EXTRACTIVE FERMENTATION OF ETHANOL FROM FRESH CASSAVA ROOTS USING VACUUM FRACTIONATION TECHNIQUE

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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การวิจัยนี้ได้ทำการศึกษาและผลิตเอทานอลจากหัวมันสำปะหลังสดด้วยการหมักแบบดั้งเดิม แบบ simultaneous saccharification and fermentation (SSF) และแบบ simultaneous liquefaction saccharification and fermentation (SLSF) โดยเน้นการสกัดเอทานอลความบริสุทธิ์สูงจากน้ำหมักด้วยเทคนิคการกลั่นลำดับส่วนแบบสูญญากาศและวิเคราะห์ประสิทธิภาพของการใช้พลังงานของระบบ พบว่าในระบบการหมักแบบแยกสกัดจากถังหมัก ด้วยวิธี SLSF ในระยะเวลาดำเนินการ 48 ชั่วโมงนั้น เอทานอลจะถูกกลั่นอย่าง ต่อเนื่อง มีความบริสุทธิ์ถึง 91 เปอร์เซ็นต์โดยน้ำหนัก ขณะเดียวกัน ปริมาณ เอทานอลในถังหมักมีความเข้มข้นคงที่ ที่ 2 เปอร์เซ็นต์โดยน้ำหนัก จากการศึกษานี้พบว่าการยับยั้งผลิตภัณฑ์ต่อเซลล์ยีสต์นั้นลดลงด้วย เมื่อสิ้นสุดกระบวนการ มีเซลล์เหลือรอด เหลือในถังหมักถึง 40% การใช้พลังงานในการดำเนินการหมักพบว่าการหมักแบบ SSF ใช้พลังงานไป 103.24 kWh ในขณะที่การหมักแบบ SLSF ใช้พลังงานเพียง 78.9 kWh และการศึกษามันสำปะหลังสดบดด้วยการ pre-treatment เพื่อเพิ่มประสิทธิภาพการหมักในผลิตเอทานอล หัวมันสดบดแบบ Very High Gravity (VHG) มีปริมาณของแข็งละลายอยู่ในปริมาณมาก (>30%) ได้ผ่าน การ pre-mashing เพื่อลดความหนืดก่อนเข้าสู่กระบวนการ liquefaction กล้องจุลทรรศอิเล็กตรอนแบบส่องกราดสามารถ ยืนยันว่า pre-mashing ทำให้ปริมาณ lignocellulosic ลดลงจากการย่อยของเอนไซม์ เซลลูเลส ในกระบวนการหมักแบบ repeated-batch เอทานอลถูกสกัดออกจากน้ำหมักอย่างต่อเนื่องจากระบบมีความเข้มข้นสูงถึง 90 เปอร์เซ็นโดยน้ำหนัก โดยใช้ปริมาณน้ำตาลรีดิวซ์เริ่มต้นที่ 250 g/L และผลการศึกษากิจกรรมของเอนไซม์ในการย่อย เพื่อให้ได้น้ำตาลรีดิวซ์ พบว่าเอนไซม์ ผสมที่ประกอบด้วยเอนไซม์เซลลูเลส แอลฟาอะไมเลสและกลูโคอะไมเลส สามารถผลิต น้ำตาลรีดิวซ์สูงสุดถึง 52.23 g/L และยังให้ปริมาณเอทานอลสูงสุดด้วย ส่วนปริมาณ ไดแอมโมเนียมฟอสเฟตที่เติม เพื่อเป็นแหล่งไนโตรเจน ที่เหมาะสม เท่ากับ 1.5 % และยังพบว่าการให้คลื่นเสียงความถี่สูง (sonicated) แก่หัวมันสดบดก่อนเข้าสู่กระบวนการหมักมีผลทำให้มีปริมาณเอทานอลสูงกว่าการไม่ให้คลื่นเสียง

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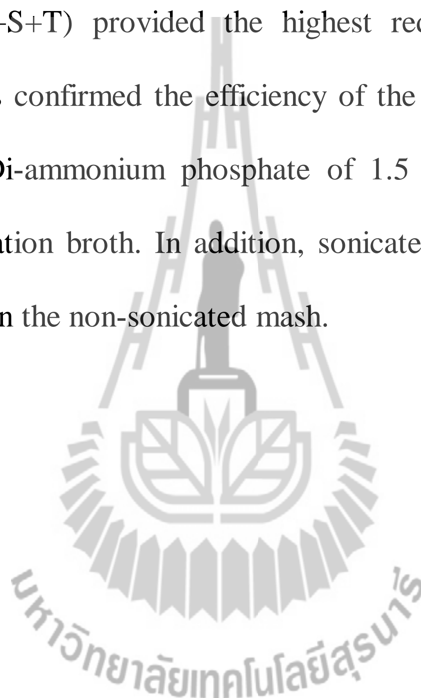
ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา_______________

SUPATHRA PHAKPING : EXTRACTIVE FERMENTATION OF ETHANOL FROM FRESH CASSAVA ROOTS USING VACUUM FRACTIONATION TECHNIQUE. THESIS ADVISOR : ASST. PROF. APICHAT BOONTAWAN, Ph.D., 83 PP.

ETHANOL/EXTRACTIVE FERMENTATION/VACUUM FRACTIONATION

Fresh cassava roots were used as a raw material for ethanol fermentation. The root was fermented into ethanol in a conventional process, simultaneous saccharification and fermentation (SSF), simultaneous liquefaction saccharification and fermentation (SLSF). This research focused on the extract of high purity ethanol from fermentation broth using a vacuum fractionation technique. The effectiveness of the system in terms of energy consumption was also analyzed. The ethanol vapor was fractionated to approximately 91 wt% leaving the system with bacth extractive fermentation in SLSF mode. Vacuum fractionating technique was successfully introduced to simultaneously remove high purity ethanol from fermentation broth whilst its concentration in the bioreactor was kept constant at 2 wt% throughout the 48 h of operation. The product inhibition effect was also reduced as cell viability was 40% at the end of the process. For energy consumption, SSF required approximately 103.24 kWh, while SLSF of the same system required only about 78.9 kWh. Fresh cassava mash was pretreated to improve ethanol concentration in the fermentation process. Very High Gravity (VHG) processes had very high soluble solid contents (>30%) in the cassava mash. Pre-mashing to reduce viscosity was required prior to enter the liquefaction process. Scanning electron microscope was used to confirm the

importance of pre-treatment step. After treatment with cellulase enzymes, the breakdown of lignocellulosic biomass was clearly observed. The optimum initial reducing sugar concentration of approximately 250 g/L was chosen for repeated-batch extractive fermentation experiment. Approximately 90 wt% produced ethanol was continuously fractionated from the system. Enzymatic activities liberated reducing sugar for 36 h of incubation time. The enzyme mixture of cellulase, alpha-amylase and glucoamylase (ST+V+S+T) provided the highest reducing sugar concentration of 52.23 g/L. The results confirmed the efficiency of the mixture enzyme on maximized ethanol production. Di-ammonium phosphate of 1.5 % was suitable as a nitrogen source in the fermentation broth. In addition, sonicated mash of cassava gave higher ethanol production than the non-sonicated mash.



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Academic Year 2013 Advisor’s Signature____________________

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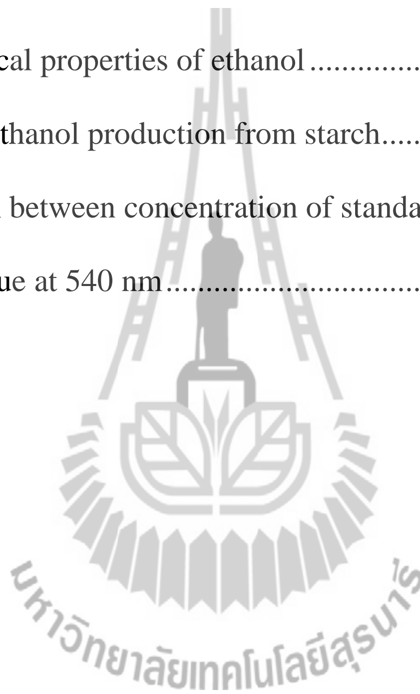

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LIST OF ABBREVIATIONS

CF = Conventional fermentation

Cm = Centimeter (s)

C = Degree Celsius

DAP = Di-ammonuim phosphate

DNS = Dinitrosalicylic acid

FBG = Fungal Betaglucanase Units

FID = Flame ionization detector

g = Gram (s)

GC = Gas chromatography

GSHU = Granular starch hydrolyzing unit

g/L = Gram (s)per liter

g/L/h = Gram (s) per liter per hour

h = Hour (s)

HPLC = High performance liquid chromatography

Kg = Kilogram (s)

kWh = Kilowatt-hour

L = Liter (s)

mg = Milligram (s)

min = Minute (s)

mL = Milliliter (s)

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LIST OF ABBREVIATIONS (Continued)

mm	=	Millimeter (s)
mM	=	Millimolar
mmHg	=	Millimatre (s) of mercury
mPa.s	=	The millipascal-seconds
nm	=	Nanometer (s)
No.	=	Number
PSI	=	Pound square per inch
%	=	Percent
%wt	=	Percent by weight
rpm	=	Round per minute
SEM	=	Scaning Eletron Microscopy
SLSF	=	Simultaneous liquefaction saccharification and fermentation
SSF	=	Simultaneous saccharification and fermentation
TSR	=	Total reducing sugar
U.S.A.	=	United States of America
VHG	=	Very high gravity
v/v	=	volume by volume
w/v	=	weight by volume
w/w	=	weight by weight
YM	=	Yeast and malt extract medium

CHAPTER I

INTRODUCTION

1.1 Rational and background

With the increasing demands of fossil fuel, renewable energy has been considered for the development of cleaner fuels, more reliable fuels will result in a reduction of the dependency on fossil fuels. Currently, bio-ethanol is one of the most viable options of non-conventional sources of energy. Refining ethanol from biomass materials is a suitable selection in order to increase energy security. In addition, the use of fuel ethanol could help to reduce the toxic exhaust emissions and greenhouse gases from vehicles. In Thailand, the main raw materials used for ethanol production are sugarcane molasses and cassava, which are used as substrates for many industries as well. Regarding the 15 year plan and target of biofuel development (year 2008–2022) of the Thai Government, ethanol production is set at 3, 6.2 and 9.0 M l/day for short-term (by 2011), medium-term (by 2016) and long-term (by 2022), respectively (Silalertruksa and Gheewala, 2010).

Cassava or tapioca (*Manihotes culenta* Crantz) is the third most important crop in Thailand after rice and sugar-cane (total production of 24, 47 and 16 $\times 10^6$ t with the farm value of 4320, 630 and 520 million US $ for rice, sugarcane and cassava, respectively in 1998)(Srirotha *et al.*, 2000). Cassava was introduced to Southern part of Thailand from Malaysia during the period 1786 to 1840, after this time it was gradually distributed throughout the country. This crop is now mainly cultivated in

the Northeast, primarily in Nakhon Ratchasima (57% of total root production), followed by the Central plains (31% of total root production) (OAE, 2006).

Typical fermentation broth contains low levels of ethanol; therefore, much of the energy consumption for commercial ethanol production is for distillation. The large amount of water carried through the process results in expensive process equipment and high construction cost. In addition, subsequent purification of ethanol is distillation which also requires considerable energy to achieve a high purity ethanol. In general, it is almost impossible to operate distillation in a small scale, because the number of active plates of the fractionating column must be more than 70 plates in order to achieve 95.6 wt% of ethanol. Significant energy savings can thus be achieved if ethanol-rich fermentation broth is used. However, the growth and production ability of cells are inhibited by high ethanol and/or sugar concentration. These constraints severely hinder the development of ethanol production as a cheap renewable source for biofuel. Recently, high ethanol concentration in fermentation broth has been achieved in commercial scale, with final ethanol concentration of 10–12% by volume (Wang *et al*., 2000). This requires fermentation at high sugar concentration, which has been developed at both laboratory and industrial scales. However, fermentation using high initial sugar concentration, especially in continuous process, results in product and substrate inhibition. For ethanol fermentation, the process is severely limited by the high toxicity of the ethanol product. The major reason is because the fermenting microorganism, usually *Saccharomyces cerevisiae*, cannot tolerate more than approximately 12 wt% of ethanol (Wang *et al*., 2000). To prevent product inhibition, ethanol product must be removed from the fermentation broth as soon as it is formed. Simultaneous removal of ethanol stimulates the growth

of yeast cells; thus, more sugars can be fermented and higher ethanol productivity was achieved consequently.

Recently, a high efficiency small scale fractionating column was successfully developed at SUT. Fractionation is based on a partial condensation concept of binary mixture (Pimkaew and Boontawan, 2010). The design is very unique in terms of the column internal, and distillation performance. When operate together with the fermentation under the vacuum pressure, it is an interesting technique to continuously obtain a high purity of ethanol. This could result in maximizing the yield, and volumetric productivity by avoid the inhibitory effect of the ethanol to the yeast cells as well as a very high gravity fermentation technology. In addition, the consequence of this operation is also to obtain a very high purity of ethanol which can be directly supplied to dehydration process without further distillation. High solid concentration batch fermentations using fresh cassava roots were investigated. Pre-mashing step was initially attempted and viscosity reduction was investigated by using a cell wall degrading enzyme. Subsequently, vacuum fractionation in batch mode was attempted and was compared with conventional batch fermentation. Finally, extractive fermentation using vacuum fractionation technique was investigated in different operation modes including conventional batch extractive fermentation (CF), simultaneous saccharification and fermentation (SSF), and simultaneous liquefaction saccharification and fermentation (SLSF), respectively.

1.2 Research objectives

This research aims to develop and optimize the extractive fermentation process, which used fresh cassava roots as a major starch source in order to increase the

ethanol production, and reduce the cost of energy consumption, with sub-objectives as follow;

1.2.1. To extract a high purity ethanol from fermentation broth using a vacuum fractionation technique.

1.2.2. To compare volumetric productivity and ethanol yield between extractive fermentation and conventional fermentation methods.

1.2.3. To analyze the effectiveness of the system in terms of energy consumption.

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CHAPTER II

LITERATURE REVIEWS

2.1 Cassava

2.1.1 Cassava in Thailand

Cassava (*Manihotes culenta*), sometimes also called manioc, is the third largest source of carbohydrates for human consumption in the world, with an estimated annual world production of 208 million tones. Africa is the largest center of cassava production. It is grown on 7.5 million hectares and produces about 60 million tons per year. It is a major source of low cost carbohydrates, and also a staple food for 500 million people in the humid tropics (Kuiper *et al*., 2007). On places where cultivation of other crops is difficult, unless considerable inputs are applied, cassava still has a reasonable yield

Figure 2.1 Cultivation of Cassava

Source: Kuiper *et al.* (2007)

The plant grows tall, some reaching 15 feet, with leaves varying in shape and size (Figure 2.1). The edible parts are the tuberous root and leaves. The tuber (root) is somewhat dark brown in color, and grows up to 2 feet long. The crop is highly efficient in producing starch, year-round available, tolerant to extreme stress conditions, and fits nicely within traditional farming systems. Fresh roots contain approximately 30 wt% starch. Cassava starch is one of the best fermentable substances for the production of ethanol. At the moment, sugar cane is the most widely used crop for bio-ethanol in tropical area. However, sugar cane requires a lot of water for growth. Consequently, sites suitable for growing sugar cane are very limited (and on most of them sugar cane plantations have ready been established). A much larger area in the Tropics is available and suitable for cassava.

Table 2.1 Chemical compositions of cassava flour.

Composition	Chemicals % (dry matter)
Moisture	6.12 ± 0.07
Protein	0.77 ± 0.01
Starch	89 ± 0.20
Fat	0.14 ± 0.01
Fiber	1.85 ± 0.10
Ash	1.54 ± 0.01

Source: Labua (2011)

The chemical composition of cassava flour is shown in Table 2.1. The most abundant component is starch at 89%. Moisture, protein, fat and fiber content

are 6.12%, 0.77%, 0.14% and 1.85%, respectively. It shows that the cassava root contains high level of starch but low levels of protein and moisture.

In fresh cassava root, the water content varies from 48 % (bitter cassava) to 69 % (sweet cassava). The carbohydrate content is about 30%. Starch is the dominant fraction of carbohydrates; it represents 85% of the total carbohydrate.

Figure 2.2 Cassava Root.

Source: Kuiper *et al*. (2007)

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Cassava starch may be produced from fresh roots, by grating the roots, mixing with water, followed by sedimentation and sun-drying or by conductive heating. The strong increased demand for cassava starch has led to a modern starch manufacturing process, in which the processing time from the grating of fresh roots to dried starch is less than 30 minutes. About 4.8 tons of fresh roots produce one ton of dry starch. 40 percent of the cassava starch produced in Thailand is used domestically (800,000 tonnes) and 60 % is exported by the Thai Tapioca Flour Industries Association. In 2004, about 1.77 million tons of starch was exported. Of various cassava-based products, mainly cassava starch and pellets are exported. In the future,

starch exports are expected to increase in volume due to the international starch market expansion.

In Asia, Thailand leads the way in the production of starches derived from cassava. Cassava starch has unique properties, such as its high viscosity and its resistance to freezing, which make it competitive with other industrial starches. Cassava is a starch-

containing root crop, and is one of the most important sources of calories in the tropics. Cassava is also widely employed as a raw material for many industrial applications in the animal feed industry and starch industry, and more recently for production of ethanol. Cassava can be cultivated on arid and semiarid land where other crops, such as corn, do not thrive (Lin *et al*., 2011). Cassava is a root crop that produces high yield with little input. Yield as high as 45 tons/hectare has been reported (Ogbonna and Okoli, 2010). Cassava pulp contains about 50%–70% starch on a dry weight basis and 20%–30% fibers, which are composed mainly of cellulose and other non-starch polysaccharides (Rattanachomsri *et al*., 2009).

In Thailand, ethanol production from cassava will not necessarily cause an over demand to the existing cassava industries, since the starch industry is not expected to grow much further and the pellets industry will decline somewhat. At an annual production of 20 million tons fresh roots in 2005, there was 4 million tons of roots available as a surplus to the feed industry and to make ethanol. The Thai agricultural policy strives to increase yields without increasing the area planted with cassava (which is now restricted to 1 million hectare). Due to continuous research and development on cassava variety improvement and cropping efficiency, Thailand has

been able to increase cassava yields from 13 tons/hectare in 1995 to an average of 17.2 tons/ hectare in 2005 (Kuiper *et al*., 2007).

The major cassava producers are located in three continental regions which are Nigeria, Brazil and Thailand, accounting approximately for 20, 11 and 12% of total world production, respectively. In the last two decades, the world production of cassava continuously increases, as primarily driven by the market demand, in particular an expansion of global starch market. The growth rate of root production in the last decade (2000-2009) is even greater than the previous one (1990-1999) due to markedly rising demand of cassava for bio-ethanol production in Asia especially in China and Thailand. Interestingly, the root productivity of cassava has been dramatically increased in some countries including Vietnam, India, Indonesia and Thailand by 8.46, 7.46, 6.22 and 5.85 tons/hectare in the past 10 years. The root productivity of India is the greatest (34.37 tons/hectare), followed by Thailand (22.68 tons/hectare) (Food and Agriculture Organization [FAO], 2011).

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2.1 Fuel-Ethanol

The use of ethanol in the internal combustion engine (ICE) began in 1897 by Nikolas Otto (Rothman, 1983). Alcohols have been used as fuels since the inception of the automobile. Fuel ethanol blends are successfully used in all types of vehicles and engines that require gasoline (Balat, 2005). Ethanol is made from a variety of products such as grain, molasses, fruit, cobs, and shell. Its production, excluding that of beverages, has been declining since the 1930s because of the lower cost of petroleum fuels (Akpan *et al*., 2005). With the oil crises of the 1970s; however, ethanol became established as an alternative fuel (Balat, 2005), and the demand of

fuel ethanol significantly increased in the last few years due to the latest oil crisis in 2007. Recently, ethanol has been considered as a potential alternative fuel in many countries especially Thailand.

Ethanol can be produced by either biological or chemical synthesis. Bio-ethanol (ethyl alcohol, $CH_3–CH_2–OH$ or EtOH) has a higher octane number, broader flammability limits, higher flame speeds and higher heats of vaporization than gasoline. These properties allow for a higher compression ratio, shorter burn time and leaner burn engine, which lead to theoretical efficiency advantages over gasoline in an internal combustion engine (Balat *et al.*, 2005). However, disadvantages of bio-ethanol include its lower energy density than gasoline (bio-ethanol has 66% of the energy that gasoline has), its corrosiveness, low flame luminosity, lower vapor pressure (making cold starts difficult), miscibility with water, and toxicity to ecosystems (MacLean and Lave, 2003). Physical properties of alcohol fuels are shown in Table 2.2.

Table 2.2 Some properties of alcohol fuels.

Fuel property	**Isooctane**	**Methanol**	**Ethanol**
Cetane number	-	5	8
Octane number	100	112	107
Auto-ignition temperature (K)	530	737	606
Latent heat of vaporization (MJ/Kg)	0.26	1.18	0.91
Lower heating value (MJ/Kg)	44.4	19.9	26.7

Table 2.3 Physico-chemical properties of ethanol.

Properties	Value
Empirical formula	CH_3CH_2OH
Molecular weight	46
Normal boiling point, °C	78.32
Critical temperature, °C	243.1
Density, d_4^{20}, g/ml	0.7893
Heat of combustion at 25°C, KJ/g	29.67
Auto ignition temperature, °C	793.0

Source: Najafpour and Lim (2002)

In some areas, ethanol is blended with gasoline to form an E10 blend (10% ethanol and 90% gasoline) it can also be used in higher concentration such as E85 or E95. Historically, due to high feedstock prices and competition from other products for its gasoline uses, the economics of the production of this renewable fuel have been marginal for many manufacturing facilities. Improvements of the fuel ethanol production process resulting in even 2-5 cents per gallon could significantly increase its demand. From a technical point of view, one approach to process improvement would be the conversion of the traditional batch to one based upon a truly continuous fermentation (O'Brien *et al.*, 1999). Table 2.3 shows physico-chemical properties of pure ethanol.

2.3 Fermentation of Ethanol

Fermentation is used to make a variety of products, including foods, flavorings, beverages, chemicals, and pharmaceuticals. At present; however, many of the simpler products such as ethanol are synthesized from petroleum feedstock at lower costs. The future of the fermentation industry, therefore, depends on its ability to utilize the high efficiency and specificity of enzyme catalysis to synthesize complex products and on its ability to overcome variations in quality and availability of raw materials.

Fermentation processes from several materials that contain sugar can produce ethanol. The different raw materials used in the manufacture of ethanol via fermentation are conveniently classified under three types of agricultural raw materials: sugar, starches, and cellulose materials. Sugars (from sugar cane, sugar beets, molasses, and fruits) can be converted to ethanol directly. Starches (from grains, potatoes, root crops) must be firstly hydrolyzed to fermentable sugars by the action of amylase enzymes from malt or molds. Cellulose from wood, agricultural residues, waste sulfite liquor from pulp and paper mills) must likewise be converted to sugars, generally by the action of mineral acids or cellulase enzymes. Once simple sugars are formed, enzymes from yeast can readily ferment them to ethanol (Yan and Shuzo, 2005).

Reaction: $C_6H_{12}O_6$ -------------> $2C_2H_5OH + 2CO_2$ + energy

Ethanol production process is operated under anaerobic conditions where glucose is converted to pyruvic acid via the glycolysis pathways. Fermentation allows the yeast to continue the production of energy and survive in the absence of oxygen,

producing ethanol and carbon dioxide from pyruvate. Moreover, glycolysis serves as the only net producer of ATP from the whole process (Hofmeyr, 1997). The step of glycolysis pathway starts from adding two phosphate groups from ATP, then splitting into two 3-carbon molecules as glyceraldehyde-3-phosphate before it is subsequently oxidized. Since one molecule of glucose produces two 3-C molecules, total yield at this stage is 4 ATPs. The resulting 3-C molecule is pyruvate (Voet, 1995). When oxygen is available, the pyruvate can be converted to acetyl-CoA and enter the Krebs cycle, where the acetyl-CoA will be completely oxidized and generate ATP through oxidative phosphorylation. However, fermentation is much less efficient than oxidative phosphorylation in making ATP, creating only 2 ATP whilst oxidative phosphorylation creates 36 ATP per one molecule of glucose. In conclusion, oxidative phosphorylation does not occur in the absence of oxygen, and yeast gains ATP under anaerobic condition via glycolysis of glucose only. Figure 2.3 shows the Embden-Meyerhof pathway of ethanol production from glucose.

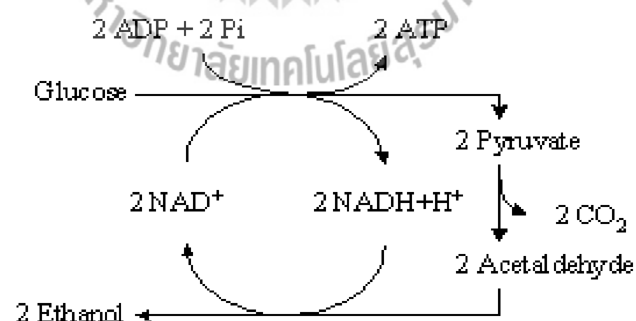

Figure 2.3 Embden-Meyerhof pathway.

Source: Bailey and Ollis (1986)

2.4 Fermentation Kinetics

More than a decade, fermentation kinetics of ethanol produced from glucose by using *Saccharomyces cerevisiae* has been studied. It was occurred to develop a fermentation model as incorporating the effects of substrate inhibition, and product inhibition (Krishnan *et al*., 1999).

2.4.1. Substrate Inhibition Kinetics

Model for substrate inhibition kinetics was typically modified Monod form expressions (Mulchandani and Loung, 1989). The following expressions were used to account for the effect of the substrate inhibition on cell growth and ethanol production using glucose as substrate (Krishnan *et al*., 1999):

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{\mu_m S}{K_s + S + S^2 / K_i} \quad (1)$$

$$\nu = \frac{1}{X}\frac{dP}{dt} = \frac{\nu_m S}{K_s' + S + S^2 / K_i'} \quad (2)$$

Where K_s = Monod constant for growth (g/L)

K_s' = Monod constant for product formation (g/L)

K_i = Inhibition constant for growth (g/L)

K_i' = Inhibition constant for product formation (g/L)

S = Substrate concentration (g/L)

P = Ethanol concentration (g/L)

X = Cell dry weight (g/L)

μ = Specific growth rate (h^{-1})

μ_m = Maximum specific growth rate (h^{-1})

ν = Specific rate of product formation (h^{-1})

2.4.2. Product Inhibition Kinetics

In addition, the correlations for modeling the inhibitory effects of ethanol on cell growth and fermentation as include exponential, linear, hyperbolic, parabolic, and nonlinear models (Uden, 1989) have been proposed in the literature. However, it has a two-constant model (Luong, 1985) is used to describe the kinetic pattern of ethanol inhibition on glucose fermentation. The model consists of the following expressions:

$$\frac{\mu}{\mu_0} = 1 - \left(\frac{P}{P_m}\right)^{\beta} \tag{3}$$

$$\frac{\nu}{\nu_0} = 1 - \left(\frac{P}{P_m^{'}}\right)^{\gamma} \tag{4}$$

Where P_m = Ethanol concentration above which cells do not grow (g/L)

$P_m^{'}$ = Ethanol concentration above which cells do not produce ethanol (g/L)

μ_0 = Specific growth rate when no initial ethanol is present (h^{-1})

ν_0 = Specific rate of product formation when no initial ethanol is present (h^{-1})

β, γ = Constants in product inhibition model (dimensionless)

The magnitude of the constant β indicate the relationship between μ and P, where as the magnitude of the constant γ indicate the relationship between v and P

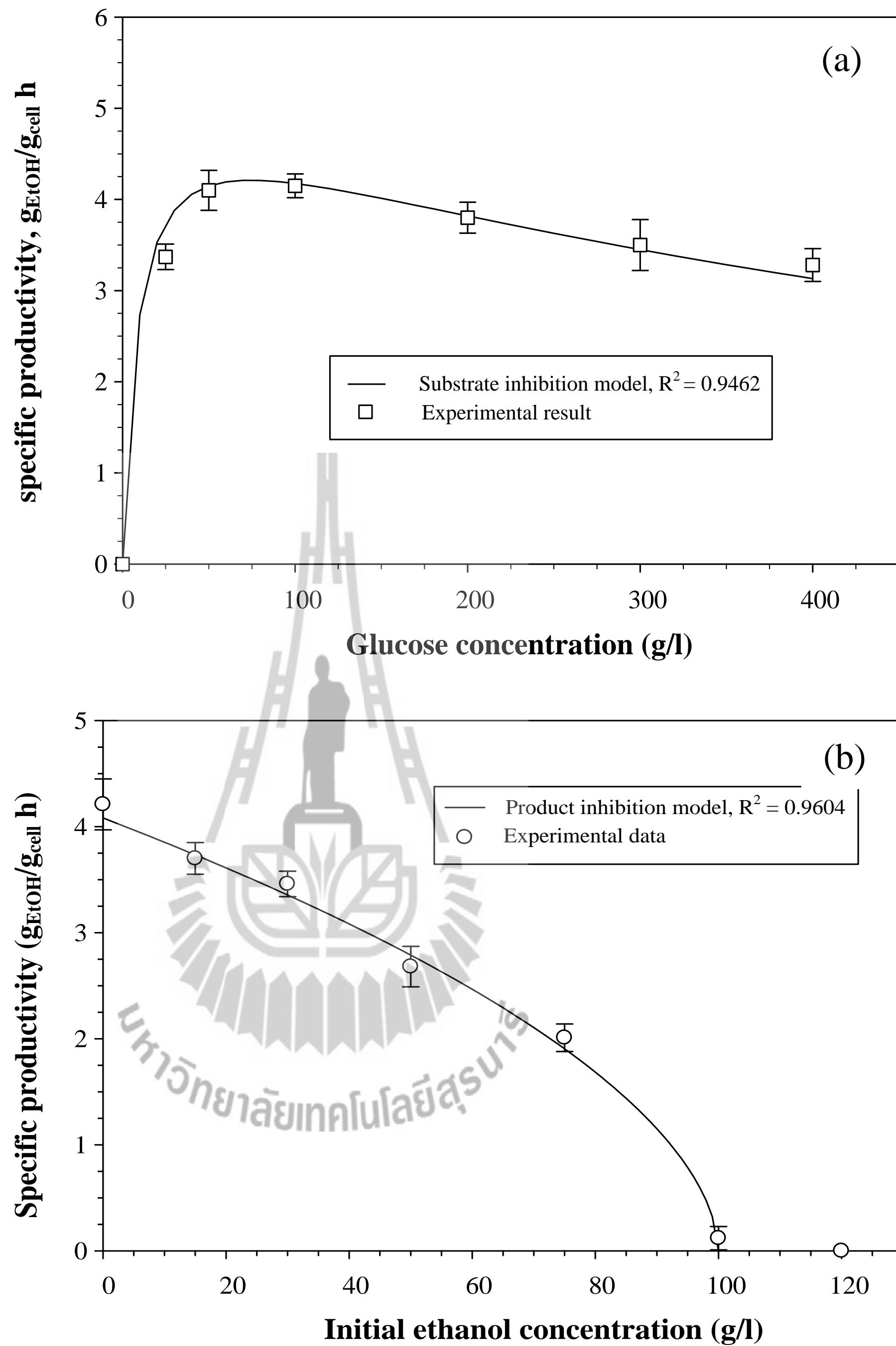

Figure 2.4 Variation of the specific productivity as a function of the substrate (a), and initial ethanol concentration (b).

Source: Samnaknit *et al* (2012)

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Substrates and product inhibition kinetics were independently investigated in order to understand the effect of each compound on fermentation performance. For the substrate inhibition kinetic, the experimental results and mathematical modeling of specific ethanol productivity are presented in Figure 2.4 (a) as a function of initial glucose concentration ranging from 0-400 g/L. The values were determined by plotting ethanol concentration versus time and yeast concentration. The period for rapid increasing of ethanol concentration was considered. The mathematical modeling for substrate inhibition on specific ethanol productivity showed a good agreement with the experimental result. The simulation result showed that the glucose inhibition effect on specific ethanol productivity was weak, partly due to the high yeast concentration used in fermentation process. The highest value of $4.08 g_{EtOH}/g_{cell}$ h was observed at glucose concentration of 100 g/L. The value was slowly decreased with the increasing glucose concentration. The saturation constant (K'_S) and the substrate inhibition constant (K'_i) of 8.92 and 620.71 g/L were reported. The high value of K'_i implying that fermentation can be carried out at high substrate concentration. The optimum glucose concentrations were suggested between 200-300 g/L.

In contrary to substrate inhibition, the product inhibition effect of ethanol to fermentation performance was very sensitive. The specific productivity was investigated at various initial ethanol concentrations ranging from 0-120 g/L whilst the initial glucose concentration was fixed at 100 g/L. The specific ethanol productivity as a function of initial ethanol concentrations was shown in Figure 2.4 (b). Experimental data confirmed that ethanol plays an important role on fermentation performance even at low concentrations. The maximum specific ethanol productivity

was observed when none of ethanol was presented in the system. The value constantly decreased with the increase of initial ethanol concentration. At 75 g/L, the value reduced to approximately 50% and the value rapidly decreased to zero when the concentration approached 100 g/L. Almost no glucose consumption was also observed at this initial ethanol concentration. At 120 g/L ethanol concentration, there was no productivity and the experiment was not investigated beyond this concentration. The critical ethanol concentration (P'_m) refers to the concentration at which the fermentation performance is severely hampered. Therefore, the concentration of 100 g/l was set as P'_min the Equation (4). The correlation was best fitted with a γ value of 0.56 (R^2 = 0.9604). This model can be used to predict the inhibitory effect in a wide range of ethanol concentrations (Samnaknit *et al*, 2012).

2.5 *Insitu* Product Removal

In situ product removal (ISPR) methodologies are a family of techniques in which a target molecule in the bio-fermentation is removed as it is synthesized during at least a portion of the biofermentation process (reviewed in Chauhan *et al*., 1997 and Freeman *et al*., 1993). Since a variety of separation principles can be used for ISPR, including those based on different volatility, solubility, size, density, charge, or specific elements (or combinations of these methods), ISPR techniques have wide applicability. A number of ISPR techniques have been reviewed (Park and Geng, 1992). These include fermentation under vacuum (Cysewski and Wilke, 1997), Pervaporation (Müller and Pons, 1991; Shabtai *et al*., 1991), liquid-liquid extraction (Matsumura and Märkl, 1984; Kühn, 1980), perstraction (Christen *et al.,* 1990), solid adsorbents (Lencki*et al*., 1983), and stripping (Walsh *et al*., 1983; Pham *et al*., 1989).

2.5.1 Vacuum fermentation

Vacuum fermentation technique was used to remove fermentation product that are more volatile than water, and to avoid the inhibitory effect on product. This is accomplished by creating a vacuum in the fermentor headspace, as shown in Figure 5. A pressure is chosen such that the fermentation broth boils at the fermentation temperature. The vapor is produced by boiling and then removed to the vacuum compressor is more concentrate in ethanol (Bazue, 1975).

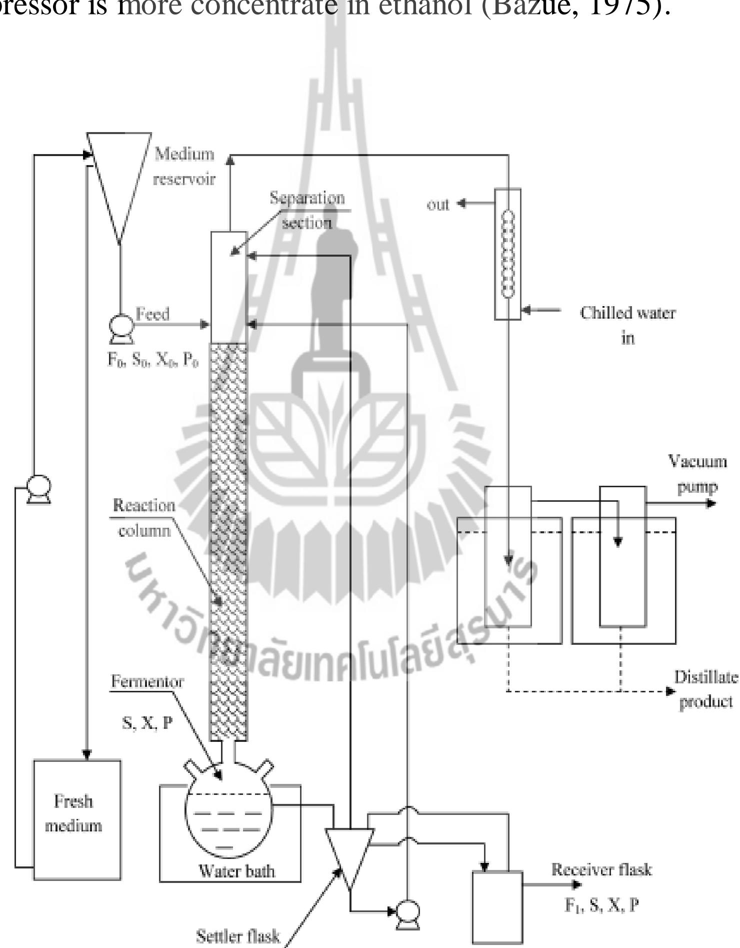

Figure 2.5 Vacuum fermentation integrated with separation process.

Source: Nguyen (2011)

Nguyen *et al.* (2011) studied continuous fermentation integrated with separation process at atmospheric and vacuum pressures as shown in Figure 2.5. An initial glucose concentration of 200 g/L was used to produce yeast cells under batch operating mode. After a period of 18–20 h, a medium of 350 g/L glucose concentration was fed continuously to the fermentation-separation column. During the continuous experiments, fermented broth was re-circulated back to the fermentation-separation column. At atmospheric pressure, the dry mass concentration decreased rapidly and remained at 2.4 g/L after long period, while glucose concentration still remained at 240 g/L. On the other hand, at vacuum pressure, a quasi-steady state condition was reached after 96 h, corresponding to 4.2 g/L of cell dry mass, 165 g/L of glucose and 44.2 g/L of ethanol levels in the fermentation broth and 33.2 wt% of ethanol concentration at the outlet. These results indicate reduced product and substrate inhibition, suggesting that the integrated process could be used to replace the first bioreactor in a fermentation cascade system. A mathematical model was then developed and used to predict the kinetic parameters. The simulation results agreed well with experimental data.

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2.5.2. Solvent Extraction

Solvent extraction is a method to separate compounds based on their relative solubilities in two different immiscible liquids, usually water and an organic solvent. It is an extraction of a substance from one liquid phase into another liquid phase. Liquid–liquid extraction is a basic technique in chemical laboratories, where it is performed using a separatory funnel. This type of process is commonly performed after a chemical reaction as part of the work up. The term partitioning is commonly used to refer to the underlying chemical and physical processes involved in liquid–

liquid extraction but may be fully synonymous. The term solvent extraction can also refer to the separation of a substance from a mixture by preferentially dissolving that substance in a suitable solvent. In that case, a soluble compound is separated from an insoluble compound or a complex matrix. Offeman (2008) studied extraction of ethanol with higher alcohol solvents and their toxicity to yeast. In a solvent extraction screening study, several alcohols show improved extractive performance to recover ethanol from aqueous solutions compared to commonly studied solvents such a solely alcohol and 1-dodecanol.

2.5.3. Pervaporation

Pervaporation processes can be coupled with fermentation to remove continuously the inhibitory products from the fermentation broth. Pervaporation is a membrane separation process based on the difference in solubility and diffusivity of the components to be separated through a dense membrane and it is not limited by the relative volatility of the components as in distillation processes. Pervaporation processes have a high potential in biotechnology and food industries for the recovery and concentration of products of high quality. By using ethanol-selective hydrophobic membranes, it is possible to produce more concentrated bio-ethanol from fermentation broths. O'Brien *et al.* (2004) studied ethanol recovery from corn fiber hydrolysate fermentations by pervaporation corn fiber, a byproduct of corn wet milling, is an attractive feedstock for biomass ethanol production. Corn fiber was hydrolyzed by dilute sulfuric acid and neutralized by the anion exchange neutralized (AEN).Coupling of a membrane pervaporation unit to a fed-batch fermentation of AEN hydrolysate maintained the ethanol concentration below 25 g/L with complete

sugar utilization. A concentrated ethanol stream of 17 wt% ethanol was produced by the pervaporation unit.

2.5.4. Stripping

Stripping is a physical separation process where one or more components are removed from a liquid stream by a vapor stream. In industrial applications the liquid and vapor streams can have co-current or countercurrent flows. Stripping is usually carried out in either a packed or trayed column. Stripping works on the basis of mass transfer. The idea is to make the conditions favorable for the component, A, in the liquid phase to transfer to the vapor phase. This involves a gas-liquid interface that A must cross. The total amount of A that has moved across this boundary can be defined as the flux of A.

Taylor *et al.* (2010) removed ethanol from the fermenter during fermentation can increase productivity and reduce the costs for dewatering the product and co product. The approach was to recycle the fermenter contents through a stripping column, where a non-condensable gas removes ethanol to a condenser. A pilot plant, that continuously fed corn meal at more than one bushel (25 kg) per day, was operated for 60 consecutive days The result can converting 95% of starch and producing 88% of the maximum theoretical yield of ethanol.

2.6 Ethanol Distillation

Distillation is defined as a process in which liquid or vapor mixture of two or more substances is separated in to its component fractions of desired purity, by the application and removal of heat. Distillation is based on the fact that the vapor of a boiling mixture will be richer in the components that have lower boiling points,

therefore when this vapor is cooled and condensed, the condensate will contain more volatile components. At the same time, the original mixture will contain more of the less volatile material.

Distillation columns are made up of several components, each of which is used either to transfer heat energy or enhance material transfer. A typical distillation contains several major components:

I. A vertical shell where the separation of fluid components is carried out

II. Column internals such as trays/plates or packings which are used to enhance components separations

III. A reboiler to provide the necessary vaporization for the distillation process

IV. A condenser to cool and condensed the vapor leaving the top of the column

V. A reflux drums to hold the condensed vapor from the top of the column so that liquid (reflux) can be recycle back to the column.

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Operation and terminology; the liquid mixture that is to be processed is known as the feed and this is introduced usually somewhere near the middle of the column to a tray known as the feed tray. The feed tray divides the column into a top (enriching) section and bottom (stripping) section. The feed flows down the column where it is collected at the bottom in the reboiler. Heat is supplied to the reboiler to generate vapor. The source of heat input can be any suitable fluid, although in most chemical plants this is normally stream. In vapor raised in the reboiler is re-introduced into the unit at the bottom of the column. The liquid removed from the reboiler is known as the bottom product. The vapor moves up the column, and as it exits the top of the unit,

it is cooled by a condenser. The condensed liquid is stored in a holding vessel known as the reflux drum. Some of this liquid is recycled back to the top of the column and this is called the reflux. The condensed liquid that is removed from the system is known as the distillate or top product. Thus, there are internal flows of vapor and liquid within the column as well as external flows of feeds and product streams, into and out of the column.A schematic of a typical distillation unit with a single feed and two product streams is shown in the Figure 2.6;

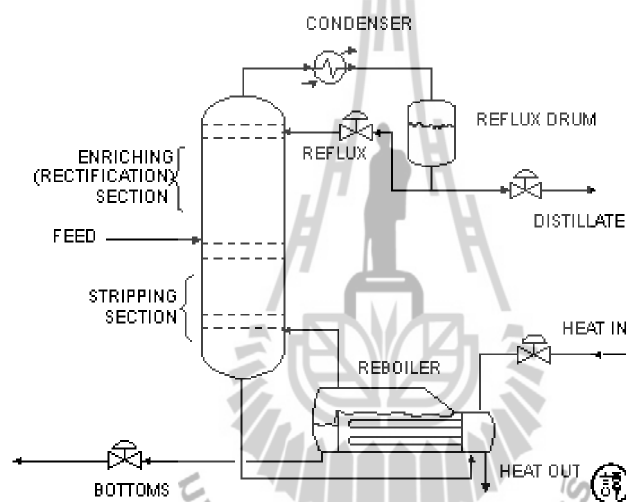

Figure 2.6 Schematic of a typical distillation unit.

Source: Tham (1990)

2.6.1 A high efficiency fractionating column.

Recently, a high efficiency lab scale distillation system using forced-mixing concept was successfully developed in SUT. The design is very unique in terms of the column internal, and distillation performance. The schematic diagram of the distillation unit based on forced-mixing concept is illustrated in Figure 2.7. In general, the column is constructed from stainless steel. A hotplate stirrer is employed as the main heating

element. The length of both rectifying and stripping sections is equally 40 cm whereas the size of the reboiler is 2.5 L. A set of internal impellers is fixed on a central shaft driven by a 100 Watts variable speed motor. The middle impeller located at the feeding point serves as a dispenser whereas the lower set of impellers has a function of stripping ethanol from fermentation broth in the upward direction. The upper set of impellers forces the rising vapor to the internal side of the column where partial condensation occurs. The high agitation rate generates close contact between the rising vapor, and descending liquid resulting in an extremely high number of condensation-vaporization cycles. In addition, a water jacket is installed below the exit point of the column where distillate temperature (T1) can be precisely controlled by re-circulation of a cooling liquid. As a result, high efficiency separation can be obtained within a short distance of rectifying column, and reflux is not necessary.

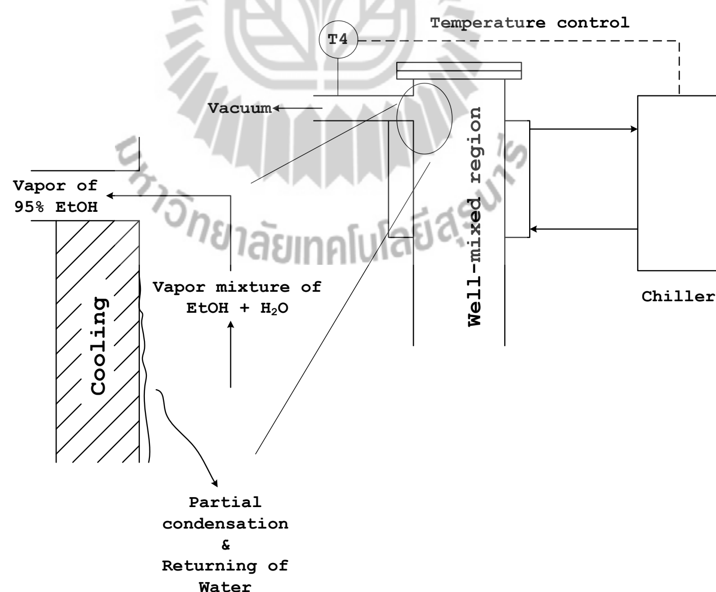

Figure 2.7 Working principle of the fractionating column based on partial condensation technique.

Source: Pimkaew (2010)

2.7 Cassava for ethanol production

Both sugar containing substrates such as sugar cane, sugar beet, Molasses, and starch-containing substrates such as cassava, and corn can be deployed for ethanol production. Although the ethanol production processes from both type of substrates are quite similar, their processing techniques are slightly different in the initial raw materials preparation stage. Sugar-containing substrates, by nature, are fermentation ready without further modification, while the starch containing ones need an additional step to convert them into fermentable sugar. Subsequent production processes are essentially the same for both types of substrates.

Starch is converted into fermentable sugar via "hydrolysis". There are two techniques for hydrolysis: enzymatic and acid hydrolysis. After fermentable sugar is obtained, ethanol can be produced directly by microbial conversion through fermentation by the same strain of yeast used with sugar-based substrate. Yeast strain used in the sugar fermentation is usually baker's yeast (*Saccharomyces cerevisiae*). It is deployed as a seeding for the fermentation.

After harvesting, the cassava roots are chopped into chips and transported to drying floors. The roots are usually dried in the sun. Once the chips are dried, they can be stored for months. However, during storage, the starch yields decreases somewhat, depending on storage temperature: typically 5% reduction of starch yield in 8 month storage (Abera *et al.*, 2007). Another advantage of chips is the easy transport. A big advantage of cassava over many other traditional crops is that it can be grown and harvested throughout the year. This results in a constant supply of cassava to the ethanol production facility in contrast to more seasonally crops. The ethanol production process consists of three basic steps as follows (Table 2.4).

Table 2.4 Main steps in ethanol production from starch.

Step	Goal	Type of process
Milling and liquefaction	Breaking down starch molecules into its building block molecules: glucose	Enzymatic
Fermentation	Convert glucose to ethanol	Yeast
Purification	Separate ethanol from other reaction products and inert materials	Distillation

On an industrial scale, the process described in Table 2.4 is carried out with two distinguishable technologies:

1. Wet milling process
2. Dry grinding process

The two processes differ with respect to complexity and associated capital costs, the numbers and types of co-products produced, and the flexibility to produce different kinds of primary products. The principal differences between the ethanol dry-grind process and the wet mill process are the feedstock preparation steps, the numbers, and types of co-products recovered. Once the starch has been recovered, the process of converting it to fuel ethanol and recovering the ethanol is similar in both wet mill and dry-grind facilities.

Currently, most new facilities use the dry grinding process. The wet milling process starts with soaking the cassava chips in an acid to soften the material which results in the separation of starch from other components. The fibers are recovered in several separation steps. Subsequently, the starch and protein are separated. In this

process, the streams are fractionated and several co-products can be recovered. Most streams are recovered before the fermentation step.

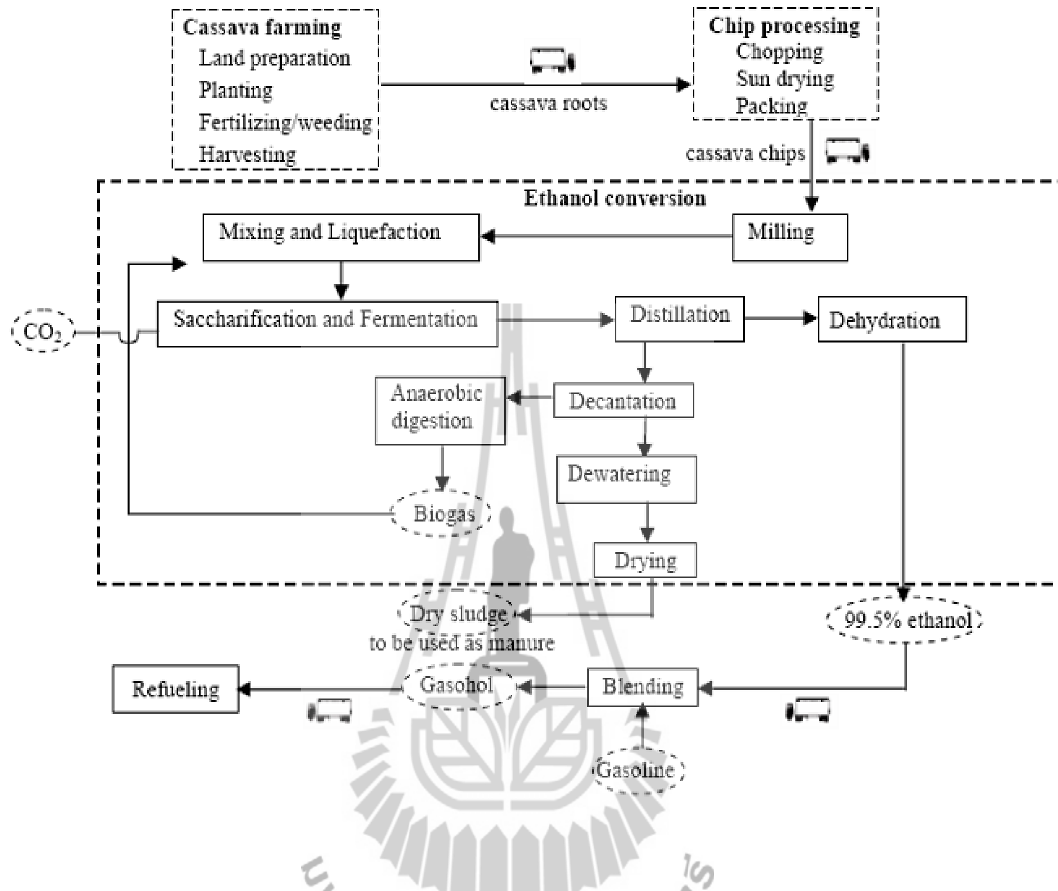

Figure 2.8 Flow chart of cassava based ethanol production process.

Source: Nguyen *et al* (2006)

The dry grinding process starts with grinding the chips. This is done by hammer mills or roller mills. Next the ground material is mixed with water, cooked and mixed with enzymes. This process produces only one co-product that is separated at the end of the whole process, after fermentation: distiller dried grains with soluble. This is mostly used as animal feed. The use as animal feed is, however, limited due to the high fiber content.

2.7.1 Simultaneous saccharification and fermentation

One of the most advanced bioprocesses in fuel ethanol production is the simultaneous saccharification and fermentation (SSF) of cellulosic materials. The SSF is a single-step process in which enzymatic hydrolysis and alcoholic fermentation are carried out in a single vessel. In the SSF, the rate of hydrolysis is much lower than the rate at which the microorganism can consume glucose. The SSF therefore proceeds under glucose-limitation, and the inhibition caused by glucose and cellobiose is eliminated. Consequently, a lower enzyme loading is required. The SSF, however, has inherent problems that need to be addressed.

The most significant one is the mismatch in optimum temperatures for hydrolysis and fermentation. The saccharification requires temperature of 45-50 °C and the fermentation is most efficient at 20-30 °C. Since the two stages are carried out simultaneously, an SSF process is normally operated at a compromised temperature of 35-38 °C. This trade-off in the temperature precludes the possibility of achieving maximum enzyme activity and the highest possible fermentation efficiency.

This has been a very well recognized problem. Substantial research effort has indeed been put forward to improve the process. Most of the research work has been focused on the identification and improvement of thermo tolerant yeast or bacteria that can produce ethanol at higher temperatures. This would allow hydrolysis to proceed at higher rates. From these studies, new strains that can withstand temperatures as high as 41strains have been identified. However, results from various studies indicate that thermo tolerant microorganisms are less tolerant against ethanol and exhibit low productivity. Furthermore the temperature range of 38-41 °C is still lower than the optimum temperature for cellulases (45-50 °C). Research efforts from

different angles have also been made. They include changing the temperature profile, varying the recipe of the enzyme, i.e., supplementing ~ glucosidase, further verifying of the kinetics, increasing substrate digestibility by employing novel pretreatment methods, and developing oligomeric fermenting microorganism. Each proposed method has its own merits and limitations. None of them, however, has provided a feasible solution for the stated problem.

Fermentable sugars can be directly converted to ethanol, while starch first has to be hydrolyzed to obtain free sugars. Next, the sugars are fermented to ethanol which is followed by a purification step yielding pure ethanol. The process of extracting starch from cassava is a well known technology. Cassava has been used as source of starch for decades. Cassava is high in starch content (70 - 85% on dry base and 28 – 35% on wet base), and the starch from cassava is of a high quality compared to other starch sources (Kuiper *et al*., 2007). Cassava starch is used as raw material in many industries, among which paper, food and textile industries. Also the technology of producing ethanol from starch is internationally well developed. Initially, alcohol derived from yeast fermented sugar has a concentration of only about 5–15% by weight. Its concentration is then further increase by separating it from water and other non-fermentable materials. The final concentration of alcohol attained is 95–96% by weight using a distillation method.

Several works investigated cassava as a substrate for the production of ethanol. Ulibarri and Hall (1997) studied the enzymatic saccharification of cassava flour starch with glucoamylase from *Aspergillus niger* using a hollow-fiber enzymatic membrane reactor (HF-EMR). The optimization of the process was also investigated. The substrate was gelatinized by extrusion that produced a substrate with a degree of

gelatinization and physical properties suitable for the enzymatic process. Typically, continuous saccharification of cassava flour starch in the HF-EMR produced a highly pure product in the permeate (i.e., 99.6% glucose) with a global starch to glucose conversion of 97.3%.

Rattanachomsri *et al.* (2008) studied cassava pulp saccharification process which utilizes the multi-activity enzyme from *Aspergillus niger.* The crude multi-enzyme composed of non-starch polysaccharide hydrolyzing enzyme activities, including cellulase, pectinase and hemicellulase act cooperatively to release the trapped starch granules from the fibrous cell wall structure for subsequent saccharification by raw starch degrading activity. The yield from multi-enzyme was greater than the yield obtained from the optimized combinations of the corresponding commercial enzymes. The multi enzyme saccharification reaction can be performed simultaneously with the ethanol fermentation process using a thermo tolerant yeast *Candida tropicalis*. The combined process produced 14.3g/L ethanol from 4% (w/v) cassava pulp after 30 h of fermentation.

Zhu *et al.* (2012) studied the process of separating hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Fermentation was performed using *S. cerevisiae* for the production of ethanol from cassava pulp without any pretreatment. A combination of amylase, cellulase, cellobiase, and glucoamylase produced the highest levels of ethanol production in both the SHF and the SSF method. The temperature of 37 °C, a pH of 5.0, and an inoculums size of 6% were the optimum conditions for SSF process. For the batch process at a pulp concentration of 20%, ethanol production level from SHF and SSF were the highest, at 23.51 and 34.67 g/L respectively. Moreover, the levels of ethanol

production from separating hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) in the fed-batch process rose to 29.39 and 43.25 g/L respectively, which were 25% and 24.7% higher than those of the batch process. Thus, SSF using the fed-batch provided a more efficient method for the utilization of cassava pulp.

Ogbonna and Okoli (2010) developed a process for conversion of cassava flour to ethanol. This involved direct inoculation of *Aspergillus awamori* spores into a cassava flour paste and incubation for some period during which hydrolytic enzymes are produced (solid state culture or koji production) and subsequent addition of water and yeast cells, during which there is simultaneous hydrolysis and ethanol production (submerged culture). Under optimum conditions, a high ethanol concentration of 120 g/L and ethanol yield of 0.309 g-ethanol/g-cassava flour were obtained. This ethanol yield corresponds to 0.44 g-ethanol/g-cassava starch.

Ocloo1 and Ayernor (2010) determined the conversion efficiency of sugar to alcohol, rate of fermentation and types of alcohol produced. Alcohol was produced from cassava flour hydrolysate with standard glucose and sucrose solutions used as controls. The effects of yeast concentrations and fermentation time on yield of alcohol from cassava flour hydrolysate were also studied. Alcohol produced constituted mostly ethanol with traces of methanol. Yeast concentrations were found not to have any significant effect ($p > 0.05$) on the alcohol yield. However, fermentation time was found to have had a significant effect ($p < 0.05$) on alcohol yield. The study suggests that high yield of alcohol could be produced from cassava flour hydrolysate.

Saoharit *et al*. (2009) employed ultrasound to improve ethanol fermentation performance from cassava chips. The effects of ultrasound and heat pretreatments on ethanol yields from cassava chips were investigated. The sonicated and non-sonicated (control) samples were then subjected to simultaneous saccharification and fermentation (SSF) of ethanol. Cassava starch- to-ethanol conversion efficiencies showed that higher ethanol yields were directly related to sonication times. The ethanol yield from the sonicated sample was 2.7-fold higher than yield from the control sample. Starch to ethanol conversion rates from solicited cassava chips were also significantly higher, and the fermentation time reduced by nearly 24 h. In conclusion, ultra sound pretreatment enhanced both the overall ethanol yield and fermentation rate. When compared to heat-treated samples, the sonicated samples produced nearly 29% more ethanol yield.

Akponah and Akpomie (2012) showed the significance of pH, and nutrient concentrations for ethanol production. Optimum fermentation duration for ethanol production in acid and enzyme hydrolysates was 24 h. The start-up fermentation experiment process with *S. cerevisiae* resulted in 1.47 and 1.00% (v/w) ethanol in acid and enzyme hydrolysates, respectively. However, appropriate adjustment in pH yielded 3.60 and 1.88% (v/w) ethanol in both acid and enzyme hydrolysates of cassava waste water.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Fresh cassava roots and hydrolytic enzymes

Fresh cassava roots were obtained from a local market (Nakhon Ratchasima, Thailand). It was washed and mashed using a grow-mill grinder. The slurry under went ultrasonic pre-treatment by using an ultrasonic unit. It was kept in 4 °C for further use. The α-amylase (Termamyl®), gluco-amylase (Spirizyme®), cellulase (Viscozyme®) were obtained from Novozymes (Denmark). The enzymes were used for liquefaction and saccharification of starch whilst the last was employed for the hydrolysis of cellulose. The enzyme *STARGEN*™ 001 was obtained from Siam Victory Chemical Co. Ltd. (Bangkok, Thailand). *STARGEN*™ 001 (456 granular starch hydrolyzing unit (GSHU)/g) is a cocktail of α-amylase (gene from *A. kawachiwas* expressed in *Trichoderm areesei*) and glucoamylase (from *A. niger*). The enzyme mixture works synergistically in hydrolyzing starch into glucose.

3.1.2 Microorganism

A commercially available dry distillery yeast (*Saccharomyces cerevisiae* EDV 493) manufactured in Denmark was used as the ethanol producer. Inoculation of 250 ml Erlenmeyer flask containing 150 ml of yeast extract and malt extract (YM) medium was carried out. The yeast suspension (approximately 3%) was transferred

into 150 ml of the YM medium. The medium consists of yeast extract, 3 g/L, peptone, 5 g/L, malt extract, 3 g/L and glucose, 10 g/L. The flask was incubated on a rotating shaker at 150 rev/min, 30°C for 24 h. (Laopaiboon *et al*., 2009). After liquefaction of cassava starch, cell inoculums were added into a fermenter and the temperature was maintained at 30°C, pH 4.5.

Figure 3.1 *Saccharomyces cerevisiae* EDV 493 colony for inoculation preparation.

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3.2 Method

3.2.1 Preparation of fresh cassava roots

Fresh cassava roots were washed, peeled, and mashed using a grow-mill grinder. Cooking procedures comprised of pre-mashing, liquefaction, and saccharification, respectively. The cassava mash was mixed with distilled water prior to add the catalase enzyme and increase the temperature to 50 °C for 45 min. Calcium chloride (dihydrate) was added to the slurry in order to stabilize the enzyme. The α-amylase was added into the slurry prior to increase the temperature to 90 °C for 1 h.

Finally, glucoamylase was added and the temperature of the solution was decreased to 70 °C for 2 h. The slurry was then pressed through a filter bag in order to remove solids.

3.2.2 Enzyme hydrolysis of starch for uncooked cassava mash.

The objective of this work is to compare the fermentation performance between cooked and uncooked cassava for different fermentation modes including conventional fermentation, simultaneous saccharification and fermentation (SSF), and simultaneous liquefaction saccharification and fermentation (SLSF), respectively.

Enzyme of liquefaction (Termamyl®, Novozymes) is an α-amylase enzyme. Enzyme of saccharification (Spirizyme®, Novozymes) is a glucoamylase enzyme. The enzymes hydrolyze of cellulose (Viscozyme®, Novozymes) is a cellulase. Enzyme mixture (a-amylase and glucoamylase enzyme) of SLSF was *STARGEN*™ 001 that was obtained from Siam Victory Chemical Co. Ltd. (Bangkok, Thailand). During hydrolysis, samples were collected at time intervals for determination of reducing sugar content by using DNS method.

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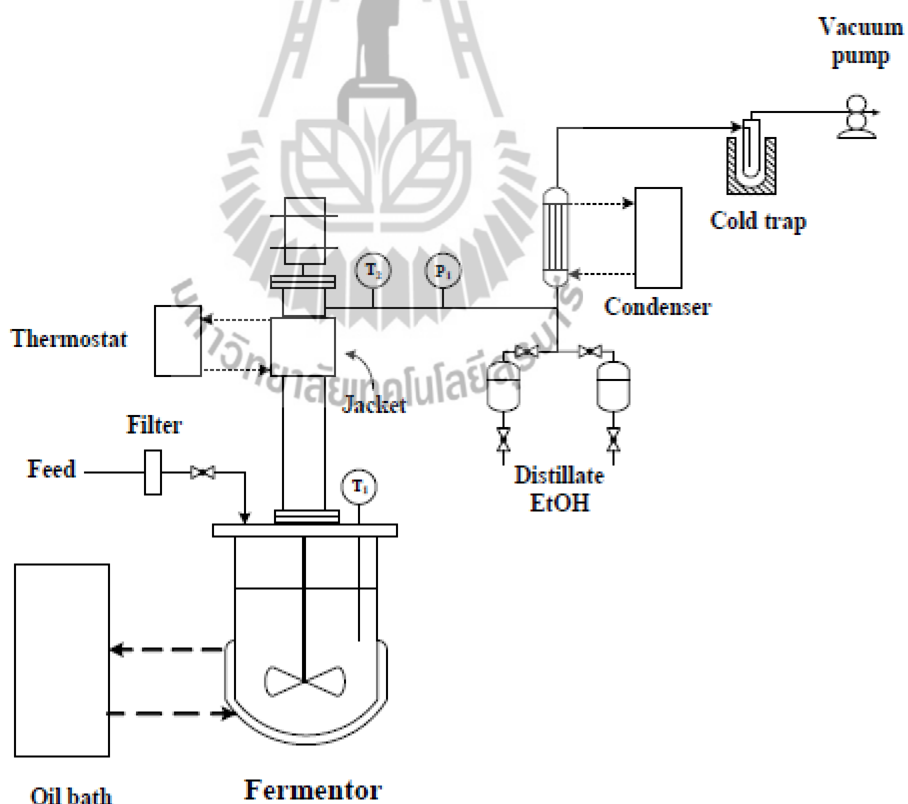
3.3 Experimental setup

Figure 3.2 shows the experimental setup for extractive fermentation of ethanol by using vacuum fractionation technique. The feed solution was introduced to the fermenter by a peristaltic pump and a temperature controller was used to adjust the desired temperature. During fermentation process, the ethanol/water vapor mixture was continuously removed from the fermenter, and entered the fractionating column. Fractionations of the ethanol/water mixture occur at the rectifying section with the help of the thermostat to control the optimum reflux. The vapor of ethanol/water

mixture contacts to the wall of the column, and some portion of water was condensed by contacting with lower temperature liquid in the jacket before reflux back to the bioreactor. The high purity ethanol vapor was flown out of the system before being condensed using a condenser and cold trap filled with liquid N_2. This series of condenser and cold trap ensured that all ethanol vapor was totally collected. In addition, two receiving units were installed so that the distillate ethanol can be removed without disturbing the vacuum condition inside the fermenter. Sample was taken from fermenter to analyze reducing sugar, starch, ethanol, water content and cell viability. Vacuum pressure was controlled at 65 mmHg by a vacuum controller, and temperature was maintained at 35 °C using an oil bath.



A.

B.

Figure 3.2 Experimental setup for extractive fermentation of ethanol using vacuum fractionation technique (A). Experimental set up in the laboratory (B).

The major components of the system comprised of;

Column – The column was made from a stainless steel, that had 89 cm long 6.4 cm inner diameter. The top of the column close by welding with the plate flange (14 cm in diameter) for installation a motor and a drive shaft. At the bottom of column welded the plate flange (14 cm in diameter) with plate flange of boiler. The midpoint of column had a pipe line for releasing the hot vapor (distillate).

Drive shaft – Drive shaft was the most important part because it fix the propellers to generate well mixed condition in both fermentation broth and rising vapor mixture. The variable speed motor (120 watts) connected directly with the top of a drive shaft by the bolts. Drive shaft had 1.4 cm in diameter and 88.5 cm long. At the top welded closing by plate flange (14 cm in diameter), and had a mechanical seal for create leak free condition.

Propeller of stripping section (upflow propellers) – The rising vapor was controlled by propellers of stripping section (upward direction), that composed three-blade paddle mixer with inclined blade (45) and 1.5 mm thickness. The three blade paddle was welded with 1.42 cm diameter and 8 mm thickness of a ring. The three-bladed propellers had the number of 10 blades, which would have equal space distance installation.

3.4 Fermentation culture condition

3.4.1 Pre mashing fresh cassava roots for fermentation

In order to reduce slurry viscosity, a pre-mashing procedure before starch liquefaction has been recommended of fresh cassava mashes preparation. Incubation of slurry with lignocellulolytic enzymes (Viscozyme) potentially reduces mash viscosity. Temperature- and pH-controlled stirred tanks could be ideal at this step for maximum viscosity reduction. SEM was used to investigate morphological change of cassava cell walls.

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3.4.2 Effect of di- ammonium phosphate

To obtain effect di- ammonium phosphate (DAP) on ethanol production from fresh cassava mash. Fermentation was operated under anaerobic condition 50% of cassava mash it mixtures with yeast, di- ammonium phosphate (DAP) in the flasks. After 24 h. the samples were taken to measure ethanol concentration.

3.4.3 Simultaneous sacchrification and fermentation

SSF were performed in shake flask with the working volume 100 ml. Cassava mash was diluted with distilled water at 10, 20, 40, 50, 60, 80, 90 and 100 %w/v. The yeast cells were added after saccharification process, and Di-ammonuim

phosphate (DAP) was used as nitrogen souse. Simultaneous saccharification and fermentation was performed in sterile 3L of the fermenter containing 2.5 L of cassava slurry, glucoamylase enzyme was added at ratio of 0.5% (v/w of starch content in cassava starch slurry). The fermenter was operated to 72 h at 55 °C and 180 rpm. After that, the enzyme and cassava starch slurry mixture was inoculated with yeast cells (*Saccharomyces cerevisiae* EDV 493). Samples were taken and analyzed for reducing sugar, biomass (free cell and immobilized cells) and starch content.

3.4.4 Simultaneous liquefaction saccharification and fermentation (SLSF)

SLSF was performed in shake flask working volume 100 ml of sample and varies cassava fresh root slurry at 10, 20, 40, 50, 60, 80, 90 and 100 %w/v. Liquefaction saccharification and fermentation at temperature 37°C. The yeast cell was added in saccharification and Di-ammonuim phosphate (DAP) was used as nitrogen souse and to control pH 5. SLSF uncooking for cassava fresh root slurry added enzyme mixture (*STARGEN*^{TM}001) at temperature 37°C.

3.4.5 Conventional batch fermentation and batch extractive fermentation.

All experiments were conducted in a 3L fermenter. The fermenter was operated to 30 h at 37 °C. The yeast (*Saccharomyces cerevisiae* EDV 493) and the enzymes were added simultaneously. Samples were taken at 4-h intervals and analyzed for reducing sugar, relative viability and ethanol content.

Figure 3.3 The 3 L fermenter for fermentation experiments.

3.5 Analytical procedure

All experiments and analytical analyses were performed in triplicates. The results of reducing sugar yield, starch content, cell viability, ethanol yield, and ethanol efficiency were analyzed. The samples were centrifuged and filtered. The filtrate was analyzed for reducing sugar and ethanol concentration.

3.5.1 Reducing sugar

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The samples were centrifuged and filtered through Whatman PP 0.45 μm syringe filters. Reducing sugar was determined by DNS method. The amount of total reducing sugar (TRS) released by the hydrolysis were measured in the supernatant. The amount of TRS released was measured by the 3, 5-dinitrosalicylic acid method (Miller, 1959). One mL of diluted broth was mixed with 1 mL of DNS solution (10 g of 3, 5-dinitrosalicylic acid, 300 g of $KNaC_4H_4O_6$ in 200 mL of 2N NaOH and adjusted to 1 L with RO water). The mixtures were mix thoroughly and development of color was conducted by boiling the reaction tube for 5 min. The concentration of

reducing sugar was calculated against standard of glucose concentration 0.2, 0.4, 0.6, 0.8, and 1.0 g/L, respectively.

3.5.2 Ethanol concentration

Ethanol concentration in the fermentation broth was analyzed by using a gas chromatography (SRI Instrument, USA) equipped with a FID detector. Ethanol concentration (g/L) was analyzed by gas chromatography with capillary column and a flame ionization detector (FID) at temperature 250 °C. The GC column was a 30 m × 0.32 mm fused silica capillary column (Carbowax®, Restek, USA). The injector and detector were set at 200, and 250 °C, respectively. The oven was operated at programmed temperature from 40 to 90 °C with the rate of 10 °C/min using He as a carrier gas and H_2 as a flaming gas. The ethanol yield (Yp/s, g/g) and volumetric ethanol productivity (g/L/h) were also calculated (Laopaiboon *et al*. 2009).

3.5.3 Determine of cell concentration

The viable suspended yeast cell numbers and total soluble solids of the fermentation broth was determined by direct counting method using haemacytometer (Zoecklein *et al.* 1995). Cell viability was measured by using the methylene blue test. The fermentation broth was diluted with DI water to an appropriate cell concentration and then mixed with methylene blue solution (0.1 g methylene blue in 100 mL water) before observation under microscope.

3.5.4 Starch content

The starch content was measured by acid hydrolysis of samples according to the procedure reported by Leonel (1998) and the starch content was determined by multiplying the content of total reducing sugar (TRS) (Leonel, 1998). Starch concentration of the sample was determined using a modified method of *Tang*

et al., 2010. A portion of 20 µL α-amylase was added to 1 mL of culture medium and then incubated at 90 °C for 3 h to hydrolyze starch in the medium to soluble dextrin. After, 8,800 µL of 0.1 M acetate buffer pH 4.5 and 100 µL of glucoamylase were added to the solution and then incubated at 58 °C for 4 h. The solution was allowed to cool down to room temperature and then transferred to a 10 mL volumetric flask followed by filling it with distilled water to the volume. Glucose concentration of this solution was determined by DNS method. Starch concentration in fermentation broth was calculated as follows:

Starch concentration (g/L) = Glucose concentration (g/L) $\times 10^{a} \times 0.9^{b}$

Where a is dilution factor and b is correction factor for glucose to starch.

3.5.5 Organic acid

Organic acids concentration was analyzed by HPLC (Thermo Scientific, USA), and quantification by UV detection was made at the wavelength of 210 nm. The mobile phase consists of 1% acetonitrile + 99% 20 mM Na_2HPO_4 (pH 2) at a flow rate of 1 mL/min. The HPLC column was ZORBAX SBAq (4.6 mm × 150 mm).

3.5.6 Kinetic analysis

Batch fermentation productivity was calculated as total ethanol concentration divided by fermentation time. Fermentation time was defined as a time period when a maximum ethanol concentration was reached. Ethanol yield, was calculate as total ethanol product (g) divided by total carbohydrates (as starch) utilized (g).

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CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Pretreatment of cassava mash

For cassava, the problem of high viscosity has been encountered during preparation of wort for fermentation due to the presence of non-soluble lignocellulosic materials. Disadvantages were observed including high power for mixing, high water consumption, resistant to solid-liquid separation, and incomplete hydrolysis of starch resulting in a low fermentation yield, respectively. These solid cellulosic materials further negatively affect downstream processing by reducing the efficiency of separation, distillation and evaporation. As a result, mash with high dissolved solid content and reduced viscosity is required prior to enter the liquefaction process. This can be achieved by using enzymatic pre-treatment to degrade the cell wall of the cassava after mashing process. According to the manufacturer, the cellulase enzyme used in this work (Viscozyme®) has the key enzyme activity of endo-β-glucanase that hydrolyzes (1, 3) - or (1, 4)-linkages in β-D-glucans. In addition, the product also contains activity of xylanase, cellulase, and hemicellulase. The activities of these enzymes results in the hydrolysis of lignocellulosic material into different major components including cellulose, hemicellulose, and pectin. Further hydrolysis of these materials produces different hexose, pentose, and acids such as glucose xylose, mannose, galactose, rhamnose, arabinose, glucuronic acid,

and galacturonic acid, respectively. In conclusion, the action of cell-wall degrading enzymes on cassava mash does not only reduce the viscosity, but also increase the reducing sugar content.

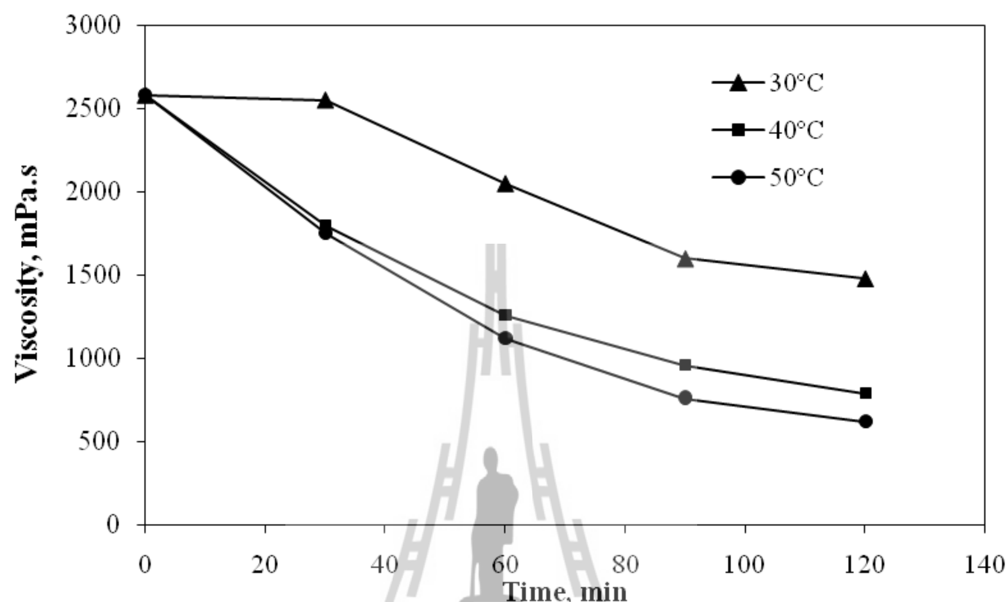

Figure 4.1 The time course of cassava mash viscosity as a function of temperature. (50% w/v of cassava mash slurry, 15 FBG/g cassava mash, stirrer 500 rpm)

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Figure 4.1 shows the viscograms of cassava mash as the function of operating temperatures. The enzyme loading for each run was fixed at 20 FBG per g cassava mash. A typical time course reduction in viscosity was observed for each experiment. At the temperature of 30 °C, viscosity started to decrease after 30 min from 2,580mPa.s to approximately 1,480 mPa.s in 2 h of operation. The pH of the mash slightly decreased from 5.9 to 5.5, which should be attributed to the de-esterification of pectin into galacturonic acid. Viscosity reduction experiments were also carried out at temperature of 40 °C and 50 °C. Although the enzyme loading was the same

dosage, the mash viscosity reduced at a higher magnitude than at 30°C. Experimental results showed that the higher the temperature, the lower the viscosity was obtained. For 40 °C, the viscosity rapidly reduced from the beginning before the reduction rate slightly decreased after 1 h of operation. The lowest viscosity of 790mPa.s was obtained after 2 h of operation. For 50 °C experiment, the experimental results followed the same trend as at 40 °C. The lowest viscosity of 620mPa.s was obtained after 2 h of operation. Although the same amount of cellulase enzyme was used, viscosity of the cassava mash varied at different temperature. In general, it is the fact that viscosity is inversely proportional to the temperature. Therefore, the temperature of 50 °C was chosen for subsequent experiments.

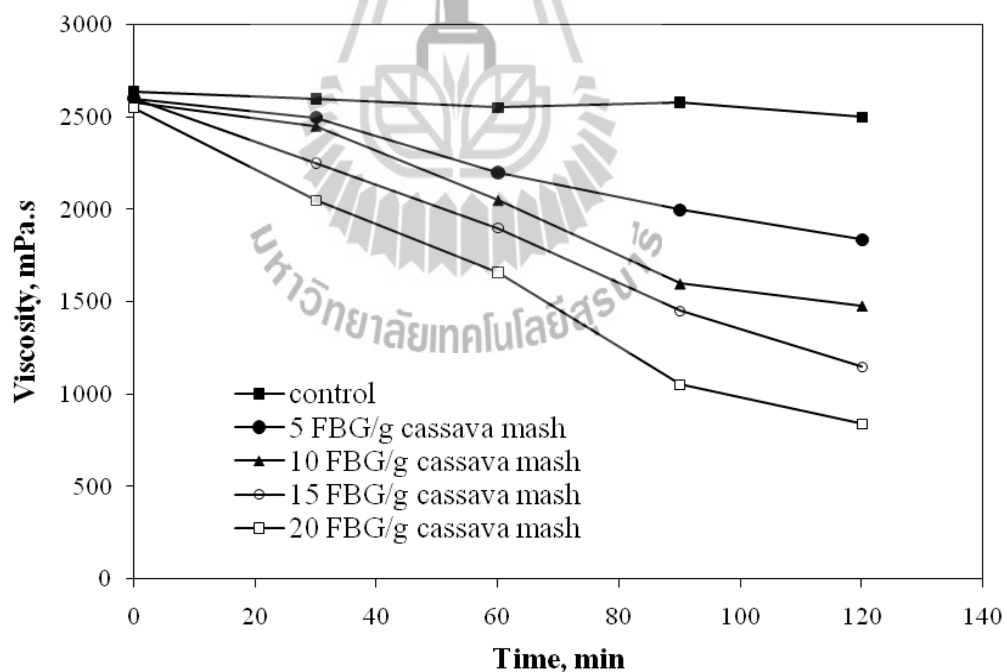
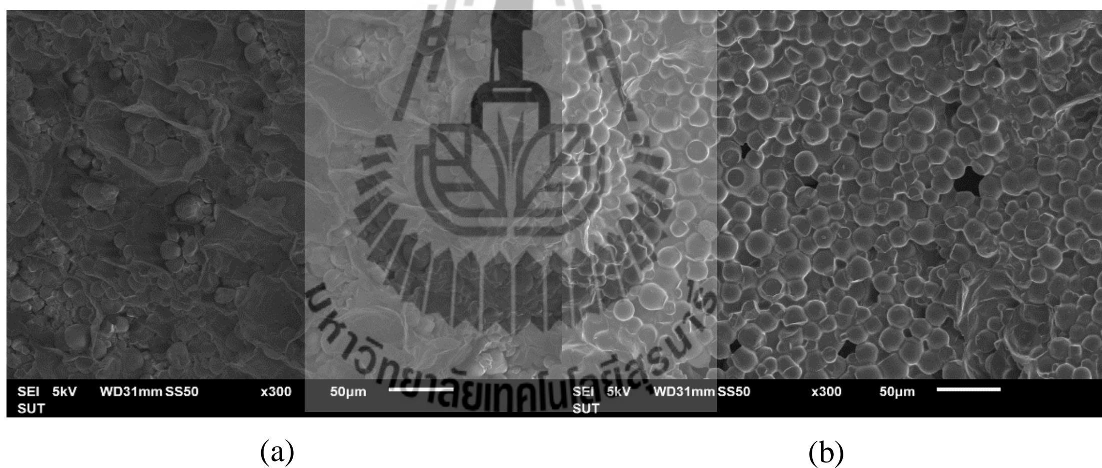


Figure 4.2 Effect of enzyme loading on the viscosity of cassava mash slurry (50% w/v); temperature 50 °C, stirrer speed 500 rpm.

Figure 4.2 shows that the viscosity reduction of cassava mash appeared according to 4 different dosages of the cellulase enzyme. For the control experiment where no cellulase enzyme was added, the viscosity of the cassava mash was constant throughout the 120 min of operation. In contrast, the rate of viscosity reduction increased from control toward the loading of 20 FBG per g cassava mash. Experimental results showed that an increase in enzyme dosage caused an increased in viscosity reduction rate. The maximum enzyme loading of 20 FBG per g cassava mash resulted in the highest viscosity reduction rate at approximately 14.83mPa.s/min during the first 60 min. After this period, the viscosity reduction rate gradually reduced until nearly reach plateau at the end of the operation. The final viscosity of 650mPa.s was obtained by using this dosage of the enzyme. For the enzyme loading of 5, 10, and 15 FBG per g cassava mash, the reduction of viscosity was also observed but at the lower rate in comparison to 20 FBG per g cassava mash. At 120 min of operation, the cassava mash viscosities were measured at 1570, 1100, and 820mPa.s, respectively. Although these values were different, these viscosities had tendency to reduce with the increasing of time. In this given condition, the final viscosities of all enzyme loading experiments would be apparently comparable. However, a long operation time was not a favorable choice for industry. Accordingly, the enzyme loading of 20 FBG per g cassava mash was selected as the optimal dosage for subsequent study. Higher dosage could result in a shorter operation time; however, it increase the cost of enzyme. Optimization for this operating step should be considered by the economical point of view. By this enzymatic pretreatment, it was possible to prepare the mash containing high solids content with a suitable viscosity.

The morphological change of cassava cell walls was also investigated using SEM. Figure 4.3 (a) shows the electron micrographs for cross-section of a fresh cassava root in which starch granules were embedded within a cell wall matrix. This structure of cell wall limits the accessibility of starch-hydrolyzing enzymes to get into the inner part of the matrix. These micrographs confirm the importance of pre-treatment step in order to enhance the starch hydrolysis yield. Figure 4.3 (b) shows the crosssectional area of fresh cassava root after treatment with the cellulose enzymes. Break down of lignocellulosic materials causing by the hydrolytic activities of cellulase was clearly observed. According to the results of these studies, it is likely that viscosity reduction of cassava mash was the result of pectin degradation.



(a) (b)

Figure 4.3 SEM micrographs of fresh cassava root cross section before (a), and after addition of cellulase enzyme (Viscozyme®), 2 hour.

4.2 Process optimization for improving ethanol fermentation

In order to obtain the optimum condition for fermentation of ethanol using fresh cassava roots as the main substrate, cassava wort was prepared as followed; viscosity reduction using cellulase, liquefaction using α-amylase, and saccharification

using glucoamylase, respectively. The last two processes followed the standard procedures for hydrolysis. After the pretreatment step, the cassava mash was heated to 80 °C prior to add the α-amylase for 2 h. The subsequent step was 3-h saccharification where glucoamylase was added after the temperature was reduced to 40 °C. Finally, liquid-solid separation was carried out by using a filter press. The liquid possessed the total reducing sugar of approximately 30 %wt, and was kept for further study.

4.2.1 Effect of initial glucose concentration.

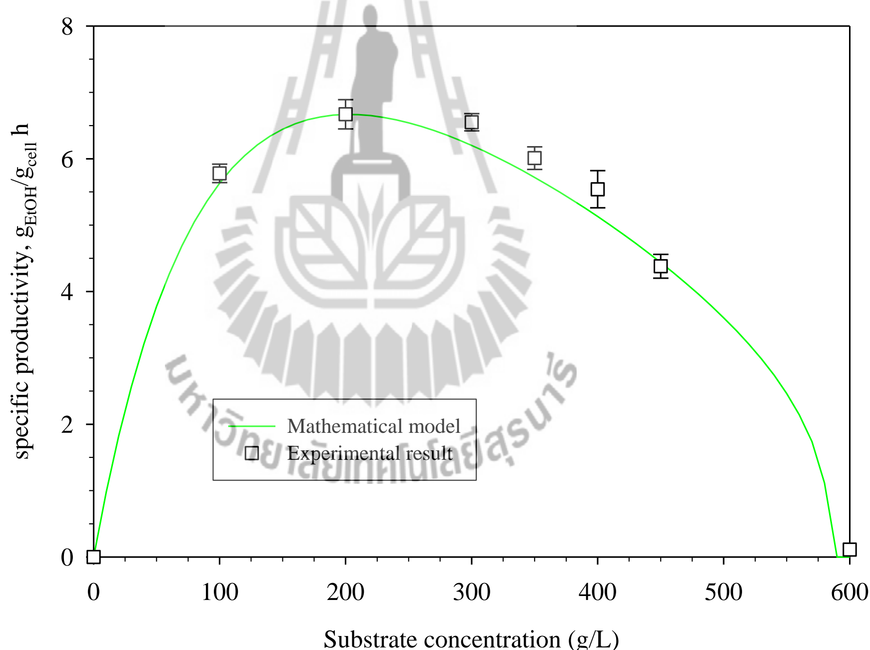


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Figure 4.4 The influence of initial glucose concentrations on the specific productivity of ethanol; temperature 35 °C, 200 rpm.

The cassava wort was either diluted with distil water or evaporated to the desired glucose concentration ranging from 150-600 g/L. Substrate inhibition kinetics

was investigated in order to understand the effect of initial glucose concentration on fermentation performance. Too low initial glucose concentration results in a low ethanol concentration whilst too high glucose concentration could result in high osmotic pressure and cell death. The experimental results and mathematical modeling of specific ethanol productivity are presented in Figure 4.4 using a modified substrate inhibition as followed (Lin *et al*, 2008);

$$v = \frac{1}{x}\frac{dP}{dt} = v_{max}\left(1 - \frac{S}{K_i'}\right)^n \frac{S}{K_S' + S + S^2/K_i'} \qquad (5)$$

The values were determined by plotting ethanol concentration versus time. Specific ethanol productivity was calculated from the ethanol production rate (slope) divided by the yeast concentration (data not shown). The period for rapid increasing of ethanol concentration was considered. The mathematical modeling for substrate inhibition showed that the glucose inhibition effect on specific ethanol productivity was strong, especially at the initial glucose concentration higher than 450 g/L. The highest value of 6.67$g_{EtOH}/g_{cell.h}$ was observed at glucose concentration of 200 g/L. After this value, the value decreased with the increasing glucose concentration. The saturation constant (K_S') and the substrate inhibition constant (K_i') of 163 and 590 g/L were calculated in this work. The high value of K_i' implying that fermentation can be carried out at a high substrate concentration (200-300 g/L) in which the size of the fermenter could be reduced associated with a high volumetric productivity.

4.2.2 Extractive fermentation using vacuum fractionation technique

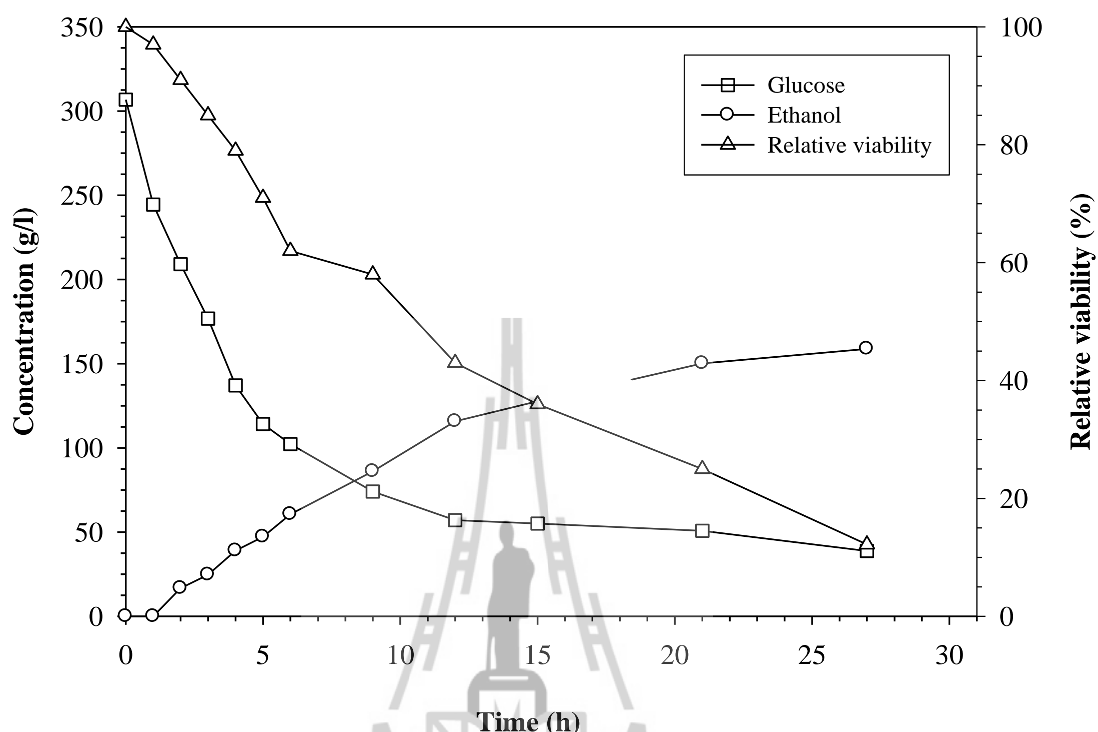

Figure 4.5 Time course for reducing sugar concentration (□), ethanol concentration (○), and relative viability of yeast cells (Δ), during batch fermentation of ethanol using very high gravity technology.

From the experimental data of the previous section, it was shown that very high gravity fermentation resulted in high glucose consumption rate, and high volumetric productivity. Figure 4.5 illustrates the changing in reducing sugar concentration, ethanol concentration, and viability of yeast cells during batch fermentation of cassava syrup. The concentration of reducing sugar rapidly decreased at the first 10 h of fermentation before the consumption rate gradually decreased. However, approximately 38.8 g/L of reducing sugar remained at the end of fermentation indicating that it was not completely consumed by the yeast cells. The

decreased reducing sugar utilization rate was inversely proportional to an increase in ethanol concentration in the fermentation broth. The consequence of this experimental result was clearly due to product inhibition effect of ethanol to the yeast cell. The ethanol concentration rapidly increased at the first 15 h with a high volumetric productivity of approximately 9.80 g/L/h. Subsequently, the value gradually increased until the maximum concentration of 155.2 g/L was obtained. In addition, product inhibition effect was evidenced by the viability test of yeast cells. The value of viability sharply decreased since the fermentation commenced. Cell viability remained only 13.6% and fermentation activity ceased. At the end of fermentation process, there was no glucose consumption, ethanol formation, and most of the yeast cells lost their viability.

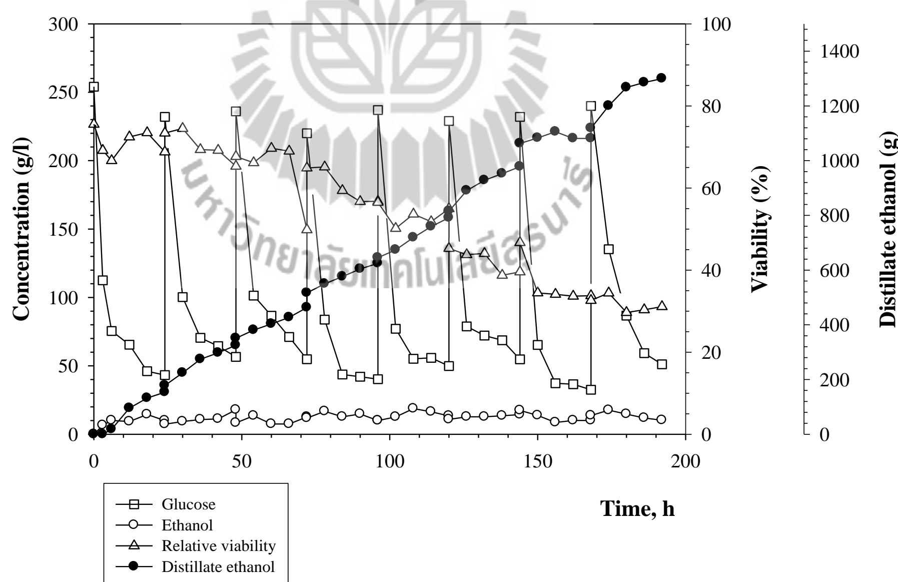

Figure 4.6 Time course for glucose concentration (□), ethanol concentration (○), relative viability of yeast cells (Δ), distillate ethanol (●) during repeated-batch extractive fermentation experiment.

The typical experimental setup for extractive fermentation using vacuum fractionation was already shown in Figure 4.6. The boiling of fermentation broth was carried out at the temperature of 35 ^{o}C where phase separation of ethanol/water vapor occurred. Due to the low ethanol concentration in the fermentation broth, its concentration at the gas/liquid interface was only approximately 20-25 % by weight. When this vapor mixture entered the fractionating column, it flow upward uniformly with a help of the rotating propellers. The cooling liquid flowing inside the jacket caused partial condensation of the vapor mixture where the high purity of ethanol flowed out of the column whilst excessive water flowed back into the bioreactor. As a result, the volume of the fermentation broth was relatively constant. The concentration of the distillate ethanol can be controlled by the adjusting of the exit vapor temperature (T_2 of Figure 3.2). Among various techniques for extractive fermentation of ethanol, pervaperation membrane bioreactor was the most studied system; however, intrinsic problems associated with separation performance of the membrane made this system not technically viable; for example, the separation must be carried out at the temperature of 30-35 ^{o}C resulting in a substantial low permeation flux of ethanol, some other fermentation by-product especially organic acids reduced the selectivity (Chovau *et al*, 2011), and most importantly the permeate concentration of ethanol is low especially at lower ethanol concentration in the fermentation broth. The permeate is then subjected to further distillation prior to dehydration processes. Unlike the other extractive fermentation systems, the separation performance of this work is not the limited by the ethanol concentration in the fermentation broth. Therefore, the distillate ethanol can be dehydrated accordingly, and the total product could be dramatically reduced because the expensive plate columns can be ignored.

The beneficial aspect of this extractive bioreactor was one stage integrated process. Separation of ethanol could be obtained in a concentrated form, and could results in an increase of the product formation. From the previous experiment, the optimum initial reducing sugar concentration of approximately 250 g/L was chosen for repeated-batch extractive fermentation experiment. Figure 4.6 shows the time courses of glucose concentration, ethanol concentration, mass of distillate ethanol, and relative viability, respectively. After 6 h for the addition of glucose and inoculation of yeast cells, the extractive fermentation was started. Experimental data showed that reducing sugar concentration rapidly decreased for the first 12 h, and the consumption rate gradually decreased until nearly constant after 24 h. Subsequently, the agitation was stopped allowing the yeast cells to sediment at the bottom of the bioreactor for 3 h. Addition of fresh cassava wort was carried out after the removal of clear supernatant. The produced ethanol was continuously fractionated from the system at the initial rate of 8.8 g/h with the concentration of approximately 90 wt%. Experimental results also showed a constant ethanol concentration in the fermentation broth below 17 g/L. The addition of fresh cassava wort was repeated for another 7 times, and system was stable for 190 h. The total ethanol concentration was obtained at 1299 g. After the seventh time of addition; however, the fermentation performance was significantly reduced since glucose consumption was low associated with a significant reduction in cell viability. The experiment ceased after 190 h when glucose concentration was constant and no ethanol was produced. In conclusion, this experiment showed that the long continuation of fermentation activity was obtained as long as the concentration of inhibitory products was kept low.

Cysewski *et al.* investigated vacuum fermentation with cell recycling for continuous ethanol production. The highest ethanol productivity of 82 g/L/h was obtained when the cell concentration reached 124 g/L. The ethanol concentrations in fermentation broth and in the condenser were 3.5 wt% and approximately 30 wt%, respectively. Ghose *et al.* studied simultaneous saccharification and fermentation of lignocellulosic materials to ethanol under vacuum cycling and step feeding. Rice straw treated with cellulase and β-glucosidase was intermittently fed into the bioreactor. The ethanol productivity of 4.5 g/L/h was reported and the maximum ethanol concentration of 13.6 wt% was collected. Lee *et al.* examined a vacuum fermentation of ethanol by using *Zymomonas mobilis.* A high productivity of 85 g/L/h was observed in the continuous cell recycle experiment. However, the condensate ethanol concentration was obtained for up to only 40 wt%. Nguyen *et al.* studied a continuous vacuum fermentation integrated with separation process. A fermentation-separation column was filled with yeast cells immobilized on biocarriers. Still, the condensate ethanol concentration was obtained for up to only 30 wt% which required further distillation in order to obtain 95 wt%.

4.3 Process improvement using uncooked cassava mash

One major characteristic for the liquefaction and saccharification of cassava mash is the use of energy for cooking to produce fermentable sugars. Reduction of heat utilization coupling with combination of operating procedures are of interest because time and production cost could be reduced. Extensive operating procedures have been studied on fermentation performance including hydrolysis of cassava mash at lower temperature, water-to-mash ratio, effect of di-ammonium phosphate as a

cheap nitrogenous source, pretreatment using ultrasound. In addition, combination of operating steps were attempted including simultaneous saccharification and fermentation (SSF), and simultaneous liquefaction saccharification and fermentation (SLSF), respectively

4.3.1 Liquefaction and saccharification in lower temperature

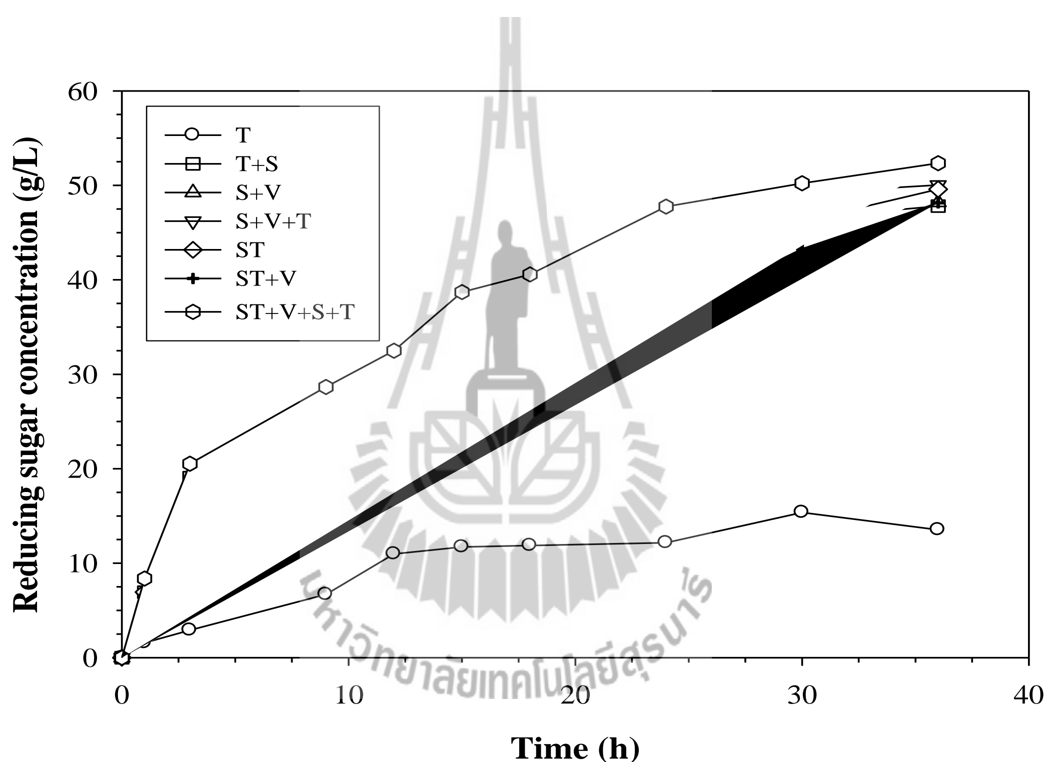

Figure 4.7 Time course of reducing sugar liberated by various enzymes. Hydrolysis was conducted at 37 °C, 200 rpm, 36 hour with 1:1 water-to-mash ratio offresh cassava mash. (ST: *STARGEN*™ 001, S: Spirizyme®, V: Viscozyme®, and T: Termamyl®).

Typical liquefaction process was operated at high temperature of 80°C since it is the optimum working temperature of the enzyme. In order to reduce the heating cost, enzymatic treatments of fresh cassava mash were attempted by using different types of enzyme. Figure 4.7 shows experimental data of enzymatic activities resulting in liberation of reducing sugar for 36 h of incubation time. Different enzyme systems show different reducing sugar productivity rates. Liquefaction and saccharification were conducted at 37 °C, 200 rpm agitation rate, and 1:1 water-to-mash ratio, respectively. Of four different enzymes tested (ST: STARGEN™ 001, S: Spirizyme®, V: Viscozyme®, and T: Termamyl®), the ST is the only enzyme with the main objective for the hydrolysis of native starch to reducing sugar. According to the manufacturer, the granular starch hydrolyzing enzymes contains *A. kawachi* α-amylase expressed in *T. reesei*, and glucoamylase from *A. niger*. For the first stage, multi-enzymatic activities involved in cassava mash saccharification were investigated systematically using different commercial enzyme combinations. Experimental results show that the obtained reducing sugar was in the range between 0.13-0.30 g/g cassava mash at 36 h of cultivation. The enzyme mixture ST+V+S+T resulted in the highest reducing sugar concentration at 52.23 g/L with the productivity of productivity of 1.34 g/L/h. The α-amylase (T) enzyme yield the minimum product of 14.87 g/L and the productivity of reducing sugar 0.4 g/L/h was obtained. The cooperative action of an array of polysaccharide hydrolyzing enzymes is thus essential for efficient reducing sugar releasing of this substrate. Enzymatic hydrolysis by individual hydrolyzing enzymes (ST, V, S, and T) at enzyme loading released relatively high levels of reducing sugar, which were derived from decomposition of the cellulose and starch polysaccharide components. The combined action of

cellulose, amylase and glucoamylase yielded more reducing sugar (242 mg/g cassava mash) owing to the efficient hydrolysis of the structure containing compounds. However, it was observed that the addition of commercial cellulose enzyme (V) did not appreciably increase the reducing sugar yield, except for the viscosity reduction during pre-mashing step. In contrast, addition of ST alone also led to a marked increase in reducing sugar yield. The higher reducing sugar yield could be due to the presence of strong raw starch-hydrolyzing side activity of the commercial enzyme, in addition to the possible direct effect from the α-amylase and glucoamylase activity on saccharide derived substrates. Nevertheless, combination of ST and cellulose enzyme (ST+V) did not show any effect on reducing sugar yield. In conclusion, the combination of α-amylase and glucoamylase resulted in an increase in reducing sugar concentration for both two manufacturers (S, T from Novozymes, and ST from Genencor). The activity of these enzymes did not differ significantly in terms of liberation of reducing sugar. However, the ST from Genencor has an advantage over Novozymes because it is a mixture of α-amylase and glucoamylase in one single dosage whilst S and T have to buy separately. In addition, ethanol fermentation was investigated using the hydrolyzed cassava mash. After the treatment of various enzyme systems, yeast was added to the prepared cassava slurry, and incubated at 37 °C for 36 h with shaking at 200 rpm. At the end of fermentation, ethanol concentration was measured. Figure 4.8 shows the experimental result for final ethanol concentration obtained with various enzyme systems. The most efficient ethanol fermentation performance was associated with the efficacy for liberation of reducing sugar using the combination of enzymes as previously shown in Figure 4.7.

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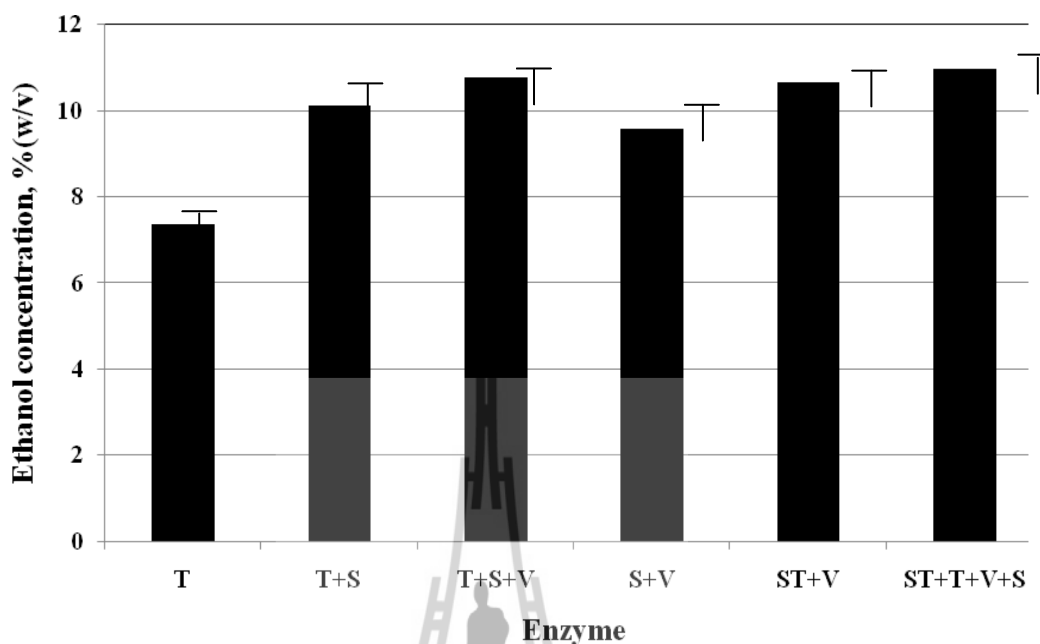


Figure 4.8 Ethanol concentration with various enzymes. Hydrolysis was conducted at 37 °C, 200 rpm, and 36 hour with 1:1 water-to-mash ratio offresh cassava mash. (ST: *STARGEN*™ 001, S: Spirizyme®, V: Viscozyme®, and T: Termamyl®).

Enzymatic hydrolysis of cassava mash is the combination of activities for granular starch and cellulose to produce fermentable sugar that was subsequently converted to ethanol. The results confirmed the efficiency of the mixture enzyme result to maximum ethanol production for saccharification and fermentation process. The quantity of ethanol obtained from the use of enzymes, each with a different significant statistical level ($p < 0.05$) were found using the enzymes ST + T + V + S. the maximum amount of ethanol (10.56 ± 0.29), T + S + V (10.52 ± 0.17), ST + V (10.15 ± 0.04), T + S (10.07 ± 0.15), S + V (9.59 ± 0.14) and T (7.47 ± 0.11)

respectively. The ST + T + V + S. to provide the maximum amount of ethanol does not vary with the enzyme T + S + V significant statistical percent confidence level 95 ($p > 0.05$). Therefore, the use of enzymes. ST + T + V + S for hydrolysis to the maximum of ethanol. However, if the cost of enzymes ST + T + V + S is high, it can be used instead of the enzymes T + S + V as well.

4.3.2 Effect of di-ammonium phosphate on ethanol production

This experiment studied the effect of di-ammonium phosphate (DAP) for ethanol fermentation performance. Di-ammonium phosphate (DAP) was used as nitrogen supplement for the yeast cells. The experiment varied the percentage of di-ammonium phosphate (DAP) at 0 (control), 0.5, 1.5, 3, and 4.5%w/v, respectively.

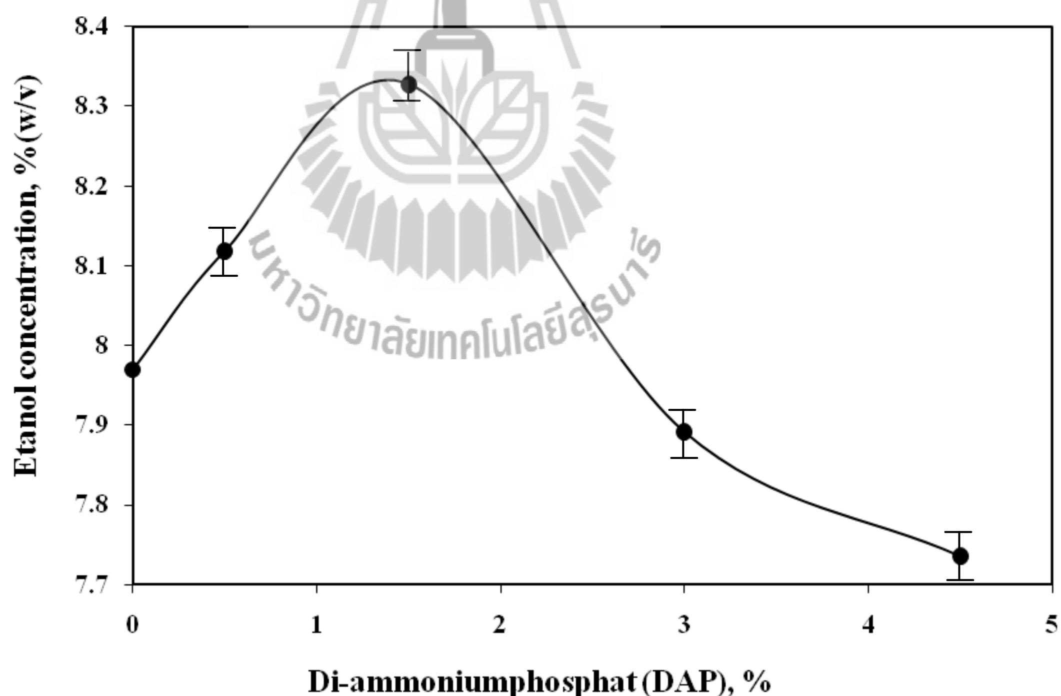


Figure 4.9 Effect of di-ammonium phosphate (DAP) concentration on final ethanol concentration % (w/v).

After supplement cassava mash with di-ammonium phosphate (DAP), yeast was added to the flask prior to incubate at 37 °C. Figure 4.9 shows the experimental results for the effect of di-ammonium phosphate (DAP) on fermentation performance. After 24 h, samples were taken and analyzed for ethanol concentration. The results of the effects of di-ammonuim phosphaton the generation of ethanol and reducing sugar utilization are presented. Supplementation with various dose of di-ammonium phosphate (DAP) for nitrogen and phosphorus sources resulted in an increase in the amount of ethanol generated in each sample. Experimental results revealed that additional ethanol generation was nutrient concentration dependent. Di-ammonium phosphate (DAP) concentrations examined in this work for the ethanol production were 0% (control), 0.5%, 1.5%, 3%, and 4.5% respectively. The resulting ethanol concentrations obtained at these concentrations were 7.98, 8.12, 8.33, 7.90, and 7.74 %w/v, respectively.

The result showed the maximum ethanol concentration of 8.33 wt% was obtained at 1.5 % of di-ammonium phosphate (DAP). After this point, the maximum ethanol concentration decreased, and the lowest concentration of 7.74 wt% was obtained at 4.5 % DAP. During fermentation process, reducing sugar concentration decreased followed by a decrease in pH. It indicated that the substrate accommodated the microorganism growth and produced ethanol. The decrease pH of substrate might due to the ionization of di-ammonium phosphate (DAP) that is used as nitrogen sources. *Saccharomyces cerevisiae* used NH_3 as a nitrogen sources and liberated H^+ into fermentation broth. Accumulation of H^+ given occasion to decrease the pH of the substrate solution (Akponah, E and Akpomie, O, 2012).

4.3.3 Effect of pretreatment by sonication on ethanol fermentation

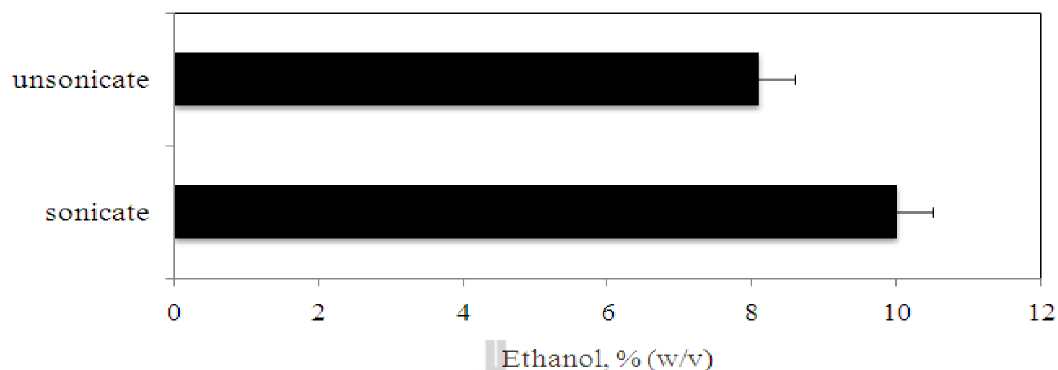


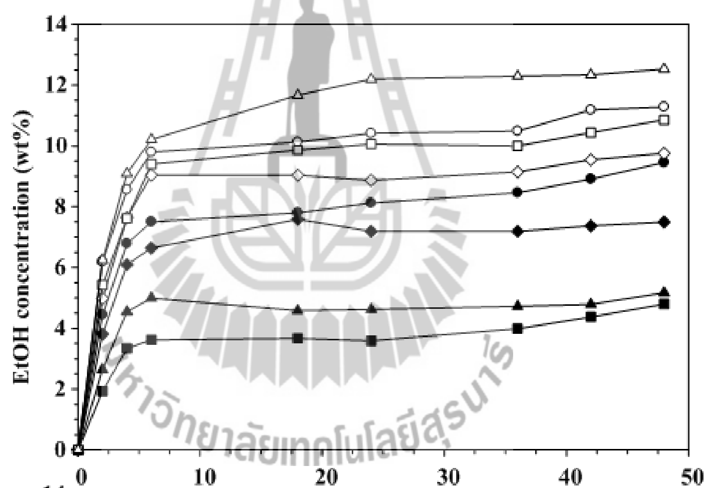
Figure 4.10 The percentage of ethanol yield profiles for sonicated samples at high power level for 30 s and the control (non-sonicated).

In this work, the effect of sonication on fermentation performance was investigated. After the preparation cassava mash, it was subjected to sonication at 30 s at each power level in order to degrade starch molecule from glanura. Commercial enzyme (*STARGEN*^{TM} 001; 0.5%, v/w) and yeast were added following sonication for liquefaction-saccharification and fermentation of the cassava starch. Comparison for the final ethanol concentration between non-sonicated and sonicated samples are presented in the Figure 4.10. The ethanol concentrations were determined after after 72 h.

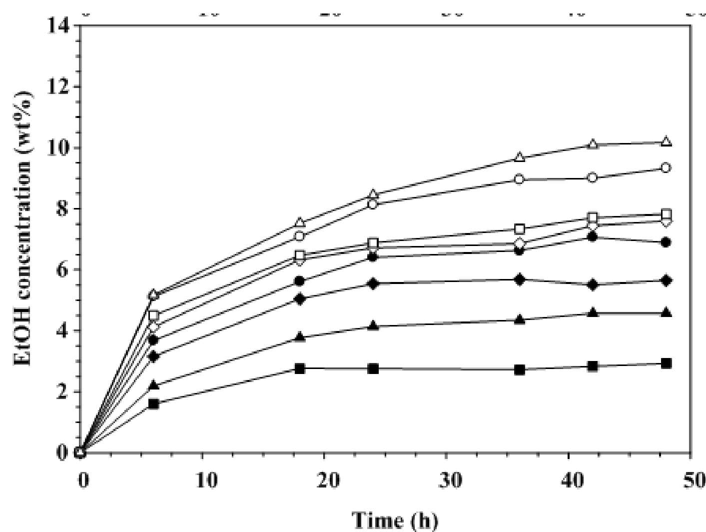
The result showed that sonicated mash of cassava resulted in a higher ethanol production than non-sonicate. The final ethanol concentration of 10.03, and 8.09 %wt for sonicated, and non-sonicated samples were obtained. The cassava cells in milled samples are generally intact, and starch granules are confined within the cell structure (Nitayavardhana *et al*., 2008). Sonication generates hydrodynamic shear

forces in the aqueous solution that facilitate the disruption of coarse particles and fibers in cassava slurry. The destruction of cassava cell structures during sonication released more starch granules in the aqueous phase, thereby exposing a much larger surface area to enzymes (Nitayavardhana *et al.*, 2010). Thus, the sonicated sample resulted in a higher reducing sugar release than the control sample. The higher ethanol yield for sonicated sample was therefore attributed to the availability of more reducing sugar in the fermentation media.

4.3.4 Effect of water-to-mash ratio



A



B

Figure 4.11 Time course of ethanol concentration from SSF (A) and SLSF (B) using uncooked fresh cassava slurry as substrate. (—■—) 10%, (—▲—) 20%, (—◆—) 40%, (—●—) 50%, (—◇—) 60%, (—□—) 80%, (—○—) 90%, and (—△—) 100%, respectively.

From the economic point of view, the water-to-mash must be at the optimum value. Too dilute mash results in low ethanol concentration and high amount of water added whilst too high solid concentration could result in difficulties in operating process as well as substrate inhibition effect. For industrial application, the substrate concentration must be high enough for enhancing the volumetric productivity of ethanol resulting in a reduced size of bioreactor. Fresh cassava mash with the water-to-mash ratio of 9:1(10% of cassava mash) to 0:10(100% of cassava mash) were incubated at 200 rpm for 48 h. The enzyme system used to hydrolyze fresh cassava mash for all experiments was S+T+V+ST. As shown in Figure 4.11, the level of ethanol production was compared using two different approaches; SSF and SLSF, respectively. In general, experimental result showed that ethanol concentration increased with an increasing in slurry concentration or decreasing water-to-mash ratio. At a fresh cassava mash of 100%, the corresponding ethanol concentrations were 12.5 and 10.2 %w/v with the initial volumetric productivities of 1.22 and 0.32 g/L/h, respectively. For SSF process, the cassava starch was partially hydrolyzed before an addition of yeast cells. As a result, higher volumetric productivities of ethanol were observed for all slurry concentrations in comparison to SLSF process. A high slurry concentration can lead to a high ethanol concentration, resulting in improved efficiency of upstream processing. However, it was reported that the

efficiency of ethanol fermentation deceased with an increase in mash concentration. When the concentration increased, the operation was difficult and a significant decrease in fermentation efficiency was observed, due to the difficulty of heat and mass transfer at high solid loading (Mitchell *et al.*, 2003).

4.4 Batch extractive fermentation using vacuum fractionation

In this work, batch extractive fermentations were operated in a 3 L bioreactor with the working volume of 2 L. The experimental results were compared with 3 conditions including conventional batch, SSF, and SLSF, respectively.

4.4.1 Conventional fermentation of ethanol in SSF mode

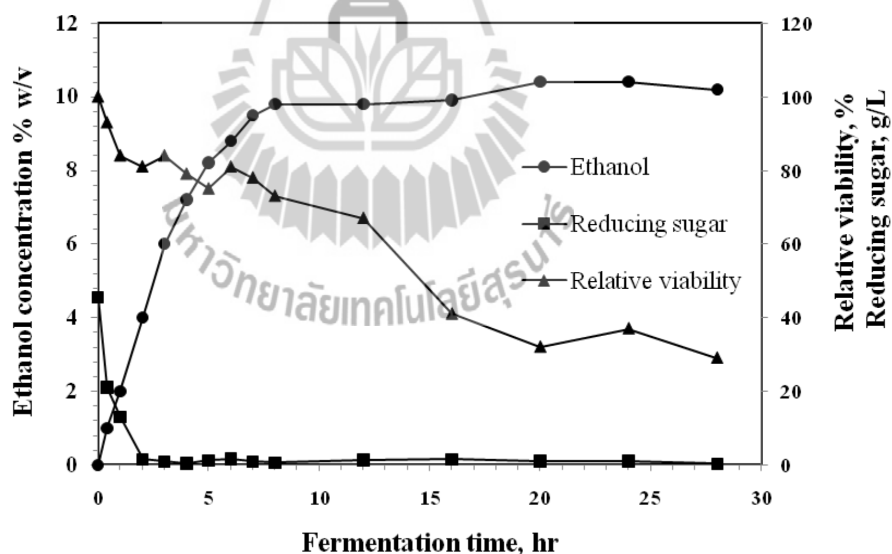


Figure 4.12 Time course for reducing sugar, ethanol concentration, relative viability of yeast cells from batch fermentation using SSF mode.

For conventional batch fermentation operated under atmospheric pressure, cassava mash was put to the bioreactor before an addition of α-amylase

enzyme (T). Temperature was increased to 90 °C for 2 h prior to add the dried distiller yeast and glucoamylase enzyme (S) at 37°C for the saccharification of hydrolyzed starch to fermentable sugar.

From the experimental data of the section 4.2.2, it is strongly indicated that keeping the ethanol concentration at low level could results in high glucose consumption rate, high volumetric productivity, and low product inhibition effect to the yeast cells. Figure 4.12 illustrated the time course for reducing sugar consumption, ethanol formation, and relative viability of the yeast cells. Because the starch was partially hydrolyzed by liquefaction process, the initial reducing sugar was measured at approximately 40 g/L. The concentration of glucose was rapidly decreased at the first 5 h of fermentation before the consumption rate gradually decreased. However, nearly 0 g/L of reducing sugar remained throughout the fermentation time indicating that it was rapidly consumed by the yeast cells after saccharification process. However, it was suggested that glucose was still produced because ethanol concentration was constantly increased before reached plateau at approximately 10.3% after 10 h of operation. The ethanol concentration rapidly increased at the first 10 h with a volumetric productivity of approximately 20 g/L/h. This high volumetric productivity was obtained as a result of high initial concentration of yeast (10 g/L). The increasing concentration of ethanol strongly effect the relative viability of yeast. At the end of fermentation, relative viability decreased to only 20%.

4.4.2 Batch extractive fermentation using SLSF mode

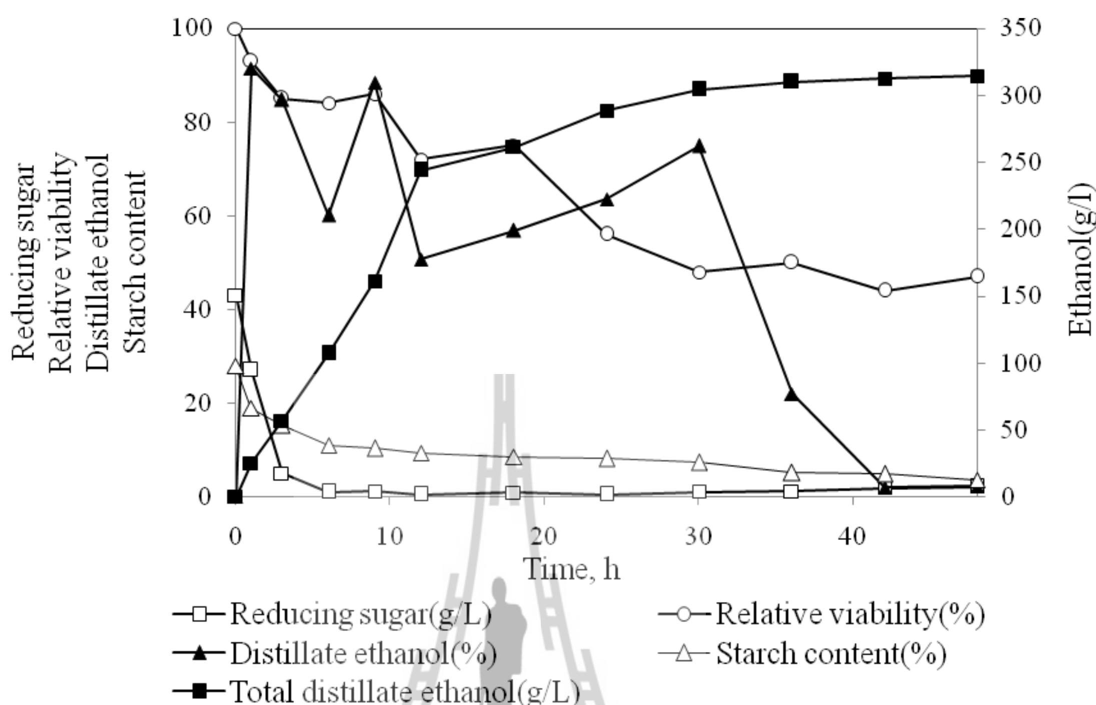


Figure 4.13 Time course for reducing sugar, ethanol concentration, relative viability and starch content from extractive batch fermentation using fresh cassava mash as substrate operated in SLSF mode.

Batch extractive fermentation operated with simultaneous liquefaction saccharification and fermentation (SLSF) with yeasts was performed. This process is expected to decrease the product inhibition on enzyme activity (i.e., glucose repression) as well as the osmotic stress on yeast cells due to high reducing sugar concentration (Sathaporn *et al.,* 2009). Figure 4.13 shows the experimental results of the batch extractive fermentation using SLSF mode. The initial cassava slurry content was 50 % (water-to-mash ratio of 1:1). In SLSF process, the starch in cassava material has to be initially uncooked, liquefied and sacchrafication prior to SLSF process. The concentration of reducing sugar in the system was rapidly decreased at the first 5 h of

fermentation before the consumption was reduced associated with the increasing ethanol concentration. Prior to reach the product inhibition regime, the vacuum pressure was gradually applied to the system at the rate of 200 mmHg/min until the pressure reached 65 mmHg. The ethanol vapor was fractionated to approximately 91 wt% before leaving the system. As a result, the ethanol concentration in the reactor was constantly below 2 %w/v while accumulate distillate ethanol reach to 314 g/L. This low level of ethanol stimulated substrate consumption as the reducing sugar concentration rapidly decreased and remained nearly 0 g/L throughout the experiment. Starch content in the reactor was gradually decreased almost completely from the hydrolysis of enzyme however also the starch remains in final. The ethanol was extracted as distillate throughout 48 h of operation. The product inhibition effect also reduced as cell viability was 40% at the end of the process. However, repeated-batch extractive fermentation of this system exhibited technical difficulties in which separation of yeast cells from the slurry was impossible due to a high solid content of the system. Therefore, only batch extractive fermentation was performed in this work.

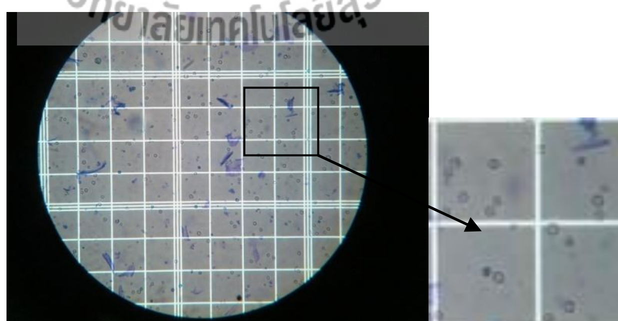


Figure 4.14 Methylene blue test for batch extractive fermentation using vacuum fractionation technique.

4.4.3 Comparison of conventional batch fermentation (CF), simultaniues saccharification and fermentation SSF, and simultaniues liquefaction saccharification and fermentation SLSF modes

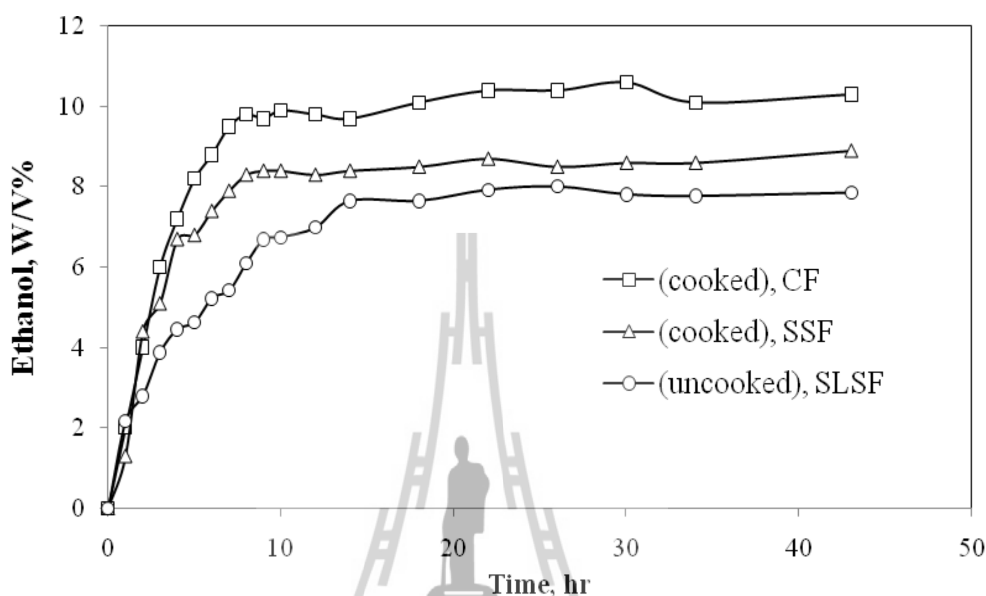


Figure 4.15 Comparison between conventional fermenation (CF), simultaniues saccharification and fermentation (SSF), and simultaniues liquefaction saccharification and fermentation (SLSF) during ethanol production from cassava fresh roots. Water-to-mash ratio 1:1, 37 °C, shaking speed 200 rpm,

In this work, three modes of experiment were compared including conventional batch fermentation (CF), SSF, and SLSF modes. As shown in Figure 4.15, the ethanol content of the CF reached 10.2 %wt whilst SSF and SLSF processes yielded ethanol about 9.1 and 7.9 %wt, respectively. Volumetric productivity also followed the same trend as CF yield the highest value followed by SSF and SLSF, respectively. Although the CF yield the highest concentration of ethanol product;

however, SSF and SLSF used lower energy and time in processing of cassava mash. In CF mode, the mash need to be liquefied and saccharified prior to undergo solid-liquid separation, and fermentation process. Removal of solid substances allowed repeated addition of cassava wort, although it was costly to prepare. The SSF used heat treatment just for liquefaction of cassava mash prior to conduct fermentation, thus saving time for saccharification. In SLSF mode, fermentation started with the same process of liquefaction and saccharification. In conclusion, choosing the fermentation mode should be considered from an economic point of view.

4.5 Energy consumption consideration of extractive fermentation

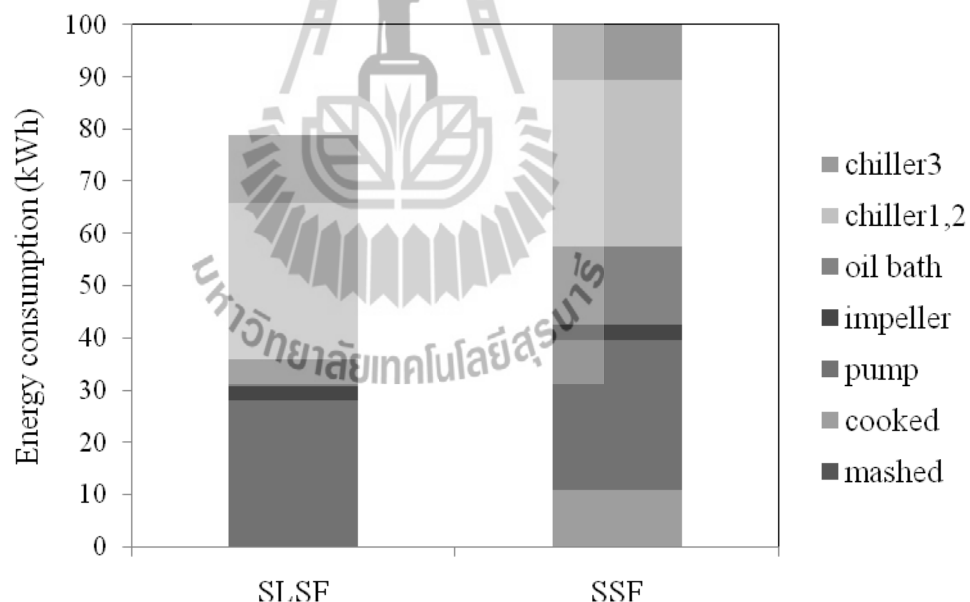


Figure 4.16 Energy consumption during extractive fermentation experiment using SSF, and SLSF mode.

The final part of this thesis focused on the analysis of energy consumption for batch extractive fermentation using SSF and SLSF processes. During the SSF and

SLSF experiments, a commercial watt-hour meter was used to measure the energy consumption of each unit operation. Watt-hour meter was set with electric equipment in process then can be calculate energy consumption per hour.

The bar charts for energy consumption of each unit operation in the extractive fermentation process are illustrated in Figure 4.16. The calculation of energy consumption showed that heat treatment of cassava mash samples (1:1 water-to-mash ratio) for gelatinization (SSF) required approximately 103.24 kWh, while SLSF (uncooked) of the same system required only about 78.9 kWh. Thus, SLSF required nearly half of the energy used in the SSF process in order to obtain similar ethanol yields. More importantly, the SSF process did not significantly improve ethanol yield in comparison to the SLSF process. This finding suggested that by integrating SLSF process into a fresh cassava mash, the overall production cost could be significantly decreased associated with a shorter processing time and less energy input. The process may even eliminate the cooking step.

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CHAPTER V

CONCLUSIONS

A high efficiency continuous distillation column using forced mixing concept was previously developed at the Suranaree University of Technology (SUT). The highest ethanol concentration of approximately 16 wt% was obtained when using a very high gravity cassava mash as the substrate. The extractive fermentation using a vacuum fractionation technique was successfully developed for continuous removal of 91 wt% ethanol from fermentation broth. Vacuum fractionating technique was successfully introduced to simultaneously remove high purity ethanol from fermentation broth whilst its concentration in the bioreactor was kept constant at 2 wt% throughout 48 h of operation. Unlike other works, the high concentration of ethanol obtained in this work requires no further distillation, and can be dehydrated directly in order to produce fuel grade ethanol. A long operation time and a high ethanol yield were attributed to a reduced product inhibition effect to the yeast cell. This particular system has advantages over conventional fuel ethanol process in terms of simpler system design, longer life of the yeast, and lower water discharge. The optimum initial reducing sugar concentration of approximately 250 g/L was chosen for repeated-batch extractive fermentation experiment. Repeated-batch extractive fermentation was operated when using solid-free cassava wort. In order to reduce fermentation cost, different approaches have been attempted including processing of native cassava starch, addition of cheap nitrogenous source, pretreatment using

sonication as well as SSF and SLSF experiments. Still, recycling of yeast cells, and more steady process operation should be further investigated for a larger scale experiment. For energy consumption, SSF required approximately 103.24 kWh, while SLSF uncooked of the same system required only about 78.9 kWh. Thus, SLSF required nearly half of the energy used in the SSF process.

Although this work showed a significant improvement on ethanol production of fresh cassava roots using vacuum fractionation technique, further studies are required if the process needs to go to the industrial application. From the current system, the fractionated ethanol vapor was extracted using a low vacuum pressure of 65 mmHg. As a result, a high energy of cooling must be used in order to condense this ethanol. Convenient ethanol vapor recovery by using an oil-free vacuum pump such as diaphragm pump, aspirator. Plant design using mathematical modeling programs is encouraged because the mass and energy balance can be calculated.

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REFERENCES

Abera, S. and Rakshit, S. K. (2007). Effect of Dry Cassava Chip Storage on Yield and Functional Properties of Extracted Starch. **Starch**. 56(6): 232 – 240.

Akpan, U.G., Kovo, A.S., Abdullahi, M. and Ijah, J.J. (2005). The production of ethanol from maize cobs and groundnut shells, Assumption University. **Journal of Technology**. 9: 106–10.

Akponah, E., and Akpomie, O. (2012). Optimization of bio-ethanol production from cassava effluent using *Saccharomyces cerevisiae.* **Affrican Journal of Biotechnology**. 11(32): 8110-8116.

Alexis, S. D., and Jean, N. G. Effect of Technological Treatments on Cassava (ManihotEsculentaCrantz) Composition, **Food and Nutrition Sciences**. 1: 19-23.

Bailey, J., and Ollis, O. (1986). **Biochemcial Engineering Fundamentals**. McGraw-Hill, New York, USA.

Balat, M. (2005). Global bio-fuel processing and production trends, **Energy Exploration and Exploitation**. 25: 195–218.

Bazue, C. (1975). Effect of alcohol concentration on the kinetics of ethanol production by *Saccharomyces cerevisiae*. **M.S. Thesis,** University of California, Berkeley.

Bullock J, Kristiansen B, editors (1987). **Basic biotechnology**. New York: Academic Press.

มหาวิทยาลัยเทคโนโลยีสุรนารี

Chauhan, R.P., Woodley, J.M. and Powell, L.W. (1997). Boron based separation for in situ recovery of L-erythrulose condensation. **Biotechnology Bioengineering.** 56: 345–351.

Chovau, S., Gaykawad, S., Straathof, A.J.J. and Van der Bruggen, B. (2011). Influence of fermentation by-products on the purification of ethanol from water using pervaporation. **Bioresource Technology**. 102: 1669–1674

Christen, M., Minier, M. and Renin, H. (1990). Kinetics of product inhibition in alcohol fermentation. **Biotechnology Bioengineering.** 10: 845-864.

Cysewski, G. R. (1976). Fermentation kinetics and process economics for the production of ethanol. **Ph.D. Thesis**, University of California, Berkeley.

Food and Agriculture Organization (FAO). 2011. Available from http://www.faostat.fao.org/site/339.

Freeman, A., Woodley, J.M. and Lilly, M.D. (1993). *In situ* removal as a tool for bioprocessing. **Biotechnology.** 11: 1007–1012.

Hofmeyr, J. H. S. (1997). Anaerobic energy metabolism in yeast as a supply-demand system in new beer in an old bottle. **Chemistry**. 1(2): 225-242.

Kosugi, A., Kondo, A., Ueda, M., Murata, Y., Vaithanomsat, P., Thanapase, W. and Mori, Y. (2008). Production of ethanol from cassava pulp via fermentation with a surface-engineered yeast strain displaying glucoamylase. **Renewable Energy**. 34(5): 1354-1358.

Krishnan, M.S., Xia, Y., Ho, N. W. Y. and Tsao, G. T. (1999). Fuel and Chemical from Biomass. **American Chemical Society Symposium Series**. 666: 74-92.

Kuiper, L., Ekmekci, B., Hamelinck, C., Hettinga, W., Meyer, S. and Koop, K.(2007). Bio-ethanol from cassava. **Ecofys Netherlands BV**, Netherlands.

Kühn, I. (1980). Alcohol Fermentation in an aqueous two-phase system. **Biotechnology Bioengineering**. 22: 2393-2398.

Labua, S., Namkang, K. and Jamrus, N. (2011). Comparison of ethanol production from cassava chips by fermentation using five yeast strains. **Rangsit Journal of Arts and Sciences**. 1(2): 113-120.

Laopaiboon, L., Nuanpeng, S., Srinophakun, P., Klanrit, P. and Laopaiboon, P. (2009). Ethanol production from sweet sorghum juice using very high gravity technology: effects of carbon and nitrogen supplementations. **Bioresource Technology**. 100: 4176–4182.

Lencki, R.W., Robinson, C.W. and Moo-Yong, M. (1983). Online extraction of ethanol from fermentation broths using hydrophobic adsorbents. **Biotechnology and Bioengineering Symposium Series**. 13: 617-628.

Lin, H.J., Xian, L., Zhang, Q.J., Luo, X.M., Xu, Q.S., Yang, Q., Duan, C.J., Liu, J.L., Tang, J.L. and Feng, J.X. (2008). Production of raw cassava starch-degrading enzyme by Penicillium and its use in conversion of raw cassava flour to ethanol. J. Ind. Microbiology. **Biotechnology**. 38(6): 733-742.

Luong, J.H.T. (1985). Kinetics of ethanol inhibition in alcohol fermentation. **Biotechnology Bioengineering**. 22: 1671-1687.

Lópes-Ulibarri, R. and Hall, G. M. (1997). Saccharification of cassava flour starch in a hollow-fiber membrane reactor, **Enzyme and Microbial Technology**. 21: 398-404.

MacLean, H.L. and Lave, L.B. (2003). Evaluating automobile fuel/propulsion system technologies. **Progress in Energy and Combustion Science**. 29: 1–69.

Matsumura, M. and Märkl, H. (1984). Application of solvent extraction to ethanol fermentation. **Applied Microbiology and Biotechnology**. 20: 371-377.

Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. **Analytical Chemistry**. 31(3): 426-428.

Mitchell, D.A., Meien, O.F.v. and Krieger, Nadia., (2003). Recent developments in modeling of solid-state fermentation: heat and mass transfer in bioreactors. **Biochemical Engineering Journal.** 13: 137–147.

Mulchandani, A. and Loung, J.H.T. (1989). Alcohol toxicity in yeasts and bacteria. **Enzyme and Microbial Technology**. 11: 66-73.

Müller, M. and Pons, M.N. (1991). Coupling of gas membrane smooth pervaporation and alcoholic fermentation. **Journal of Chemical Technology and Biotechnology**. 52: 343-358.

Najafpour, G.D. and Najafpoand Lim, J. (2002). Evaluation and Isolation of Ethanol Producer Strain SMP-6. **Regional Symposium on Chemical Engineering**. 229–236.

Nguyen, T. L. T, Gheewala, S. H. and Garivait, S. (2006). Life cycle cost analysis of fuel ethanol produced from cassava in Thailand. **Int J Life Cycle Assess. 13: 564-573.**

Nguyen, V. D., Auresenia, J., Kosuge, H., Tan, R. R and Brondial, Y. (2011). Vacuum fermentation integrated with separation process for ethanol production. **Biochemical Engineering Journal**. 55(3): 208-214.

มหาวิทยาลัยเทคโนโลยีสุรนารี

Nitayavardhana, S., Rakshit, S.K., Grewell, D., Pometto III, A.L., van Leeuwen, J.(Hans). And Khanal, S.K. (2008). Ultrasound pretreatment of cassava chip slurry to enhance sugar release for subsequent ethanol production. **Biotechnol. Bioeng**. 101: 487–496.

Nitayavardhana, S., Shrestha, P., Rasmussen, M. L., Lamsal, B. P., van Leeuwen, J. H. and Khanal, S. K. (2010). Ultrasound improved ethanol fermentation from cassava chips in cassava based ethanol plants. **Bioresource Technology**. 101(8): 2741-2747.

OAE. (2006). **Agricultural statistics of Thailand**. Center for agriculture in formation. Office of Agricultural Economics, Ministry of Agriculture and Cooperatives Agriculural Statistics.

Oclool, F. C. and Ayernor, G. S. (2010). Production of alcohol from cassava flour Hydrolysate. **Journal of Brewing and Distillation**. 1(2): 15-21

Offeman, R. D., Stephenson, S. K., Franqui, D., Cline, J. L., Robertson, G. H. and Orts, W.J. (2008). Extraction of ethanol with higher alcohol solvents and their toxicity to yeast. **Separation and Purification Technology**. 63(2): 444-451.

Ogbona, C. N and Okoli, E. C. (2010). Convertion of cassava flour to fuel ethanol by sequential solid state and submerged cultures. **Process Biochemistry**. 45(7): 1196-1200.

O'Brien, D. J., Lorie, H. R. and Andrew, J. M. (1999). Ethanol production by continuous fermentation-pervaporation: a preliminary economic analysis. **Journal of Membrane Science**. 166: 105-111.

O'Brien, D. J., Senske, G. E., Kurantz, M. J. and Craig, J C. (2004). Ethanol recovery from corn fiber hydrolysate fermentations by Pervaporation. **Bioresource Technology**. 92(1): 15-19.

Park, C. H and Geng, Q. (1992). Simultaneous fermentation in the ethanol and ABE fermentation. **Separation and Purification Methods**. 21(2): 127-174.

Pham, C. B., Motoki, M., Matsumura, M. and Kataoka H. (1989). Simultaneous ethanol fermentation and stripping process coupled with rectification. **Journal Fermentation and Bioengineering**. 68(1): 25-31.

Pimkaew, S. and Boontawan, A. (2010). Process Optimization for Motor Fuel Grade Ethanol Production using Hybrid Vapor Permeation and Pressure Swing Adsorption Technique. **European Journal of Scientific Research**. 644-646.

Ramasamy, A. and Paramasamy, G. (2001). Production of Ethanol from Liquefied Cassava Starch Using Co-Immobilized Cells of *Zymomona smobilis* and *Saccharomyces diastaticus.* **Journal of bioscience and bioengineering**. 92(6): 560-564.

Rattanachmsri, U., Tanapongpipat, S., Eurwilaichitr, L and Champreda, V. (2008). Simultaneous non-thermal saccharification of cassava pulp by multi-enzyme activity and ethanol fermentation by *Candida tropicalis*. **Bioscience Bioengineering**. 107(5): 488-493.

Rothman, H., Greenshields, R. and Calle, F.R. (1983). **The alcohol economy: fuel ethanol and the Brazilian experience**. London: Francis Printer. 198-199.

Samnaknit, W., Kongkaew, A. and Boontawan, A. (2012). Extractive fermentation of ethanol using vacuum fractionation technique. *In preparation*.

Srichuwong, S., Fujiwara, M., Wang, X., Seyama, T., Shiroma, R., Arakane, M., Mukojima, N. and Tokuyasu, Ken. (2009). Simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash for the production of ethanol. **Biomass and bioenergy**. 33: 890 – 898.

Shabtai, Y., Chaimovitz, S., Freeman, A. and Kathalski-Katzir, E. (1991). Continuous ethanol production by immobilized yeast reactor couled with membrane pervaporation unit. **Biotechnology Bioengineering**. 38: 869.

Silalertruksa, T. and Gheewala, S.H. (2010). Security of feedstocks supply for future bio-ethanol production in Thailand. **Energy Policy**. 38: 7476–7486.

Srirotha, K., Piyachomkwanb, K., Wanlapatitc S. and Oates, C.G. (2000). Cassava Starch Technology: The Thai Experience. **Starch**. 52: 439–449

Taylor, F., Marquez, M. A., Johnston, D. B., Goldberg, N. M. and Hicks, K. B. (2010). Continuous high-solids corn liquefaction and fermentation with stripping of ethanol. **Bioresource Technology**. 101(12): 4403-4408.

Tham, M.T., Willis, M.J., Massimo, C.D., Montague, G.A. and Morris, A.J. (1990). Artificial Neural Networks in Process Engineering. **Institution of Electrical Engineers Proceedings**. 138(3): 256-266.

Uden, N.V. (1989). **Alcohol toxicity in yeasts and bacteria**. Boca Raton, FL., CRC Press.

Voet, V.A. (1995). **Glycolysis in the diversity of metabolism in prokaryotes**. Vol. 1. University of Wisconsin-Madison.

Walsh, P.K., Liu, C.P., Findley, M.E., Liapis, A.I. and Siehr, D. (1983). Ethanol separation from water in a two-stage adsorption process. **Biotechnology and Bioengineering Symposium**. 13: 629-647.

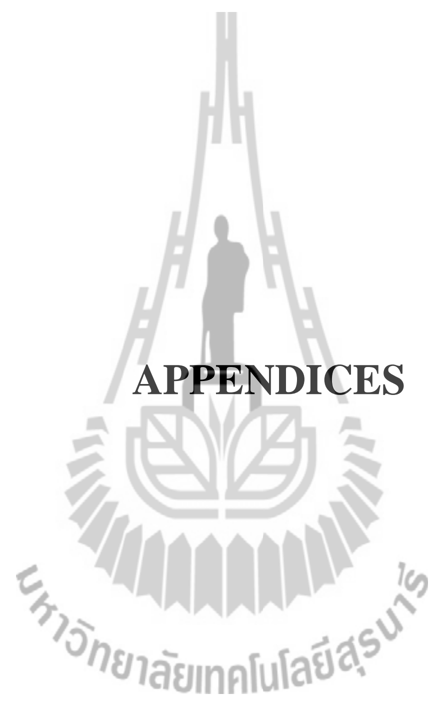
Wang, D., Li, K. and Teo, W.K. (2000). Highly permeable polyethersulfone hollow fiber gas separation membranes prepared using water as non-solvent additive. **Journal of Membrane Science**. 176: 147-158.

Yan, L. and Shuzo, T. (2005). Ethanol fermentation from biomass resources: current state and prospects. **Applied Microbiology and Biotechnology**. 69(6): 627-642.

Zamora, L.L., Amir, J., Calderón, G., Vázquez, E.T. and Reynoso, E.B. (2010). Optimization of Ethanol Production Process from Cassava Starch by Surface Response. **Sociedad Química de México**. 54(4): 198-203.

Zhu, M., Li, P., Gong, X. and Wang, J. (2012). A comparison of the Production of Ethanol between Simultaneous Saccharification and Fermentation and Separate Hydrolysis and Fermentation Using Unpretreated Cassava Pulp and Enzyme Cocktail, Bioscience. **Biotechnology and Biochemistry**. 76(4): 671-678.

APPENDICES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APPENDIX A

STANDARD CURVES

1. Glucose Standard calibration curve

The standard curve of glucose was prepared by dissolving glucose in distilled water and diluted to obtain various concentrations (Table 1A). Take the sample take each samples for 0.5 mL into test tube then add Dinitrosalicylic (DNS) solution 0.5 mL (raio 1:1) gently mixed. Take the reaction mixed into water bath 100 °C for 15 min then cool down in cool water immediately, add DI water 4 mL into reaction mixed then gently mixed. Take the sample for measurement at OD 540 nm and standard curve was made by plotting between different concentrations of standard glucose against their absorbance values as shown as in figure 1A.

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Table 1A The correlation between concentration of standard glucose and their absorbance value at 540 nm.

Glucose concentration (mg/mL)	**OD_{540} (Average)**
0	0
0.2	0.103
0.4	0.236
0.6	0.359
0.8	0.479
1.0	0.612
2.0	1.195

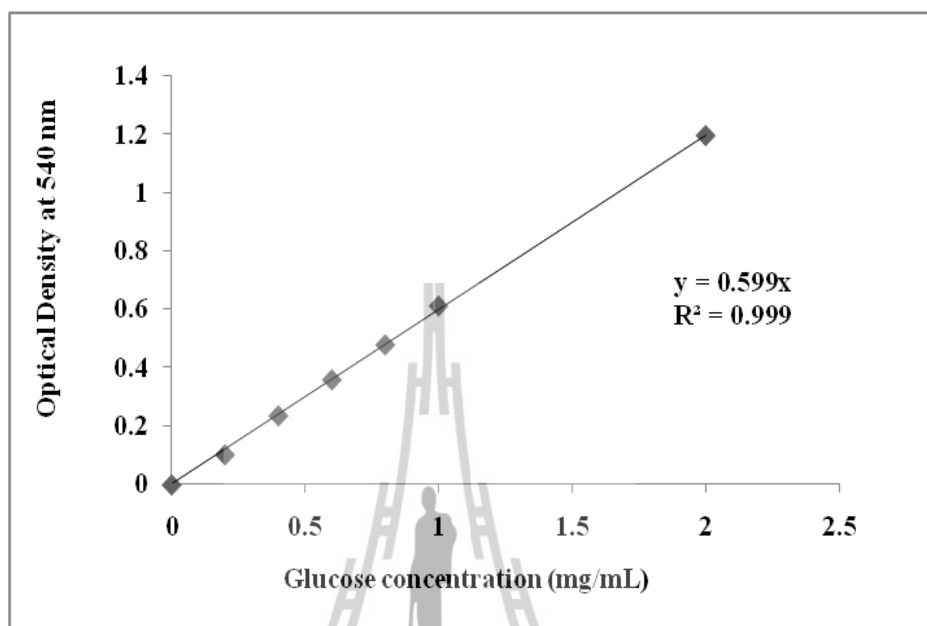

Figure 1A Standard curve of standard glucose using DNS method.

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2. Ethanol Standard calibration curve

The standard curve of ethanol was prepared by ethanol stock solution dilution 99.99 % of ethanol with some distilled water, then transferred the solution into a 100ml volumetric flask and filled to the line. Prepare 0%, 1%, 3%, 5%, 7%, 9% and 11% standard ethanol solutions. Then the standard ethanol was measure by GC injection.

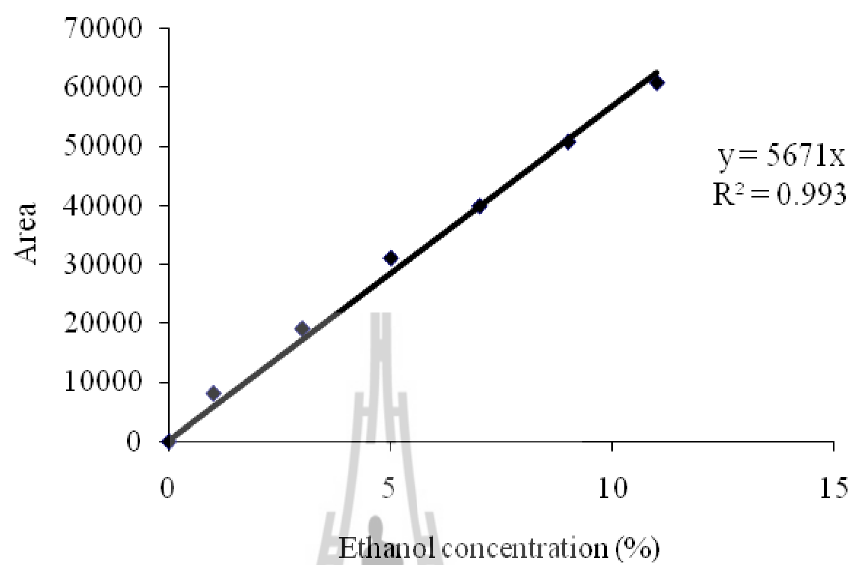

Figure 2A The correlation between concentration of standard ethanol and their graph area from GC.

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APPENDIX B

PRESENTATION

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Extractive fermentation of ethanol from fresh cassava roots using vacuum fractionation technique.

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Keywords: Cassava, Ethanol, Simultaneous Saccharification and Fermentation (SSF), Simultaneous Liquefaction, Saccharification and Fermentation (SLSF), Extractive fermentation, Vacuum fractionation.

Abstract. Fresh cassava roots were used as a raw material for ethanol fermentation. Conventional batch mode (CF), simultaneous saccharification and fermentation (SSF) and simultaneous liquefaction, saccharification and fermentation (SLSF) were investigated with various enzyme systems. The ethanol concentrations obtained in batch fermentations ranged from 8-12 wt%. In addition, vacuum fractionating technique was successfully introduced to simultaneously remove high purity ethanol from fermentation broth in batch mode. The distilled ethanol concentration was approximately 86 wt% whilst its concentration in the bioreactor was kept lower than 2 wt%. As a result, the product inhibition effect to yeast cells was reduced.

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Introduction

Cassava is an important energy crops for fuel ethanol production. Cassava starch can be fermented into ethanol in a conventional process of liquefaction, saccharification, and fermentation. Recently, numerous research works focus on simultaneous saccharification and fermentation (SSF), and simultaneous liquefaction saccharification and fermentation (SLSF) as a one step process. In addition, Very High Gravity (VHG) processes having very high soluble solid contents (>30%) are gaining attentions. Combination of VHG and SLSF processes can reduce the energy consumption during the fermentation and distillation process. The main advantage over conventional fermentation process is the reduction in water usage as well as the energy used in the manufacturing process [1]. In addition, these particular processes yield approximately 10-14 wt% of ethanol in the fermentation broths. However, product inhibition effect can occur during fermentation. Then, *in situ* ethanol removal from fermentation can reduce this problem as well as enhance the ethanol production. In order to increase fermentation performance, different methods have been introduced to simultaneously separate ethanol from fermentation broths including pervaporation membrane bioreactor [2], membrane distillation bioreactor [3], gas stripping [4], solvent extraction [5], and vacuum fermentation [6]. Nevertheless, the ethanol products obtained from these techniques contain a large amount of water typically in the range between 20-40 wt%. As a result, additional distillation step is required in order to obtain azeotropic ethanol prior to dehydration step. In this work, extractive fermentation by using a vacuum fractionation technique was investigated. Batch fermentations of fresh cassava roots as the sole carbon source were investigated with different enzymes. A vacuum fractionation coupled with conventional fermentation was attempted in a 2-L bioreactor. The aims of this research were to increase the yield of ethanol production from cassava, and obtain a high purity of ethanol in the distillate.

BIOGRAPHY

Mr. SupathraPhakping was born in KhonKaen, Thailand. In 2010, she studied in Environmental Science, Faculty of Science, Khonkaen University, Khonkaen. She graduated with bachelor degree of Science in 2010. In 2011, she decided to further study for master degree in the field of Bioprocess engineering at school of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, NakornRatchasima,Thailand. Her research topic wasextractive fermentation of ethanol from fresh cassava roots by using vacuum fractionation technique. The results from part of this study have been presented and published Phakping, S., Ketudat-Cairns, M. and Boontawan, A.(2014).Extractive fermentation of ethanol from fresh cassava roots by using vacuum fractionation technique. In **The 5th KKU international engineering conference.** (pp1096-1100). Pullman KhonKaen raja orchid hotel, Khonkaen, Thailand.

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