COMPARISON OF BIOBUTANOL PRODUCTION FROM RAIN TREE AND GOLDEN RAIN TREE PODS

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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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บทคัดย่อ

การผลิตพลังงานชีวภาพจากแหล่งพลังงานหมุนเวียนนับว่าเป็นทางออกที่มีแนวโน้มในการแก้ปัญหาที่เกี่ยวข้องกับพลังงานและปัญหาสิ่งแวดล้อม สำหรับการผลิตพลังงานชีวภาพจากชีวมวลประเภทลิกโนเซลลูโลส ได้รับการยอมรับอย่างกว้างขวางว่าเป็นวัตถุดิบที่ยั่งยืน เนื่องจากมีราคาต่ำ มีปริมาณมาก และแพร่กระจายอย่างกว้างขวาง ในงานวิจัยนี้ได้ทำการศึกษาการผลิตไบโอบิวทานอลโดยการหมักทางชีวภาพ เพื่อศึกษาความเป็นไปได้ของฝักจามจุรีและฝักราชพฤกษ์เพื่อผลิตไบโอบิวทานอลโดยใช้วิธีการสกัดด้วยความร้อน และหมักร่วมกับแบคทีเรียสายพันธุ์ *Clostridium acetobutylicum* TISTR 2375 ในการสกัดด้วยความร้อนฝักใช้อุณหภูมิ 30, 63 และ 96 องศาเซลเซียส เป็นระยะเวลา 20, 40 และ 60 นาที และใช้สถิติพื้นผิวตอบสนอง (Response Surface Methodology, RSM) แบบเซ็นทรัลคอมโพสิท (Central Composite Design, CCD) เพื่อประเมินและศึกษาสภาวะที่เหมาะสมของอุณหภูมิและเวลา ซึ่งเป็นตัวแปรอิสระต่อผลผลิตน้ำตาลทั้งหมดและน้ำตาลรีดิวซ์ที่ได้ตามการตอบสนองของฟังก์ชัน และทำการศึกษาปฏิสัมพันธ์ของผลกระทบและตัวแปร โดยใช้ซอฟต์แวร์ Design Expert 11.1.0 (Stat-Ease Inc., Minneapolis, USA) ผลการทดลองพบว่า สภาวะที่เหมาะสมของการสกัดด้วยความร้อนที่ส่งผลให้ปริมาณน้ำตาลทั้งหมดและน้ำตาลรีดิวซ์มากที่สุดในฝักจามจุรี ได้แก่ ที่อุณหภูมิ 63 องศาเซลเซียส เป็นเวลา 40 นาที และในฝักราชพฤกษ์ที่อุณหภูมิ 96 องศาเซลเซียส เป็นเวลา 60 นาที ซึ่งแสดงให้เห็นว่าทั้งสองปัจจัยมีผลกระทบอย่างมีนัยสำคัญต่อปริมาณน้ำตาลทั้งหมดและน้ำตาลรีดิวซ์ จากนั้นวัตถุดิบที่ผ่านการสกัดด้วยความร้อนที่สภาวะที่เหมาะสมถูกนำไปย่อยสลายด้วยเอนไซม์เซลลูเลส 2 เปอร์เซ็นต์ ที่อุณหภูมิ 50 องศาเซลเซียส เป็นระยะเวลา 24 ชั่วโมง การย่อยสลายโดยใช้เอนไซม์เซลลูเลสนั้นพบว่า ฝักจามจุรีมีประสิทธิภาพในการย่อยสลายประมาณ 51 เปอร์เซ็นต์ และฝักราชพฤกษ์มีประสิทธิภาพในการย่อยสลายประมาณ 41 เปอร์เซ็นต์ สำหรับการผลิตไบโอบิวทานอลโดยใช้แบคทีเรียสายพันธุ์ *Clostridium acetobutylicum* TISTR 2375 จากฝักจามจุรีและฝักราชพฤกษ์ที่ผ่านการย่อยสลายด้วยเอนไซม์และเจือจางน้ำตาลรีดิวซ์ประมาณ 80 กรัมต่อลิตร ผลการศึกษาพบว่า ฝักจามจุรีให้ผลผลิตไบโอบิวทานอลที่ความเข้มข้น 1.1718 กรัมต่อลิตร และฝักราชพฤกษ์ให้ผลผลิตไบโอบิวทา

นอลที่ความเข้มข้น 0.0628 กรัมต่อลิตร จากผลการศึกษาแสดงให้เห็นว่าฝักจามจุรีผลิตไบโอบิวทานอลได้สูงกว่าฝักราชพฤกษ์ ซึ่งฝักจามจุรีและฝักราชพฤกษ์อาจมีสารยับยั้งต่อการเจริญเติบโตของเซลล์ที่ส่งผลให้ไบโอบิวทานอลที่ผลิตได้ต่ำ ดังนั้นพืชทั้งสองนี้ควรได้รับการศึกษาเพิ่มเติมเพื่อเพิ่มประสิทธิภาพในการผลิตไบโอบิวทานอล อย่างไรก็ตามฝักจามจุรีและฝักราชพฤกษ์นับว่าเป็นวัตถุดิบใหม่ที่น่าสนใจ สามารถลดต้นทุนด้านพลังงานและเอนไซม์ที่ใช้อยู่ในกระบวนการแปลงชีวมวลสู่กระบวนการผลิตเชื้อเพลิงชีวภาพ

คำสำคัญ : ฝักจามจุรี, ฝักราชพฤกษ์, การสกัดด้วยความร้อน, การผลิตไบโอบิทานอล

Title COMPARISON OF BIOBUTANOL PRODUCTION FROM RAIN TREE AND GOLDEN RAIN TREE PODS

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ABSTRACT

The production of bioenergy from renewable resources is a promising solution to energy issues as well as the associated environmental problems. For bioenergy production, lignocellulosic biomass is widely considered as a sustainable feedstock because it is inexpensive, highly abundant and broadly distributed. In this thesis, biobutanol production by biological fermentation was studied analyzing the feasibility and thermal extraction method of rain tree and golden rain tree pods for biobutanol production by *Clostridium acetobutylicum* TISTR 2375. Rain tree and golden rain tree pods were extracted by thermal extraction method using 30, 63 and 96 °C at 20, 40 and 60 min. Furthermore, the Response Surface Methodology (RSM) was used based on Central Composite Design (CCD) in order to evaluate and optimize the effect of temperature and time as an independent variable on the total sugar and reducing sugar concentration. The interaction effects and optimal parameters were obtained using Design Expert 11.1.0 software (Stat-Ease Inc., Minneapolis, USA). The results showed that the optimal condition of thermal extraction in the amount of total sugar and reducing sugar was at 63 °C for 40 min (rain tree pods) and 96 °C for 60 min (golden rain tree pods). Both variables have a significant effect on the total sugar and reducing sugar concentration. After which, the raw materials that were thermally extracted at the optimal condition were enzymatic hydrolyzed with 2% (v/v) cellulase enzyme at a temperature of 50 °C for 24 hours. The enzymatic hydrolysis found that the rain tree pods have the efficiency of hydrolysis of 51% and golden rain tree pods have the efficiency of hydrolysis of 41%.

The production of biobutanol by *Clostridium acetobutylicum* TISTR 2375 from rain tree and golden rain tree pods that have been enzymatic hydrolyzed diluted approximately 80 g/L of reducing sugar. The results showed that rain tree pods could produce biobutanol concentration of 1.1718 g/L and golden rain tree pods could produce biobutanol concentration of 0.0628 g/L. Henceforth rain tree pod produced a higher biobutanol yield than golden rain tree pods. Rain tree and golden rain tree pods may contain inhibitors for cell growth that results in low biobutanol. Therefore, these two plants should be further studied to increase the efficiency of biobutanol production. However, rain tree and golden rain tree pods are interesting new feedstock that can potentially reduce the cost of energy and enzyme inputs currently used in the conventional biomass-to-biofuel processes.

Keywords : Rain tree pods, Golden rain tree pods, Thermal extraction, Biobutanol production

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ABBREVIATION

ABE	Acetone Butanol Ethanol
ANOVA	Analysis of Variance
CCD	Central Composite Design
Df	Degree of freedom
DNS	3,5-Dinitrosalicylic acid
DP	Degree of polymerization
GRT	Golden Rain Tree
OD	Optical Density
RCM	Reinforced Clostridia Medium
RSM	Response Surface Methodology
RT	Rain Tree

CHAPTER 1
INTRODUCTION

Background

The rapid development of our society, the current environmental, economic and social concerns regarding sustainability energy have pushed researchers towards discovering cleaner, renewable and sustainable energy resource. Biofuels produced from living biomass (such as biodiesel, biogas, bioethanol, biobutanol, etc.) is a clean and efficient technology that does not adversely affect the environment. It can be used in replacing fossil fuels for sustaining growing energy demands (Kumar et al., 2017). At present, butanol is considered to be a relatively new biofuel, which has some advantages over ethanol for use as a transportation fuel. Several researches showed that the use of butanol has a high calorific value, which is 29.2 MJ/L (melting point -89.5 °C, boiling point 117.2 °C, flash point 36 °C, the self-ignition 340 °C) that is closer to that of gasoline (32 MJ/L) and ethanol (19.6 MJ/L). Furthermore, butanol can be blended with gasoline to any proportion for better engine performance (Gomez-Flores et al., 2018; Kaminski et al., 2011).

Biobutanol can be produced from fermentation route using means of bacteria of the genus Clostridium such as *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* (Kumar et al., 2017). This process occurs under anaerobic conditions through acetone-butanol-ethanol (ABE) fermentation. Aside from solvent, Clostridium bacteria can also produce acids (acetic and butyric acid) and gases (hydrogen and carbon dioxide) (Ibrahim et al., 2017; Kaminski et al., 2011). The first phase of ABE fermentation is the acidogenic phase in which the acid-forming trunks are stimulated, yielding acetic and butyric acids as main products. This commonly occurs during the exponential growth phase. The second phase is the solventogenic phase; the acids derived from the first phase are used as substrates for the production of acetone, butanol, and ethanol (Wechgama et al., 2017).

Renewable materials of biofuel production are available in the country. It is further subcategorized into different generation biofuels which are based on raw material being utilized and technological processes done during the conversion into biofuels. The first generation of biofuels, the source is derived from edible oil bearing crop plants such as palm oil, corn, soybean, sunflower, etc. However, there is a high cost of production and the food with fuel dispute is the main constraints on using those materials for the production of biofuel. Issues over feedstock sourcing, impact on biodiversity, land availability for growing agricultural crops, and global food crisis are among the firm criticisms lambasted by environmentalists and non-government organizations. With that, lignocellulosic feedstock from plant biomasses came into the development of second generation biofuels.

Indeed, the second generation biofuels cover a wider range of feedstock in the sense that they are mainly derived from non-edible feedstock such as lignocellulosic plant biomasses, agriculture residues (e.g., bagasses, straws, etc.), and waste products such as waste cooking oil. These feedstocks are advantageous because they can counter the food-versus-fuel issues present in the first generation biofuels. To date, the exploitations of various oleaginous microorganisms (microalgae, bacteria, yeast, and fungi) have resulted in the rise of third-generation biofuels. However, pretreatment of the material is needed to obtain enough six-carbon sugar before it can be fermented and use for the production of biobutanol. The easiest way to produce biobutanol is to covert six-carbon sugar through fermentation (Leong et al., 2018; Siano et al., 2017).

Rain tree (Samanea saman) and golden rain tree (Cassia fistula) are widely interspersed in the tropics. It is cultivated as an ornamental shade tree, yielding dark brown and large leathery pods. Rain tree pods have been generally used as a feed for goats and other ruminants because they comprise a high amount of protein (Siano et al., 2017). The rain tree pod was reported to be having high total sugar and protein. It contains 10.00-17.30% total sugar and 15.31-18.00% protein (Hosamani et al., 2005; Semae et al., 2013). Meanwhile, golden rain tree pods were reported to be having glucose (42.5%) and protein (11.94%) (Danish et al., 2011). However, it was

observed that most of the pods are unutilized and remain to the ground until they are putrid. Most of the rain tree and golden rain tree pods are planted along roadside and university vicinities which defoliate and pounded to the ground and become glutinous that invites flies when rotting (Siano et al., 2017). Therefore, rain tree and golden rain tree pods can be a promising material for biobutanol production because it contains an appreciable amount of sugar and has a significant volume of production during its fruiting season in Thailand. This study aims to investigate of feasibility and thermal extraction method of rain tree and golden rain tree pods for biobutanol production. The results will further undergo energy analysis to determine the possibility of the material for sustainable energy use in the future.

Research objectives

1. To assess the pretreatment process of rain tree and golden rain tree pods by thermal extraction method for biobutanol production.
2. To compare the potential of biobutanol production from rain tree and golden rain tree pods.
3. To evaluate the feasibility of biobutanol production using mass and energy balance analysis and energy engineering process.

Scope of research

1. Rain tree and golden rain tree pods were investigated for possible production of biobutanol.
2. Use of pretreatment method by thermal extraction method before biobutanol production for future applications. This extraction method was optimized using RSM and engineering aspects.
3. The potentiality of biobutanol production from sugar presents from rain tree and golden rain tree pods.
4. The biobutanol production process was verified with mass balance and energy balance for energy sustainability and usage.

Significance of research

1. The result of this study will provide an appropriate pretreatment method and fermentation process for rain tree and golden rain tree pods.
2. This study will lead to economic progress along with the development of greener production processes in Thailand.
3. The industry can utilize the data from this study to apply commercially.
4. Finally, the discovery of new material available in the country for possible production of biofuel.

CHAPTER 2

LITERATURE REVIEW

Overview on butanol

Butanol, or butyl alcohol and 1-butanol, is a four primary carbon alcohol with the molecular formula of C_4H_9OH, a molecular weight of 74.12 g/mol and a colorless liquid. Butanol is primarily used as a solvent, and as an intermediate in chemical synthesis, and more importantly a potential biofuel. It is completely miscible with organic solvents and partly miscible with water (Lee et al., 2008; Moradi et al., 2013). Essential properties of butanol are concluded in Table 1.

Table 1 Properties of butanol

Properties	**Values**
Melting point (°C)	-89.3
Ignition temperature (°C)	35
Flash point (°C)	365
Critical pressure (hPa)	48.4
Critical temperature (°C)	287

(Lee et al., 2008)

Biobutanol has a great potential and some advantages over ethanol (shown in Table 2). It is estimated that demand for biobutanol is about 122 million tons per year by 2020. In 2013, butanol prices were around USD 4.00 per gallon equivalent to about USD 1.05 per liter (Ibrahim et al., 2017).

Table 2 Comparison of characteristics of butanol and other fuels

Properties	**Butanol**	**Gasoline**	**Ethanol**	**Methanol**
Structure		Average C_8H_{15}		
Molecular weight	74.12	111.19	46.06	32.04
Oxygen content (%w/w)	22	0	35	50
Boiling point (°C)	117.7	27-225	78	64.7
Density at 20°C (g/mL)	0.8098	0.72-0.78	0.794	0.792
Energy density (MJ/L)	29.2	30-33	21.4	15.8
Solubility in water at 25 °C (%wt)	18.1	Negligible	Miscible	Miscible
Air-fuel ratio	11.17	14.58	8.98	6.46
Heat of vaporization (MJ/kg)	0.43	0.36	0.92	1.2
Research octane number	96	91-99	129	136
Motor octane number	78	81-89	102	104

(Lee et al., 2008; Satsangi et al., 2018)

One of the advantages of biobutanol is that it can be used as fuel in the engine directly compared to ethanol that needs to be modified first to be suitable for use as fuel (Ibrahim et al., 2017).

Chotwichien et al., (2009) studied the use of biodiesel as an additive in stabilizing ethanol-diesel and butanol-diesel blends. The study proposed that the use of butanol in diesohol can solve the problem of fuel instability at 30 °C as the result of its higher solubility in diesel fuel. In addition, the fuel properties results showed that blends containing butanol have properties closer to diesel than those of mixes containing ethanol as shown in Figure 1.

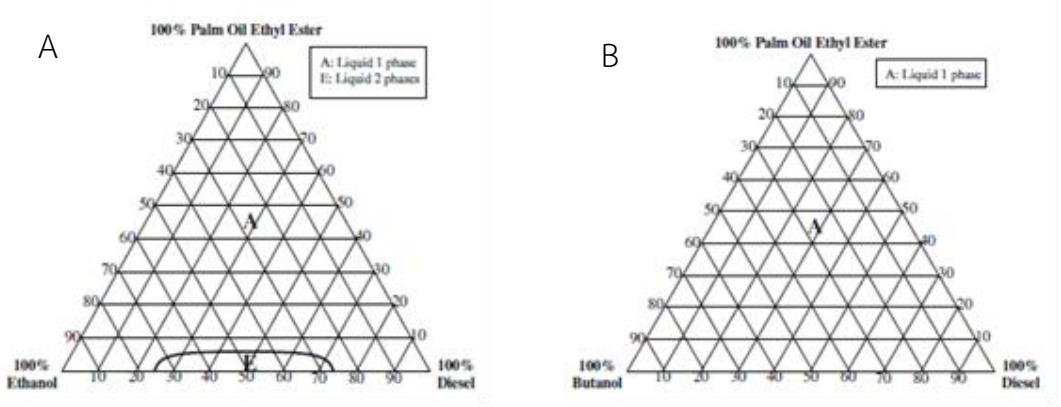


Figure 1 Phase behavior of (A) diesel-palm oil ethyl ester-ethanol and (B) diesel-palm oil ethyl ester-butanol systems at 30 °C

(Chotwichien et al., 2009)

Butanol is also used as fuel to the engine and is important for many industrial chemical applications. Most of the butanol is converted to compounds such as Butyl acrylate which is used as an intermediary in chemical reactions for coating and mixture in color. Butanol is also widely used as a solvent for wood coatings and materials in the furniture industry. Also, butanol can also be used as an extract in the manufacture of drugs and natural substances such as antibiotics, hormones, vitamins, etc. Moreover, it can be used in other areas such as glass, cleaning agents, cosmetics industry (such as eyelashes, nail polish products, and shaving products), health products, etc. (Dürre, 2007; Lee et al., 2008).

Rain tree and golden rain tree

Rain tree (*Samanea saman*) with a family name of Fabaceae is a large deciduous tree of 15-25 m height with a short bole and broad spreading crown. It is widely distributed in the tropics and grown all along the roadsides and gardens as avenue trees. It is propagated by seeds, cuttings and thrives best in hot moist localities and dry barren lands. Leaves are bipinnate, shining above and downy beneath, folding and dropping at night or on the approach of rain. The tiny flowers are massed in pinkish heads and the leaves are alternately arranged along twigs.

Mature pods are black-brown, oblong, 4-8 inch long, 0.6-0.8 inch wide, 0.25 inch thick, straight or slightly curved but eventually cracking irregularly, containing 10-12 seeds embedded in a brownish pulp that is sweet and edible as shown in Figure 2. A mature tree can yield about 500-600 kg green forage foliage and 250-300 kg pods per annum. The leaves and pods of the trees are esteemed as fodder for livestock. Ripe pods are available from February to May. It is reported that the pods consist of 15.31-18.00% crude protein, 10.0-17.30% total sugars 9.72% hemicellulose and 17.48% cellulose. Chemical composition revealed that the pods are high in dry matter protein which suggests the potential as a component of feed for livestock (Hosamani et al., 2005; Raja et al., 2017; Semae et al., 2013).

Figure 2 Flowers and fruit of ripeness of rain tree

However, the rain tree pods fall on the ground and go waste. Rain tree pods were seen to be a promising source of biofuel since it contains a significant amount of fermentable sugars. As to date, bioethanol production from rain tree pods is only available from past researches such as from the study of Siano et al., (2017). The study was conducted to establish the complete procedure in processing rain tree pods for bioethanol production. Production processes were done for bioethanol production from the collection, drying, storage, shredding, dilution, extraction,

fermentation, and distillation. The feedstock was sundried, and moisture content was determined at a range of 20% to 26% before storage. Dilution ratio was 1:1.25 (1 kg of pods = 1.25 L of water) and after the extraction process yielded a sugar concentration of 22 °Bx to 24 °Bx. The dilution period was three hours. After three hours of diluting the samples, the juice was extracted using extractor with a capacity of 64.10 L/hour. 150 L of rain tree pods juice was extracted and subjected to a fermentation process using anaerobic bioreactor. Fermentation with yeast (*Saccharomyces cerevisiae*) can fasten up the process, thus producing more ethanol at a shorter period; however, without yeast fermentation, it also produces ethanol at lower volume with the slower fermentation process. Distillation of 150 L of fermented broth was done for six hours at 85 ℃ to 95 ℃ temperature (feedstock) and 74 ℃ to 95 ℃ temperature of the column head (vapor state of ethanol). The highest volume of ethanol recovered was established at with yeast fermentation at five-day' duration with a value of 14.89 L and lowest actual ethanol content was found at without yeast fermentation at three-day duration having a value of 11.63 L. The results suggested that rain tree pods had very good potential as a feedstock for bioethanol production. Fermentation of rain tree pods juice can be done with yeast and without yeast.

Golden rain tree (*Cassia fistula* Linn.) with a family name of Caesalpiniaceae commonly known as Amulthus is a deciduous tree with greenish grey bark, compound leaves, leaflets are each 5-12 cm long pairs as shown in Figure 3. A semi-wild tree is known for its beautiful bunches of yellow flowers. The fruit pods are 40-70 cm long and 20-27 mm in diameter, straight or slightly curved, smooth but finely striated transversely, the striations appearing as fine fissures. The rounded distal ends bear a small point marking the position of the style. The dorsal suture appears as a single vascular strand and the ventral suture as two strictly applied strands. Internally the pod is divided by thin, buff-colored, transverse dissepiments at intervals of about 0.5 cm. Each compartment contains one seed which is flat, oval, reddish brown with a well-marked raphe. The seed contains a whitish endosperm in which the yellowish embryo is embedded.

The long pods which are green, when unripe, turn black on ripening after flowers shed. The pulp is dark brown in color, sticky, sweet and mucilaginous, odor characteristic, and somewhat disagreeable. It is reported that the golden rain tree pods consists 19.94% proteins, 31.3% sucrose, 26.2% fructose and 42.5% glucose (Danish et al., 2011). Moreover, there are still no reports of golden rain tree pods use for the production of biofuels.

Figure 3 Flowers and fruit of golden rain tree

Processes for extraction of sugar from sugar plant material

Rain tree and golden rain tree pods contained a sugar-rich juice that can be readily utilized for biobutanol production. Most of the sugar is stored inside the brownish pulp of the pods.

Extraction of sugar from each agricultural source requires unique operating conditions developed based on sugar and water content, fiber structure and composition, and geometric size. The traditional method to extract sugar from sugar plant is to squeeze the stalks through a roller mill, releasing the sugar-rich juice in a process derived from sugar cane sugar extraction. The main drawbacks of crushing are: 1) there is substantial fermentable sugar remaining after a single crushing (less

than half of the total sugar in the stalks typically is recovered) and 2) it is labor and energy intensive (Jia et al., 2013). For example, to extract sugar from cashew apple bagasse, the optimum extraction conditions of liquid: solid 3.26 (mL/g), pH 6.42, extraction time 6.30 h and temperature 52.27 ℃ (Kuila et al., 2011). This method of extraction is called the water extraction method. It has the draw backed is that the sugar concentration in the extraction water typically is relatively low making (Jia et al., 2013). Therefore, improved water extraction methods were used the thermal in the process of extraction sugar which developed and assessed in this research to overcome the low sugar concentration.

Enzymatic hydrolysis

Enzymatic hydrolysis is the unit operation in the lignocellulose conversion process that utilizes enzymes to depolymerize lignocellulosic biomass. The saccharide components released are the feedstock for fermentation (Modenbach and Nokes, 2013). Enzymatic hydrolysis of cellulose materials for produce reducing sugar has long been pursued its potential for higher yields, higher selectivity, lower energy costs, and milder operating conditions than chemical processes (Dai et al., 2011). Hydrolytic enzymes are also available as commercial preparations such as Celluclast, Cellic CTec2, Speczyme CP, Novozyme 188, Cytolase CL, and Accellerase (F and Shastri, 2016). A cellulase is a group of enzymes that contribute to the degradation of cellulose to glucose. Most investigated cellulases were extracted from fungal species like *Trichoderma viride*, *Trichoderma reesei*, and *Fusarium solani*. It is generally that cellulase consist of endoglucanases, exoglucanases and cellobiase (β-glucosidase) (Gan et al., 2003). The proportion of each of these components in the enzyme mixture is determined depending on the source of the enzyme. The endoglucanases bind to the cellulose and expose the reducing and non-reducing ends resulting in the formation of cellooligomers. The exoglucanases bind to the reducing and non-reducing ends of the cellooligomers converting the same to cellobiose. The final component that acts is the β-glucosidase which converts cellobiose to glucose (Wilson, 2012). The insufficient quantity of β-glucosidases in

the enzyme mixture leads to accumulation of cellobiose which inhibits the hydrolysis reactions. Apart from cellobiose, glucose, cellooligomers, and xylose also inhibit the hydrolysis reaction. Lignin reduces the enzyme available for hydrolysis by non-productive adsorption. In addition to the quantity of enzyme, maintaining optimal operating conditions like temperature and pH is also important (F and Shastri, 2016). The optimum operating temperature for cellulose hydrolysis ranges between 40 to 55 °C and pH ranges from 4.5 to 5.5. The enzymes are susceptible to degradation upon exposure to high temperature, and mixing speed (Caminal et al., 1985; Gan et al., 2003). After enzymatic hydrolysis, a relatively clean sugar stream (mainly glucose and xylose) can be obtained at a reasonably high yield with economically relevant enzyme dosages. The resulting mixed sugar stream can be a suitable feedstock for biofuel production, especially when targeting butanol via solvent-producing bacteria which, unlike most yeast strains used in ethanol production, can utilize both hexose and pentose (Gao and Rehmann, 2014).

The process of biobutanol production

Butanol can be produced using several chemical technologies. It is also possible to produce butanol in the process of fermentation by means of bacteria of the genus *Clostridium*. This process is called ABE (acetone-butanol-ethanol) fermentation. This fermentation process occurs under anaerobic conditions. The general ratio of these compounds being 3:6:1. The final concentration of butanol is about 3% (Kaminski et al., 2011).

The history of the ABE process

Pasteur (1861), first reported butanol production in a microbial fermentation. Later, Schardinger (1905) was discovered the production of acetone by fermentation. Butanol was used as the starting material for the production of butadiene or isoprene while acetone was used as a raw material for the production of explosives in World War I. After the war ended, the demand for acetone is reduced. However, the demand for acetone prospers again for automotive and paint industries (Jones

and Woods, 1986). During the early 20th century the production of ABE fermentation boosted and became after ethanol the second largest industrial fermentation process in the world due to the rising world crude oil prices (Lee et al., 2008). However, the fermentation process has a relatively high cost of production compared to the petroleum synthesis process.

Microbial strain and media

Butanol is obtained from the ABE fermentation process under anaerobic conditions by anaerobic bacteria. Most of the bacteria are in the genus *Clostridium* (such as *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, *C. saccharoperbutylacetonicum*, etc.) are primary solvent producers (Lee et al., 2008). Table 3 shows biobutanol producing by bacteria of the genus *Clostridium*. *Clostridium* bacteria can be used for many types of carbon sources such as glucose, sucrose, lactose, xylose, xylan, flour and glycerol (Andrade and Vasconcelos, 2003; Mitchell, 1997). These carbon sources are available or can be produced from a wide variety of biomass.

Table 3 Biobutanol producing from sugarcane bagasse by *Clostridium*

Species name	Butanol concentration (g/L)	ABE concentration (g/L)	ABE yield (g/g raw biomass)	Ref.
C. acetobutylicum ATCC 824	7.68	12.12	0.092	(Li et al., 2017)
C. acetobutylicum GX01	14.17	21.11	0.33	(Pang et al., 2016)
C. beijerinckii NCIMB 8052	6.86	11.86	0.20	(Su et al., 2015)
C. beijerinckii TISTR 1461	0.27	-	-	(Jonglertjunya et al., 2014)

Species name	Butanol concentration (g/L)	ABE concentration (g/L)	ABE yield (g/g raw biomass)	Ref.
C. sporogenes NCIM 2337	4.11	11.01	0.173	(Sivanarutselvi et al., 2017)

Table 3 shows that *C. acetobutylicum* can produce the most ABE yield. *C. acetobutylicum* (Figure 4) are members of genus *Clostridium*. They are gram-positive, spore-forming rods that are obligate anaerobic bacteria.

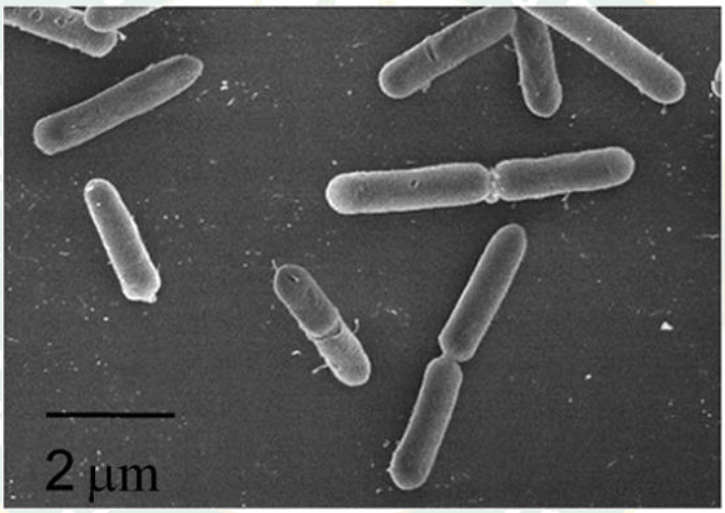


Figure 4 Shape of *Clostridium acetobutylicum*

(Tracy et al., 2011)

Figure 4 is a picture of the bacteria visualized under a Scanning Electron Microscope (SEM). The image was captured for batch cultures that were 76 hours old (late stationary phase of cultures) - this image is showing the rod-shaped, vegetative-like cells. There were no swollen, clostridial-cell-form morphologies observed (Tracy et al., 2011).

The life cycle and growth of *C. acetobutylicum* can be divided into 4 phases. It has distinctly different growth characteristics and is consistent with product

creation as shown in Figure 5. Phase 1 is a vegetative cell, the cell is found in the rods shaped, which can be found in a single cell or a pair as well as in a long chain. Phase 2 is clostridia which look like a cigar shape. In this stage, the cells will build up the granulose accumulation within the cell causing the cells to swell. Phase 3 is forespores that occurs in cases in which environment is not suitable for growth. The cells start to produce forespores and will be developed as spores. Moreover, phase 4 is spore, the stage in which cells create structures called spores that allow them to live in an unsuitable environment (Schuster et al., 1998).

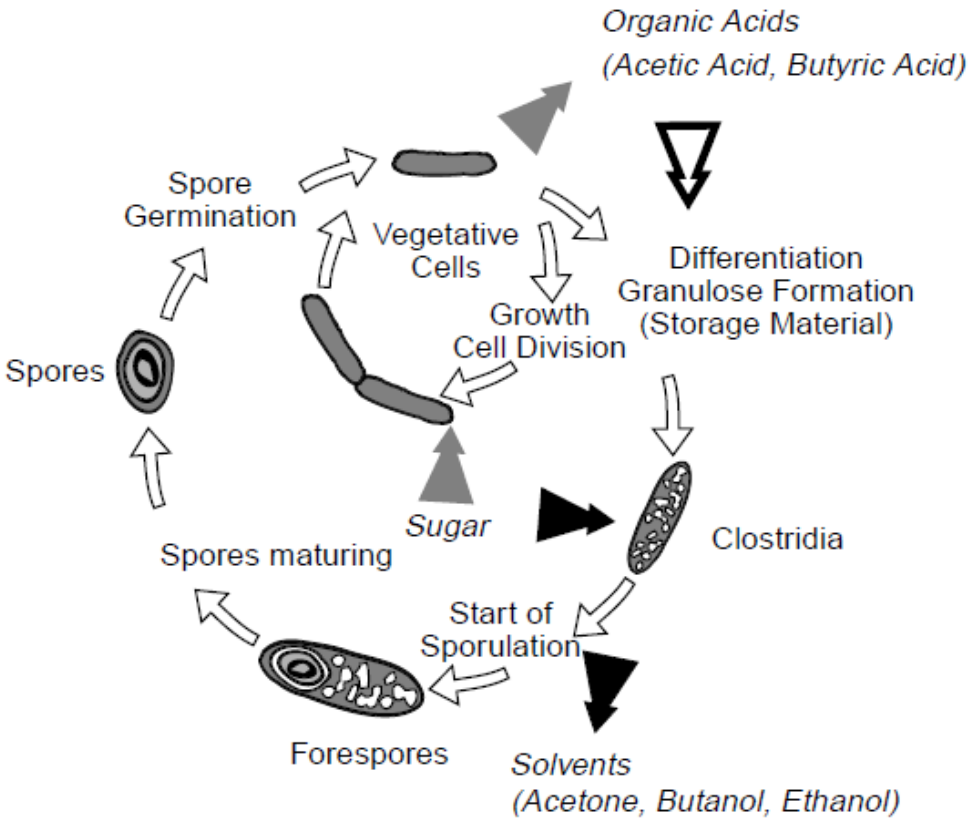


Figure 5 Cell cycle of *Clostridium acetobulyticum*

(Schuster et al., 1998)

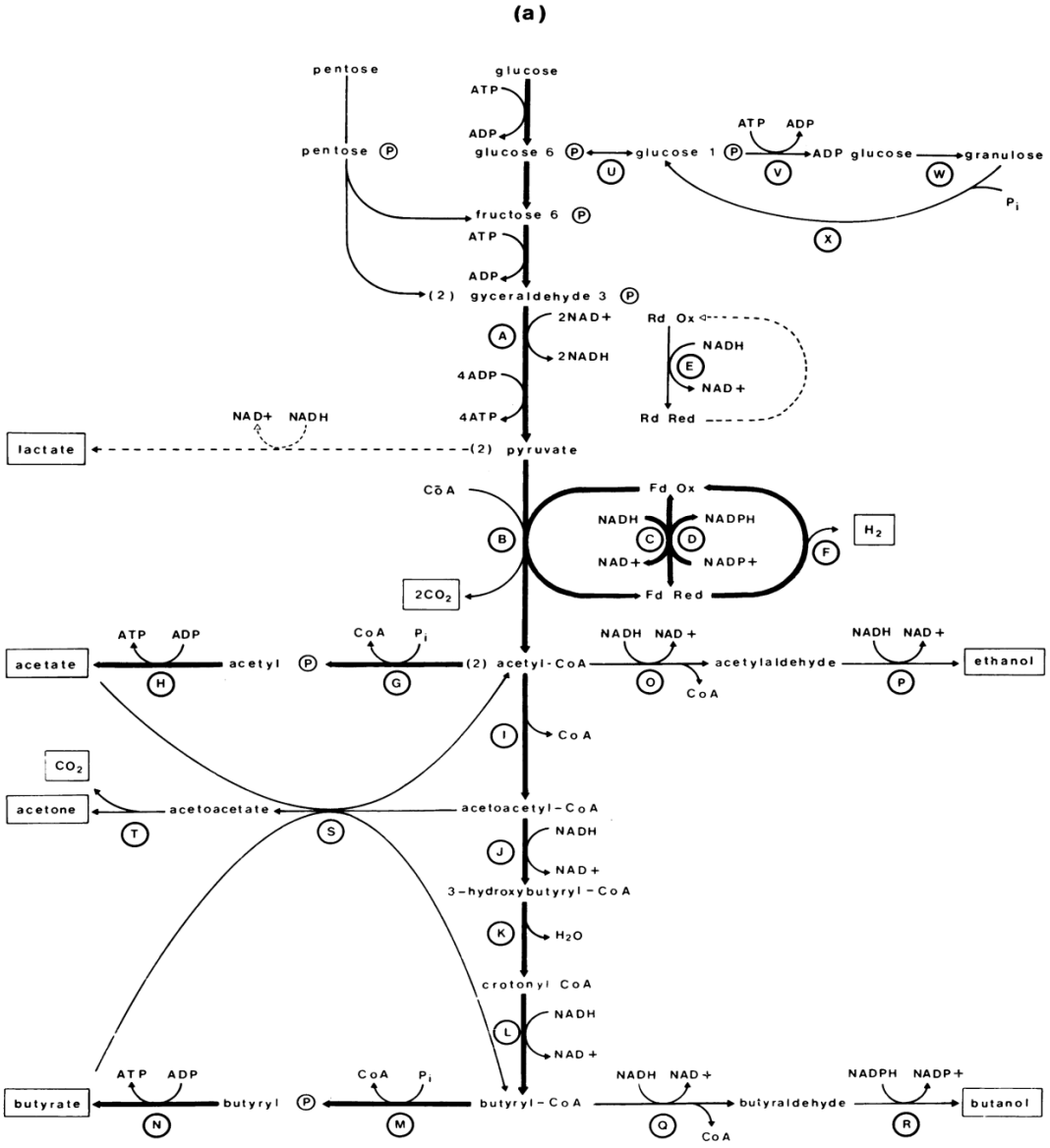
Biochemistry of the fermentation

The form of batch fermentation of *Clostridium* species can be divided into 2 phases. The first phase is producing hydrogen, carbon dioxide, acetate, and butyrate during the initial growth phase (acidogenic phase) occurs during the 7-18 hours of fermentation, which results in a decrease in the pH of the culture medium. And the second phase is the culture enters the stationary growth phase, the metabolism of the cells undergoes a shift to solvent production (solventogenic phase). The solvent production includes acetone, butanol, and ethanol. This usually occurs after fermentation for 18 hours to 36 or 60 hours, which results in a slight increases in the pH of the culture medium and the organic acids produced in the first phase to be used in part.

The biochemical pathway of this bacterium involves the conversion of carbohydrate compounds into organic acids and organic solvents, as well as carbon dioxide and hydrogen, as shown in Figure 6. The solvent-producing clostridia metabolize hexose sugars (including mono-, di-, tri-, and polysaccharides) by way of the Embden-Meyerhof pathway (EMP). The conversion of 1 mol of hexose to 2 mol of pyruvate, with the net production of 2 mol of adenosine triphosphate (ATP) and 2 mol of reduced nicotinamide adenine dinucleotide (NADH). Pentose sugars are metabolized via the pentose phosphate pathway with the conversion to pentose 5-phosphate and dissimilated using the transketolase-transaldolase sequence, resulting in the production of fructose 6-phosphate and glyceraldehyde 3-phosphate, which enter the glycolytic pathway. The fermentation of 3 mol of pentose yields 5 mol of ATP and 5 mol of NADH. The pyruvate caused by glycolysis is cleaved by pyruvate ferredoxin oxidoreductase in the presence of coenzyme A (CoA) to produce carbon dioxide, acetyl-CoA, and reduced ferredoxin.

Acetyl-CoA caused by the phosphoroclastic cleavage is the central intermediate in the branched fermentation pathways that leads to the production of acids and solvents. The product of 2 mol of acetyl-CoA are converted into acetoacetyl-CoA after that it will be used in the construct of butyric acid, which results in a reduction in the pH of the fermented water. In addition to acetoacetyl-CoA is also used to build acetate after that it will be converted to acetone and

carbon dioxide with an enzyme in the acetoacetate decarboxylase system. Bacteria have a mechanism of change butyrate into butyryl-CoA, and then it will be further reduced to butanol. For ethanol, it is produced from acetoacetyl-CoA. Acetoacetyl-CoA is converted to acetaldehyde by the acetaldehyde dehydrogenase enzyme after that acetaldehyde is converted to ethanol by the ethanol dehydrogenase enzyme.



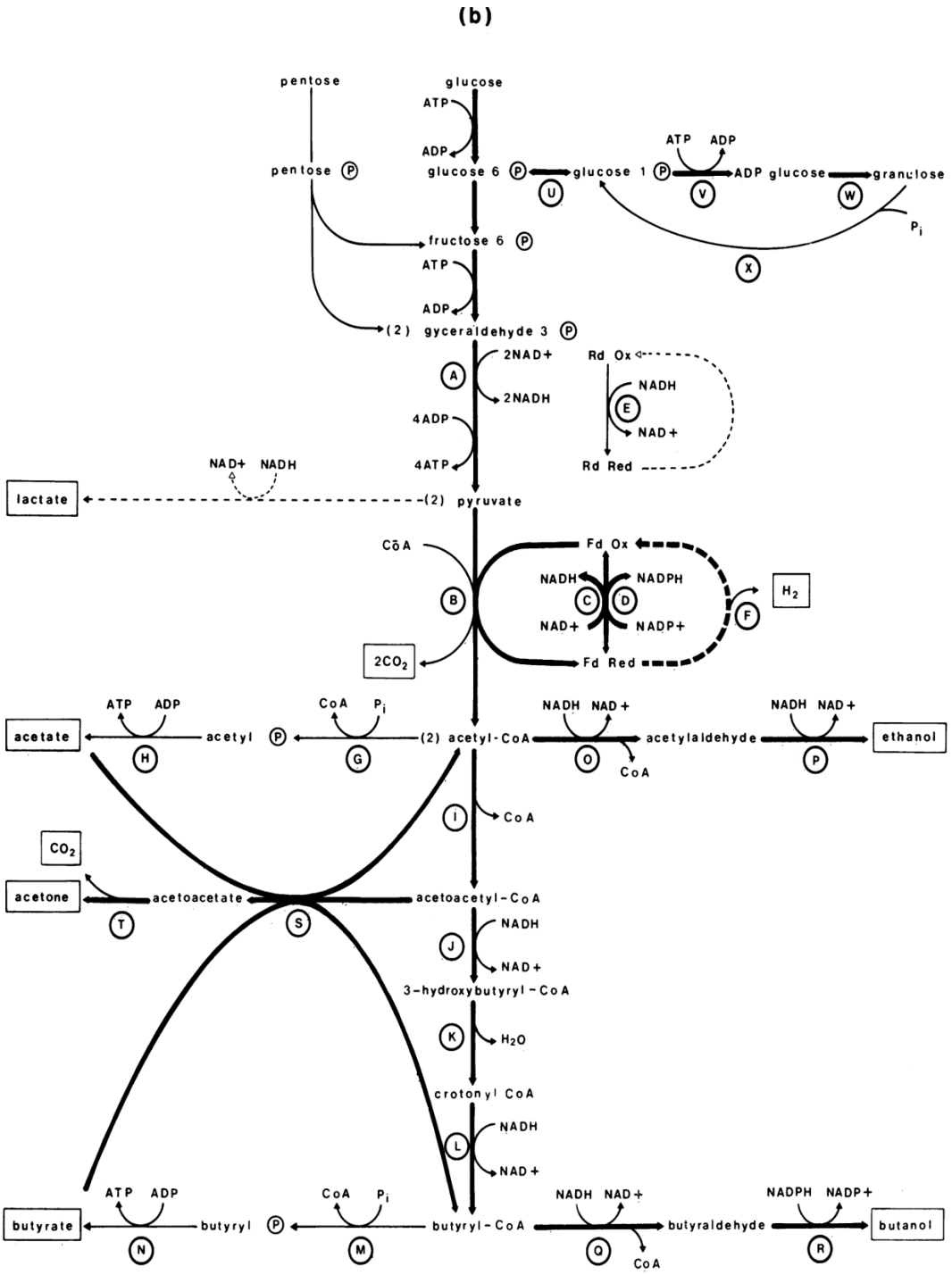


Figure 6 Biochemical pathways of *C. acetobutylicum*. Reactions of the fermentation during the acidogenic phase (a) and the solventogenic phase (b) are shown by thick arrows

(Jones and Woods, 1986)

Enzymes are showed by letters hereinafter: (A) glyceraldehyde 3-phosphate dehydrogenase; (B) pyruvate-ferredoxin oxidoreductase; (C) NADH-ferredoxin oxidoreductase; (D) NADPH ferredoxin oxidoreductase; (E) NADH rubredoxin oxidoreductase; (F) hydrogenase; (G) phosphate acetyltransferase (phosphotransacetylase); (H) acetate kinase; (i) thiolase (acetyl-CoA acetyltransferase); (J) 3-hydroxybutyryl-CoA dehydrogenase; (K) crotonase; (L) butyryl-CoA dehydrogenase; (M) phosphate butyltransferase (phosphotransbutyrylase); (N) butyrate kinase; (O) acetaldehyde dehydrogenase; (P) ethanol dehydrogenase; (Q) butyraldehyde dehydrogenase; (R) butanol dehydrogenase; (S) acetoacetyl-CoA:acetate/butyrate:CoA transferase; (T) acetoacetate decarboxylase; (U) phosphoglucomutase; (V) ADP-glucose pyrophosphorylase; (W) granulose (glycogen) synthase; (X) granulose phosphorylase (Jones and Woods, 1986).

During the fermentation process, the cell of bacterial will change shape over time of fermentation and are associated with product creation. Acidogenesis phase, the bacteria grow rapidly (log phase). The cell shape of the rod and move faster. Bacteria produce acetic acid and butyric acid, causing the pH to decrease to the point where the lowest pH is called the breakpoint. This is the point that indicates that the fermentation started the second phase. The accumulation of these acids is an induction, causing the bacteria to use acids as substrates to convert acids to solvents such as acetone, butanol, and ethanol. The cells of bacteria in this stage are swollen and slow to move. For the duration of the fermentation process, the bacteria can produce other substances such as hydrogen and carbon dioxide. Solventogenesis phase, bacteria convert acidic products into solvent products. In this period, the bacteria are reduced in growth because they are inhibited by the acid that was created in the first phase. Both acids (acetic and butyric) are used as carbon sources, resulting in higher acidity of media. The end of acidogenesis phase and beginning of solventogenesis phase called transition point (Oshiro et al., 2010).

The factors affecting the fermentation process

At present, the researches focus on the development of production processes that can increase the amount of acetone, butanol, and ethanol, and can

reduce the cost of production. Therefore, the study on factors affecting the growth and ability of each substrate and conversion of acid production to the solvents production of bacteria is. The study on the factors affecting batch and continuous fermentation is aimed in order to enhance the production of biobutanol. The details are as follows.

Substrate and concentration

One of the most important factors to consider when developing an ABE solution is the cost of production. The choice of agricultural raw materials to cultivate in the country as a substitute for fermentation can reduce production costs. The production of acetone, butanol, and ethanol, can be used for various substrates including starch and sugar, such as fermented rice straw (Li et al., 2018), sugarcorn juice (Gomez-Flores et al., 2018), etc. Also, agricultural raw materials such as lignocellulose can produce biobutanol. However, it requires the pretreatment and hydrolysis of raw materials before the sugar can be used to ferment which resulted in higher production cost. The easiest way to produce biobutanol is to convert six-carbon sugar through ABE fermentation (Siano et al., 2017).

The initial concentration of sugar is important for ABE fermentation. If the initial sugar concentration is lower (less than 20 g/L), the fermentation is directed towards the acidic phase. It will produce only a few organic solvents (Lee et al., 2008). However, if high concentration (higher than 60 g/L), the process produces more organic matter (Madihah et al., 2001). At concentrations above 80 g/L, sugar is not fermented as a result of product inhibition. At concentrations up to 120 g/L, fermentation occurs only slightly (Qadeer et al., 1980). This may be due to substrate inhibition.

Temperature

The fermentation temperature affects the production of organic solvents, the yield rate of the organic solvent, and the products formed in the fermentation.

McNeil and Kristiahsen, (1985) studied the effect of temperature in the range 25 to 40 °C on the solvent production by *Clostridium acetobutylicum*. It was found that when the temperature increased, the total solvent yield decreased. This seems

to be due to the reduction in acetone production. It appeared that the yield of the other major solvent, butanol, was not affected by the temperature. Considering total solvent yield and productivity, the optimum fermentation temperature is 35 °C.

Oxygen

C. acetobutylicum can live in an anaerobic condition. The optimal growth occurs in Redox potassium hydroxide (Eh) between -250 to -400 mV. Exposure to oxygen in anaerobic digestion is not harmful if it occurs only briefly. Exposure to short oxygen is not lethal, however, if the cell is exposed to a high concentration of oxygen, it will result to decrease in the rate of glucose consumption and deoxyribonucleic acid (DNA), ribonucleic acid, and protein syntheses stops. Aerobic conditions appear to be a reducing factor and stop the production of butyrate (Jones and Woods, 1986).

The pH

In fermented water, the pH determines the degradation of sugar. There are many reports that if the pH of the fermented water is kept high, it makes most products of fermentation an organic acid. On the other hand, if the pH is maintained at a low value, most of the products are organic solvents. However, the range of pH that will produce organic solvents is very wide. It depends on the species of microorganisms and the conditions of fermentation. The range of organic fermentation was in the range of pH 3.8 to 5.5 (Lee et al., 2008). However, industrial-grade bacteria such as *C. acetobutylicum* P262 can produce organic solvents at pH 6.5 (Jones and Woods, 1986).

Design of experiment

Design of experiment (DOE) is a fundamental tool in the field of engineering. This technique can be used as a statistical methodology to analyze data and predict product property performance under all possible conditions within limits selected for the experimental design. This leads to improving performance, minimize the number of experiments necessary to obtain the answer to a problem or minimizing the variance of estimated coefficients obtained through regression (Wagner et al., 2014).

Response Surface Methodology, or 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 (Sarrai et al., 2016). RSM will answer questions about how to choose the level for the applied factors to obtain desirable, smallest or most significant, the value of the response function in the reduced number of experiments (Bai et al., 2015). This method was presented by (Box and Wilson, 1951) and since then it has been widely used as a technique for experimental design.

Six stages (Figure 7) in the application of RSM as an optimization technique are as follows (Witek-Krowiak et al., 2014): (1) selection of independent variables and possible responses, (2) selection of experimental design strategy, (3) execution of experiments and obtaining results, (4) fitting the model equation to experimental data, (5) obtaining response graphs and verification of the model (ANOVA), (6) determination of optimal conditions.

Several design methods for RSM, the most popular being the central composite design (CCD), Box–Behnken design (BB), Doehlert Matrix (D), as well as Plackett–Burman (PB) design, full or fractional factorial designs for optimizations with many variables. The statistical software such as Design Expert (Stat-Ease, Inc.), Minitab (Minitab Inc.), Statistica (StatSoft), JMP (SAS) and Matlab (MathWorks).

A central composite design (CCD) is often used for building a second-order polynomial for the response variables in RSM. To establish the coefficients of a polynomial with quadratic terms, the experimental design must have at least three levels of each factor. In CCD, there are three different points, namely factorial points (-1 and +1), central points (0), which corresponds to the middle level of the factors, and axial points ($-\alpha$ and $+\alpha$), which in turn depends on specific properties desired for the design and the number of parameters related (Sahoo and Barman, 2012; Witek-Krowiak et al., 2014).

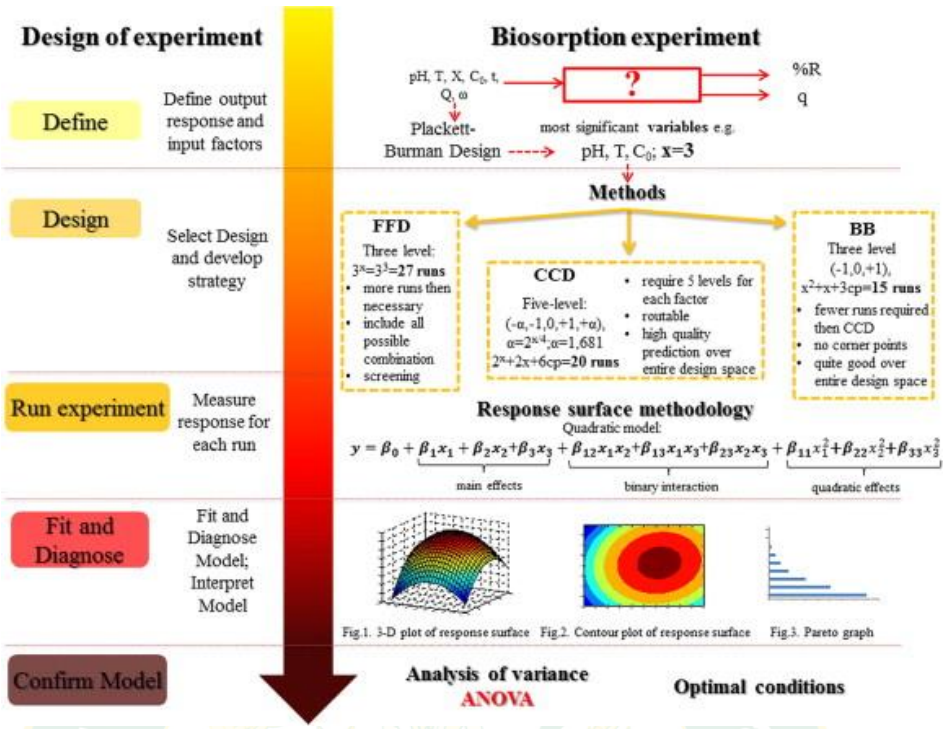


Figure 7 Design of experiment in RSM methodology

(Witek-Krowiak et al., 2014)

Lin et al., (2011) studied the produce butanol from corn straw hydrolysate by *Clostridium acetobutylicum* CICC 8008. Plackett-Burman (P-B) design has adopted to screen crucial factors during fermentation hat among the seven factors, namely, Yeast extract, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $FeSO_4$, $CuSO_4$, and $CaCO_3$. The results show that only $CaCO_3$ was selected as the most critical factor in the produce butanol. After that, studied the optimization of experimental results for $CaCO_3$ usage, temperature and reaction time by Central Composite Design (CCD) were determined to be 5.04 g/L, 35 ℃ and 70 hours, respectively. A corresponding mathematical model was established to predict the fermentation experiment and maximum butanol yield of 6.57 g/L was acquired. The result of a confirmation experiment under the optimum conditions shows that 6.20 g/L is the maximum butanol yield. Therefore, this demonstrated that the statistical method was a powerful tool for the optimization of butanol production from enzymatic hydrolysis of corn stalk.

CHAPTER 3

MATERIALS AND METHODS

This study aimed to produce biobutanol from rain tree and golden rain tree pods using *Clostridium acetobutylicum* TISTR 2375 through ABE fermentation. Figure 8 illustrates the methodology of the study. Rain tree pods and golden rain tree pods were collected and dried to reduce the moisture content of the samples. The dried samples were milled using mortar and pestle and were used in the thermal extraction method. After thermal extraction method, response surface methodology (RSM) was used for the optimization of the factors such as temperature and time. The best condition for thermal extraction that was identified with the use of RSM was applied in the enzymatic hydrolysis of the samples. Afterward, ABE fermentation was done with *Clostridium acetobutylicum* TISTR 2375 to produce biobutanol. Energy and mass balance analysis was also done in this study.

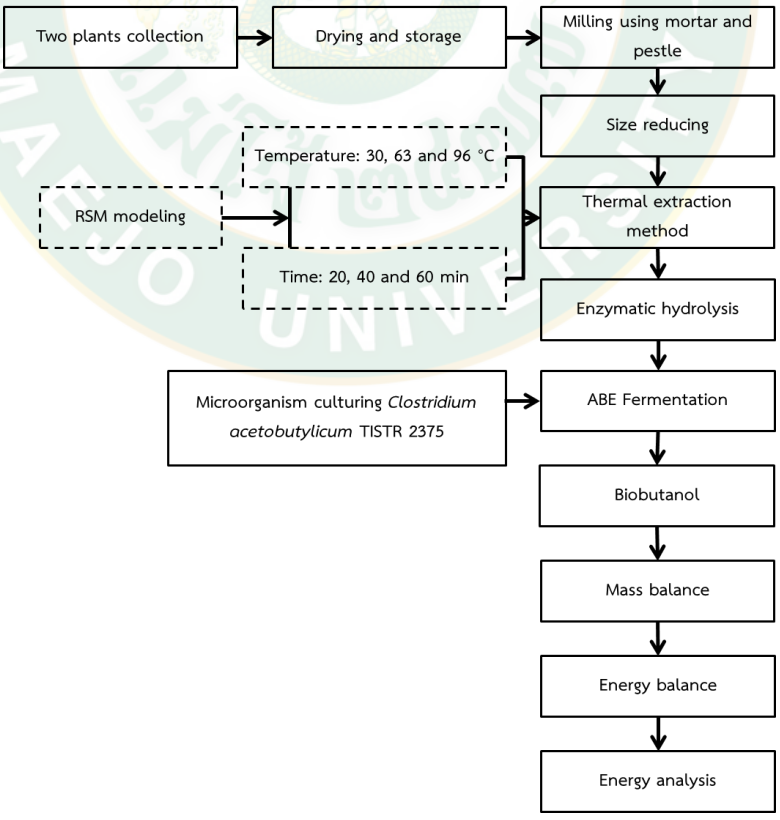


Figure 8 The experimental procedure of the study

Material collection and preparation

Rain tree (*Samanea saman*) and golden rain tree (*Cassia fistula*) were grown at Maejo University campus, Chiang Mai, Thailand (Figure 9). The ripened pods of rain tree and golden rain tree that were fallen on the ground were collected from March to April of 2018. Then, the samples were dried at 50 °C for 48 hours in an oven to prevent mold growth, seed germination, and rotting (Figure 10). Dried samples were stored in a plastic bag until experiments are performed.

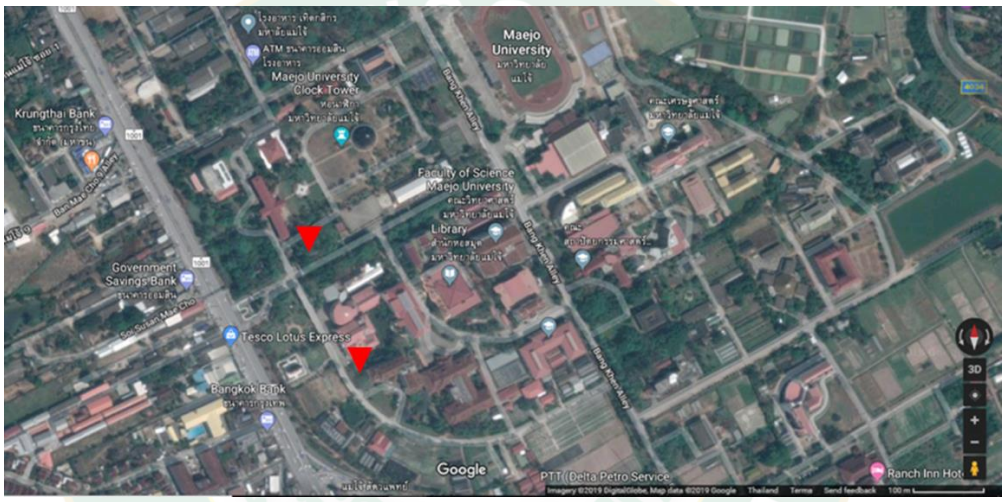


Figure 9 Sampling location (Red triangle) inside Maejo University campus

Figure 10 Rain tree and golden rain tree pods (A, B); Oven drying (C, D); Storing of samples (E, F)

Biomass yield

Biomass yield was calculated by total mass of pods collected within a given unit of environment area, since both rain tree and golden rain tree pods ripe and fall on the ground at Maejo University campus, Chiang Mai, Thailand (18°53'45"N, 99°00'37"E and 18°53'39"N, 99°00'40"E). A 1m x 1m quadrat was placed randomly on the ground where the pods fell (Figure 11). Two types of pods were counted, collected and weighted as fresh samples followed by drying in hot air oven unit until it reached a constant weight. The recorded data were used to calculate the density (pods/m^2) and biomass yield (kg/tree/year).

Figure 11 A 1m x 1m quadrat for counting and collected sample

Lab scale experiment of sugar extraction

The lab scale experiments were carried out to investigate the optimal condition of sugar extraction from rain tree and golden rain tree pods (Figure 12). The extraction processes were tested in a different value of temperature and time.

In this method were performed using a dilution ratio of 1:10 (25 g of pods = 250 ml of distilled water). The pods were soaked in distilled water, which controls

the temperature and time on sugar yield during the extraction process, a study of RSM was carried out using the software, namely design of the experiment. According to Sarrai et al., (2016), 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. The Design Expert software 11.1.0.1 (Stat-Ease Inc., Minneapolis, USA) was used to build and analyze the experimental design. The two factors, namely temperature (°C) and time (min), were statistically optimized with RCM using CCD (Box and Wilson, 1951). The low (-1), middle (0), and high (+1) levels for each factor were given in Table 4. The central composite design was used to present total sugar (g/L) and reducing sugar (g/L) in rain tree and golden rain tree pods. A 2^3 factorial CCD, with three axial points and three replications at the center points leading to a total number of 27 experiments were employed for the optimization of the extraction conditions for each plant.

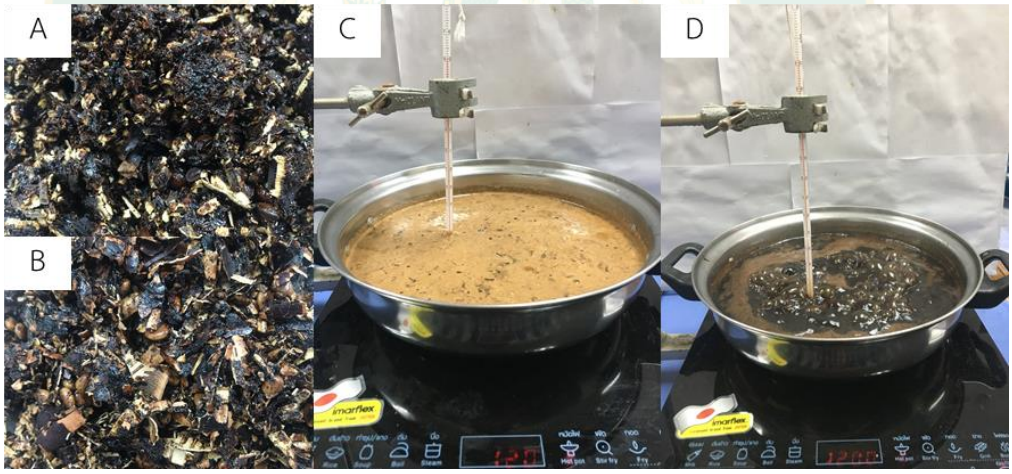


Figure 12 Thermal extraction; Reduced size of materials (A, B); Adding distilled water and controls temperature and time (C, D)

Table 4 Factors and levels by central composite design for rain tree and golden rain tree pods

Factor	Unit	Symbol coded	Coded levels		
			-1	0	+1
Temperature	℃	A	30	63	96
Time	min	B	20	40	60

Analysis of variance (ANOVA) was used for the analyses of the data to define the interaction between the process variables and the responses to estimate the statistical parameters. The F-test checked the statistical significance in the software. The coefficient of R^2 determined the preciseness of the fitted polynomial model. The significant model terms were evaluated by the probability value (P-value) less than 0.05 which confidence interval level above 95% (Behera et al., 2018). Three-dimensional (3-D) surface plots and two-dimensional (2-D) contours plot were achieved to demonstrate the effects of independent factors on sugar concentration. The second order polynomial model proposed for the response surface analysis as follows:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{12} AB + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{112} A^2 B + \beta_{122} AB^2 \quad \text{Equation 1}$$

where Y is the predicted response, A and B are independent variables studied, β_0 is the offset term, β_1 and β_2 are linear effects, β_{11} and β_{22} are the quadratic terms and β_{12}, β_{112} and β_{122} are interaction coefficients.

Enzymatic hydrolysis

The enzymatic hydrolysis was used for converting cellulose into fermentable sugar. The best condition of thermal extraction form rain tree and golden rain tree pods were carried out with cellulase enzyme for hydrolysis process (Figure 13). Enzyme assays used were cellulase 2398 units/g, β-glucosidase 577 units/g, and pH 4

provided by Union Science Company, Chiang Mai, Thailand. The thermal extraction samples with solid were adjusted to pH 5.0 by addition hydrochloric acid (HCl) or sodium hydroxide (NaOH) and added 2% (v/v) of cellulase enzyme. After that, the samples were kept at 50 °C for 24 hours in an oven. The sample was filtered to measure the total sugar and reducing sugar.

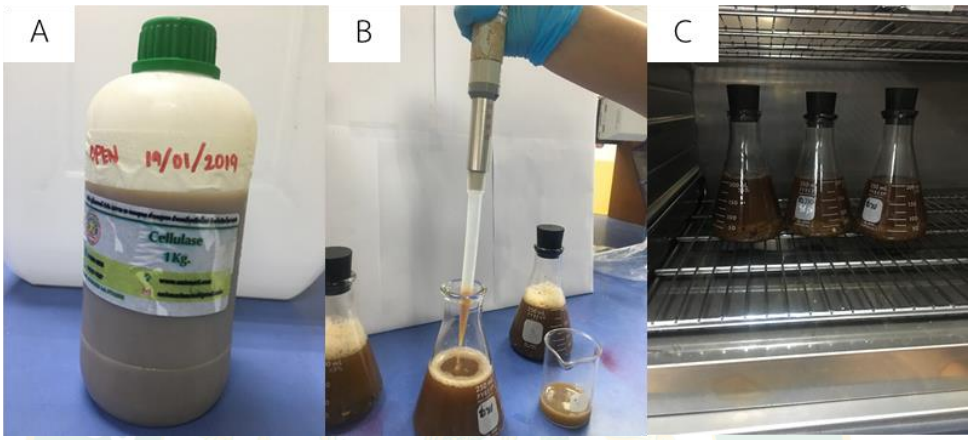


Figure 13 Enzymatic hydrolysis: cellulose enzyme (A); hydrolysis (B, C)

Microorganism culturing *Clostridium acetobutylicum* TISTR 2375

C. acetobutylicum TISTR 2375 (freeze-dried cultures) was purchased from the Culture Collection of the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand (Figure 14).

Figure 14 The freeze-dried cultures of *C. acetobutylicum* TISTR 2375

Culture medium

Reinforced Clostridia Medium or RCM medium (BD Difco™, New Jersey, USA) was used to prepare for the stock culture of *C. acetobutylicum* TISTR 2375. RCM medium was composed of (g/L): peptone 10.0, beef extract 10.0, yeast extract 3.0, dextrose 5.0, sodium chloride 5.0, soluble starch 1.0, cysteine hydrochloride 0.5, sodium acetate 3.0 and agar 0.5. These were mixed with distilled water to form a homogeneous solution. RCM medium was sterilized at 121 °C for 15 minutes under a pressure of 15 pounds per square inch.

P2 medium was used as a synthetic medium for biobutanol production. It reported by Qureshi and Blaschek, (1999) which was the component as follow (g/L): glucose, 60 and yeast extract, 1 as well as stock solutions A, B and C. Stock solution A consisted of KH_2PO_4, 50 g/L; K_2HPO_4, 50 g/L and ammonium acetate, 220 g/L. Stock solution B consisted of para-amino-benzoic acid, 0.1 g/L; thiamine, 0.1 g/L and biotin, 0.001 g/L. Stock solution C was composed of $MgSO_4 \cdot 7H_2O$, 20 g/L; $MnSO_4 \cdot H_2O$, 1.0 g/L; $FeSO_4 \cdot 7H_2O$, 1.0 g/L and NaCl, 1.0 g/L. Stock solutions A and C were autoclaved at 121 °C for 15 min under a pressure of 15 pounds per square inch, whereas the stock solution B was sterilized by filtration through a syringe filter (PVDF) with a pore size of 0.2 µm (Whatman, USA).

Anaerobic incubation

C. acetobutylicum TISTR 2375 was incubated in an anaerobic jar (Merck™ Anaerobic jar 2.5 L-volume, US). Their disposable sachets of GasPak (BD GasPak™ EZ, USA) were used to produce an atmosphere free of elemental oxygen gas (O_2) or anaerobic condition in the jar. Besides, a catalyst made of palladium pellets in wire gauze bag was placed in the anaerobic jar. The palladium catalyst was re-generated either by heating in an oven at 50 °C for overnight. Anaerobic incubation for microorganism culturing *C. acetobutylicum* TISTR 2375 is shown in Figure 15.

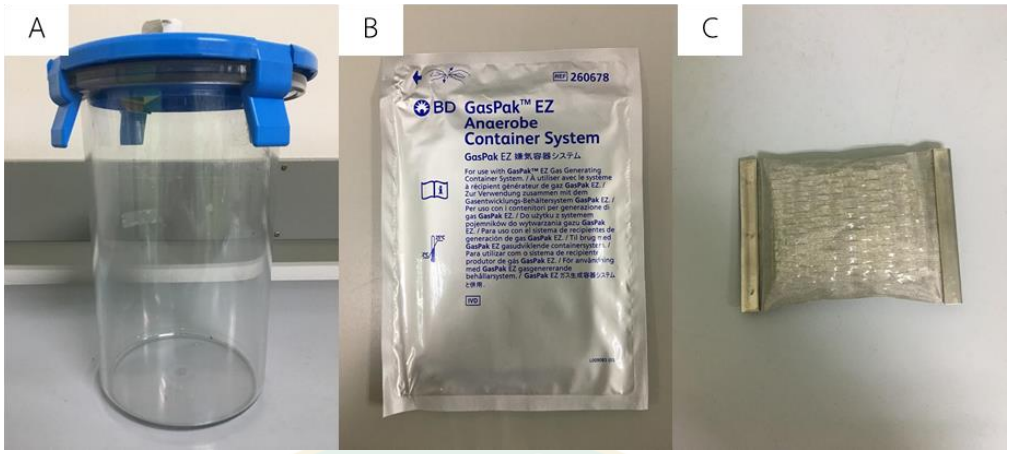


Figure 15 Anaerobic incubation: anaerobic jar (A); GasPak (B); palladium catalyst (C)

Preparation of spore suspensions

C. acetobutylicum TISTR 2375 was activated following the manufacturer's procedure (TISTR). The stock culture was maintained in the form of a spore suspension in 20% glycerol in 1.5 ml Eppendorf microtube and froze at -20 °C (Figure 16).

Figure 16 The stock culture of *C. acetobutylicum* TISTR 2375 in 20% glycerol

Growth curve

A growth curve is an empirical model of the evolution of a quantity increases over time. Growth curve of *C. acetobutylicum* TISTR 2375 was used to determine the quantity of growth pattern in term of optical density (OD) over time. This result was used to determine the OD value and the culture time suitable for fermentation. Five-

milliliter spore suspension of *C. acetobutylicum* TISTR 2375 was transferred into 50 ml of RCM medium for spore activation and incubated at 35 °C for 24 hours. Then, 10 ml of the activated spore suspensions were transferred into RCM medium and incubated at 35 °C for 18 hours to have initial inoculum at 660 nm (OD660) of 1.0. One ml of initial inoculum was transferred into 10 ml of RCM medium and incubated at 35 °C for 48 hours. Cell density was analyzed at 660 nm every 3 hours.

Gram staining

Gram staining method is the most important procedure in Microbiology. This is usually used for differentiating bacterial species into two large groups namely Gram-positive and Gram-negative microorganisms. The method was developed by Danish scientist Hans Christian Gram in 1884. A drop of distilled water was placed in a clean microscope glass slide and a loop of pure culture colony of bacteria was smeared. This smeared glass slide was air dried and flame-heated to fix the cells in the surface of the slide. After that, 1 drop of crystal violet staining reagent was used to stain the cells, which stains all bacterial cells blue. After one minute, the slide was washed gently by running tap water for 2 seconds. After washing, a dropped iodine-potassium iodide solution was applied for 1 minute. After 1 minute, cells were treated with 95% of ethyl alcohol for 15 seconds or add drop by drop to the slide until decolorizing agent running from the slide runs clear. Lastly, a drop of safranin O was added for 15-30 seconds and washed with water and dried with the use of tissue paper. The slide was then examined microscopically using a 100x objective (Olympus BX41, Japan). Counterstained gram-negative cells appeared red, and gram-positive cells remained violet (Sandle, 2004).

Spore activation and inoculum preparation

10% (v/v) of a spore suspension of *C. acetobutylicum* TISTR 2375 was transferred into RCM medium for spore activation in an incubator at 35 °C for 24 hours. Then, 10% (v/v) of the activated spore suspensions were transferred into RCM medium and incubated at 35 °C for 24 hours to obtain highly motile vegetative cells.

Vegetative cells (10%, v/v) with an optical density at 660 nm (OD660) of 1.5〜2 were used as inocula for biobutanol production.

Batch fermentation

All batch experiments of *C. acetobutylicum* TISTR 2375 was carried out in 100 ml bottle with rubber cap covered with an aluminum cap containing previously sterilized medium for 120 hours of fermentation time at 35 ℃ under anaerobic and static conditions. For each batch experiments, the fermentation medium was adjusted to 6.5 using 2 M NaOH and 1 M HCl. To generate an anaerobic condition, the medium was spared with oxygen-free nitrogen gas for 4 min and closed with a rubber cap covered with an aluminum cap. The medium was sterilized at 121 ℃ for 15 min. After the medium cold, the inoculum culture (OD660 = 1.5〜2.0) of *C. acetobutylicum* TISTR 2375 (4.5 ml cell suspension in 45 ml medium) was injected by sterilizing syringe and followed by supplements. The fermentation was operated at 35 ℃ for 120 hours. The samples were withdrawn at time intervals of 24 hours for analysis. Before analysis, the samples were centrifuged at 8,000 rpm for 15 min.

Experiment I: Potential biobutanal production from *C. acetobutylicum* TISTR 2375

To study the potential biobutanol production from *C. acetobutylicum* TISTR 2375 before fermentation from rain tree and golden rain tree pods. This study was using P2 medium (60 g/L glucose and 1 g/L yeast extract) as carbon sources supplemented with 0.5 ml cysteine HCl·H_2O, 0.5 ml buffer, 0.25 ml mineral, and 0.25 ml vitamin solutions (Figure 17).

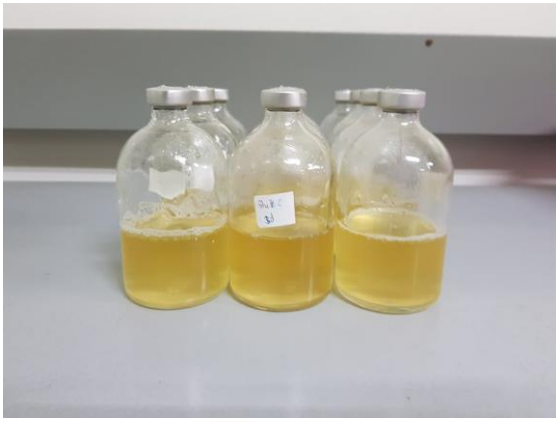


Figure 17 Fermenter of biobutanol production using P2 medium as carbon sources

Experiment II: Biobutanol production from rain tree and golden rain tree pods

To study the high sugar concentration from thermal extraction through enzymatic hydrolysis of rain tree and golden rain tree pods. The fermentation medium was composed of prehydrolysate (high reducing sugar concentration) from two plants as carbon sources supplemented with 0.5 ml cysteine $HCl{\cdot}H_2O$, 0.5 ml buffer, 0.25 ml mineral, and 0.25 ml vitamin solutions (Figure 18).

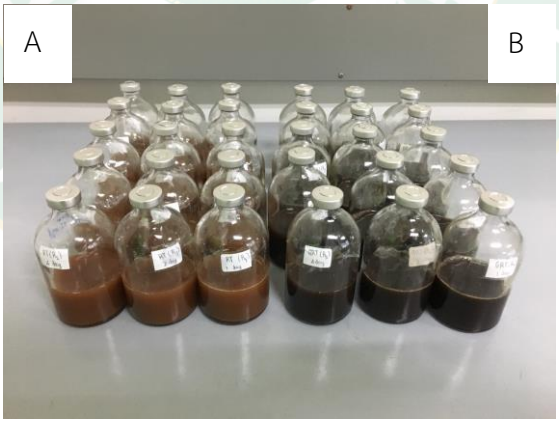


Figure 18 Fermenter of biobutanol production using prehydrolysate (high reducing sugar concentration) from rain tree (A) and golden rain tree (B) pods

Experiment III: Dilute prehydrolysate juice of rain tree and golden rain tree pods for biobutanol production

To study the low sugar concentration from thermal extraction through enzymatic hydrolysis of rain tree and golden rain tree pods. The prehydrolysate juice from two plants was diluted approximately 80 g/L of reducing sugar for biobutanol fermentation. The medium was supplemented with 0.5 ml cysteine HCl·H_2O, 0.5 ml buffer, 0.25 ml mineral, and 0.25 ml vitamin solutions (Figure 19).

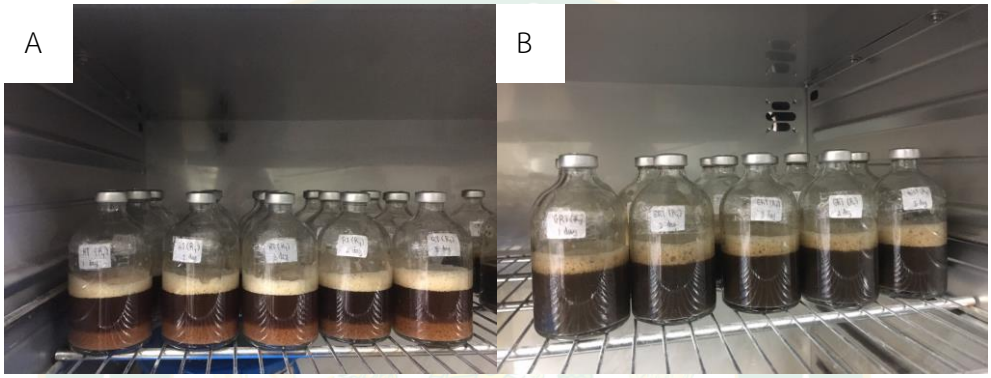


Figure 19 Fermenter of biobutanol production using prehydrolysate (~80 g/L of reducing sugar concentration) from rain tree (A) and golden rain tree (B) pods

Analytical method

Total sugar and reducing sugars were analyzed by the phenol-sulfuric procedure (DuBois et al., 1956) and DNS method (Miller, 1959). Cell growth was determined by measurement of the optical density at 660 nm (OD660) by a spectrophotometer (Thermo Fisher scientific, Genesys 10-S, USA). For sampling, during the fermentation period (120 hours), a sample was taken every 24 hours and centrifuged at 8,000 rpm for 10 min to remove particulate matter. The supernatant was used to analyze for solvents (acetone, butanol, and ethanol) and residual sugar concentrations. The solvents were measured using gas chromatography (GC) (Agilent Technologies 7890A GC, China) connected to a flame ionization detector (FID). ABE fermentation products were separated in a DB-FFAP column (30 m × 0.25 mm, 0.25 µm, China) and a flame ionization detector with helium as the carrier gas. The oven

temperature program varied from 60 to 200 °C at a fixed rate of 10 °C/min. The temperature of the front detector and front injector were maintained at 250 and 150 °C, respectively. The internal standard was used 99.7% acetone (Dr. Ehrenstorfer, Germany), 99.9% 1-butanol (Dr. Ehrenstorfer, Germany) and 99.9% ethanol (Dr. Ehrenstorfer, Germany). The supernatant was sterilized by filtration through a syringe filter (PVDF) with a pore size of 0.2 μm (Whatman, USA) before analytical by gas chromatography. The pH was determined using a pH meter (Eutech, Singapore). The butanol yield ($Y_{B/S}$) and butanol productivity (Q_B) were calculated as follows:

$$Y_{B/S} = P_B / RS \qquad \text{Equation 2}$$

$$Q_B = P_B / t \qquad \text{Equation 3}$$

In the above equation, $Y_{B/S}$ is butanol yield in terms of gram butanol per gram substrate, P_B is the total butanol concentration (g/L), RS is the reducing sugar utilized (g/L), and t is the fermentation time (h) giving the highest butanol concentration. Total ABE (P_{ABE}) was also determined (Wechgama et al., 2017).

Statistical analysis

All the experiments of sugar extraction were performed in triplicate. Data are presented as means with error bar and ± indicating standard deviations.

Mass and energy balance

Material quantities as they pass through processing operations can be described by material balances. Such balances are statements on the conservation of mass. Similarly, energy quantities can be explained by energy balances which are stated on the conservation of energy. If there is no accumulation, what goes into a process must come out. This principle is applicable to batch operation. It is equally true for continuous operation over any chosen time interval (Earle, 1966).

Mass balance calculations were performed for the ABE process under optimal conditions. The mass balance equation can be written simply as:

Mass In = Mass Out Equation 4

Energy takes place in 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 (Earle, 1966). Therefore, this study was focusing on the heat balance for ABE fermentation under optimal conditions. The energy balance equation can be written simply as:

Energy In = Energy Out Equation 5

Techno-economic analysis

A techno-economic analysis was conducted to validate the commercial viability of lab-scale bio-butanol production from rain tree and golden rain tree pods by pretreatment and hydrolysis using a concentrated enzyme and batch fermentation. Techno-economic models assess the potential of research developments to reduce the production cost by process designs. The average total costs for the production of biobutanol can be calculated following as:

Average total costs = Total fixed costs / Quantity (Butanol production) Equation 6

CHAPTER 4
RESULTS AND DISCUSSION

Biomass yield

The research was conducted at Maejo University campus, Chiang Mai, Thailand in which golden rain tree and rain tree were dominant. The average density of rain tree pods and golden rain tree pods were 65 pods/m^2 and 58 pods/m^2, respectively. Rain tree pods resulted in 280 kg/tree per year, while golden rain tree pods produce 307 kg/tree per year. The yield varies according to season and planting area.

Lab scale experiment of sugar extraction

Sugar analysis

Using thermal extraction method as a pretreatment has been widely in recent studies due to its less harmful, less cost and less energy. Before sugar extraction of rain tree and golden rain tree pods, samples were dried at moisture content reached to a range of 8% to 15% and 4% to 10%, respectively. The pods of rain tree were conditioned under a certain temperature (30, 63 and 96 ℃) and time (20, 40 and 60 min) of extraction. The results from experiments in term of gram sugar per gram substrate were illustrated in Figure 20. The result of the analysis revealed that the rain tree pods contained about 3.64±0.005 to 5.04±0.011 g/g of total sugar and 0.61±0.006 to 1.10±0.005 g/g of reducing sugar at 30 to 96 ℃ and 20 to 60 min condition. The high sugar concentration can frond at 63 ℃ for 40 min condition and the thermal extraction efficiency compared with non-thermal showed that the thermal extraction results the total sugar increased by 22.9%. Sarrai et al., (2016) reported that the rain tree pods contained about 9.13% of sucrose, 11.3% of fructose, 11.2% of glucose, and 0% of maltose.

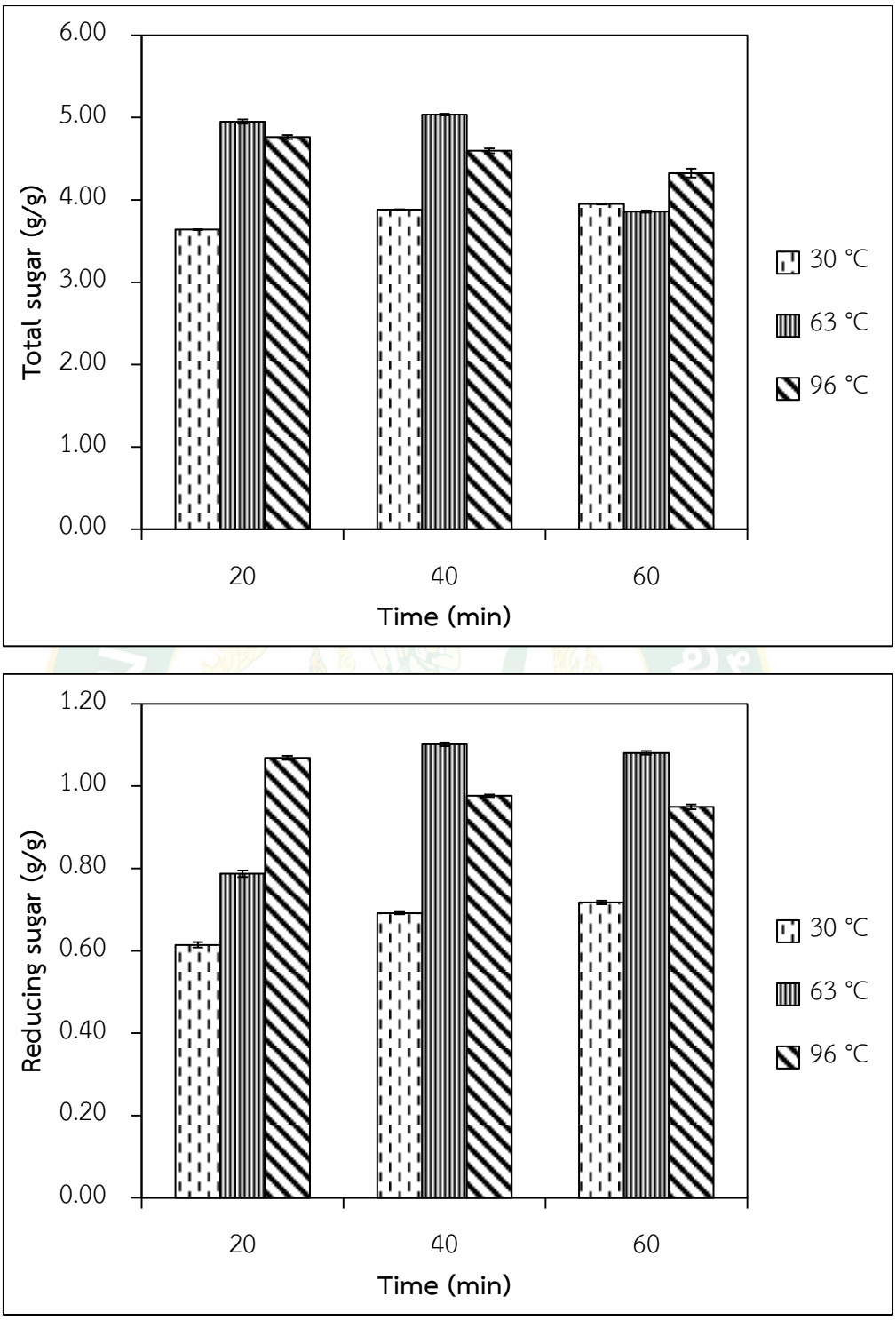


Figure 20 Sugar production from rain tree pods with thermal extraction method

Figure 21 shows the sugar production in term of gram sugar per gram substrate of the golden rain tree pods with temperature (30, 63 and 96 ℃) and time (20, 40 and 60) of extraction. At 30-96 ℃ and 20-60 min condition, the result of the analysis revealed that golden rain tree pods contained about 0.55±0.007 to 2.58±0.014 g/g of total sugar and 0.07±0.002 to 0.56±0.012 g/g of reducing sugar.

Moreover, the study of the sugar extraction at 96 ℃ was done with 20, 40, 60 and 80 minutes extraction time. The results from the experiments performed were shown in Figure 22. It was found out that the total sugar was increased to 3.71±0.060 g/g and the reducing sugar is reduced to 0.39±0.065 g/g. Therefore, the maximum sugar concentration of golden rain tree pods (2.58±0.014 g/g of total sugar and 0.56±0.012 g/g of reducing sugar) was found at 96 ℃ for 60 min of thermal extraction condition. The thermal extraction efficiency compared with non-thermal showed that the thermal extraction result the total sugar increased by 38.4%. Danish et al., (2011) investigated the contents of fruit pod and it was reported to have sucrose (31.3%), glucose (42.5%), fructose (26.2%), proteins and minerals.

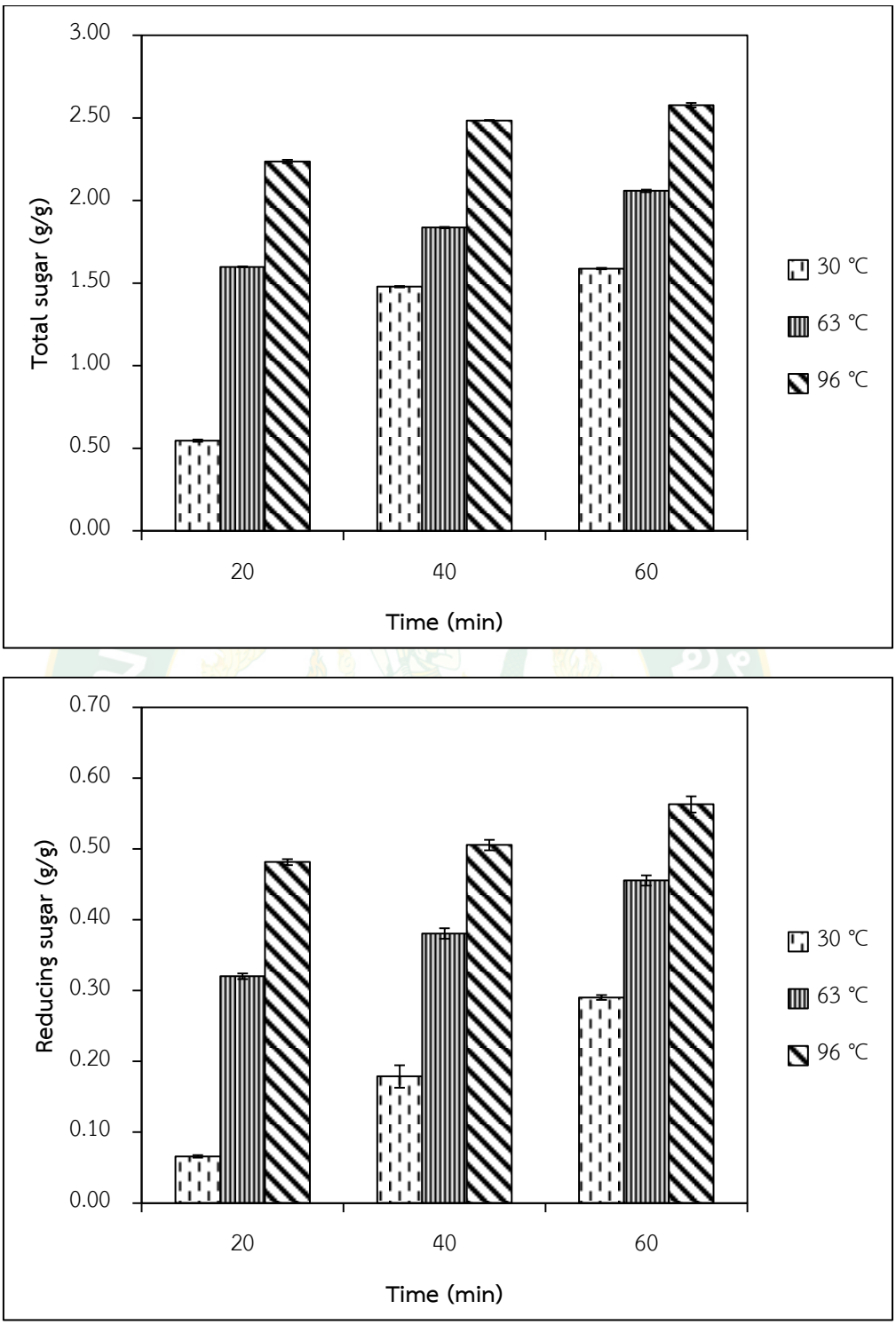


Figure 21 Sugar production from golden rain tree pods with thermal extraction method

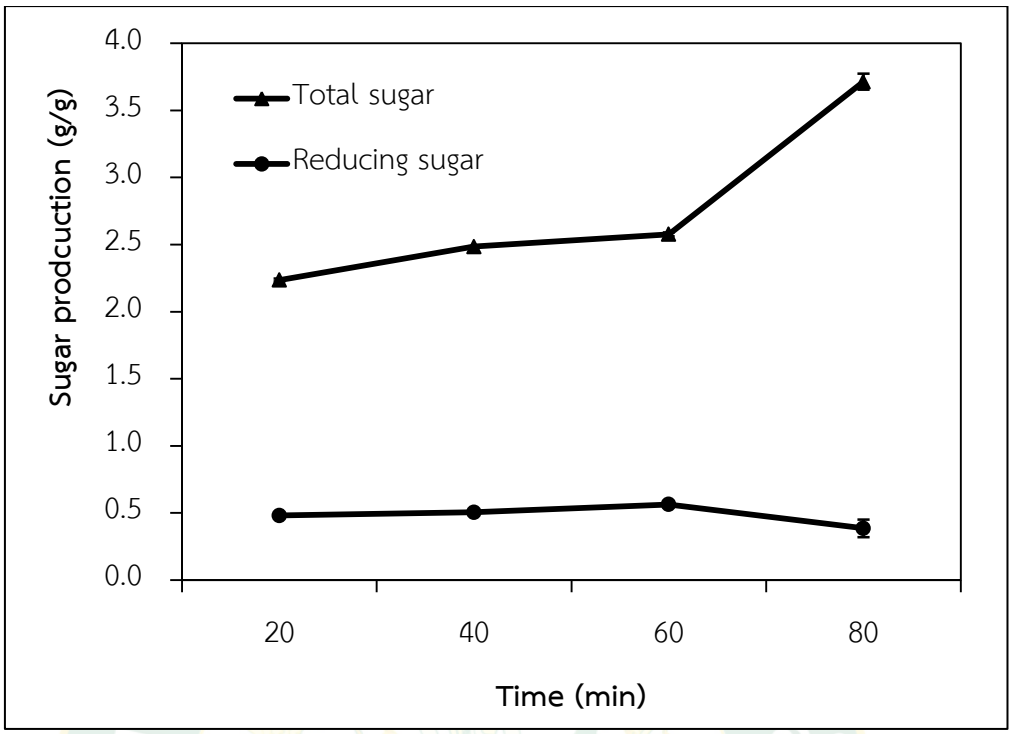


Figure 22 Sugar production from golden rain tree pods with thermal extraction method at 96 °C

Optimization of sugar content by Central composite design

The two materials, rain tree and golden rain tree pods were investigated as a promising feedstock for biobutanol production. To study the interaction effects of the thermal extraction method at each condition on total sugar and reducing sugar, CCD was applied to optimize the sugar production after thermal extraction process with two independent variables namely temperature and time. The results from sugar analysis in term of gram sugar per liter solution were used for RSM modeling.

The Design Expert software 11.1.0 (Stat-Ease Inc., Minneapolis, USA) was used to calculate the coefficients of the second-order fitting equation in order to predict the optimal condition divided into the liner, quadratic and interactive components as below. The following second-order quadratic model equation describing the influence of different considered variables on total sugar and reducing sugar responses after thermal extraction was obtained. The equation in terms of coded factors can be used to make predictions about the response for given levels of each

factor. By default, the high levels of the factors were coded as +1, and the low levels were coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

$$Y_1 = 478.63 + 35.64A - 54.63B - 18.69AB - 42.16A^2 - 25.70B^2 + 51.49A^2B + 1.86AB^2 \quad \text{Equation 7}$$

$$Y_2 = 102.55 + 14.25A + 14.65B - 5.55AB - 15.35A^2 - 5.35B^2 - 15.05A^2B + 2.90AB^2 \quad \text{Equation 8}$$

where Y_1 and Y_2 are total sugar (g/L) and reducing sugar (g/L) from rain tree pods, respectively; A and B are temperature (hour) and time (min) for thermal extraction method, respectively.

The complete design with experimental and predicted values of the total sugar and reducing sugar (g/L) from rain tree pods are presented in Table 5. The model suitability was tested using the analysis of variance (ANOVA) test. Results were assessed with various descriptive statistics such as the p-value, F-value, and the degree of freedom (df); the determination coefficient (R^2) of each coefficient was determined by Fisher's F-test and values of probability >F. The ANOVA analysis was conducted to determine the significance of model equation and model term of total sugar and reducing sugar from rain tree pods are given in Table 6 and Table 7.

As shown in Table 6, the Model F-value of 38.47 implied that the model is significant. There was 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, AB, A^2, B^2, A^2B were significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The Lack of Fit F-value of 669.07 implied that the Lack of Fit is significant. There was only a 0.01% chance that a Lack of Fit F-value this large could occur due to noise. Significant lack of fit is bad. The Predicted R^2 of 0.8802 was in reasonable agreement with the Adjusted R^2 of 0.9098; i.e. the

difference is less than 0.2. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 18.238 indicated an adequate signal. This model can be used to navigate the design space.

As shown in Table 7, the Model F-value of 54.97 implies the model is significant. There was 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, AB, A^2, B^2, A^2B were significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The Lack of Fit F-value of 1462.05 implied that the Lack of Fit is significant. There was only a 0.01% chance that a Lack of Fit F-value this large could occur due to noise. Significant lack of fit is bad. The Predicted R^2 of 0.9147 was in reasonable agreement with the Adjusted R^2 of 0.9356; i.e. the difference is less than 0.2. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 21.081 indicated an adequate signal. This model can be used to navigate the design space.

Furthermore, Figure 23 shows the very high correlation between the actual and predicted value which emphasizes the reliable of the model.

The design of the experiment in CCD was used to produce three-dimensional (3D) response surfaces and two-dimensional (2D) contour plots. Figure 24 and Figure 25 show the interaction effect of temperature and time concentration on total sugar and reducing sugar. As can be seen in the plots, there was an increase in total sugar and reducing sugar with an increase of temperature. On the other hand, the effect of time on total sugar and reducing sugar has a similar trend, regardless of the temperature. The total sugar was achieved from the regression equation (357.74 to 507.56 g/L) which is near to the experimental value (363.44 to 504.71 g/L). And the experimental value of reducing sugar ranged from 60.75 to 110.55 g/L and its corresponding predicted values are 59.55 and 111.85 g/L, respectively. The maximum of total sugar (504.71 g/L) and reducing sugar (110.55 g/L) from rain tree pods were obtained using thermal extraction at 63℃ for 40 min. On the other hand, the corresponding condition for minimum total sugar (363.44 g/L) and reducing sugar

(60.75 g/L) was temperature at 30°C for 20 min. The optimal condition of selected factors for the thermal extraction from rain tree pods was at 63°C for 40 min.

Table 5 Experimental design with actual and predicted value from rain tree pods

Run	A:Temperature (°C)	B:Time (min)	Total sugar (g/L)		Reducing sugar (g/L)	
			Actual	Predicted	Actual	Predicted
1	30	40	388.53	400.84	69.15	72.95
2	63	60	386.35	398.29	108.15	111.85
3	96	60	433.8	426.43	94.95	93.05
4	96	40	456.71	472.11	97.95	101.45
5	96	40	459.44	472.11	97.65	101.45
6	30	20	363.44	357.74	60.75	59.55
7	30	40	387.98	400.84	69.45	72.95
8	63	20	493.8	507.56	78.15	82.55
9	30	60	395.62	388.83	72.15	69.85
10	30	60	394.53	388.83	71.25	69.85
11	30	20	364.53	357.74	61.65	59.55
12	30	40	388.53	400.84	68.85	72.95
13	30	20	363.98	357.74	61.95	59.55
14	63	20	493.25	507.56	78.45	82.55
15	63	40	502.53	478.63	110.25	102.55
16	96	20	475.8	470.10	107.25	104.95
17	63	20	498.16	507.56	79.65	82.55
18	96	20	479.07	470.10	106.35	104.95
19	30	60	395.07	388.83	71.85	69.85
20	63	40	503.62	478.63	110.55	102.55
21	96	40	462.71	472.11	97.35	101.45
22	63	60	384.16	398.29	107.55	111.85
23	63	60	386.89	398.29	108.45	111.85
24	96	60	426.71	426.43	94.35	93.05

Run	A:Temperature (°C)	B:Time (min)	Total sugar (g/L)		Reducing sugar (g/L)	
			Actual	Predicted	Actual	Predicted
25	63	40	504.71	478.63	109.65	102.55
26	96	60	437.53	426.43	95.55	93.05
27	96	20	474.16	470.10	106.95	104.95

Table 6 ANOVA analysis for quadratic model of total sugar from rain tree pods

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	61333.53	7	8761.93	38.47	< 0.0001	significant
A-Temperature	7619.83	1	7619.83	33.45	< 0.0001	
B-Time	17909.90	1	17909.90	78.63	< 0.0001	
AB	4191.05	1	4191.05	18.40	0.0004	
A^2	10662.55	1	10662.55	46.81	< 0.0001	
B^2	3964.14	1	3964.14	17.40	0.0005	
A^2B	10605.57	1	10605.57	46.56	< 0.0001	
AB^2	13.76	1	13.76	0.0604	0.8085	
Residual	4327.88	19	227.78			
Lack of Fit	4214.50	1	4214.50	669.07	< 0.0001	significant
Pure Error	113.38	18	6.30			
Cor Total	65661.41	26				
Std. Dev.	15.09		**R^2**	0.9341		
Mean	433.39		**Adjusted R^2**	0.9098		
C.V. %	3.48		**Predicted R^2**	0.8802		
			Adeq Precision	18.2376		

$p < 0.05$ is considered as significant

Table 7 ANOVA analysis for quadratic model of reducing sugar from rain tree pods

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	7992.60	7	1141.80	54.97	< 0.0001	significant
A-Temperature	1218.38	1	1218.38	58.65	< 0.0001	
B-Time	1287.74	1	1287.74	61.99	< 0.0001	
AB	369.63	1	369.63	17.79	0.0005	
A^2	1413.74	1	1413.74	68.06	< 0.0001	
B^2	171.74	1	171.74	8.27	0.0097	
A^2B	906.01	1	906.01	43.62	< 0.0001	
AB^2	33.64	1	33.64	1.62	0.2185	
Residual	394.68	19	20.77			
Lack of Fit	389.88	1	389.88	1462.05	< 0.0001	significant
Pure Error	4.80	18	0.2667			
Cor Total	8387.28	26				
Std. Dev	4.56			**R^2**		0.9529
Mean	88.75			**Adjusted R^2**		0.9356
C.V. %	5.14			**Predicted R^2**		0.9147
				Adeq Precision		21.0811

$p < 0.05$ is considered as significant

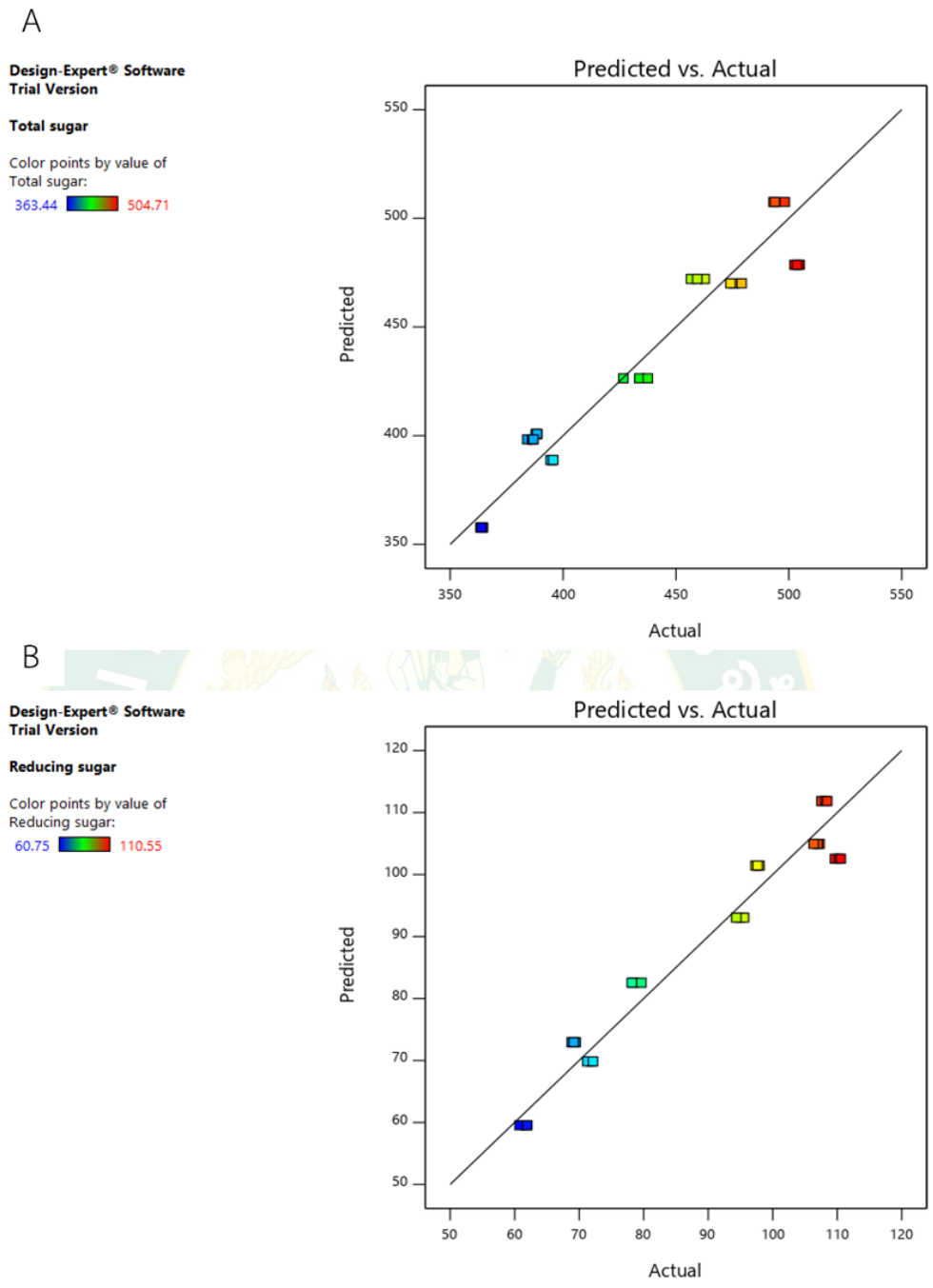


Figure 23 Comparison of predicted and actual value of (A) total sugar and (B) reducing sugar from rain tree pods

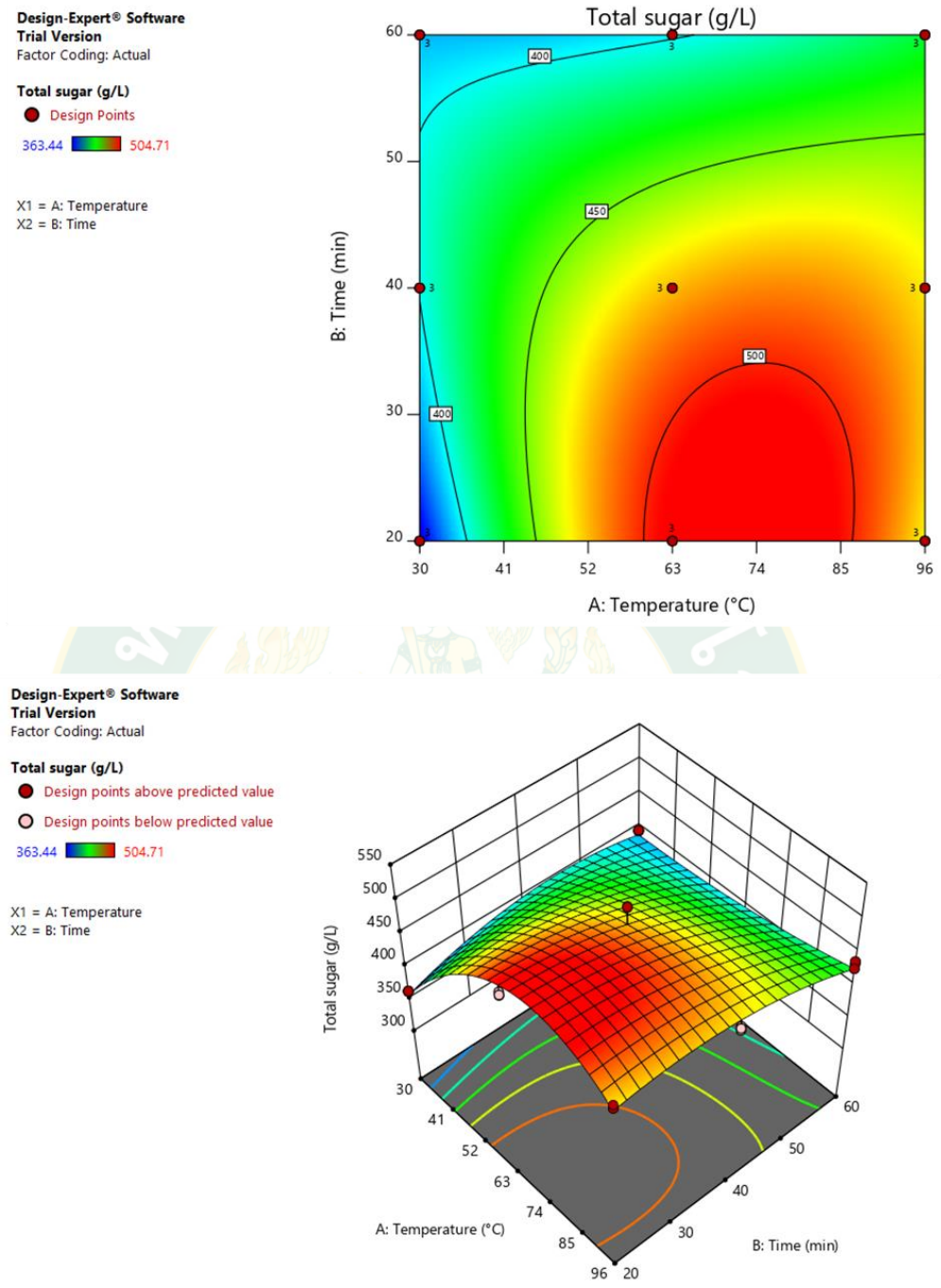


Figure 24 Effects of temperature and time on total sugar from rain tree pods

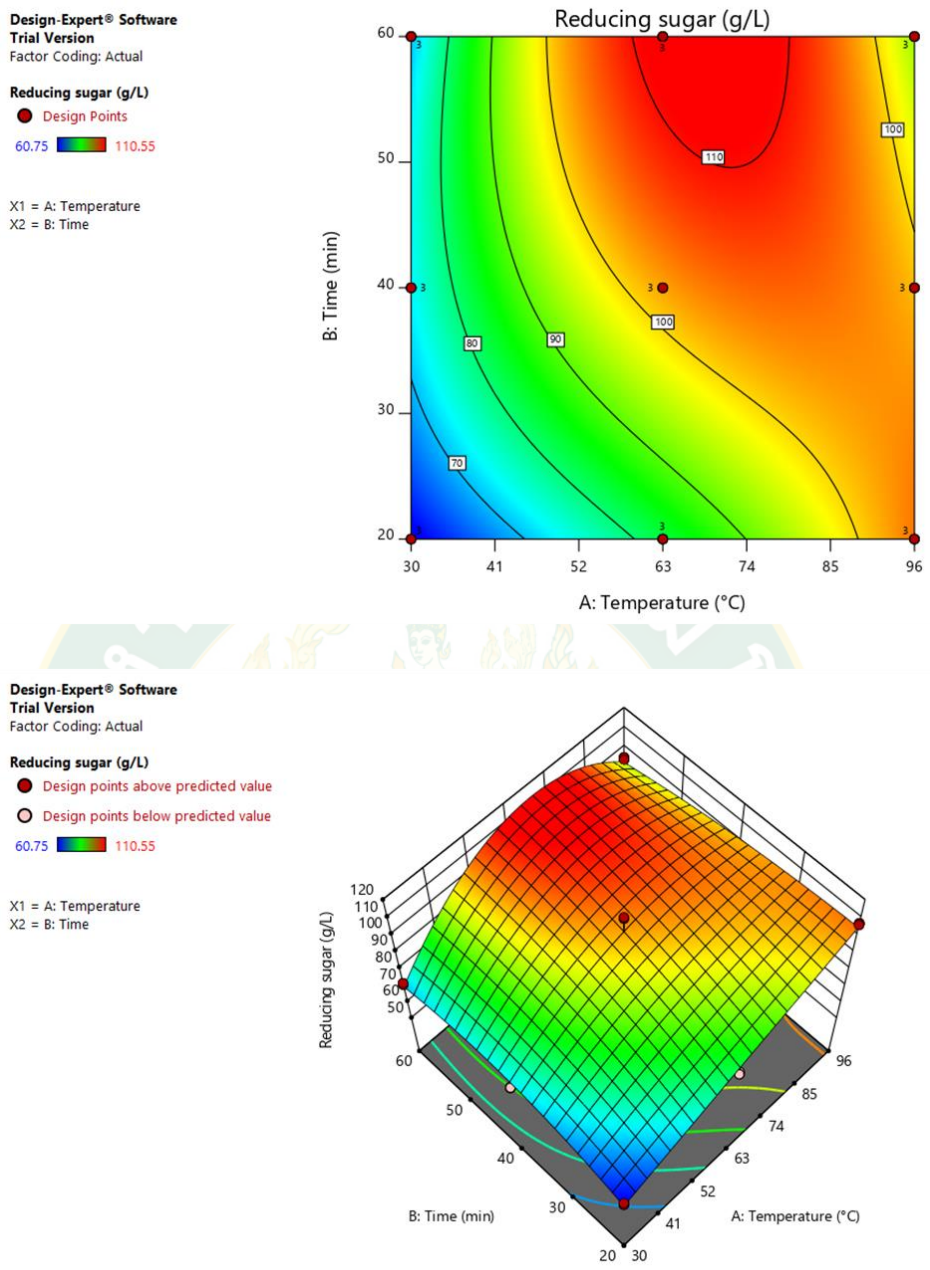


Figure 25 Effects of temperature and time on reducing sugar from rain tree pods

Based on CCD and experimental data, the following second order quadratic model equation described the influence of different considered variables on total sugar and reducing sugar from golden rain tree pods obtained:

$$Y_3 = 194.10 + 50.33A + 23.02B - 17.52AB - 1.24A^2 - 16.57B^2 + 11.53A^2B + 16.66AB^2 \quad \text{Equation 9}$$

$$Y_4 = 38.02 + 16.34A + 6.77B - 3.58AB - 3.80A^2 + 0.7750B^2 + 0.8800A^2B + 0.8700AB^2 \quad \text{Equation 10}$$

where Y_3 and Y_4 are total sugar (g/L) and reducing sugar (g/L) from golden rain tree pods, respectively; A and B were temperature (hour) and time (min) for thermal extraction method, respectively.

The ANOVA analysis of total sugar from golden rain tree pods is given in Table 9. The Model F-value of 329.86 implied that the model is significant. There was 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, AB, B^2, A^2B, AB^2 were significant model terms. Values higher than 0.1000 indicate the model terms are not significant. The Lack of Fit F-value of 1375.13 implied that the Lack of Fit is significant. There was only a 0.01% chance that a Lack of Fit F-value this large could occur due to noise. Significant lack of fit is terrible. The Predicted R^2 of 0.9852 was in reasonable agreement with the Adjusted R^2 of 0.9888; i.e., the difference is less than 0.2. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 59.141 indicated an adequate signal. This model can be used to navigate the design space.

Moreover, the ANOVA analysis of reducing sugar from golden rain tree pods is shown in Table 10. The Model F-value of 1458.72 implied 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, AB, A^2, B^2, A^2B, AB^2 were significant model terms. Values greater than 0.1000 indicate the

model terms are not significant. The Lack of Fit F-value of 0.01 implied that the Lack of Fit was not significant relative to the pure error. There was a 92.74% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good. The Predicted R^2 of 0.9962 was in reasonable agreement with the Adjusted R^2 of 0.9975; i.e., the difference is less than 0.2. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 115.578 indicated an adequate signal. This model can be used to navigate the design space.

This trend can be found on the interaction of temperature and time due to the very small value of coefficient (Figure 26). The design of the experiment in CCD was used to produce three-dimensional (3D) response surfaces and two-dimensional (2D) contour plots. Figure 27 and Figure 28 show the interaction effect of temperature and time concentration on total sugar and reducing sugar from golden rain tree pods. By comparing the experiments of total sugar and reducing sugar, it was increased from 53.9 to 258.82 g/L and 6.44 to 57.22g/L, respectively. The predicted value from the regression equation of total sugar (57.23 to 260.31 g/L) and reducing sugar (6.57 to 56.29 g/L) were found to be near to the experiment value. The optimal condition of selected factors for the thermal extraction from golden rain tree pods was at 96℃ for 60 min.

Table 8 Experimental design with actual and predicted value from golden rain tree pods

Run	A:Temperature (°C)	B:Time (min)	Total sugar (g/L)		Reducing sugar (g/L)	
			Actual	Predicted	Actual	Predicted
1	30	20	55.32	57.23	6.44	6.57
2	63	60	205.15	200.55	44.72	45.57
3	30	20	53.9	57.23	6.5	6.57
4	30	60	159.09	161.38	29.33	29.01
5	96	60	256.13	260.31	57.22	56.29
6	96	40	248.28	243.19	50	50.57
7	63	20	159.53	154.50	31.94	32.03

Run	A:Temperature (°C)	B:Time (min)	Total sugar (g/L)		Reducing sugar (g/L)	
			Actual	Predicted	Actual	Predicted
8	96	20	223.28	226.24	48.06	48.14
9	63	40	184.07	194.10	38.33	38.02
10	30	40	148.28	142.53	16.11	17.88
11	96	20	224.75	226.24	48.61	48.14
12	96	60	258.09	260.31	56.67	56.29
13	63	20	160.02	154.50	32.44	32.03
14	63	40	183.58	194.10	38.61	38.02
15	96	60	258.82	260.31	55	56.29
16	30	60	158.95	161.38	29.06	29.01
17	96	20	222.79	226.24	47.78	48.14
18	63	20	159.73	154.50	31.67	32.03
19	30	60	158.21	161.38	28.67	29.01
20	63	40	183.09	194.10	37.22	38.02
21	96	40	248.77	243.19	51.39	50.57
22	30	20	54.58	57.23	6.78	6.57
23	96	40	248.28	243.19	50.28	50.57
24	63	60	206.62	200.55	45.83	45.57
25	63	60	205.64	200.55	46.11	45.57
26	30	40	147.79	142.53	19.17	17.88
27	30	40	147.3	142.53	18.33	17.88

Table 9 ANOVA analysis for quadratic model of total sugar from golden rain tree pods

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	91888.27	7	13126.90	329.86	< 0.0001	significant
A-Temperature	15196.64	1	15196.64	381.87	< 0.0001	
B-Time	3179.98	1	3179.98	79.91	< 0.0001	
AB	3683.05	1	3683.05	92.55	< 0.0001	
A^2	9.17	1	9.17	0.2304	0.6367	
B^2	1647.61	1	1647.61	41.40	< 0.0001	
A^2B	532.15	1	532.15	13.37	0.0017	
AB^2	1109.89	1	1109.89	27.89	< 0.0001	
Residual	756.11	19	39.80			
Lack of Fit	746.34	1	746.34	1375.13	< 0.0001	significant
Pure Error	9.77	18	0.5427			
Cor Total	92644.38	26				
Std. Dev.	6.31		**R^2**	0.9918		
Mean	182.22		**Adjusted R^2**	0.9888		
C.V. %	3.46		**Predicted R^2**	0.9852		
			Adeq Precision	59.1409		

$p < 0.05$ is considered as significant

Table 10 ANOVA analysis for quadratic model of reducing sugar from golden rain tree pods

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	6378.50	7	911.21	1458.72	< 0.0001	significant
A-Temperature	1602.63	1	1602.63	2565.58	< 0.0001	
B-Time	274.86	1	274.86	440.02	< 0.0001	
AB	153.37	1	153.37	245.52	< 0.0001	
A^2	86.49	1	86.49	138.46	< 0.0001	
B^2	3.60	1	3.60	5.77	0.0267	
A^2B	3.10	1	3.10	4.96	0.0382	
AB^2	3.03	1	3.03	4.85	0.0403	
Residual	11.87	19	0.6247			
Lack of Fit	0.0056	1	0.0056	0.0085	0.9274	not significant
Pure Error	11.86	18	0.6591			
Cor Total	6390.37	26				
Std. Dev.	0.7904		**R^2**	0.9981		
Mean	36.01		**Adjusted R^2**	0.9975		
C.V. %	2.19		**Predicted R^2**	0.9962		
			Adeq Precision	115.5776		

$p < 0.05$ is considered as significant

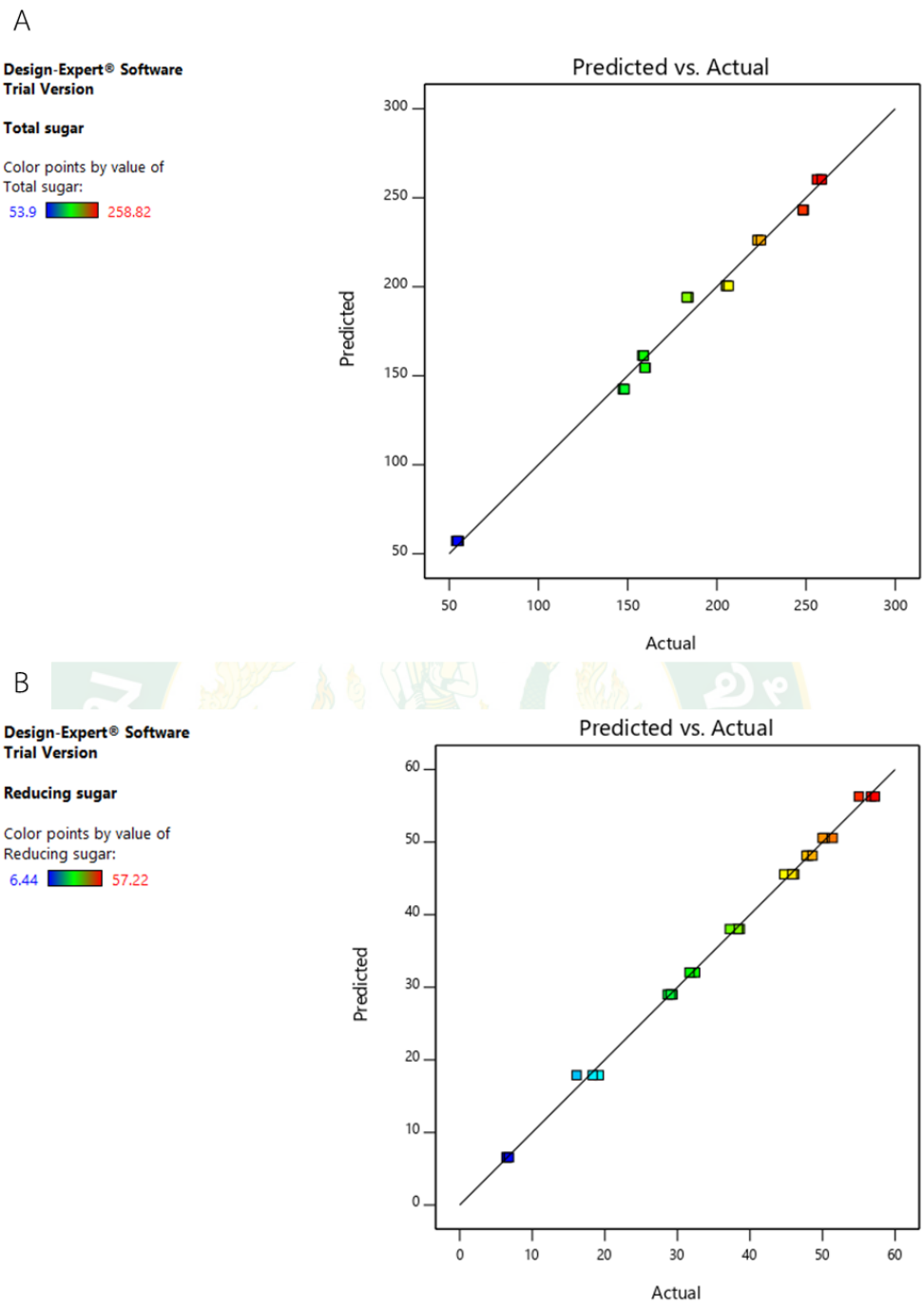
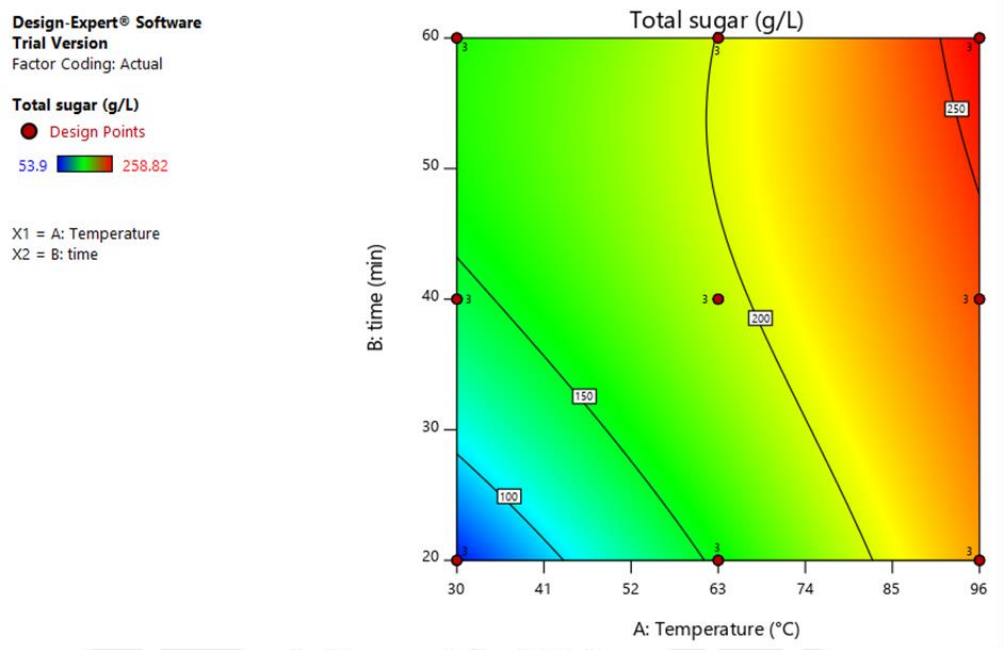


Figure 26 Comparison of predicted and actual value of (A) total sugar and (B) reducing sugar from golden rain tree pods



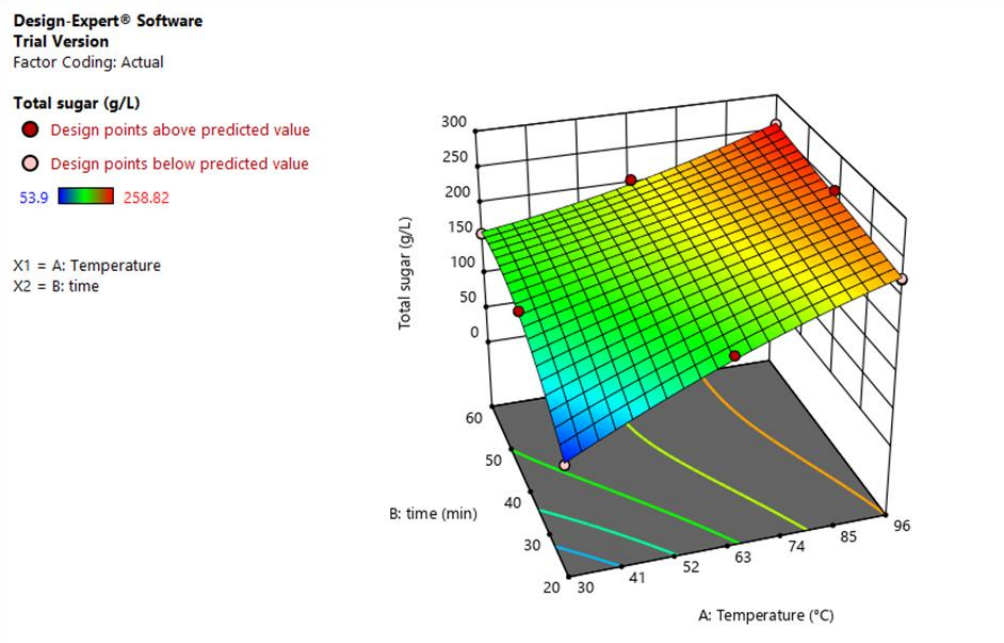


Figure 27 Effects of temperature and time on total sugar from golden rain tree pods

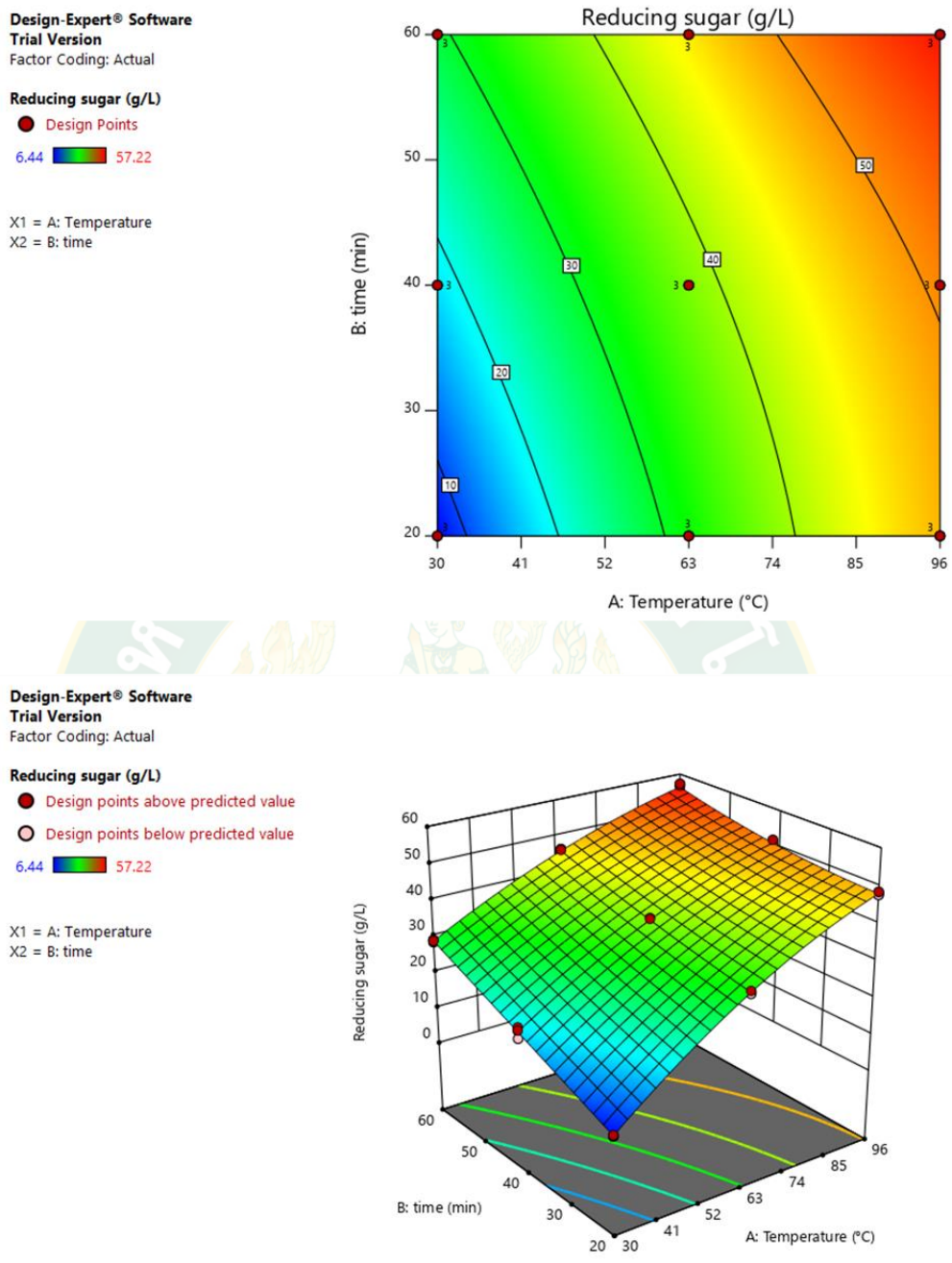


Figure 28 Effects of temperature and time on reducing sugar from golden rain tree pods

Similar extraction method has been used for sugar extraction from sweet sorghum (Jia et al., 2013), cashew apple bagasse (Kuila et al., 2011), sugar beets (López et al., 2009), and carob (Carlos Roseiro et al., 1991). All the method of extraction has a perspective to release sugars for biofuel production. Extraction of sugar from each biomass source requires unique operating conditions developed based on sugar and water content, fiber structure and composition, and geometric size (Jia et al., 2013). Based on the above information, it showed that the thermal extraction method of rain tree and golden rain tree pods is more effective in obtaining the sugars, which could be the source of useful biofuel products.

Enzymatic hydrolysis

Hydrolysis process is used to decrease the degree of polymerization (DP) of cellulose by hydrolysis of large polysaccharides to fermentable sugar. The optimal conditions from CCD were used for enzymatic hydrolysis. Rain tree and golden rain tree pods as thermal extraction at 63 ℃ for 40 min and 96 ℃ for 60 min (with solid fraction) were carried out enzymatic hydrolysis process for 24 hours. The results of sugar concentration without enzymatic hydrolysis (control) and enzymatic hydrolysis are shown in Table 11.

Table 11 Sugar yield after enzymatic hydrolysis

Feedstocks	Total sugar (g/g)	Reducing sugar (g/g)	DP
Rain tree pods			
Control	5.07±0.03	1.12±0.02	4.5
Enzymatic hydrolysis	5.55±0.01	2.81±0.01	2.0
Golden rain tree pods			
Control	2.72±0.02	0.58±0.02	4.7
Enzymatic hydrolysis	3.51±0.02	1.44±0.02	2.4

Note: DP means the degree of polymerization

After 24 hours of enzymatic hydrolysis, reducing sugar produced was 2.81±0.01 and 1.44±0.02 g/g of rain tree and golden rain tree pods, respectively. While the yield from thermal extraction was only 1.12±0.02 and 0.58±0.02 g/g of rain tree and golden rain tree pods, respectively. The degree of polymerization present the number of monomer of sugar presents in solution. In other words, the reduction of DP showed very clear evidence of enzyme activities on breaking down the big sugar chains into smaller chains. Enzymatic hydrolysis of pretreated rain tree and golden rain tree pods resulted in the highest efficiency enzyme of 51% and 41%, respectively.

Microorganism culturing *Clostridium acetobutylicum* TISTR 2375

Before using the spore suspension of *C. acetobutylicum* TISTR 2375 in 20% glycerol were transferred into RCM medium for spore activation in an incubator at 35 °C for 24 hours. Figure 29 illustrates the spore suspension after activated in RCM medium. It was seen as a bacterial colony. Then, 10% (v/v) of the activated spore suspensions were transferred into RCM medium and incubated at 35 °C for 24 hours to obtain highly motile vegetative cells. Vegetative cells (10%, v/v) with an optical density at 660 nm (OD660) of 1.5～2 were used as inocula for biobutanol production. Before transferring the activated spore suspensions into RCM medium, the medium has a clear yellow color. After being incubated, it was found that the medium looked more turbid due to the growth of bacteria.

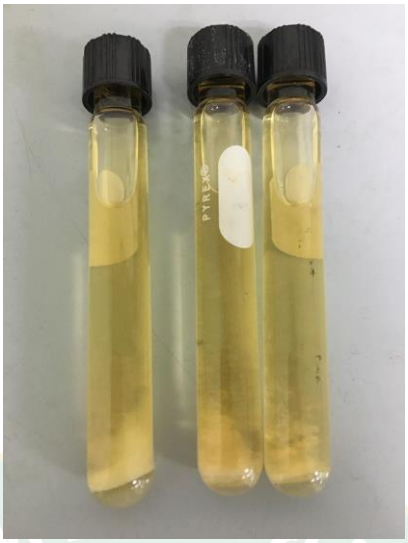


Figure 29 Observed turbidity occurring in RCM medium after activated 24 hour

Batch fermentation

In general, the production process of ABE from the *Clostridium* bacterial consists of two steps: the first step was the production of acid (Acidogenesis) and the second step was the production of solvents (Solventogenesis). For the first step, the bacteria were used sugar to create cells and produced a metabolic acid, which are a butyric acid, acetic acid, and ethanol. This period resulted in the pH of the fermentation broth is decrease. Bacteria grow in multiples of the phase (Log phase). In the second step is changing the butyric acid and acetic acid into butanol and acetone to reduce the toxicity of butyric acid and acetic acid to bacteria cells. This period resulted in the pH of the fermentation broth is slightly decrease. Bacteria are growing at a constant amount of cells (Stationary phase). ABE production mechanism is shown in Figure 30 (Batstone et al., 2002).

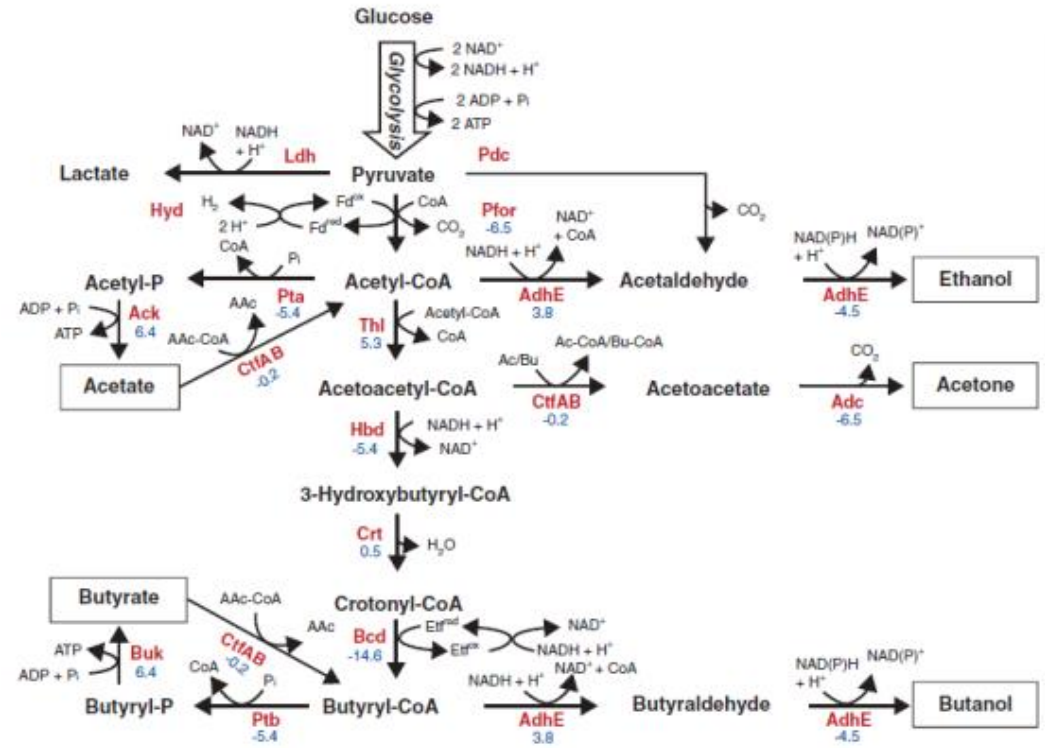


Figure 30 The mechanism for ABE production

(Batstone et al., 2002)

Potential biobutanal production from *C. acetobutylicum* TISTR 2375

ABE production using glucose at initial concentration is 60 g/L, shown in Figure 31. The results showed that within 120 hours of fermentation found that glucose decreased by 62.5%. Therefore, it was concluded that glucose is reducing sugar that bacteria are easily used. In addition, at 24 hours of fermentation, butanol was detected in a small amount of fermentation broth (0.178 g/L). Within 48 to 120 hours of fermentation, butanol concentration is increasing and the maximum in 120 hours of fermentation is 0.274 g/L. It was found that the sugar content reduced to 37.5 g/L. Figure 32 presents a typical chromatogram for the ABE from P2 medium within 120 hours. The pH of the fermented broth decreased from 6.50 to 4.93 in 24 hours corresponding to acid production, and the pH slightly fluctuated then at 36 hours until the end of fermentation (Figure 33).

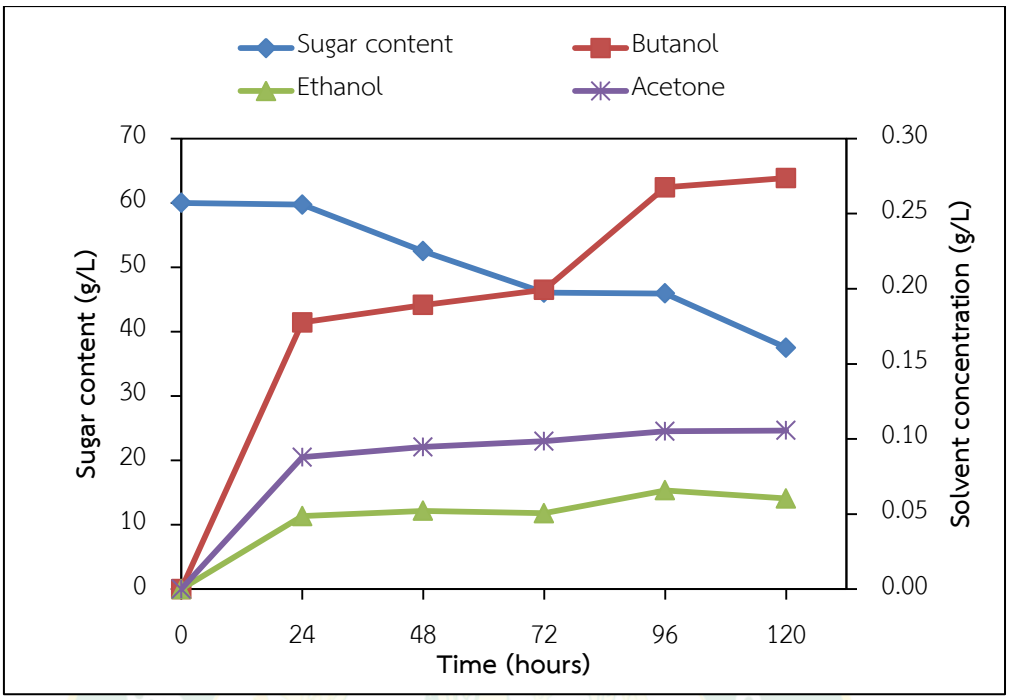


Figure 31 Butanol production using P2 medium as substrate

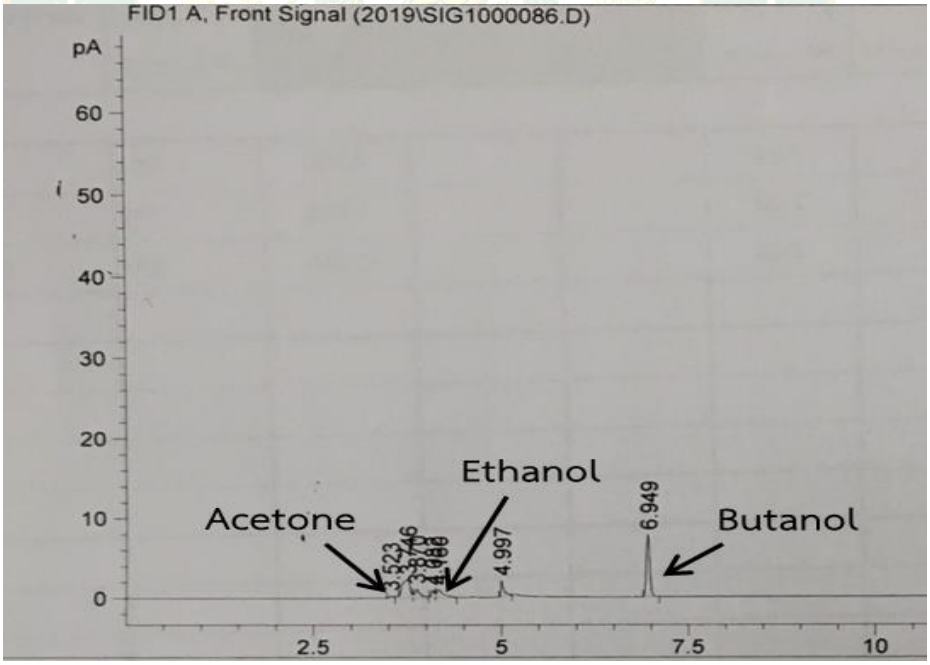


Figure 32 Chromatography of acetone butanol and ethanol from P2 medium within 120 hours

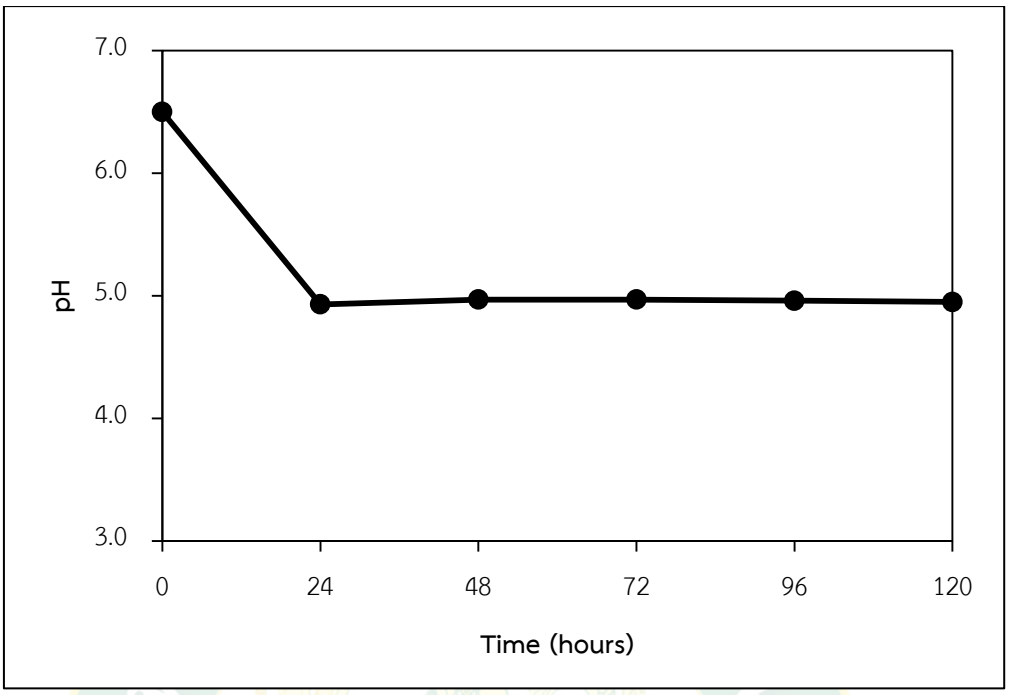


Figure 33 The pH of batch butanol fermentation from P2 medium within 120 hours

Biobutanol production from rain tree and golden rain tree pods

Batch fermentation was carried out in 100 ml bottle. In this section, the ABE fermentation from rain tree pods juice as a carbon source by *C. acetobutylicum* TISTR 2375 to convert to butanol. The initial sugar concentration was 556.42 g/L of total sugar and 281.43 g/L of reducing sugar. Biobutanol concentration within 24, 48, 72, 96 and 120 hours were recorded in the range of 0.0457 to 0.0641 g/L (Figure 34). The maximum butanol concentrations obtained was 0.0641 g/L within 24 hours of fermentation and slightly fluctuated until 96 hours. Reducing sugar during the fermentation was estimated in the meantime to observe the sugar consumption of *C. acetobutylicum* TISTR 2375. It was observed by the amount of reducing sugar decreased after 24 hours. The pH of the fermented broth decreased from 6.50 to 4.68 in 24 hours corresponding to acid production, and the pH slightly fluctuated then at 36 hours until the end of fermentation (Figure 38). Figure 35 presents a typical chromatogram for the ABE from rain tree pods within 24 hours.

With regards to golden rain tree pods, the initial sugar concentrations was 340.00 g/L of total sugar and 140.00 g/L of reducing sugar. Biobutanol concentration

within 24, 48, 72, 96 and 120 hours were recorded in the range of 0.0632 to 0.0645 g/L (Figure 36). The maximum butanol concentration obtained was 0.0645 g/L within 72 hours of fermentation and slightly fluctuated until the end of fermentation. The pH of the fermented broth decreased from 6.50 to 5.08 in 24 hours corresponding to acid production, and the pH slightly fluctuated then at 36 hours until the end of fermentation (Figure 38). Figure 37 presents a typical chromatogram for the ABE from golden rain tree pods within 72 hours.

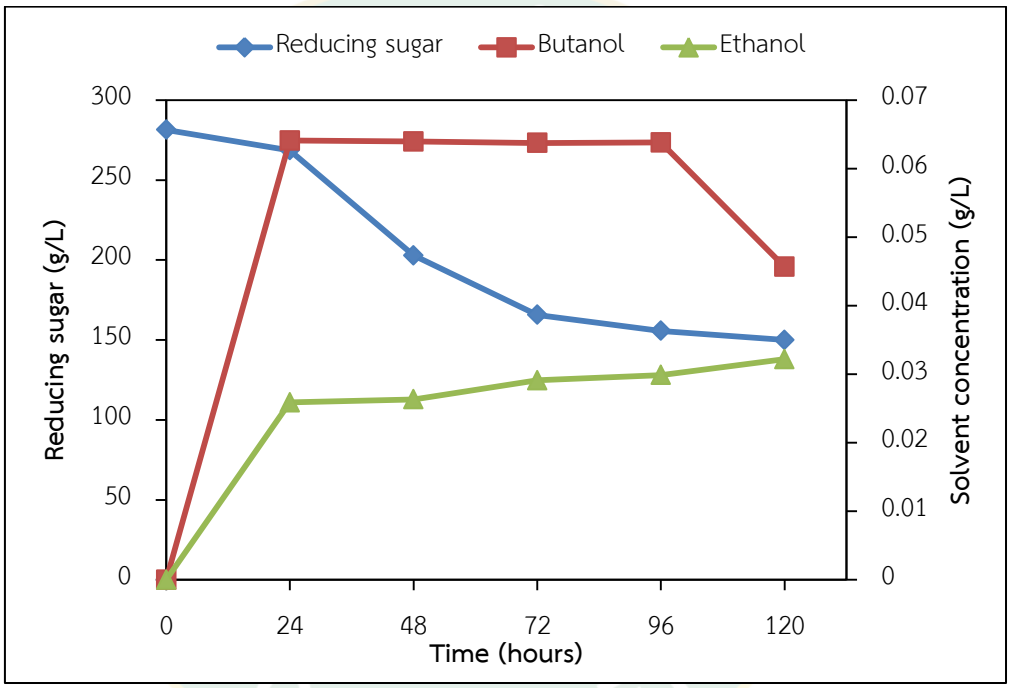


Figure 34 Batch butanol fermentation from rain tree pods in 50 ml fermenter

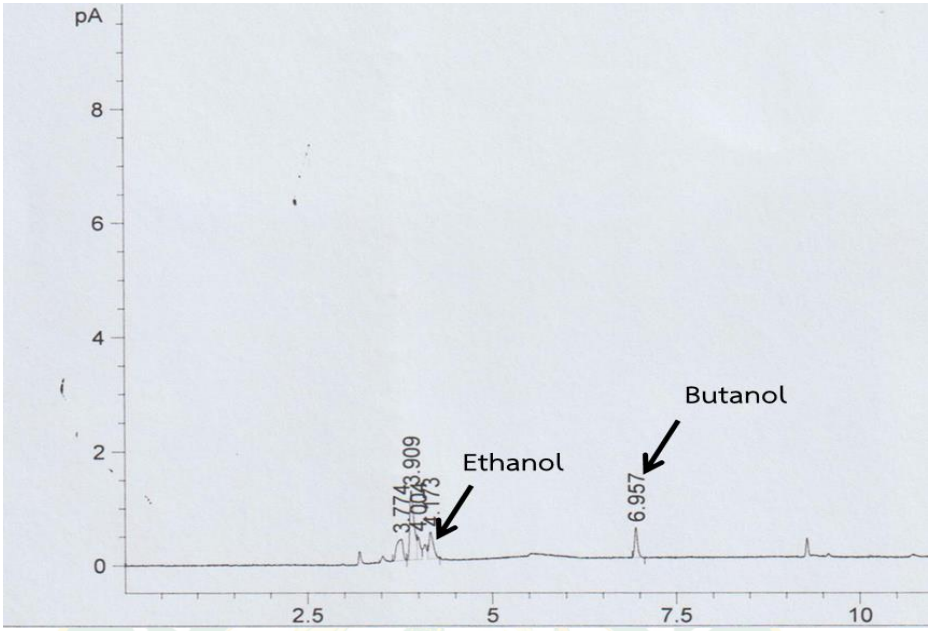


Figure 35 Chromatography of butanol and ethanol from rain tree pods within 24 hours

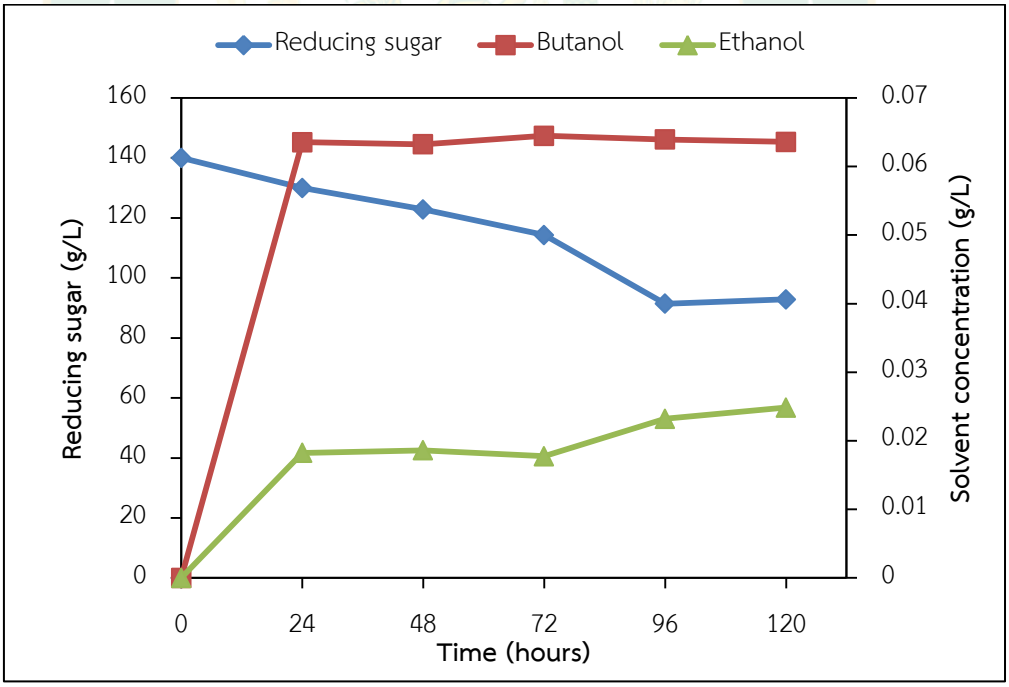


Figure 36 Batch butanol fermentation from golden rain tree pods in 50 ml fermenter

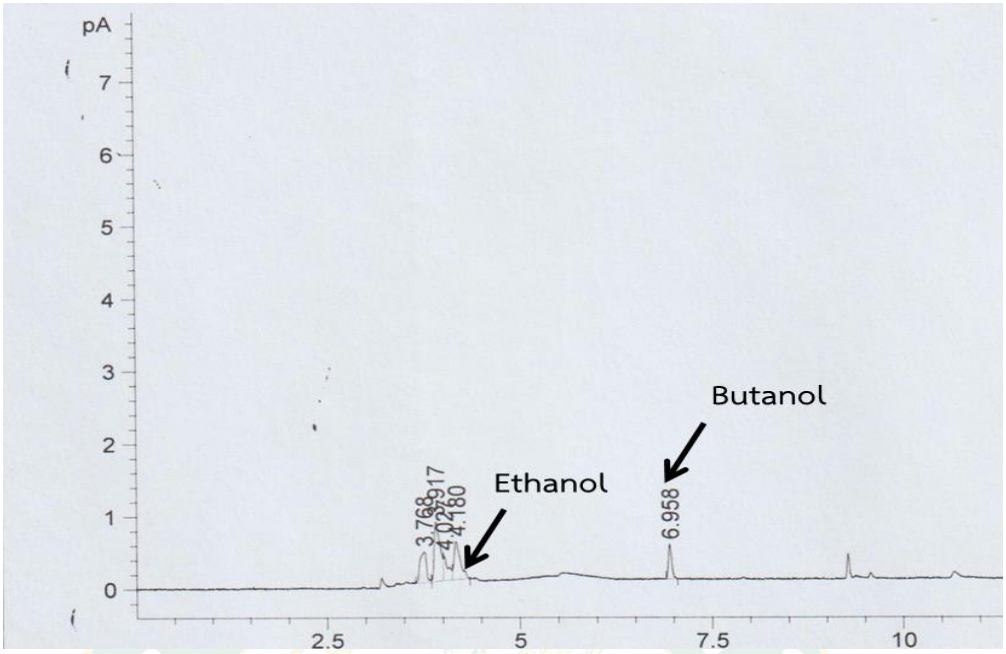


Figure 37 Chromatography of butanol and ethanol from golden rain tree pods within 72 hours

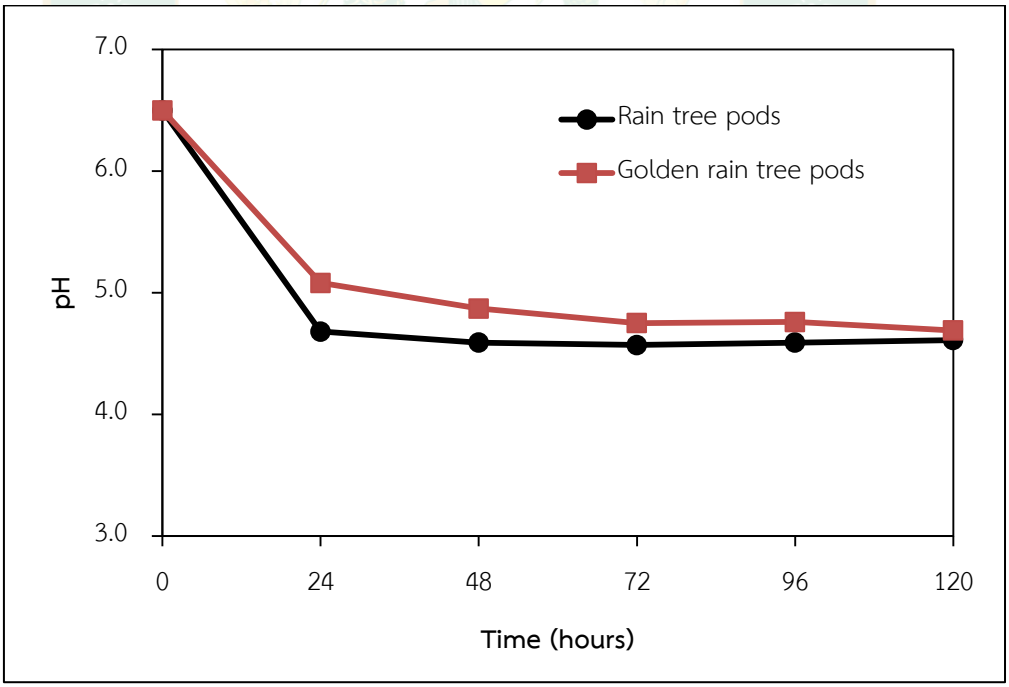


Figure 38 The pH of batch butanol fermentation in 50 ml fermenter

Table 12 indicated the ABE fermentation using various fruit residues as a substrate. This study was produced a small amount of butanol compared to other studies. It was reported that the rain tree and golden rain tree pods juice contained a supernumerary amount of sugars. (Ezeji et al., 2004) stated that the concentration of fermentation medium components including carbon source significantly affects the biobutanol fermentation. The amount of sugar supplied for *Clostridia* in the batch system should not be over 160 g/L. After 48 h of fermentation with the initial sugar concentration lower than 40 g/L could yield more acids than solvents and inhibited the growth of the cells (Ibrahim et al., 2015). The initial sugar concentration supply should not be over 100 g/L to prevent substrate inhibition of the growth of the cell (Maddox et al., 2000). Besides, highly inhibitory compounds that are usually present in the biomass hydrolysate inhibit the growth of cells during the early stage, which then limits the fermentation with high substrate concentration (Ibrahim et al., 2018). However, optimization in terms of sugar dilution for butanol production by *C. acetobutylicum* TISTR 2375 from rain tree and golden rain tree pods was examined in the next section to incused the biobutanol yield of addition.

Table 12 List of selected feedstocks used from fruit residues for ABE production by using *Clostridia*

Fruit residues	Pretreatment/ Hydrolysis	Substrate concentration	Microorganism	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	ABE (g/L)	$Y_{Butanol}$ (g/g)	Ref.
Date fruit	N/A	30 g/L date fruit	*C. acetobutylicum* NCIMB 13357	1.1	3.1	0.1	4.3	0.32	(Khamaiseh et al., 2013)
Mango peel waste	Mechanical and enzyme hydrolysis	30% (w/w) reducing sugars	*C. acetobutylicum* NCIM 2878	N/A	10.5	N/A	15.13	N/A	(Avula et al., 2015)
Sugarcane juice		90 g/L reducing sugar	*C. acetobutylicum ATCC824*	6.1	15.9	1.9	23.9	0.18	(Kittithanesuan and Phisalaphong, 2015)
Acorn	Milling	20 g/L treated acorn starch	*C. acetobutylicum* NRRL B-591	N/A	N/A	N/A	5.81	N/A	(Heidari et al., 2016)
Apple pomace	Steam explosion and enzyme hydrolysis	42.0 g/L total sugar	*C. beijerinckii* CECT 508	3.55	9.11	0.26	12.92	0.276	(Hijosa-Valsero et al., 2017)
Pineapple waste	Acid hydrolysis and detoxification using activated carbon	60.66 g/L	*C. acetobutylicum* B 527	N/A	N/A	N/A	5.23	N/A	(Khedkar et al., 2017)
Sago waste	Acid hydrolysis and enzyme hydrolysis	-	*C. bifermentans* (SBI 4) and *Bacillus coagulans*	N/A	3.36	N/A	9.01	N/A	(Johnravindar et al., 2017)

Fruit residues	Pretreatment/ Hydrolysis	Substrate concentration	Microorganism	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	ABE (g/L)	$Y_{Butanol}$ (g/g)	Ref.
Pea pod waste	Acid hydrolysis and detoxification	60.14 g/L	*C. acetobutylicum* NRRL B-527	N/A	3.82	N/A	5.94	0.20	(Nimbalkar et al., 2018)
Pineapple waste juice	N/A	39.11 g/L reducing sugar	*C. beijerinckii* TISTR 1461	1.03	3.14	N/A	4.17 (A + B)	0.08	(Sanguanchaipaiwong and Leksawasdi, 2018)
Rain tree pods	Thermal extraction method and enzyme hydrolysis	268.57 g/L reducing sugar	*C. acetobutylicum* TISTR 2375	N/A	0.0641	0.0259	0.09 (A + B)	0.0002	This study
Golden rain tree pods	Thermal extraction method and enzyme hydrolysis	114.29 g/L reducing sugar	*C. acetobutylicum* TISTR 2375	N/A	0.0645	0.0177	0.0822 (A + B)	0.0006	This study

Note: *A + B means the results are calculated based on acetone and butanol.*

Dilute juice of rain tree and golden rain tree pods for biobutanol production

For this study, the medium that contained sugar from thermal extraction through enzymatic hydrolysis of rain tree (temperature of 63 °C and time of 40 min) and golden rain tree pods (temperature of 96 °C and time of 60 min) diluting approximately 80 g/L of reducing sugar was chosen. Figure 39 shows the results of butanol fermentation of the rain tree pods prehydrolysate juice using *C. acetobutylicum* TISTR 2375. At the beginning of the fermentation, 79.17 g/L of reducing sugar was present in rain tree pods prehydrolysate juice. Biobutanol concentration within 24, 48, 72, 96 and 120 hours were recorded in the range of 0.063 to 1.172 g/L. Within 24 hours of fermentation, there is about 0.063 g/L of butanol production. However, ethanol and acetone were observed to be very low (< 0.9 g/L). The culture produced 0.899 g/L acetone, 1.172 g/L butanol, and 0.077 g/L ethanol in 120 hours. It was found that reducing sugar reduced to 10 g/L. The pH of the fermented broth decreased from 6.50 to 4.40 in 48 hours corresponding to acid production, and the pH slightly fluctuated then at 72 hours until the end of fermentation (Figure 43). Figure 40 presents a typical chromatogram for the ABE from rain tree pods within 120 hours.

In the light of golden rain tree pods, the initial sugar concentration was 80.00 g/L of reducing sugar. Biobutanol concentration within 24, 48, 72, 96 and 120 hours were recorded in the range of 0.0624 to 0.0628 g/L (Figure 41). The maximum butanol concentration obtained was 0.0628 g/L within 120 hours of fermentation and slightly fluctuated until the end of fermentation. This result showed that golden rain pods produced little butanol compared to rain tree pods and slightly reduced fermented sugar (57.50 g/L reducing sugar within 120 hours). The pH of the fermented broth decreased from 6.50 to 6.01 in 24 hours, and the pH slightly fluctuated then at 36 hours until the end of fermentation (Figure 43). Therefore, rain pods contain inhibitors for high cell growth, resulting in low production of butanol. Figure 42 presents a typical chromatogram for the ABE from golden rain tree pods within 120 hours.

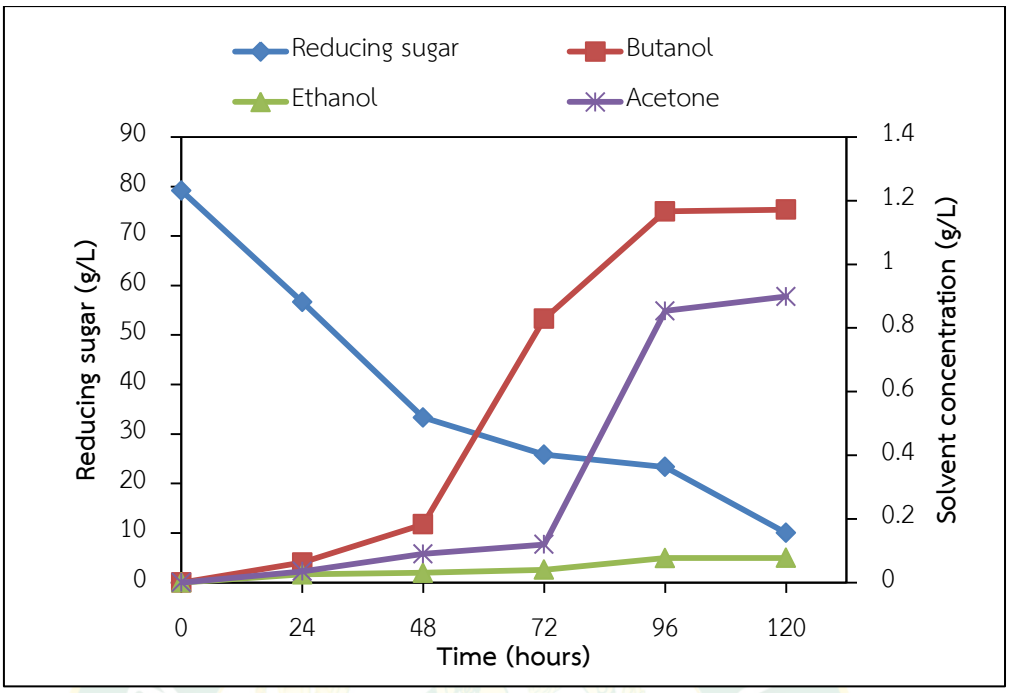


Figure 39 Biobutanol production using dilute juice of rain tree pods as substrate

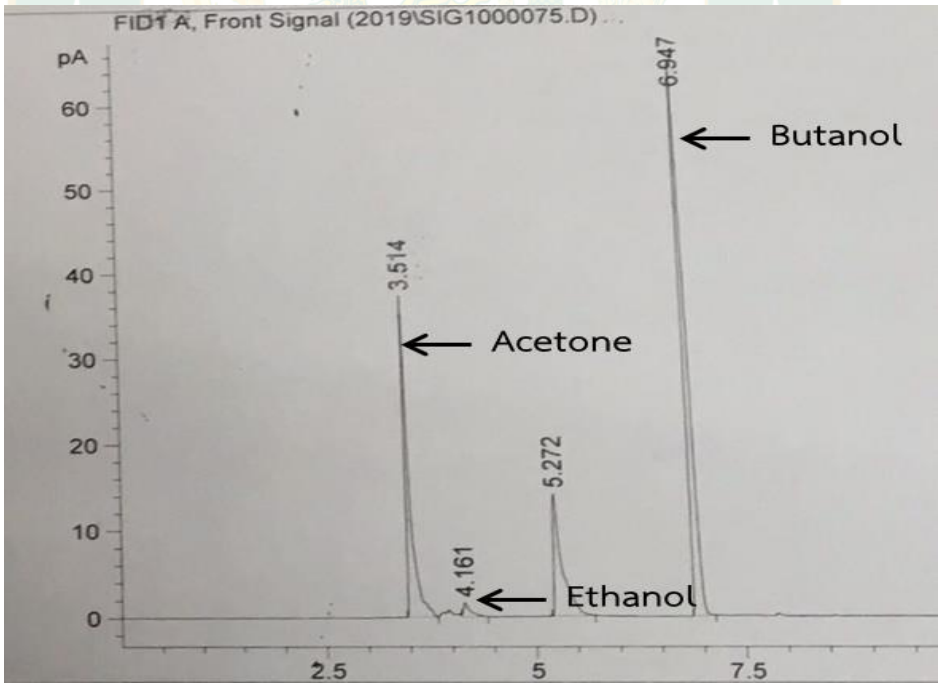


Figure 40 Chromatography of acetone-butanol-ethanol from dilute juice of rain tree pods within 120 hours

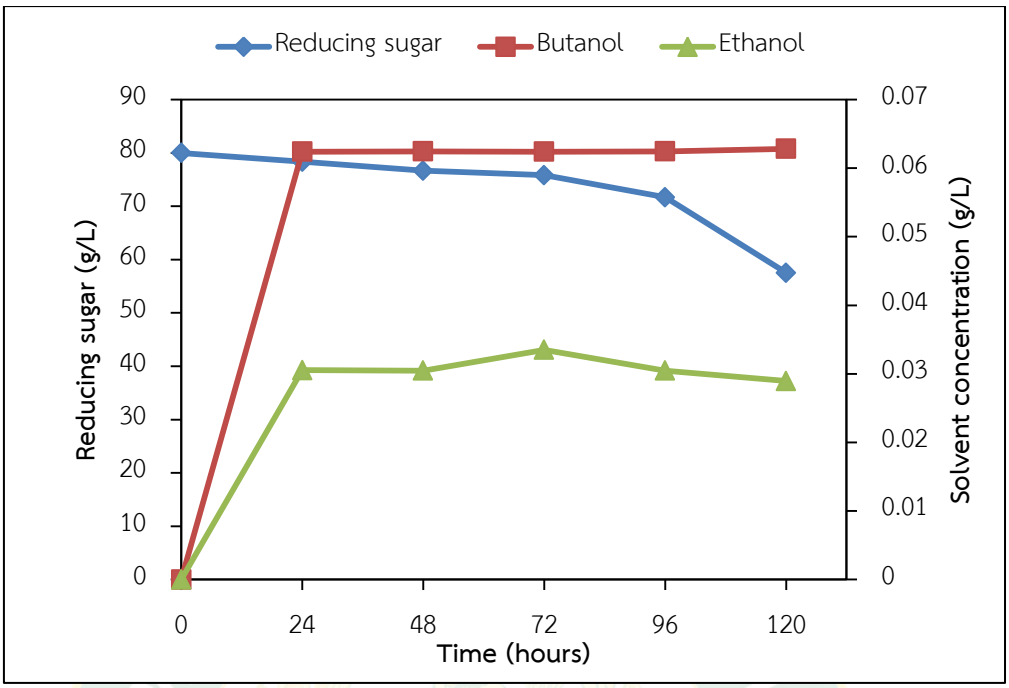


Figure 41 Biobutanol production using dilute juice of golden rain tree pods as substrate

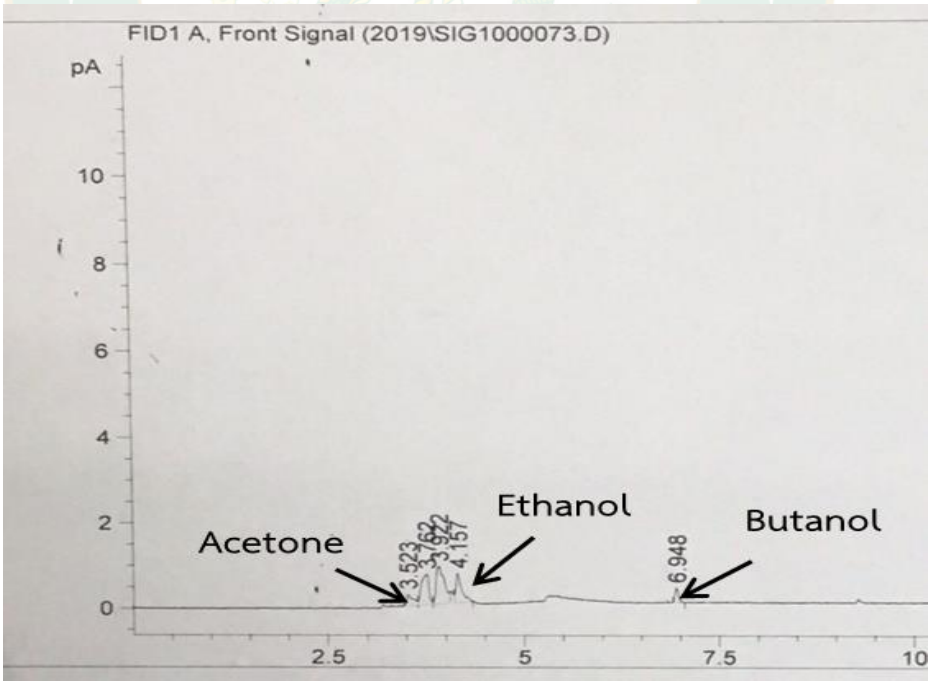


Figure 42 Chromatography of acetone-butanol-ethanol from dilute juice of golden rain tree pods within 120 hours

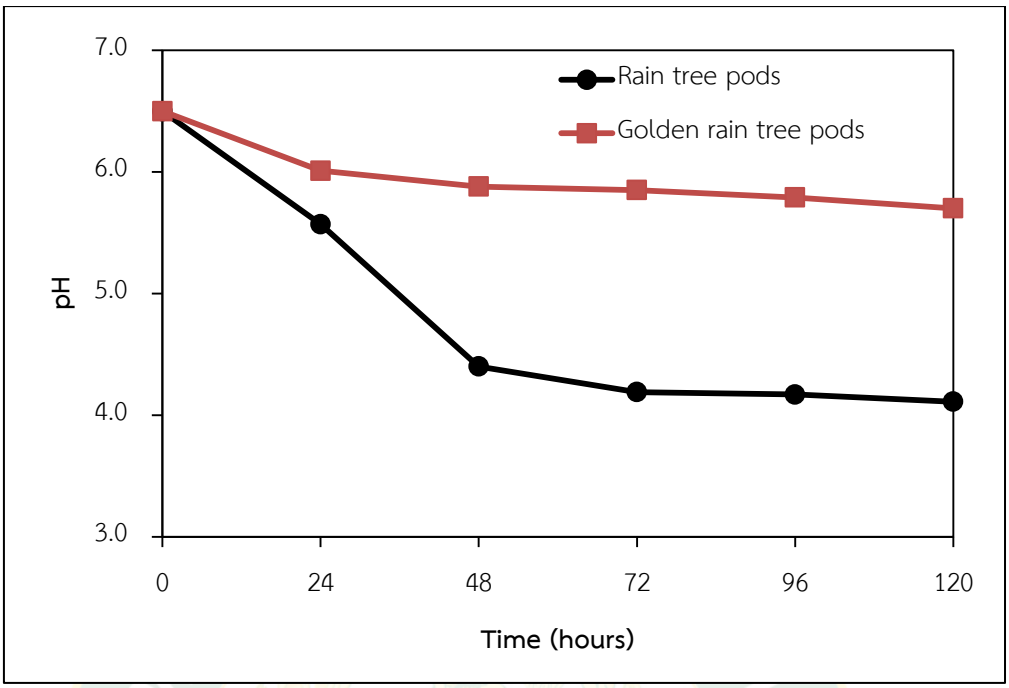


Figure 43 The pH of batch butanol fermentation from dilute juice of rain tree and golden rain tree pods

It can be seen that the use of rain tree and golden rain tree pods prehydrolysate juice did not bring benefit to the butanol production. This low acetone-butanol-ethanol productivity was presumably because low cell growth occurred in the experiments. The cause for this low cell growth may be that the bacteria prefer to use only glucose as a substrate rather than other sugar (Jonglertjunya et al., 2012). In addition, this rain tree and golden rain tree pods prehydrolysate juice may have an inhibitive effect on bacterial growth, resulting in low productivity. Several researchers have reported the effect of inhibitors observed in lignocellulosic biomass hydrolysate on fermentation products (García et al., 2011; Jonglertjunya et al., 2014; Qureshi et al., 2008; Wang and Chen, 2011). According to Martinez et al., (2001); Sun and Liu, (2012); Wang and Chen, (2011), the enzymatic hydrolysis can produce some amount of phenolic compounds, acids, and furfural that inhibit the cells during fermentation.

Table 13 shows the substrate consumed, P_B, Q_B and $Y_{B/S}$ of the experiment results of dilute juice from rain tree and golden rain tree pods. The rain tree pod was found to have a total P_B values ranged from 0.0627 to 1.172 g/L and the $Y_{B/S}$ values from sugar were in the range of 0.001 to 0.117 $g_{Butanol}/g_{Sugar}$. With regards to golden rain tree pods, the total P_B values ranged from 0.0624-0.0628 g/L and the $Y_{B/S}$ values from sugar were in the range of 0.0008-0.0011 $g_{Butanol}/g_{Sugar}$.

Table 13 Fermentation parameters of batch butanol production from dilute juice of rain tree and golden rain tree pods at the fermentation time of 120 hours

Plants	Sugar consumed (g/L)	Total P_B (g/L)	Q_B (g/L·h)	$Y_{B/S}$ (g/g)
Rain tree pods	79.17	1.172	0.010	0.117
Golden rain tree pods	80.00	0.0628	0.0005	0.0011

Note: *P_B, butanol concentration; Q_B, productivity and $Y_{B/S}$, yield*

Mass and energy balance as lab scale

Based on the batch fermentation (50 ml of fermented broth), a simple mass balance for ABE production from dilute juice of rain tree and golden rain tree pods were estimated (Figure 44). When 5 gram of dried samples were used through a thermal extraction method, enzymatic hydrolysis, and fermentation, the mass input of 50 g of fermented broth, it contains 0.059 g acetone, 0.059 g butanol, and 0.004 g ethanol for rain tree pods and 0 g acetone, 0.004 g butanol, and 0.001 g ethanol.

On the other hand, energy balance is a significant index to evaluate energy performance in biobutanol production. In other words, to evaluate if biobutanol production produces gain or loss of energy (Quiroz-Ramírez et al., 2018). The energy required in the steps of the investigated flowsheets (heat, electricity, etc) was made homogeneous and expressed as fuel equivalents. In particular, 1 MJ of electrical energy was set as 3 MJ of fuel-derived energy (Salemme et al., 2016). A simple

energy balance for ABE production from dilute juice of rain tree and golden rain tree pods was estimated in Figure 45. An energy balance in this process used the total energy requirements for the production of biobutanol from rain tree 2,624.97 MJ of fuel-derived energy and it can produce biobutanol of 0.00202 MJ. For the golden rain tree pods, it was used total energy requirements for the production of biobutanol from rain tree 2,631.09 MJ of fuel-derived energy and it can produce biobutanol 0.00011 MJ.

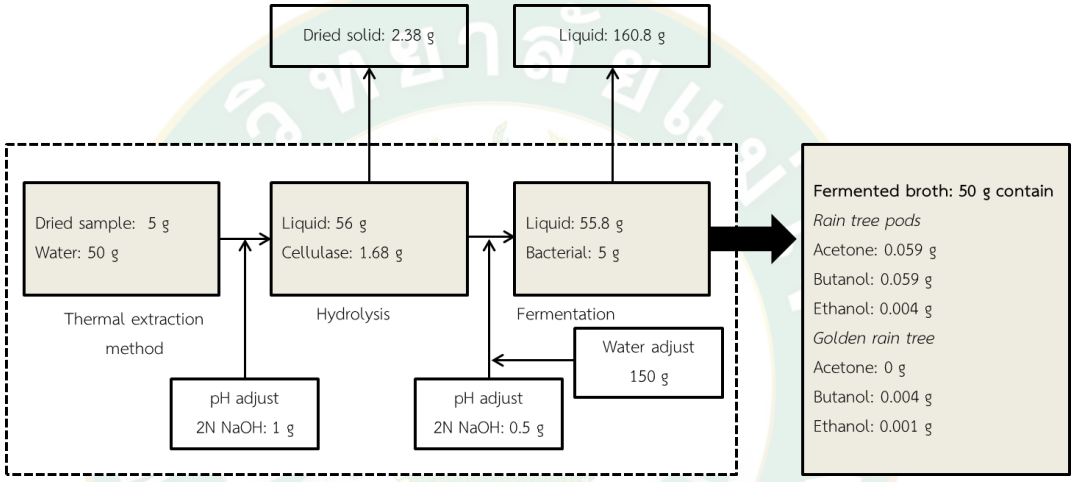
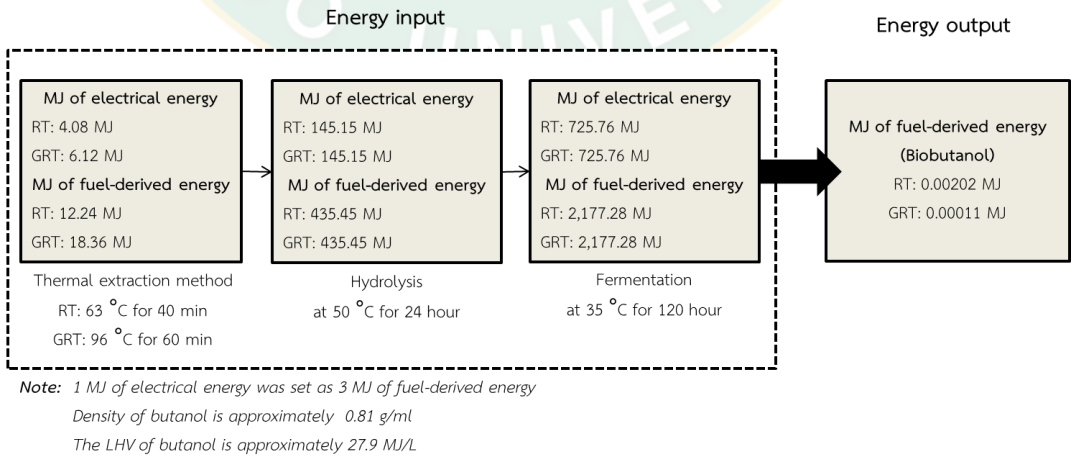


Figure 44 Mass balance based on the batch fermentation for ABE production as lab scale (50 ml); RT: Rain tree pods; GRT: Golden rain tree pods



Note: *1 MJ of electrical energy was set as 3 MJ of fuel-derived energy*
Density of butanol is approximately 0.81 g/ml
The LHV of butanol is approximately 27.9 MJ/L

Figure 45 Energy balance based on the batch fermentation for ABE production as lab scale (50 ml); RT: Rain tree pods; GRT: Golden rain tree pods

Techno-economic analysis of different pods for batch fermentation

In the methodology of this work, the techno-economic analysis has been considered as decision variables for produce biobutanol from dilute juice of rain tree and golden rain tree pods in scale-up. Once those conditions are determined in the lab scale model, the average total cost was calculated by the total fixed cost which is equal to the total cost (Bath/L) divided by the high of produce or the output quantity (ml). In this section, 1 L of fermentation can produce biobutanol 1.1718 g for rain tree pods and 0.0628 g for golden rain tree pods. Determine the density of butanol equal to 0.81 g/ml. Total fixed costs for produce biobutanol 1 L was 702.36 Baht (rain tree pods) and 704.22 Baht (golden rain tree pods). It was found out that the average total cost for the production of biobutanol from rain tree and golden rain tree pods were 485.50 Baht/ml (Table 14) and 9,083.11 Baht/ml (Table 15). The result of the average total cost in terms of baht per milliliter of butanol is high due to the high-cost value of the reagents and chemicals used in this study. The fermented sugar from prehydrolysate juice present on the substrate inhibits cell growth. Therefore, low biobutanol production was observed. If the high-cost reduction is possible in this part, biobutanol production from rain tree and golden rain tree pods are more interesting to produce at the industrial level.

Table 14 The average total cost for biobutanol production from dilute juice of rain tree pods

Items	Price		Amount		Cost	
Feedstocks	0	Baht/g	25.00	g	0	Baht
Water	1.02	Baht/L	1.10	L	1.12	Baht
Induction cooker using 40 min	3.25	Baht/kWh	1.70	kW	3.68	Baht
Enzyme cellulase	185.73	Baht/L	20.00	ml	3.71	Baht
Medium for fermentation (38 g for 1000 ml) using 100 ml	10,200	Baht/kg	3.80	g	38.76	Baht
Nitrogen gas	4.50	Baht/L	50.00	ml	0.23	Baht
Hot air oven using 120 hours	3.25	Baht/kWh	1.68	kW	654.86	Baht
Total fixed costs					12.60	Baht
Quantity (Butanol production) 1 L produce 1.1718 g of butanol (Density=0.81 g/ml)			1.45	ml		
Average total costs = Total fixed costs / Quantity (Butanol production)					485.50	Baht/ml

Table 15 The average total cost for biobutanol production from dilute juice of golden rain tree pods

Items	Price		Amount		Cost	
Feedstocks	0	Baht/g	25.00	g	0	Baht
Water	1.02	Baht/L	1.12	L	1.14	Baht
Induction cooker using 60 min	3.25	Baht/kWh	1.70	kW	5.52	Baht
Enzyme cellulate	185.73	Baht/L	20.00	ml	3.71	Baht
Medium for fermentation (38 g for 1000 ml) using 100 ml	10,200	Baht/kg	3.80	g	38.76	Baht
Nitrogen gas	4.50	Baht/L	50.00	ml	0.23	Baht
Incubator using 120 hours	3.25	Baht/kWh	1.68	kW	654.86	Baht
Total fixed costs					704.22	Baht
Quantity (Butanol production) 1 L produce 0.0628 g of butanol (Density=0.81 g/ml)			0.08	ml		
Average total costs = Total fixed costs / Quantity (Butanol production)					9,083.11	Baht/ml

CHAPTER 5

SUMMARY

The success and sustainable operation of a commercial cellulosic biorefinery are highly dependent on price, quality, and availability of feedstocks. Currently, rain tree and golden rain tree pods are primary feedstock chose for cellulosic ethanol production and the same feedstock can be used for butanol production, which is a potential alternative biofuel. In this study, rain tree and golden rain tree pods were characterized and evaluated as substrates for butanol production.

The results of the thermal extraction in rain tree and golden rain tree pods revealed that the optimal condition at temperature 63 °C for 40 min and 96 °C for 60 min, respectively. The sugar concentration at the optimal condition has 5.04±0.011 g/g total sugar and 1.10±0.005 g/g reducing sugar from rain tree pods and 2.58±0.014 g/g total sugar and 0.56±0.012 g/g reducing sugar from golden rain tree pods. The high sugar concentration from rain tree pods can frond at 63 °C for 40 min condition and the thermal extraction efficiency compared with non-thermal showed that the thermal extraction results the total sugar increased by 22.9%. In the light of the high sugar concentration form golden rain tree pods was found at 96 °C for 60 min of thermal extraction condition. The thermal extraction efficiency compared with non-thermal showed that the thermal extraction result the total sugar increased by 38.4%. These raw materials that have been thermally extracted and continued hydrolysis with cellulase enzymes. It was found out that the total sugar concentrations were increased from 5.07±0.03 g/g to 5.55±0.01 g/g from rain tree pods and 2.72±0.02 g/g to 3.51±0.02 g/g from golden rain tree pods. The reducing sugar concentrations were increased from 1.12±0.02 g/g to 2.81±0.01 g/g from rain tree pods and 0.58±0.02 g/g to 1.44±0.02 g/g from golden rain tree pods. Also, the degree of polymerization (DP) was reduced from 4.5 to 2.0 from rain tree pods and 4.7 to 2.4 from golden rain tree pods. This result shows that the reduction of DP showed very clear evidence of enzyme activities on breaking down the big sugar chains into smaller chains. Enzymatic hydrolysis of pretreated rain tree and golden

rain tree pods resulted in the highest efficiency enzyme of 51% and 41%, respectively.

Then, the raw materials underwent thermal extraction in optimal condition and enzymatic hydrolysis was used for ABE fermentation via *C. acetobutylicum* TISTR 2375. It was found out that the sugar from extraction and enzymatic hydrolysis can be utilized by *C. acetobutylicum* TISTR 2375, which can produce butanol of 0.0641 g/L from rain tree pods. For golden rain tree pods, sugar from extraction and enzymatic hydrolysis can be used by *C. acetobutylicum* TISTR 2375, butanol 0.0645 g/L. This result showed that the high concentration of sugar resulting produces a small amount of butanol. Therefore, the prehydrolysate juice of two plants was diluted about 80 g/L of reducing sugar to produce butanol again. It was found that rain tree pods (1.172 g/L butanol) can produce higher butanol concentration than golden rain tree pods (0.0628 g/L butanol).

A simple mass balance for ABE production from dilute juice of rain tree and golden rain tree pods were estimated. The mass input of 50 g of fermented broth. It can produce 0.059 g acetone, 0.059 g butanol, and 0.004 g ethanol for rain tree pods and 0 g acetone, 0.004 g butanol, and 0.001 g ethanol. An energy balance in this process used the total energy requirements for the production of biobutanol from rain tree 2,624.97 MJ of fuel-derived energy, and it can produce biobutanol of 0.00202 MJ. For the golden rain tree pods, it was used total energy requirements for the production of biobutanol from rain tree 2,631.09 MJ of fuel-derived energy, and it can produce biobutanol 0.00011 MJ. The evaluate feasibility of biobutanol production from dilute juice of rain tree and golden rain tree pods. It was found total fixed costs for produce biobutanol 1 L of 702.36 Baht (rain tree pods) and 704.22 Baht (golden rain tree pods). It was found out that the average total cost for the production of biobutanol from rain tree and golden rain tree pods were 485.50 Baht/ml and 9,083.11 Baht/ml.

This study produced a small amount of butanol, indicating that the rain tree and golden rain tree pods prehydrolysate juice contained inhibitors such as phenolic compounds, acids, and furfural that may affect the cell growth during the early stage,

of fermentation. This result showed that the concentration of fermentation medium components including carbon source significantly affects the biobutanol fermentation. Additionally, the cost of the feedstock is low compared to other agricultural raw materials used for alternative energy production. Hence, rain tree and golden rain tree pods hold a promise as a raw material for butanol production. However, optimization for butanol production by *C. acetobutylicum* TISTR 2375 from rain tree and golden rain tree pods should be further investigated to increase the biobutanol yield. The results of this study may lead to future research with particular attention of increasing the butanol productivity of rain tree and golden rain tree pods prehydrolysate juice. The presence of inhibitors may be toxic to bacterial cells. Therefore, future studies may focus on butanol production by eliminating of fermentation inhibitors. Another, for the fermentation process equipment such as shaking incubator, can be used.

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APPENDIX

APPENDIX A Standard sugar

Total sugar determination by phenol sulfuric method (DuBois et al., 1956)

Reagents

- Sulfuric acid (H_2SO_4) concentrated 98% (v/v)
- 5% (w/v) Phenol solution

Standard glucose preparation

0.1 g of glucose was dissolved in distilled water in a 100 ml volumetric flask to get 1000 µg/ml glucose solution.

Method

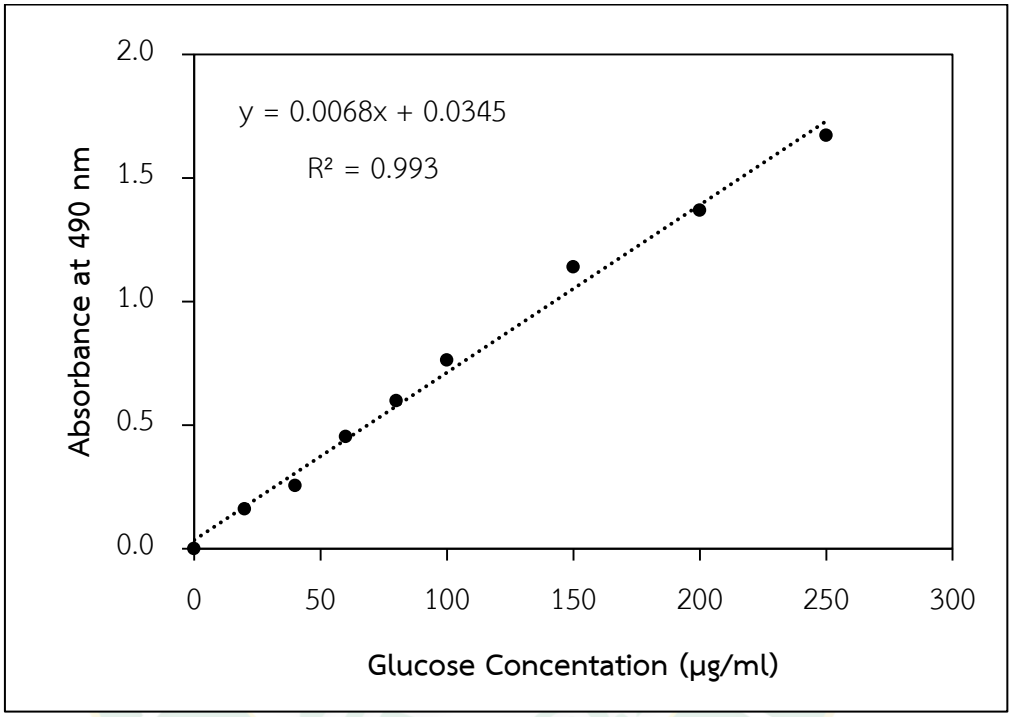
Standard curve of total sugar was prepared using the serial concentration of glucose solution (0-250 µg/ml) in distilled water. The 500 µl of each concentration was transferred to test tube and added with 500 µl of 5% phenol solution. The mixtures were shaken and followed by the addition of 2.5 ml conc. sulfuric acid. All mixtures were homogenized by vortex and subsequently stand for 10 min. The absorbance (490 nm) of the reaction mixture was measured. Finally, the relation between A_{490} and glucose concentration was plotted.

Determination of total sugar in the samples, sugar concentration in sample solution was determined as the method described above. The reaction mixture composed with 500 µl of sample solution, 500 µl of 5% phenol solution and 2.5 ml conc. sulfuric acid.

Appendix Table 1 Absorbance at 490 nm by glucose solution at several concentrations

Glucose concentration (µg/ml)	A_{490}
0	0.000
20	0.161
40	0.255
60	0.453
80	0.599
100	0.763

Glucose concentration (µg/ml)	A_{490}
150	1.140
200	1.370
250	1.673



Appendix Figure 1 Standard curve of total sugar by phenol sulfuric method using glucose as standard sugar

Reducing sugar determination by DNS method (Miller, 1959)

Preparation of DNS solution

- Dissolving 5 g of 3,5 Dinitrosalicylic acid in 100 ml of 2N NaOH
- Adding 150 g of sodium potassium tartrate and stir unit completely dissolve
- Adjusting the volume up to 500 ml

Preparation of glucose solution

0.1 g of glucose was dissolved in distilled water in a 100 ml volumetric flask to get 1000 µg/ml glucose solution.

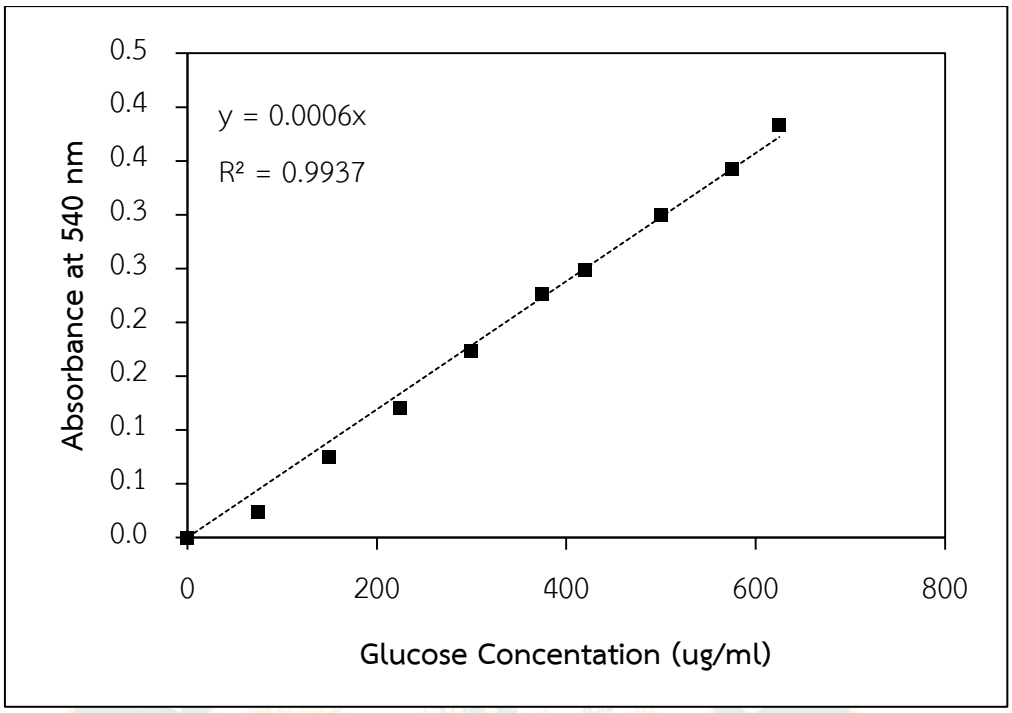
Method

Standard curve of reducing sugar was prepared using the serial concentration of glucose solution (0-1000 µg/ml) in distilled water. The 500 µl of each concentration was filled into test tube and added with 500 µl of DNS solution and subsequently boiled for 15 min. After that, cooling and addition with 4.0 ml of distilled water was performed. After homogenizing of reaction mixture, the absorbance at 540 nm was measured. The relation between glucose concentration and A_{540} was plotted.

To determine amount of reducing sugar in sample solution, the 500 µl of sample solution was determined with the method as described above similar to standard curve preparation. After A_{540} measurement, reducing sugar concentration was calculated by comparing to standard curve.

Appendix Table 2 Absorbance at 540 nm by glucose solution at several concentrations

Glucose concentration (µg/ml)	A_{540}
0	0.000
75	0.024
150	0.075
225	0.120
300	0.173
375	0.227
420	0.249
500	0.299
575	0.343
625	0.383



Appendix Figure 2 Standard curve of reducing sugar by DNS method using glucose as standard sugar

APPENDIX B Standard ABE

ABE determination

Reagents

- Acetone solution
- 1-Butanol solution
- Ethanol solution

Standard ABE preparation

633 µl of acetone was dissolved in distilled water in a 10 ml volumetric flask to get 50 g/L acetone solution.

127 µl of ethanol was dissolved in distilled water in a 10 ml volumetric flask to get 10 g/L ethanol solution.

Method

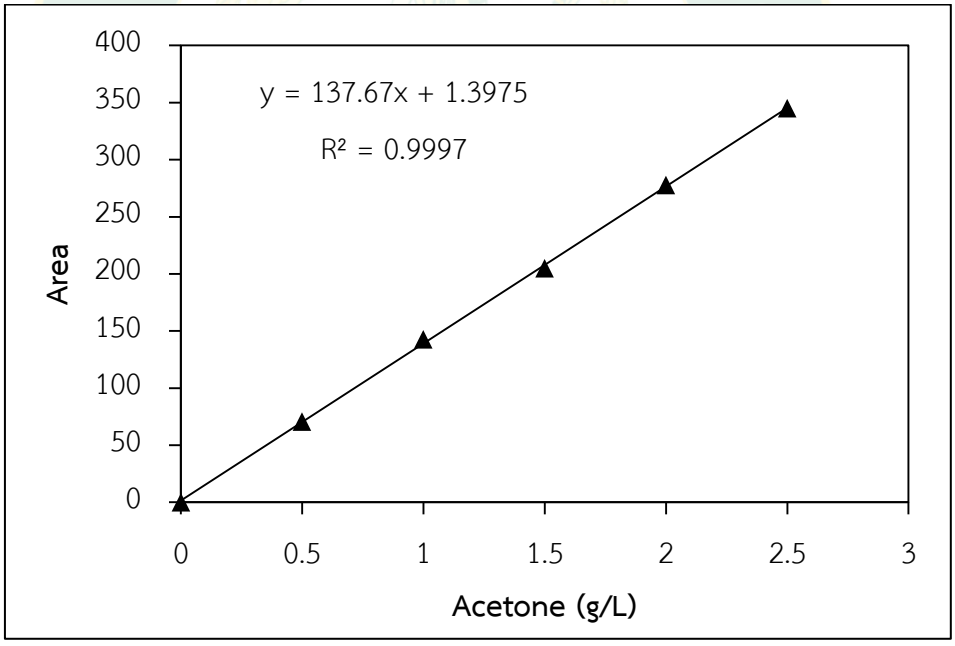
Standard curve of ABE were prepared using the serial concentration of acetone solution (0.5-2.5 g/L), 1-butanol solution (2.0-10.0 g/L) and ethanol solution (0.1-0.5 g/L) in distilled water. ABE mixtures were homogenized and measured using a gas chromatography. Finally, the relation between area and acetone, butanol and ethanol concentration was plotted. Determination of ABE in the samples, ABE concentration in sample solution was determined as the method described above.

Appendix Table 3 The several concentrations of ABE

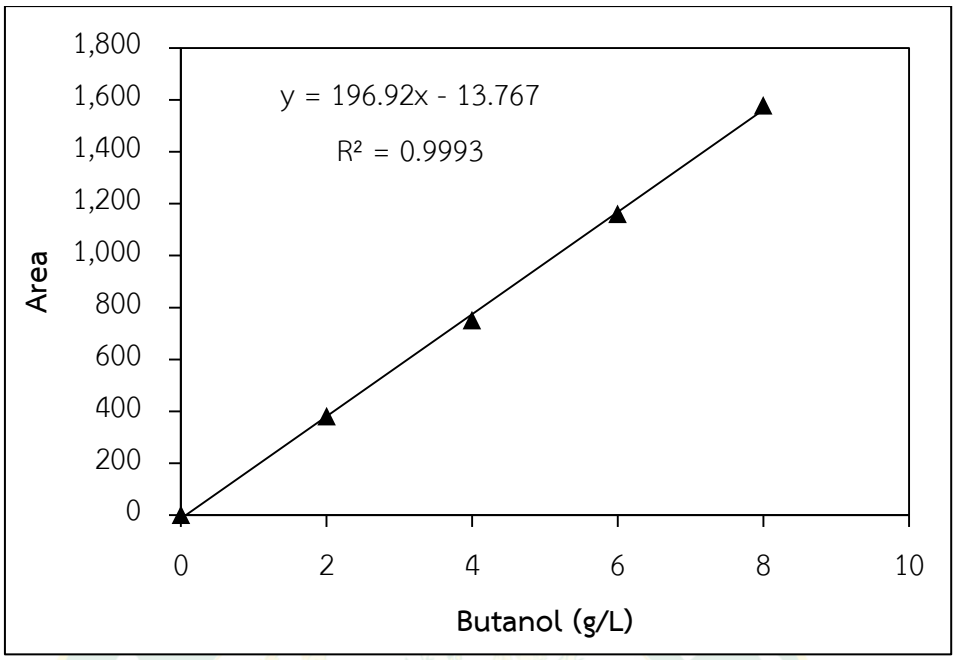
Vial	Concentration (g/L)			Standard preparation (µl) in 10 ml of distilled water		
	Acetone	Butanol	Ethanol	Acetone[1]	Butanol	Ethanol[2]
1	0.5	2.0	0.1	100	25	100
2	1.0	4.0	0.2	200	50	200
3	1.5	6.0	0.3	300	74	300
4	2.0	8.0	0.4	400	99	400
5	2.5	10.0	0.5	500	124	500

[1] *Acetone solution was prepared from 50 g/L acetone solution*

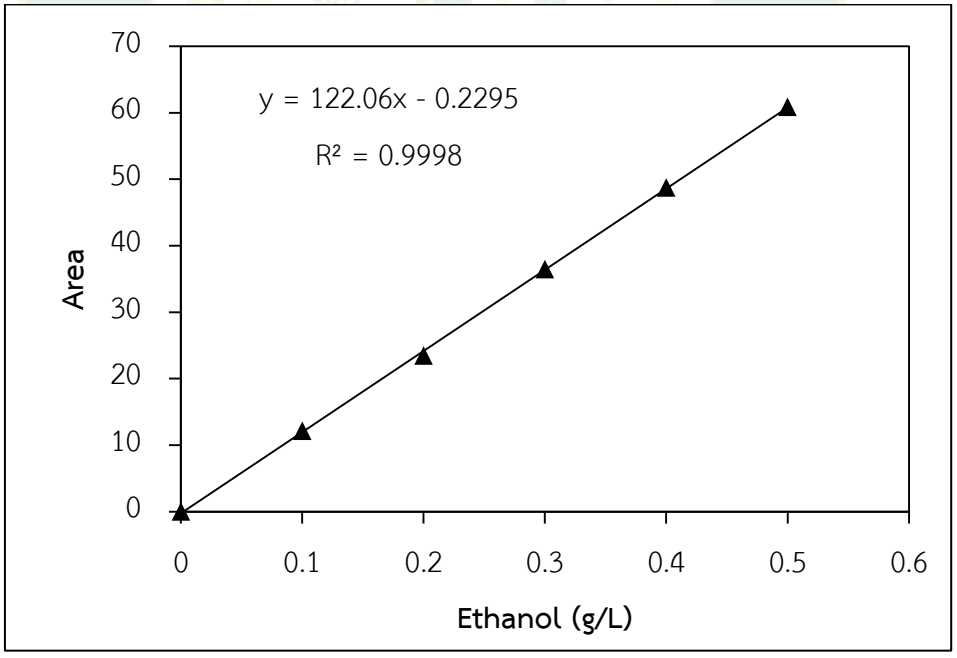
[2] *Ethanol solution was prepared from 10 g/L ethanol solution*



Appendix Figure 3 Standard curve of acetone by gas chromatography



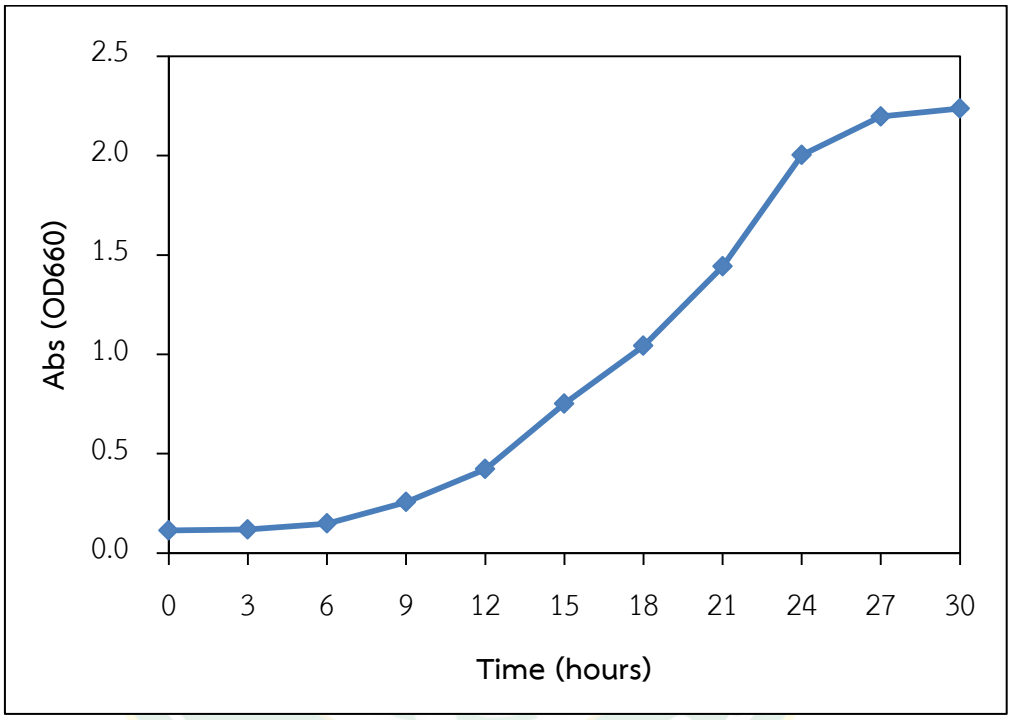
Appendix Figure 4 Standard curve of butanol by gas chromatography



Appendix Figure 5 Standard curve of ethanol by gas chromatography

APPENDIX C Growth curve

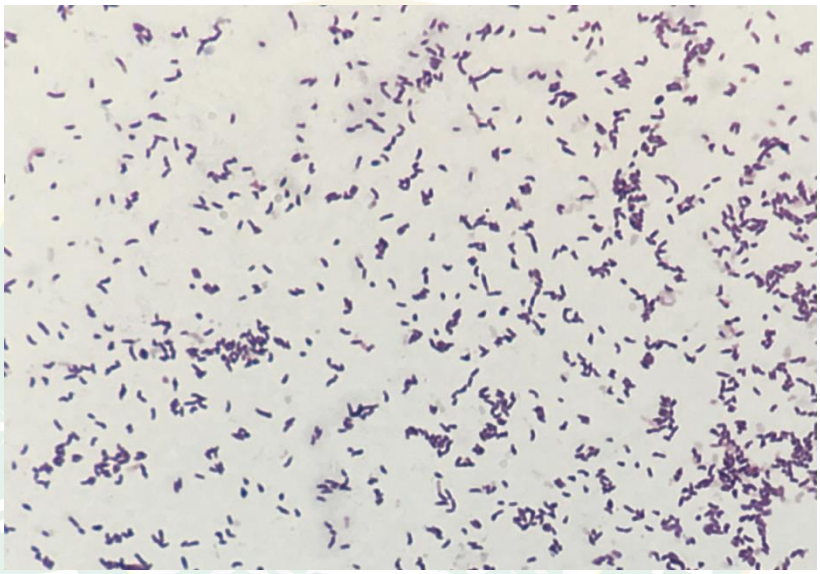
C. acetobutylicum TISTR 2375 was cultured in RCM medium to study the growth curve of the microorganism (Appendix Figure 6). The cell density was analyzed at OD 660 nm. Under anaerobic conditions, the culture reached the OD660 values of 1.5～2.0 after just 24 hour.



Appendix Figure 6 Growth curve of *C. acetobutylicum* TISTR 2375

APPENDIX D Gram strain

Clostridium is a rod-shaped, gram-positive bacterium. They move using the peritrichous flagella as observed by (Tracy et al., 2011). Appendix Figure 7 shows the microscopic appearance of *C. acetobutylicum* TISTR 2375 at 100x objective lens after gram staining. This illustration shows the normal cells that grows during fermentation in RCM medium



Appendix Figure 7 Characteristics of *C. acetobutylicum* TISTR 2375 after gram staining

APPENDIX E Research publication

The 6th ASCON-IEEChE 2018
November 4-7, 2018, Sun Moon Lake, Taiwan

Optimization of sugar production from rain tree pods by thermal extraction method for biobutanol production

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Abstract

Sugar plays an important role in biobutanol production. Extraction and production of sugar in the form of glucose from renewable materials would be a great solution to impending demand to biofuels. In this study, rain tree pods *(Samanea saman)* were used to produce sugar through thermal extraction. The objective of this study was to analyze the factors affecting the amount of total sugar and reducing sugar by using Design of Experiment: Response Surface Methodology (RSM) on Design Expert software 11.1.0 (Stat-Ease Inc., Minneapolis, USA) for model predictions. The important factors are temperature and time for the pretreatment. Two levels of temperatures (30 and 96 °C) along with two levels of time (20 and 60 min) were applied. All the factors were significant at p-value < 0.05. The results showed that these two factors affected the amount of total sugar and reducing sugar. Consequently, the best results recorded in total sugar was 504.71 g/L and reducing sugar of 110.55 g/L at conditions 63 °C for 40 min. In general, the results suggested that rain tree pods possess a very good potential as feedstock for biobutanol production in the future.

Keywords: Sugar Production, Rain Tree Pods, Thermal Extraction, Central Composite Design.

1. Introduction

The research on biofuel aims to produce bioenergy such as biobutanol, bioethanol, biodiesel, biohydrogen, and biogas from biomass[1-2]. At present, biobutanol is a relatively new biofuel which has more advantages than ethanol for transportation fuels. In addition, it can be mixed with gasoline to achieve better engine performance[3-4]. First generation of biofuels (such as soybean, palm oil, corn, sunflower, etc.) is a raw material with high cost of production and the food with fuel dispute is the main constraints. Lignocellulosic feedstock from plant biomasses, agriculture residues (such as bagasses, straws, etc.), and waste products (such as waste cooking oil) are the second generation biofuels. It is non-edible feedstock and not counters the food-versus-fuel issues present in the first generation biofuels. Third generation biofuels including microalgae, bacteria, yeast, and fungi[5-6]. The most important and most active part of biofuel research is converting biomass material into sugar to produce biofuel[7]. Sugar in the biomass can be produced by pretreatment process (such as physical, chemical, physical-chemical, biological methods)[8-9]. The easiest way is to use raw materials with sugar as the main component.

Rain tree is widely distributed in the tropics and grown all along the road sides at Thailand. Mature pods are black-brown and brownish pulp that is sweet and edible. Ripen pods are available from February to May every year in Thailand. It is reported that the pods consists of 15.31-18.00% crude protein, 10.00-17.30% total sugars 9.72% hemicellulose and 17.48%

Appendix Figure 8 Proceeding I-I

cellulose[10-11]. Most of pods are unutilized and defoliate to the ground and becomes glutinous that invites flies when rotting[12]. Moreover, rain tree pods produced significant amount during fruiting season. In addition, it can be easily extracted by thermal extraction method with low production cost. Therefore, rain tree pods can be a promising material for biobutanol production.

Response surface methodology (RSM) is a combination of mathematical and statistical technique used for the modeling and analyzing the results of several independent variables. The central composite design (CCD) for analysis of experimental information is the most popular RSM design[13-14]. In this study, the total sugar and reducing sugar of rain tree pods by thermal extraction method were investigated. The effect of temperature and time were evaluated using a Central Composite Design (CCD) combined with RSM. The optimal operating conditions to achieve maximum total sugar and reducing sugar were obtained and validated experimentally. The method of analysis can be useful for production of biobutanol from rain tree pods in the future.

2. Materials and Methods

2.1 Materials

Rain tree (*Samanea saman*) was grown at Maejo University campus, Chiang Mai, Thailand (18°53'45" N, 99°00'37" E). The pods of rain tree were collected from March to April 2018. It was dried at 50 °C for 48 hours in a hot air oven to prevent mold growth, seed germination, and rotting of the sample prior to storage. After drying, the rain tree pods were stored in plastic bag under ambient condition for further analysis.

2.2 Thermal Extraction Method

The dried samples were pulverized and subjected to thermal extraction. The thermal extraction method was operated using a dilution ratio of 1:10 (25 g of pods = 250 mL of water). The pods were soaked in water, which controls the temperature and time.

2.3 Analytical Methods

Rain tree pods juice was analyzed by phenol-sulfuric procedure[15] and DNS method[16] for determining its total sugar and reducing sugar, respectively.

2.4 Experimental Design and Statistical Analysis

The RSM was used to optimize sugar production from rain tree pods and investigated the influence of different factor variables on the sugar yield. The best experimental conditions were used for biobutanol production in the future. All analytical tests were carried out in triplicate. Statistical analysis was performed using the Design Expert software 11.1.0 (Stat-Ease Inc., Minneapolis, USA) on CCD. The data of the experiment were analyzed by the analysis of variance (ANOVA), and p-value lower then 0.05 was considered significant in surface response analysis.

The independent variables were applied to investigate the effect of temperature and time by thermal extraction method and the dependent variable were total sugar and reducing sugar. A total of 27 experiments were found to be sufficient to calculate the coefficients of the second-order polynomial regression model for two variables. The two identified design independent variables, namely, temperature (A) and time (B). Each variable was investigated at two levels: low (−1) and high (+1). The design factors (variables) with low (−1) and high (+1) levels, are, namely, A (30 and 96 °C) and B (20 and 60 min). The central values; zero level chosen for experimental design were 63 °C and 40 min for A and B respectively, as shown in Table 1.

Appendix Figure 9 Proceeding I-II

Table 1 Variables and their levels used in the experimental design

Variables	Symbol coded	Range and levels		
		Low level (−1)	Center (0)	High level (+1)
Temperature (°C)	A	30	63	96
Time (min)	B	20	40	60

The next step was to perform a RSM experiment to create a prediction model to maximum yield of total sugar and reducing sugar from rain tree pods. In this study, the model used to estimate the response surface is the second order polynomial equation as follows:

$$Y = \beta_0 + \beta_1 A - \beta_2 B - \beta_{12} AB - \beta_{11} A^2 - \beta_{22} B^2 + \beta_{112} A^2 B + \beta_{122} AB^2 \quad (1)$$

Where Y is the total sugar or reducing sugar (g/L), β_0 is the interception coefficient, β_{11} and β_{22} are the quadratic terms, β_{12}, β_{112} and β_{122} are the interaction coefficients, and A and B are the independent variables studied (temperature and time, respectively).

3. Results and Discussions

3.1 Characterization of Rain Tree Pods

Feedstock was dried at moisture content range of 8% to 15%. The pods were conditioned by temperature and total sugar and reducing sugar was monitored using phenol sulfuric procedure and DNS method. Figure 1 shows the sugar production of the rain tree pods.

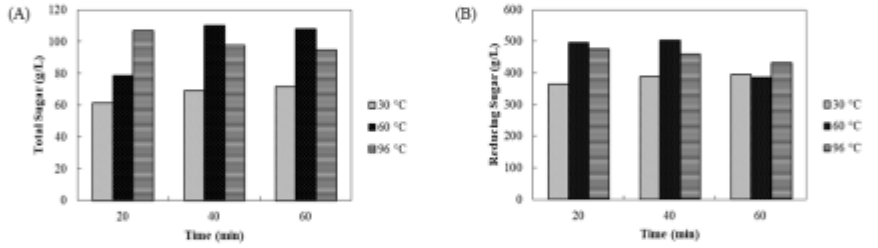

Figure 1 The sugar production of the rain tree pods: (A) total sugar and (B) reducing sugar

The result of the analysis revealed that the rain tree pods contained about 363.44-504.71 g/L of total sugar and 60.75-110.55 g/L of reducing sugar at 30-96 °C and 20-60 min condition. It was reported that the rain tree pods contained about 9.13% of sucrose, 11.3% of fructose, 11.2% of glucose, and 0% of maltose[17].

3.2 RSM Model Development

Temperature and time were selected as factors in the CCD. As a response, the total sugar and reducing sugar were chosen. A total number of 27 experiments were employed for the response surface modeling (Table 2), and the order of experiments was arranged randomly. The actual and predicted value for the total sugar and reducing sugar were also depicted in Table 2. Where, the actual total sugar ranged from 363.44 to 504.71 g/L and its corresponding predicted values are 357.74 and 507.56 g/L, respectively. And the actual reducing sugar ranged from 60.75 to 110.55 g/L and its corresponding predicted values are 59.55 and 111.85 g/L, respectively.

The Design Expert software 11.1.0 (Stat-Ease Inc., Minneapolis, USA) was used to calculate the coefficients of the second-order fitting equation and the model suitability was tested using the analysis of variance (ANOVA) test. Therefore, the polynomial equation obtained is as follows:

$$\text{Total sugar} = 478.63 + 35.64A - 54.63B - 18.69AB - 42.16A^2 - 25.70B^2 + 51.49A^2B + 1.86AB^2 \quad (2)$$

$$\text{Reducing Sugar} = 102.55 + 14.25A + 14.65B - 5.55AB - 15.35A^2 - 5.35B^2 - 15.05A^2B + 2.90AB^2 \quad (3)$$

Table 2 Experimental designs of the three levels and their experimental results and predictive values

Run	Factor 1 A:Temperature (℃)	Factor 2 B:Time (min)	Total sugar (g/L)		Reducing sugar (g/L)	
			Actual	Predicted	Actual	Predicted
1	30	40	388.53	400.84	69.15	72.95
2	63	60	386.35	398.29	108.15	111.85
3	96	60	433.8	426.43	94.95	93.05
4	96	40	456.71	472.11	97.95	101.45
5	96	40	459.44	472.11	97.65	101.45
6	30	20	363.44	357.74	60.75	59.55
7	30	40	387.98	400.84	69.45	72.95
8	63	20	493.8	507.56	78.15	82.55
9	30	60	395.62	388.83	72.15	69.85
10	30	60	394.53	388.83	71.25	69.85
11	30	20	364.53	357.74	61.65	59.55
12	30	40	388.53	400.84	68.85	72.95
13	30	20	363.98	357.74	61.95	59.55
14	63	20	493.25	507.56	78.45	82.55
15	63	40	502.53	478.63	110.25	102.55
16	96	20	475.8	470.10	107.25	104.95
17	63	20	498.16	507.56	79.65	82.55
18	96	20	479.07	470.10	106.35	104.95
19	30	60	395.07	388.83	71.85	69.85
20	63	40	503.62	478.63	110.55	102.55
21	96	40	462.71	472.11	97.35	101.45
22	63	60	384.16	398.29	107.55	111.85
23	63	60	386.89	398.29	108.45	111.85
24	96	60	426.71	426.43	94.35	93.05
25	63	40	504.71	478.63	109.65	102.55
26	96	60	437.53	426.43	95.55	93.05
27	96	20	474.16	470.10	106.95	104.95

Where A and B are temperature and time for pretreatment by thermal extraction method.

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors were coded as +1 and the low levels were coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

3.3 Effects of Model Parameters and Their Interactions

The Design Expert software was used to produce three-dimensional (3D) response surfaces and two-dimensional (2D) contour plots. The results of the interactions between two independent variables and the dependent variable are shown in Figure 2 and Figure 3.

Appendix Figure 11 Proceeding I-IV

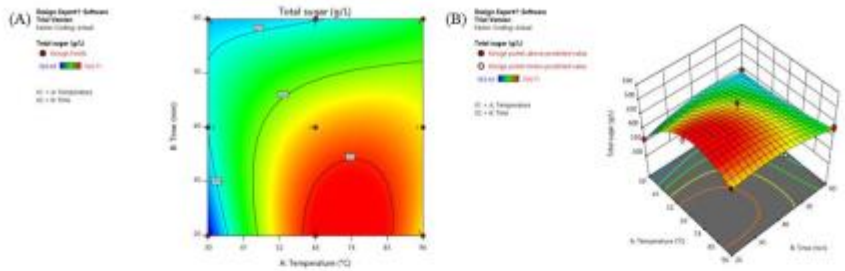

Figure 2 Response surface plots showing the effect of temperature and time on total sugar

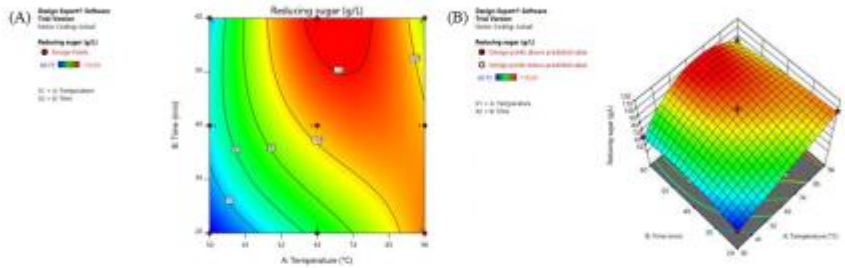

Figure 3 Response surface plots showing the effect of temperature and time on reducing sugar

Figure 2A,B shows the interaction effect of temperature and time concentration on total sugar. As it can be seen in the plots, there is an increase in total sugar with an increase of temperature, with the maximum total sugar in the temperature range of 55 to 90 °C. On the other hand, the effect of time on total sugar has similar trends, regardless of the temperature, with the maximum total sugar in the time rang of 20 to 40 min.

Figure 3A,B shows the interaction effect of temperature and time concentration on reducing sugar. The contour plots show that the optimum region for the reducing sugar is in the temperature range of 59 to 79 °C and time is in the range of 35 to 60 min.

4. Conclusion

RSM based on CCD was used to optimize sugar production from rain tree pods by thermal extraction method for biobutanol production. It was found that total sugar and reducing sugar increased with the increase of temperature and time. The optimal conditions found for total sugar (504.71 g/L) and reducing sugar (110.55 g/L) were temperature 63 °C and time 40 min respectively. The result corresponds to that predicted by the model. Hence, it can be concluded that pods have high sugar content and can be use to produce biobutanol and other biofuel in the future.

Acknowledgements

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Appendix Figure 13 Proceeding I-VI

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International Education and Training Center, Maejo University, Chiang Mai, Thailand

Response Surface Optimization for Thermal Extraction Method of Total Sugar and Reducing Sugar from Golden Rain Tree Pods

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Abstract: The aim of this study is to find new material to produce biofuel and optimize the amount of total and reducing sugar by thermal extraction. The two independent variables namely temperature (30-96 °C) and time (20-60 min) were optimized using the central composite design (CCD) with a quadratic regression model built using response surface methodology (RSM). The experiments were carried out using 2 factors (27 runs) with 3 levels. The optimal values of the influencing parameters that affect the production of sugar from golden rain tree pods to be as follows: 96 °C and 60 min. Under these optimum operating conditions the maximum sugar production was recorded as 260.31 g/L of total sugar and 56.29 g/L of reducing sugar. The optimum sugar was statistically significant, from the predicted value obtained by RSM which suggests that RSM could be efficiently used to optimize a sugar production from golden rain tree pods using thermal extraction method. This approach will add value to golden rain tree pods by converting waste into a clean for produce biofuel in future.

Keywords: Central composite design (CCD), Response surface methodology (RSM), Golden rain tree pods, Thermal extraction.

Appendix Figure 14 Proceeding II-I

1. Introduction

Our sources of energy are mainly from fossil fuels for transportation and industry causing environmental pollution attracted the researchers and scientists to explored for alternative renewable sources of energy especially biomass based biofuels (Patel et al., 2015, Sangyoka et al., 2016). They can be derived from various biomass feedstocks that available in the country which are classified according to the source of the raw materials used such as sugar, starch and lignocellulosic biomass (Leong et al., 2018). In this present investigation, we have explored golden rain tree pods as an easily available, unutilized, non-edible lignocellulosic biomass feedstock as a raw material for sugar production.

Golden rain tree (*Cassia fistula* Linn.) family Caesalpiniaceae commonly is known as Amulthus and in english popularly called Golden Shower or Indian Laburnum. It is widely distributed especially in the tropics namely Asian countries, South Africa, West Indies Brazil and Thailand (Rajagopal et al., 2013). It is a deciduous, medium sized tree up to 24 m in height and 1.8 m (Danish et al., 2011). The fruit pods are dark brown in colour, sticky, sweet, pendulous, cylindrical, septate with long pods (25-50 cm) having 1.5–3.0 cm diameter, possessing 25–100 seeds and almost 400–500 fruiting bodies per tree (Patel et al., 2015). It is reported that the fruit pods consists of 31.3% sucrose, 26.2% fructose and 42.5% glucose (Danish et al., 2011). In addition, golden rain pods are produced significant amount during fruiting season in Thailand. It is unutilized and defoliate to the ground. It can be extracted easily and economically by using thermal extraction method. Therefore, golden rain tree pods can be a promising material for produce biofuels in future.

The extraction of sugar from each biomass feedstocks requires unique operating conditions developed based on the amount of sugar and water, fiber structure and composition, and geometric size. The traditional method of extract sugar is to squeeze the stalks through a roller mill, releasing the sugar rich juice. This method is called the water extraction method. It has the sugar concentration in the extraction water typically is fairly low making (Jia et al., 2013).

Response Surface Methodology (RSM) is an important implement for process optimization. It is statistical technique used for design, improve, and formulate the process parameters. RSM is selected as a technique that has the proficient to enhance such practices. The most popular RSM design for analysis of experimental information is central composite design (CCD) (El-Gendy et al., 2013, Zaidon et al., 2014, Sablania et al., 2018).

This study investigates the effects of temperature and time on thermal extraction method from golden rain tree pods. A central composite experimental design was employed in planning the experiment in order to find out which experiment variables affect total sugar and reducing sugar by using RSM and a predictive polynomial quadratic equation.

2. Materials and methods

The study aims to optimize the amount of total sugar and reducing sugar from golden rain tree pods by thermal extraction method. Figure 1 is the summarized methodology of the study:

Appendix Figure 15 Proceeding II-II

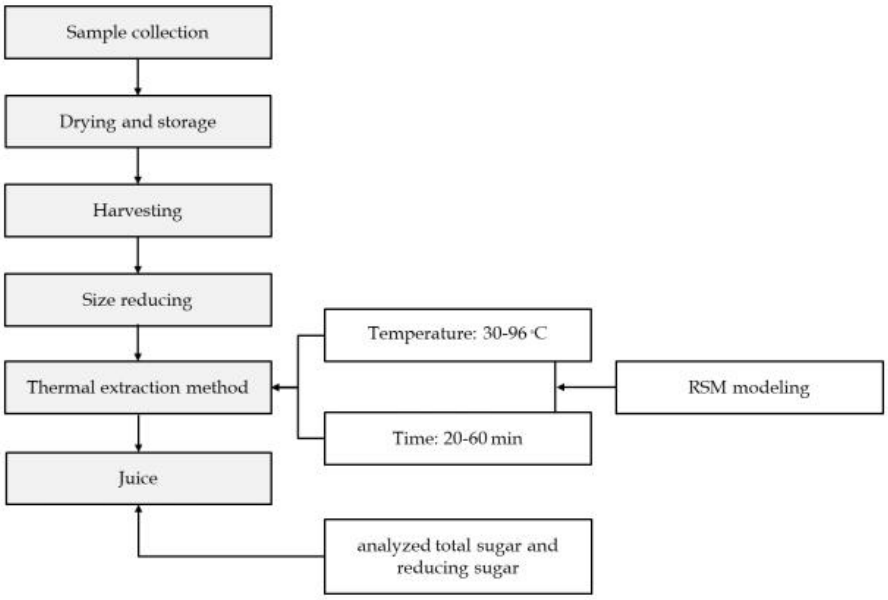

Figure 1 Conceptual framework and methodology

2.1. Feedstock

Golden rain tree pods (Figure 2) used in this study was collected from Maejo University campus, Chiang Mai, Thailand (18°53′39″N, 99°00′40″E) during March to April 2018. The pods of golden rain tree collected have average weight of 75.07 g per pods. It was air dried at 50 °C for 48 hour in an oven before being stored at room temperature prior to usage.

Figure 2 Golden rain tree pods

2.2 Thermal extraction method and analytical method

The dried samples were shredded to obtain desired size and subjected to thermal extraction. It was operated using a dilution ratio of 1:10 (25 g of pods = 250 mL of water). The pods were soaked in water, which controls the temperature (30-96 °C) and time (20-60

Appendix Figure 16 Proceeding II-III

min). The juice from the pods was analyzed total sugar and reducing sugar by phenol-sulfuric procedure (Dubois et al., 1956) and DNS method (Miller, 1959), respectively.

2.3 Experimental design and statistical analysis

The experimental design and statistical analysis were performed according to the response surface analysis methodology (RSM) using Design Expert software 11.1.0.1 (Stat-Ease Inc., Minneapolis, USA). It was used to optimize sugar production process from golden rain tree pods and investigate the influence of different sugar production process variables on the sugar yield. The central composite design or CCD (Box and Wilson, 1951) with quadratic model was employed to study the combined effect of two independent variables namely temperature (X_1, °C) and time (X_2, min), with low (−1) and high (+1) levels for thermal extraction method. The dependent variables (Y) measured were total sugar (g/L) and reducing sugar (g/L) in golden rain tree pods. In CCD, the range and the levels of variables employed in this study are listed in Table 1. A 2^2 factorial CCD, with three axial points and three replications at the center points leading to a total number of 27 experiments were employed for optimization of the thermal extraction conditions. Experimental data were fitted to the following second order polynomial model and regression coefficients were obtained to estimate the response of the dependent variable. In this study, the second order polynomial model proposed for the response surface analysis as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{112}X_1^2X_2 + b_{122}X_1X_2^2 \quad (1)$$

where Y is the predicted response, X_1 and X_2 are independent variables studied, b_0 is the offset term, b_1 and b_2 are linear effects, b_{11} and b_{22} are the quadratic terms and b_{12}, b_{112} and b_{122} are interaction coefficients.

The data of the experiment were analyzed by the analysis of variance (ANOVA), and F-value at a probability (p) lower then 0.05 was considered significant in surface response analysis. The regression coefficients were then used to generate contour maps from the regression models. The three-dimensional (3D) response surfaces contour plots were generated by keeping one variable constant at the center point and varying the other variables within the experimental range (Mohan et al., 2013).

Table 1 Variables and their levels for central composite design

Independent variables	**Unit**	**Symbol coded**	**Coded levels**		
			-1	**0**	**+1**
Temperature	°C	X_1	30	63	96
Time	min	X_2	20	40	60

3. Results and discussion

3.1 Sugar production from golden rain tree pods

The present study aims for produce sugar from golden rain tree pods by using thermal extraction method. The pods were conditioned by temperature and time of extraction method. The sugar production was monitored using phenol sulfuric procedure (for total sugar) and DNS method (for reducing sugar). Figure 3 shows the sugar production from the golden rain tree pods by using thermal extraction method.

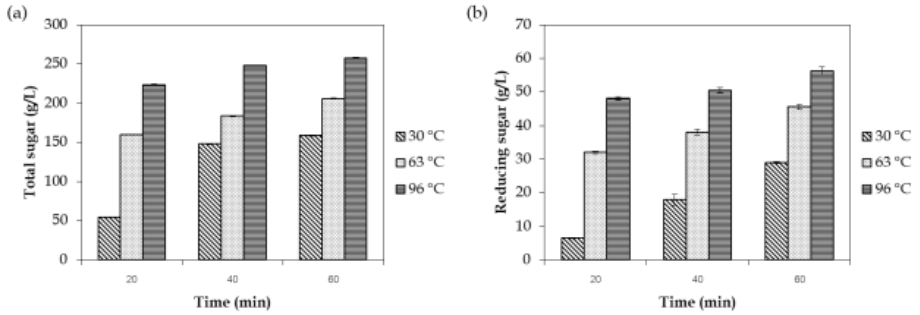

Figure 3 Sugar production of golden rain tree pods: (a) total sugar and (b) reducing sugar.

Feedstock was dried at moisture content range of 4% to 10%. The result of the analysis revealed that golden rain tree pods contained about 54.60±0.71 to 257.68±1.39 g/L of total sugar and 6.57±0.18 to 56.30±1.16 g/L of reducing sugar at 30-96 °C and 20-60 min condition. The sugar content of golden rain tree pods is from Danish et al., 2011. The variation may be due to the influence of different factors on golden rain tree cultivation and golden rain tree pods juice extraction. The result also shows that the fruit pods consist of 31.3% sucrose, 26.2% fructose and 42.5% glucose (Danish et al., 2011).

3.2 Regression model and its validation

The complete design matrix with experimental and predicted values of the total sugar and reducing sugar (g/L) are presented in Table 2. Based on CCD and experimental data, the following second order quadratic model equation describing the influence of different considered variables on sugar production was obtained:

$$\text{Total sugar} = 194.10 + 50.33X_1 + 23.02X_2 - 17.52X_1X_2 - 1.24X_1^2 - 16.57X_2^2 + 11.53X_1^2X_2 + 16.66X_1X_2^2 \quad (2)$$

$$\text{Reducing sugar} = 38.02 + 16.34\,X_1 + 6.77\,X_2 - 3.58\,X_1X_2 - 3.80\,X_1^2 + 0.7750\,X_2^2 + 0.8800X_1^2X_2 + 0.8700\,X_1X_2^2 \quad (3)$$

where X_1 and X_2 are temperature and time for thermal extraction method. The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

The validity of the fitted model was evaluated and the statistical significance was controlled by F-test. The ANOVA for the response surface full quadratic model of total sugar and reducing sugar are given in Table 3 and Table 4.

Total Sugar is a measurement of sucrose and reducing sugars (Mustafa et al., 2018). In this study, from the regression model (Y_1) of total sugar, the value of the determination coefficient ($R^2 = 0.9918$) indicates that only 0.82% of the total variations were not explained

by the model. The value of the adjusted determination coefficient (Adjusted R^2 = 0.9888) was also high in supporting high significance of the model. Among the model terms X_1 and X_2 were significant with a probability of 99% (Table 3). The interaction between X_1 and X_2 had significant influence on increase in total sugar in golden rain tree pods.

Reducing sugar is any sugar that is capable of acting as a reducing agent. The most common reducing sugars are glucose, fructose, and galactose as monosaccharides and lactose and maltose as disaccharides (Pratt and Cornely, 2013, Mustafa et al., 2018). From the experiments, the determination coefficient of reducing sugar (Y_2) is R^2 = 0.9981, only 0.19% of the total variations were not explained and the adjusted R^2 = 0.9975 of the model have high significance. The model terms X_1 and X_2 are significant with a probability of 99% (Table 4). The reducing sugar in golden rain tree pods was significantly influenced by the interaction between X_1 and X_2.

Table 2 Experimental design matrix with experimental results and predicted values of total sugar and reducing sugar.

Run	Factor 1 X_1:Temperature (°C)	Factor 2 X_2:Time (min)	Total sugar (g/L)		Reducing sugar (g/L)	
			Actual	Predicted	Actual	Predicted
1	30	20	55.32	57.23	6.44	6.57
2	63	60	205.15	200.55	44.72	45.57
3	30	20	53.9	57.23	6.5	6.57
4	30	60	159.09	161.38	29.33	29.01
5	96	60	256.13	260.31	57.22	56.29
6	96	40	248.28	243.19	50	50.57
7	63	20	159.53	154.50	31.94	32.03
8	96	20	223.28	226.24	48.06	48.14
9	63	40	184.07	194.10	38.33	38.02
10	30	40	148.28	142.53	16.11	17.88
11	96	20	224.75	226.24	48.61	48.14
12	96	60	258.09	260.31	56.67	56.29
13	63	20	160.02	154.50	32.44	32.03
14	63	40	183.58	194.10	38.61	38.02
15	96	60	258.82	260.31	55	56.29
16	30	60	158.95	161.38	29.06	29.01
17	96	20	222.79	226.24	47.78	48.14
18	63	20	159.73	154.50	31.67	32.03
19	30	60	158.21	161.38	28.67	29.01
20	63	40	183.09	194.10	37.22	38.02
21	96	40	248.77	243.19	51.39	50.57
22	30	20	54.58	57.23	6.78	6.57
23	96	40	248.28	243.19	50.28	50.57
24	63	60	206.62	200.55	45.83	45.57
25	63	60	205.64	200.55	46.11	45.57

Appendix Figure 19 Proceeding II-VII

Run	Factor 1 X_1:Temperature (°C)	Factor 2 X_2:Time (min)	Total sugar (g/L)		Reducing sugar (g/L)	
			Actual	Predicted	Actual	Predicted
26	30	40	147.79	142.53	19.17	17.88
27	30	40	147.3	142.53	18.33	17.88

Table 3 ANOVA for reduced cubic model (Equation 2) of total sugar from golden rain tree pods.

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	91888.27	7	13126.90	329.86	< 0.0001	significant
X_1-Temperature	15196.64	1	15196.64	381.87	< 0.0001	significant
X_2-Time	3179.98	1	3179.98	79.91	< 0.0001	significant
X_1X_2	3683.05	1	3683.05	92.55	< 0.0001	significant
X_1^2	9.17	1	9.17	0.2304	0.6367	not significant
X_2^2	1647.61	1	1647.61	41.40	< 0.0001	significant
$X_1^2X_2$	532.15	1	532.15	13.37	0.0017	significant
$X_1X_2^2$	1109.89	1	1109.89	27.89	< 0.0001	significant
Residual	756.11	19	39.80			
Lack of Fit	746.34	1	746.34	1375.13	< 0.0001	significant
Pure Error	9.77	18	0.5427			
Cor Total	92644.38	26				
Std. Dev.	6.31		**R^2**		0.9918	
Mean	182.22		**Adjusted R^2**		0.9888	
C.V. %	3.46		**Predicted R^2**		0.9852	
			Adeq Precision		59.1409	

$p < 0.05$ is considered as significant.

Table 4 ANOVA for reduced cubic model (Equation 3) of reducing sugar from golden rain tree pods.

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	6378.50	7	911.21	1458.72	< 0.0001	significant
X_1-Temperature	1602.63	1	1602.63	2565.58	< 0.0001	significant
X_2-Time	274.86	1	274.86	440.02	< 0.0001	significant
X_1X_2	153.37	1	153.37	245.52	< 0.0001	significant
X_1^2	86.49	1	86.49	138.46	< 0.0001	significant
X_2^2	3.60	1	3.60	5.77	0.0267	significant
$X_1^2X_2$	3.10	1	3.10	4.96	0.0382	significant
$X_1X_2^2$	3.03	1	3.03	4.85	0.0403	significant

Appendix Figure 20 Proceeding II-VIII

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Residual	11.87	19	0.6247			
Lack of Fit	0.0056	1	0.0056	0.0085	0.9274	not significant
Pure Error	11.86	18	0.6591			
Cor Total	6390.37	26				
Std. Dev.	0.7904		**R^2**		0.9981	
Mean	36.01		**Adjusted R^2**		0.9975	
C.V. %	2.19		**Predicted R^2**		0.9962	
			Adeq Precision		115.5776	

$p < 0.05$ is considered as significant.

3.3 Effect of temperature and time on thermal extraction of total sugar and reducing sugar

The response surface plots showed the effect of temperature and time on total sugar and reducing sugar in golden rain tree pods. For total sugar, comparison of the predicted values with the experimentally obtained actual values indicated that these data are in reasonable agreement (Figure 3a). The highest level of total sugar was achieved at higher temperature (96°C) and higher time (60 min) of thermal extraction method (Figure 4a). For reducing sugar obtained and the results indicates that there is good correlation between the actual and predicted values (Figure 3b). The surface plot of reducing sugar indicates that maximum yield of reducing sugar in golden rain tree pods was attained at the higher temperature with higher time (Figure 4b). The minimum yield of total sugar and reducing sugar was obtained under the optimization of thermal extraction conditions of temperature (30°C) and time (20 min) was 53.9 and 6.5 g/L, respectively. For selection of the optimum conditions and range, the models were analyzed separately. The maximum yield of total sugar and reducing sugar was obtained with the optimization of conditions of temperature (96°C) and time (60 min) was 258.82 and 57.22 g/L, respectively. The maximum response predicted from the model was 260.31 and 56.29 g/L. By comparing the experiments the cellulose and hemicellulose yield increased from 53.9 to 258.82 g/L and 6.44 to 57.22g/L, respectively.

The maximum reducing sugar content was achieved at the temperature of 96 °C at time of 60 min. The reducing sugar content of 57.22 g/L indicates that the thermal extraction at 196°C for 60 min effectively increase the reducing sugar content in golden rain tree pods. Therefore, it can be concluded that when the temperature and time of extraction increase, the sugar production is higher, which corresponds to the research of Kuila et al., 2011. They studied the effects of temperature and time of extraction on reducing sugar from cashew apple bagaase, a potential low cost substrate. Response Surface Methodology (RSM) based Box Behnken Design (BBD) was employed to obtain the best possible combination of extraction time (4-8 hours) and extraction temperature (40–60 °C) for maximum reducing sugar extraction. The results showed that reducing sugar increased from 30.89 to 46.02 g reducing sugar per 100 g of dry substrate at 4 hours for 40 °C and 8 hours for 60 °C conditions, respectively (Kuila et al., 2011).

Appendix Figure 21 Proceeding II-IX

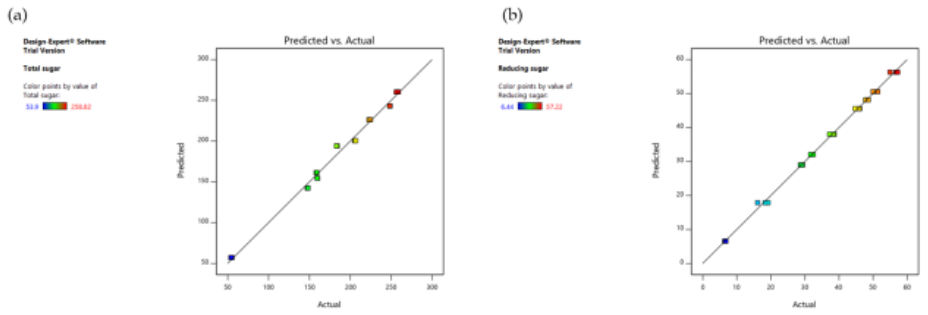

Figure 3 Predicted vs. actual for total sugar (a) and reducing sugar (b).

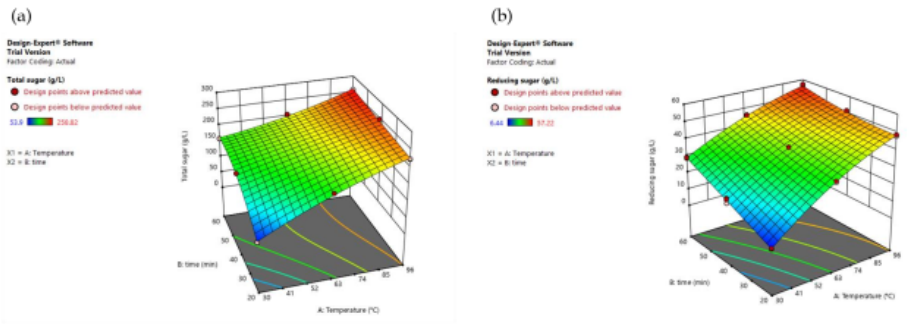

Figure 4 Response surface plot show the interactive effect of temperature and time on total sugar (a) and reducing sugar (b).

3.4 Optimization and verification of model

The optimal conditions for thermal extraction of total sugar and reducing sugar from golden rain tree pods were estimated by using Design Expert software 11.1.0.1 (Stat-Ease Inc., Minneapolis, USA). In order to optimize the independent variables of thermal extraction the following constraints were selected such as temperature (30-96 °C) and time (20-60 min). The independent variables were defined in range and responses were attributed to be maximized or minimized according the maximum desirability functions. The optimized thermal extraction conditions with most of sugar production of golden rain tree pods were obtained as shown in Figure 5. Taking costs and efficiency into consideration, the optimum operating parameters was temperature 96 °C and time 60 min. Under these conditions, the maximum sugar produced was about 260.31 g/L of total sugar and 56.29 g/L of reducing sugar at desirability level of 1.000. Therefore, the optimization of thermal extraction of total sugar and reducing sugar in golden rain tree pods was developed by RSM. Jia et al., (2013) reported that using water extraction method to optimize of sugar in sweet sorghum. Operating parameters investigated include temperature, stalk size, and solid–liquid ratio. The most desirable conditions include 30 °C, 0.6 ratio of solid to liquid (w/w), which collects 90 % of the available sugar. It has reported that the particle size of the feedstock were affects by the extraction. By decreasing the particle size of raw extraction material from 406.33 to 24.93 μm, the extraction yield increased from 15.81% to 20.50%

(Huang et al., 2018). All the method of extraction has prospective to release sugars for biofuel production. But extraction of total sugar and reducing sugar by varying liquid: material, pH, extraction time and extraction temperature is efficient and has numerous advantages. Thus, thermal extraction of total sugar and reducing sugar is more effective in obtaining the sugars, which could be the source of useful biofuel products.

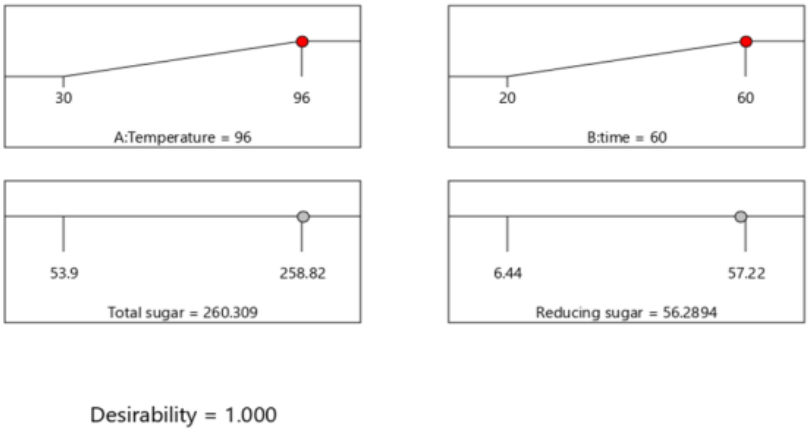

Figure 5 The optimize condition of thermal extraction for golden rain tree pods according to desirability function

4. Conclusions

Analysis of sugar production from golden rain tree pods showed that it contains moisture 4-10 %, total sugar 54.60±0.71 to 257.68±1.39 g/L and reducing sugar 6.57±0.18 to 56.30±1.16 g/L at 30-96 °C and 20-60 min condition. Statistical optimization for thermal extraction of total sugar and reducing sugar was successfully carried out using RSM based on the 2^2 factorial CCD. A total experiment of 27 runs was conducted to study the effect of variables. All the responses were significantly affected by the independent factors. When the temperature and time of thermal extraction increase, the total sugar and reducing sugar are increased. The optimum conditions for extraction of sugar were determined as extraction time 60 min and temperature 96 °C. Under these optimum operating conditions the maximum sugar production was recorded as 260.31 g/L of total sugar and 56.29 g/L of reducing sugar.

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Appendix Figure 24 Proceeding II-XII

Appendix Figure 25 Certificate I

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