value. During harvesting, leaves were removed to collect the sugar cane stalks; the wastes from the postharvest were then burned. This activity causes air pollution and can contribute to the world's most alarming problem—climate change. Since sugarcane leaves were the major waste component of the sugarcane industry, this study focused on the potential of sugarcane leaves as chemical feedstock to produce bioethanol by biotechnology process (Boochapun et al., 2014) In this study, chemical, physical and biological pretreatment methods were used to breakdown the chemical bonds of lignin and cellulose of sugarcane leaves.

MATERIAL AND METHODS

Feedstock

Sugarcane leaves used in this study were gathered during harvest from the farm of Program in Agronomy, Faculty of Agricultural Production, Maejo University, Chiang Mai, Thailand (18° 8' 98" N 99°0' 13" E) (Figure 2.A). It was originally dried by sunlight (Figure 2.B, C) then rolled to a size of < 1–4 cm by a rolling machine (Figure 3.A,B) and blended up to a size of < 1 mm diameter using a house blender. The final product was collected as powder. Finally, it was dried then at ambient temperature before being used for the experiments. Sugarcane leaves chemical composition including cellulose, hemicelluloses, lignin, extractives and ash of the solid materials was determined according to Gouveia et al. (2009).

Pretreatment method

The pretreatment was done by using distilled water (control), silage, 2% NaOH, and 1% of *T.* spp. from the Institute of Product Quality and Standardization, Maejo University(Figure 4.A). And with some chemical and biological addition solid to liquid ratio of 1:3 were conducted at room temperature for 3 days, measured for comparison total sugar and reducing sugar analyzed by phenol – sulfuric procedure and DNS method. Before analyzed to take distilled water solid to liquid ratio of 1:4 to the condition sample.

Hydrolysis method

The enzymatic hydrolysis of sugarcane leaf powder from the best condition pretreatment sample was performed in a 500 mL Beaker, containing 350 mL mixture of water and solid substrate. Two percent (2%) of cellulose enzyme was utilized for enzymatic hydrolysis without detoxification before hydrolysis (Figure 4.B). The pH were adjusted to 5.0 by adding diluted HCl and temperature were adjusted to room temperature overnight. And assayed total sugar and reducing sugar. Then filtered and evaporated until 100 mL on the hot plate to fermentable sugar and checked sugar concentration Figure 4.C,D.

Appendix Figure 10 Proceeding II-III

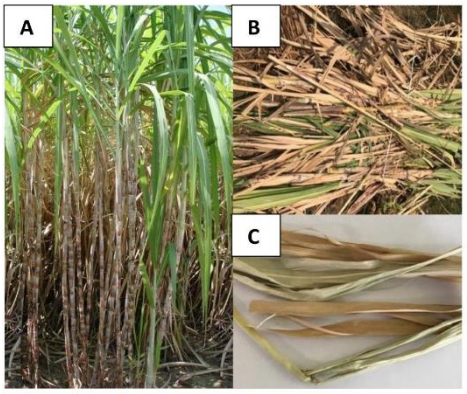

Figure 2 Sugarcane Raw Materials

A: Sugarcane, B: Sugarcane leaves were dried, C: Sugarcane leaves before rolling

Figure 3 Material preparation

A: Sugarcane leaves were rolling, B: Sugarcane leaves size during < 1–4 cm., C: Sugarcane leaves size during < 1 mm.

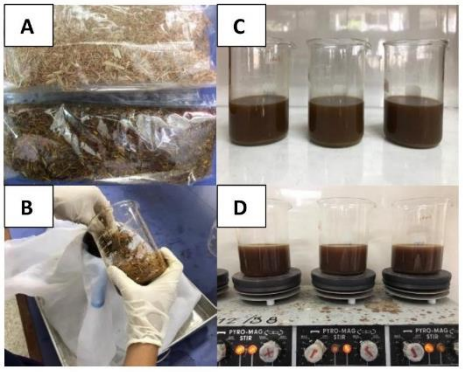

Figure 4 Pretreatment and Hydrolysis

A: The sample pretreatment for 3 days, B: The sample filtration, C: The sample before evaporation, D: The sample evaporation

Figure 5 Fermentation

Appendix Figure 11 Proceeding II-IV

Microorganism and media

The microorganism used in this study was *Saccharomyces cerevisiae* TISTR5020 that obtained from Thailand Institute of Scientific and Technology Research (TISTR). *S. cerevisiae* was maintained on YM agar containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 15 g/L agar with a pH 5.6 achieved by putting by diluted NaOH. The media was sterilized at 121 °C, 15 psi for 15 min. For the inoculum preparation, yeast was inoculated to YM broth and been put to a shaker with 150 rpm for 24 hat 35°C. The yeast biomass was harvested by centrifugation at 7,000 rpm for 10 mins at 4 °C and used as inoculum.

Fermentation

Cellulosic hydrolysate, obtained from batch hydrolysis, was utilized as a fermentation medium. The fermentation medium was not sterilized. Ethanol fermentations were carried out with 1% of *S. cerevisiae* ina 250 mL flask at 30 °C with a pH adjusted to 5.6 under anaerobic conditions. It was then incubated for five (5๓/ days (Figure 5). The ethanol content of each samples were measured using an ebulliometer for 1, 3, and 5 days of fermentation.

RESULTS AND DISCUSSION

In this study, *T.* spp. was applied in the biological pretreatment process. The biological and chemical reaction of sugarcane leaf was studied. The effect of amount of biomass, Pretreatment by the using of silage, distilled water, NaOH 2% and *T.* spp. 1% addition solid to liquid ratio of 1:3 were conducted at roomtemperature for 3 days by experiment was repeated 3 times. The chemical composition of the raw sugarcane leaves contained 37.21±0.02% cellulose, 28.53±0.11% hemicellulose, 23.24±0.04% lignin (), 7.25±0.01% extractives (and 3.125±0.03% ash. (Figure 6).

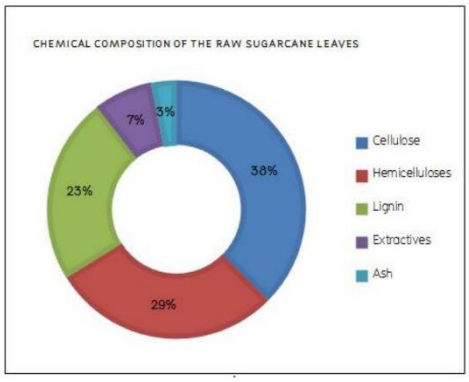

Figure 6 chemical composition of the raw sugarcane leaves

The sugar concentration with various the biological and chemical pretreatments performed total sugar and reducing sugar from three different pretreatments comparing with control (without any pretreatment) of sugarcane leaf were shown in Table 1. The lowest amount of total sugar and reducing sugar were 7.530±1.117 g/L, 1.527±0.306 g/L which observed from silage while the highest amount was obtained from pretreatment

with NaOH 2% wth 8.957±0.250 g/L, 2.193±0.221 g/L, respectively. This proves that sodium hydroxide adequately affect the structure of material and released more sugar. Total sugar and reducing sugar from pretreated by H_2O and 1% *T.* spp. were 7.393±0.944 g/L, 1.680±0.106 g/L and 6.017±0.182 g/L, 1.247±0.311 g/L, respectively. Meanwhile, amounts of reducing sugar of sugarcane leaves from hydrolysis have ranged from 1.247±0.311 g/L to 2.193±0.221 g/L, as most of sugar exits as long chain polymers like cellulose, understandably the values obtained in this pretreatment was low (Table 2). The sample was hydrolyzed by 2% cellulase enzyme the result showed reducing sugar 4.400±0.529 g/L and total sugar 27.807±1.350 g/L, Table 2 presented reducing sugar and total sugar from evaporated for fermentable sugar after hydrolysis by 2% cellulase released reducing sugar and total sugar 9.267±0.702 g/L and 51.57 g/L, ethanol yields were presented in Figure 5 at the 3th day there is the highest ethanol concentration is 5.234±0.907 g/L Table 3 compared ethanol concentration of similar and different biomass this study with sugarcane leaf was pretreated by NaOH 2% and hydrolyzed by 2% cellulaes enzyme there are higher than sugarcane leaf was pretreated by H_2O_2 from (Dawson and Boopathy, 2007) is 5 time.

Table 1 Reducing Sugar and Total Sugar from Sugarcane Leaves Pretreatments

	Pretreatments	Reducing Sugar (g/L)	Total Sugars (g/L)
Sugarcane Leaves	Control (H_2O)	1.680±0.106	7.393±0.944
	Silage	1.527±0.306	7.530±1.1172
	2% NaOH	2.193±0.221	8.957±0.250
	1% *Trichoderma* spp.	1.247±0.311	6.017±0.182

Table 2 Reducing sugar and total sugar content obtained from cellulase hydrolysis of pretreated sugarcane leaves by 2% NaOH

	Hydrolysis	Reducing Sugar (g/L)	Total Sugars (g/L)
Sugarcane Leaves	2% Cellulase Enzyme	4.400±0.529	27.807±1.350
	Evaporating After Hydrolyzed By 2% Cellulase Enzyme	9.267±0.702	51.404±2.373

Appendix Figure 13 Proceeding II-VI

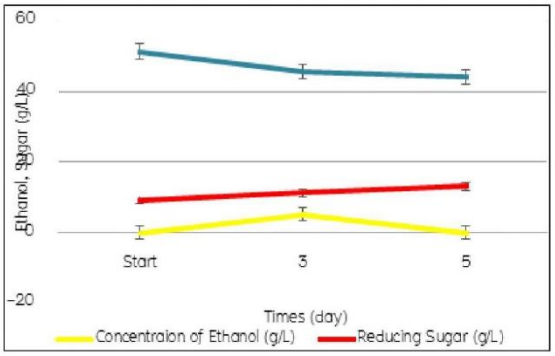

Figure 5 Ethanol concentration, total sugar, and reducing sugar throughout fermentation

Table 3 Ethanol concentration of similar and different biomass

Substrate	Pretreatment and Hydrolysis	Ethanol Concentration (g/L)	References
Sugarcane leaf	H_2O_2	1.30	(Dawson and Boopathy, 2007)
Sugarcane leaf	H_2SO_4	3.35	(Dawson and Boopathy, 2007)
Sunflower straw	HCl 0.1%	2.52	(Antonopoulou et al., 2016)
Sunflower straw	NaOH 0.1%	2.34	(Antonopoulou et al., 2016)
Sugarcane leaf	NaOH-Enzyme	5.23	This Study

CONCLUSION

In conclusion, this study observation implies that sugarcane leaves could be applicable for large scale ethanol production. Also this experimental results suggested that relatively similar amounts of fermentable sugars would be released from their suitable pretreatment and enzymatic hydrolysis for bioethanol production.

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Appendix Figure 15 Proceeding II-VIII

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