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EXTRACTIVE FERMENTATION OF ETHANOL USING A VACUUM FRACTIONATION TECHNIQUE

Weeraya Samnuknit

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EXTRACTIVE FERMENTATION OF ETHANOL USING A VACUUM FRACTIONATION TECHNIQUE

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

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เทคนิคการแยกภายใต้ความดันสุญญากาศถูกนำมาใช้พร้อมกับการหมัก เพื่อแยกเอทานอลความเข้มข้นสูงในสถานะก๊าซออกจากน้ำหมัก เอทานอลที่ผลิตได้ในระหว่างกระบวนการหมักที่ความเข้มข้น 100 กรัมต่อลิตร สามารถยับยั้งการเจริญของเซลล์ยีสต์ได้ ในการทดลองนี้น้ำหมักถูกทำให้เดือดที่อุณหภูมิ 35 องศาเซลเซียส ด้วยการลดความดันลงจนถึง 70 มิลลิบาร์ เอทานอลถูกแยกได้สูงถึง 90 เปอร์เซ็นต์โดยน้ำหนัก ก่อนที่จะออกจากคอลัมน์ ผลที่ได้สามารถแยกน้ำออกจากเอทานอลโดยไม่ต้องผ่านขั้นตอนการกลั่นอีก ความเข้มข้นของเอทานอลในน้ำหมักจะรักษาไว้ที่ความเข้มข้นต่ำกว่า 25 กรัมต่อลิตร ดังนั้นความเข้มข้นของเอทานอลที่ลดลงจึงส่งผลดีต่อการอยู่รอดของเซลล์ยีสต์ สำหรับกระบวนการหมักแบบกะ ที่ความเข้มข้นเริ่มต้นของน้ำตาล 280 กรัมต่อลิตร อัตราการใช้น้ำตาลสูงถึง 26.6 กรัมต่อลิตรต่อชั่วโมง และน้ำตาลถูกใช้เกือบหมดภายในเวลา 21 ชั่วโมง สำหรับกระบวนการหมักภายใต้ความดันสุญญากาศแบบกะซ้ำ จึงมีการเติมน้ำตาลลงไปใหม่ได้ถึง 9 รอบ และสามารถผลิตเอทานอลได้มากกว่าการหมักแบบกะถึง 8 เท่า กระบวนการหมักภายใต้ความดันสุญญากาศแบบกะซ้ำหยุดลง เนื่องจากผลของการสะสมของผลิตภัณฑ์ข้างเคียงที่เป็นกรดแลคติค

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EXTRACTIVE FERMENTATION/ETHANOL/VACUUM FRACTIONATION/ INHIBITION EFFECT/ETHANOL PRODUCTION

A vacuum fractionation technique was introduced to simultaneously remove a significant amount of very high ethanol concentration in vapor phase from fermentation broth. The ethanol concentration of 100 g/L produced during the fermentation process could inhibit the growth of yeast cell. In this experiment, the broth was boiled at 35^{o}C by reducing the pressure to 70 mBar. The ethanol was fractionated for up to 90%wt before leaving the column. As a result, the obtained ethanol can be dehydrated without any further distillation. Ethanol concentration in the broth was kept below 25 g/L, thus it minimized the product inhibition effect on the survival ability of yeast cells. For batch extractive fermentation at the initial glucose concentration of 280 g/L, a high substrate utilization rate was obtained at 26.6 g/L.h and most of glucose was consumed within 21 h. For repeated batch extractive fermentation, addition of glucose was carried out up to 9 times and ethanol was produced 8 fold higher than that obtained from the batch fermentation. The fermentation was ceased due to accumulation of lactic acid by-products.

School of Biotechnology Student's Signature _____

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LIST OF ABBREVIATIONS

g/L	=	Gram per liter
α	=	Separation factor
K_i'	=	Substrate inhibition constant
K_S'	=	Saturation constant
P_m'	=	Maximum product concentration
$/m^3	=	US dollar per cubic meter
% wt	=	Percent by Weight
°C	=	Degree Celsius
µm	=	Micrometer
ai	=	Exponential constant
C_2H_5OH	=	Ethanol
$C_6H_{12}O_6$	=	Glucose
cm	=	Centimeter
Co	=	Cobalt
CO_2	=	Carbon dioxide
Cu	=	Copper
dP/*dt*	=	Maximum product rate
et al.	=	And others
EtOH	=	Ethanol
g	=	Gram
GC	=	Gas chromatography

LIST OF ABBREVIATIONS (Continued)

$g_{EtOH}/g_{cell}.h$	=	Gram ethanol per gram cell per hour
H	=	Hour
HPLC	=	High pressure liquid chromatography
J/g	=	Joule per gram
kPa	=	Kilopascal
L/ton	=	Liter per ton
mJ/kg	=	Millijoule per kilogram
mM	=	Milli molar
Mn	=	Manganese
P	=	Pressure
P	=	Product concentration
Psia	=	Pounds per square inch absolute
rpm	=	Revolutions per minute
S	=	Substrate concentration
SUT	=	Suranaree University of Technology
T	=	Temperature
T_2	=	Temperature of the exit vapor
V	=	Sample volume
VLE	=	The vapor/liquid equilibrium
YE	=	Yeast extract
$Y_{P/S}$	=	Ethanol yield factor of ethanol production and glucose utilization
Zn	=	Zinc

CHAPTER I

INTRODUCTION

1.1 Background

Ethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels, and is probably the most promising future fuel for transportation due to its high energy value and its simplicity of production process. Fermentation derived ethanol received a wide popularity as a motor fuel additive. However, a major challenge in the production of ethanol is the separation including high energy cost associated with the distillation of ethanol from the large excess of water. In the case of ethanol fermentation from glucose, the limitation of conventional process comes from high initial glucose concentration and high ethanol concentration inhibition. When initial glucose concentration in the medium is over 300 g/L and ethanol concentration in the fermentation broth reaches 10-14% by weight, both of specific growth rate and specific production rates of yeast decline, the cell mass in the fermentation broth decreases and glucose cannot be converted completely to ethanol (Cysewski and Wilke, 1997). For single substrate and multiple by-products, the specific production rate (ν) can be expressed in terms of inhibitions as follows equation (1) (Lin *et al*., 2008).

$$\nu = \nu_{max}\left[\frac{S}{K_S' + S + (S^2/K_i')}\right]\left[\Pi\left(1 - \frac{P}{P_m'}\right)\right]^{ai} \quad (1)$$

Where; ν_{max} is the maximum specific production rate, S is substrate concentration, K_S' is the saturation constant, K_i' is the substrate inhibition constant, P is the product concentration, and P_m' is the maximum product concentration, respectively. In addition, the superscript *ai* represents the exponential constant of the inhibitory product. Ethanol fermentation coupling with *in situ* product separation has attracted considerable interests over the past few decades. It combines biochemical reaction with selective mass transport of ethanol from the reaction site resulting in an increase of the product yield (Lye and Woodley, 1999). In order to increase fermentation performance, different methods have been introduced to simultaneously separate ethanol from fermentation broths including pervaperation membrane bioreactor (Chen *et al*., 2012; O'Brien *et al*., 2004; Thongsukmak and Sirkar, 2009), membrane distillation bioreactor (Gryta *et al*., 2000; Lewandowicz *et al*., 2011), gas stripping (Tayler *et al*., 2010), solvent extraction (Offeman *et al*., 2008), and vacuum fermentation (Cysewski and Wilke, 1977; Ghose *et al*., 1984; Lee *et al*., 1981; Nguyen *et al*., 2011). Nevertheless, the distillate or permeate ethanol products obtained from these techniques contain a large amount of water typically in the range between 20-40% by weight. As a result, additional distillation step is required in order to azeotrope mixture of ethanol solution prior to dehydration step.

In this work, extractive fermentation by using a vacuum fractionation technique was investigated. A convention bioreactor was equipped with an in-house fractionating column. The ethanol/water vapor mixture was fractionated allowing only high concentration of ethanol to leave the column. The consequence of this operation was not only to enhance yields and volumetric productivity, but also to obtain a high

concentration of ethanol that can be directly supplied for dehydration without any further distillation. The system was studied for both batch and repeated batch fermentations.

1.2 Research objectives

Extractive fermentation by using vacuum fractionation technique was developed in order to accelerate the product formation, improve the product yield, and facilitate downstream processing.

1.3 Scope and limitation of the study

1.3.1 Substrate and product inhibition characteristic

1.3.1.1 Substrates and product inhibition kinetics were investigated in order to understand the effect of each compound on fermentation performance.

1.3.1.2 In order to determine the effect of ethanol on the specific ethanol productivities, experiments with a range of initial ethanol concentrations in the fermentation media were performed and initial glucose concentration was selected in order to separate ethanol inhibition effects from those of substrate or nutrient limitation.

1.3.2 Conventional batch fermentation and batch extractive fermentation

1.3.2.1 Substrate consumption rate, specific productivity, percentage of survival ability of yeast cell was performed with the purpose of comparing the conventional batch fermentation with the extractive fermentation.

1.3.2.2 Batch extractive fermentation was interested in order to accelerate the product formation, enhance high production yield, and facilitate downstream processing.

1.3.2.3 Batch extractive fermentation was carried out at 70 mBar in order to remove high purity ethanol from fermentation broth, minimize the effect of product inhibition, and keep the temperature of fermentation broth close to 35^{o}C.

1.3.2.4 In order to minimize the effect of product inhibition on fermentation performance, the concentration of ethanol in the broth was kept lower than 25 g/L.

1.3.2.5 Under vacuum pressure operation, the temperature of the partial reflux condenser was controlled at 0^{o}C in order to control the ethanol leave the column at 90% by weight.

1.3.3 Extractive fermentation of ethanol in repeated-batch mode

1.3.3.1 For repeated-batch extractive fermentation, glucose was added into the system in order to avoid substrate inhibition effect and long continuation of fermentation activity was obtained.

1.3.3.2 The addition of glucose was carried out when the glucose concentration of the system depleted.

1.3.3.3 The addition of glucose was repeated until glucose concentration in the system was constant and no ethanol was produced.

1.3.4 Comparisons of extractive fermentation technology

The comparisons of this study with other technology were mentioned to reveal the advantage of this study.

CHAPTER II

LITERLATURE REVIEWS

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 natural resources such as grains, molasses, fruits, cobs, and shells. 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 *et al*., 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 (ethyl alcohol, $CH_3–CH_2–OH$ or EtOH) is a clear colorless liquid; it is biodegradable, low in toxicity and causes little environmental pollution if spilt. The physic-chemical properties of pure ethanol are show in Table 2.1 The ethanol is an oxygenated fuel that contains 35% oxygen, which reduces particulate and NO_x emissions from combustion. Ethanol has a higher octane number (Rahman *et al*., 2007), broader flammability limits, higher flame speeds and higher heats of vaporization. These properties allow for a higher compression ratio and shorter

burn time, which lead to theoretical efficiency advantages over gasoline in an internal combustion engine (Balat, 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). For the alcohol fuels, the physical properties are shown in Table 2.2.

Table 2.1 Physico-chemical properties of pure ethanol (Najafpour and Lim, 2002).

Properties	**Value**
Empirical formula	CH_3CH_2OH
Molecular weight	46
Normal boiling point, °C	78.32
Critical temperature, °C	243.1
Density, g/mL	0.7893
Heat of combustion at 25°C, J/g	29676.69
Auto-ignition temperature, °C	793.0

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, °C	253	464	333
Latent heat of vaporization, MJ/kg	0.26	1.18	0.91
Lower heating value, MJ/kg	44.4	19.9	26.7

In some areas, ethanol is blended with gasoline to form an E10 blend (10% ethanol and 90% gasoline) but it can be used in higher concentration such as E85 or E95 (For motor fuel grad ethanol was shown in Table 2.3). 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.

Table 2.3 Motor fuel grade ethanol (ASTM International 2004).

Components	**Unit**	**Min.**	**Max.**	**Test method**
Ethanol	wt%	98.7	-	EC/2807/2000 method I
Higher Saturated mono-alcohol	wt%	-	2	EC/2807/2000 method II
Methanol	wt%	-	1	EC/2807/2000 method III
Water	wt%	-	0.3	EN 15489
Inorganic chloride	mg/L	-	20	EN 15484
Copper	mg/kg	-	0.1	EN 15488
Total acidity (acetic acid)	wt%	-	0.007	EN 15491
Phosphorus	- mg/L - mg/100mL	-	0.5 10	EN 154887 EC/2807/2000 method II
Nonvolatile material	mg/kg	-	10	EN 15485, EN 15486
pH	-	6.5	9	EN 15490
Appearance	-	Clear and bright		Visual inspection

2.2 Ethanol production process

2.2.1 The overview of ethanol production process from starchy materials

The overview of ethanol production process as shown in Figure 2.1 involves the step of milling, hydrolysis of starch to release fermentable sugars, follow by inoculation with yeast. Chemically starch is a polymer of glucose. Yeast cannot use starch directly for ethanol production. Therefore, starch has to be completely broken down to glucose by a combination of two enzymes (amylase and amyloglucosidase), before it is fermented by yeast to produce ethanol. And then, go through the process of distillation and dehydration.

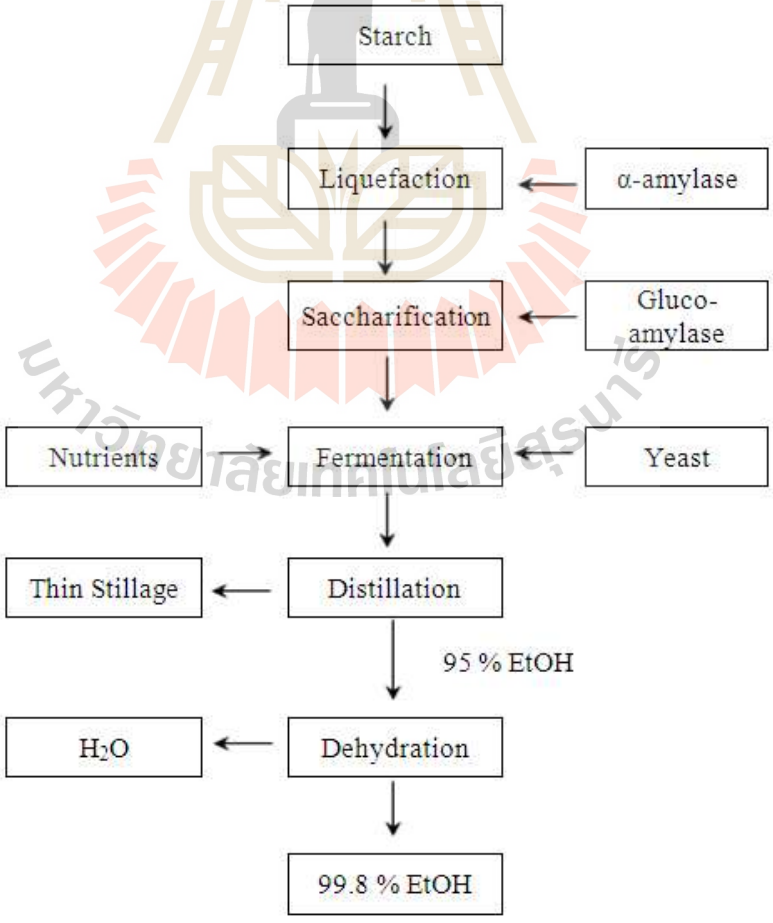

Figure 2.1 Ethanol production process from starch.

2.2.2 Ethanol fermentation

Fermentation, one of the oldest chemical processes known to man, 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.

Biological feed-stocks for ethanol fermentation are the materials that contains appreciable amounts of sugar or materials that can be converted into sugar, such as starch or cellulose (Malca and Freire, 2006). Bio-ethanol feed-stocks can be conveniently classified into three types: (i) sucrose-containing feed-stocks (e.g. sugar beet, sweet sorghum and sugarcane), (ii) starchy materials (e.g. wheat, corn, and barley), and (iii) lignocellulosic biomass (e.g. wood, straw, and grasses). Different feed-stocks that can be utilized forbio-ethanol production and their comparative production potential are given in Table 2.4 (Linoj *et al*., 2006), and the cost levels and comparison of bio-ethanol yield produced from different feed-stocks are presented in Table 2.5 (Dutch Sustainable Development Group, 2005; Wang, 2002).

Table 2.4 Different feedstock for bio-ethanol production and their comparative production potential (Linoj *et al*., 2006).

Feedstock	**Bio-ethanol production potential (L/ton)**
Sugar cane	70
Sugar beet	110
Sweet potato	125
Potato	110
Cassava	180
Maize	360
Rice	430
Barley	250
Wheat	340
Sweet sorghum	60
Bagasse and other cellulose biomass	280

In industrial operations, the microorganism of primary interest in fermentation of ethanol included *S. cerevisiae, S. uvarum, Schizosaccharomyces pombe* and *Kluyueromyces* sp. The microorganism to be used in ethanol fermentation process should have specific well defined characteristics, such as ability to ferment carbohydrates with high performance, high fermentation speed, osmotolerance, tolerance to ethanol, ability to produce high concentrations of ethanol, tolerance to acid medium, and high cellular viability for repeated recycling, resistance to high temperatures (Ferrari *et al*., 1980).

Table 2.5 Comparison of production cost and bio-ethanol yield from different feed stocks (Dutch Sustainable Development Group, 2005; Wang, 2002).

Type	Yield (ton/ha/year)	Conversion rate to sugar or starch (%)	Conversion rate to bio-ethanol (L/ton)	Bio-ethanol yield (kg/ha/year)	Coast[a] ($/m^3)
Sugar cane	70	12.5	70	4900	~160
Cassava	40	25	150	6000	700
Sweet-sorghum	35	14	80	2800	200-300
Corn	5	69	410	2050	250-420
Wheat	4	66	390	1560	380-480

Yeast, under anaerobic conditions, metabolizes glucose to ethanol primarily by way of the Embden-Meyerhof pathway (Figure 2.2). So, one glucose molecule breaks down into two pyruvates (1). The energy from this exothermic reaction is used to bind inorganic phosphates to ADP and convert NAD+ to NADH. The two pyruvates are then broken down into two acetaldehydes and give off two CO_2 as a waste product (2). The two acetaldehydes are then converted to two ethanol by using the H- ions from NADH; converting NADH back into NAD+ (3).

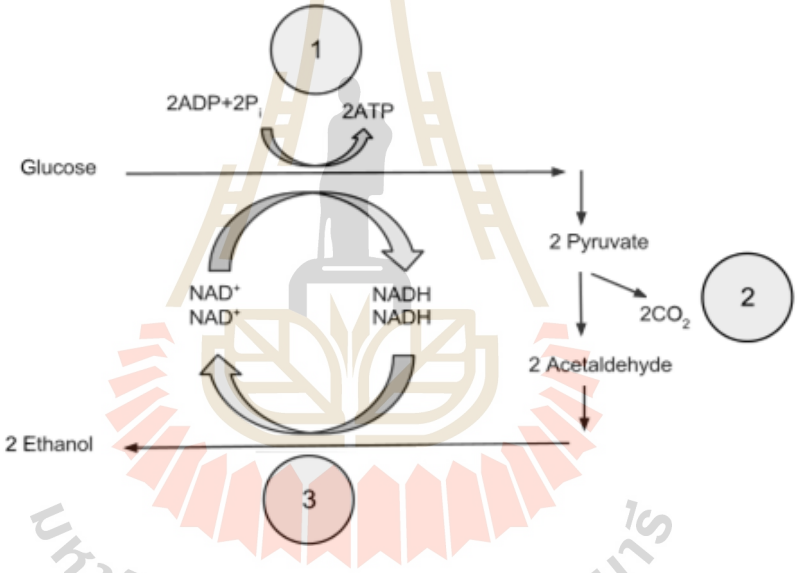

Figure 2.2 Embden-Meyerhof pathway (Bailey and Ollis, 1986).

The overall net reaction of ethanol fermentation as in equation (2) rely on yeasts that convert 1 mole of six-carbon sugars to 2 mole of ethanol and 2 mole of carbon dioxide. However, other substances such as glycerol and acetic acid are produced in smaller amounts (Cook, 1958; Conn and Stumpf, 1972; Stupiello and Horii, 1981; Scriban, 1985; Souza and Queiroz, 1995). In practical fermentations the yield however does not usually exceed 95% of theoretical (Najafpour and Lim 2002). This is partly due to the requirement for some nutrient to be utilized in the synthesis

of new biomass and other cell maintenance related reactions. The relative requirements for nutrients not utilized in ethanol synthesis are in proportion to the major components of the yeast cell. These include carbon, oxygen, nitrogen and hydrogen. To lesser extent quantities of phosphorus, sulfur, potassium, and magnesium must also be provided for the synthesis of minor components. Minerals (i.e. Mn, Co, Cu, Zn) and organic factors (amino acids, nucleic acids, and vitamins) are required in trace amounts.

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

Like any other microorganism, yeasts have high water requirements in order to grow and maintain metabolic activity. Osmotolerance is the ability of yeast to grow in media with high solute concentrations or in a low water activity environment. There are reports on yeast strains able to grow at high substrate concentration (around 250 g/L). However, the specific ethanol production rates were rather low (Panchal, 1990; Ortiz-Zamora *et al*., 2009; Walker, 1998). A maximum substrate concentration of 200 g/L has been recommended (Alfenore *et al*., 2002) because under this stress condition stimulation of glycerol production, needed to maintain water activity inside the cell, has been observed (Walker, 1998).

On an industrial scale it recommended to perform yeast cell recycling in order to improve process productivity. However, ethanol is a toxic compound that increases permeability and fluidity of the plasma membrane, causing viability loss (Charpentier, 1993). Additionally, high ethanol concentration can cause important metabolic changes in yeasts such as ATPase inhibition, denaturation of several glycolytic enzymes (Petrov and Okorokov, 1990) and changes in the cell wall (Francois and Aguilar-Uscanga, 2003).

2.2.3 Kinetics of ethanol fermentation

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).The specific production rate express in terms of product and substrate inhibition (Ghose and Tyagi, 1979) and (Nguyen *et al*., 2009) as follow:

$$\nu = \nu_{max}\left[\frac{S}{K_S' + S + (S^2/K_i')}\right]\left[\Pi\left(1 - \frac{P}{P_m'}\right)\right]^{ai} \quad (3)$$

$$Y_{P/S} = -\frac{dP/dt}{dS/dt} = \frac{Mass\ of\ ethanol\ produced}{Mass\ of\ glucose\ consumed} \quad (4)$$

Where; ν_{max} is the maximum specific production rate, S is substrate concentration, K_S' is the saturation constant, K_i' is the substrate inhibition constant, P is the product concentration, P_m' is the maximum product concentration which dP/dt is closed to zero, and dS/dt is substrate consumption rate, the superscript ai represents the exponential constant of the inhibitory product, respectively. In addition, $Y_{P/S}$ is defined as ethanol yield factor of ethanol production and glucose utilization.

2.2.3.1 Glucose and ethanol inhibition effect

The study of Benigno *et al*.in the influence of glucose and ethanol concentration on growth and fermentative activity shown that the duration of *S. cerevisiae* ITV-01 lag growth phase increased when initial ethanol concentration and glucose concentration was increased in culture media, and at 100 and 250 g/L of

initial glucose concentrations is not affected on the specific growth rate. Najafpour and Lim (2002) mentioned about at ethanol concentration of 1-2% weight by volume are sufficient to retard microbial growth, and at 10% weight by volume the growth rate of the organism is nearly halted.

2.2.3.2 Nutrient, pH, temperature and bacterial contamination effect

In order to discard the substrate inhibition effect, fermentations using enriched media were performed. Benigno *et al*. reported that yeast extract (YE) can stimulate the consumption rate of glucose because it provides important cofactors like biotin and riboflavin (Aguilar-Uscanga *et al*., 2000; Jorgensen, 2009), and no inhibition effect was observed at YE concentration of ≥2 g/L due to nutritional requirements.

However, there is an inhibition effect or limitation by other factors like pH or temperature. The tolerance of pH to low values and high temperature are related, since pH changes can increase temperature tolerance to the maximum (Coote *et al*., 1991). Temperature tolerance has also been related to media composition and other physical factors such as water activity. High temperatures cause a decrease in cell viability, as well as changes in mitochondria and fluidity of the plasma membrane (Swan and Watson, 1997). Based on this experiment of Benigno *et al*., the best substrate consumption occurred in the pH range 3.0-3.5. Biomass yield did not vary significantly in the pH range between 3.0 and 6.5, indicating that yeast growth is not affected by initial pH. The highest ethanol yield was obtained at pH values lower than 4.5. Almost all reports of *S. cerevisiae* strains (Sanchez-Gonzalez *et al*., 2009; Ortiz-Zamora *et al*., 2009; Alfenore *et al*., 2002; Moulin *et al*., 1984; Campos, 2008;

Turhan *et al*., 2010; Chin *et al*., 2010) used pH values from 4.5 to 5.5 with optimum temperature range between 35-39^{o}C.

Moreover, Bacterial contamination is an ongoing problem in commercial Fuel ethanol production facilities. Lactic and acetic acid are found in fermenter as byproducts produced by contaminated bacteria. Lactic acid is produced by *Lactobacilli* bacteria, and acetic acid is produced by *Acetobacter* and *Gluconobacter* bacteria. These acids can cause loosing activity of yeast with high ethanol and sugars concentrations. To avoid these acids, producers have to remove contaminated bacteria. However, it is very difficult to achieve because the condition suitable for yeast is also suitable for those bacteria. Also, their growth rates are much faster than yeast. Currently, the best way to deal with them is to take care of the pH, temperature and liquefaction very closely and to keep clean all equipments.

2.2.4 Extractive fermentation

Extractive fermentation is an alternative technique for reducing end product inhibition by removing the fermentation product *in situ* (Kollerup and Daugulis, 1985; Daugulis *et al*., 1991; Gyamerah and Glover, 1996; Kang *et al*., 1990; Offeman *et al*., 2005). In ethanol production process, ethanol should be extracted from the fermentation broth as it is formed for prevent product inhibition (Nguyen *et al*., 2009). Many proposed configurations using this integration approach are related to ethanol removal by different means such as vacuum fermentation, gas stripping, pervaperation combining fermentation and liquid extraction. Among them, the vacuum fermentation has been widely investigated for the ethanol fermentation with *S. cerevisiae* and *Z. mobilis* (Cysewski and Wike, 1977; Ghose *et al*., 1984;

Le *et al*., 1981; Ishida and Shimizu, 1996). The researches have shown that the fermentation under vacuum pressure is an effective alternative method for continuous ethanol removal. Basically, the theory of fermentation process running under vacuum pressure is based on both physical properties of ethanol-water mixture and biochemical properties of fermentation reaction by yeast. The fermentation process by yeast carried out in a temperature of 30-35^{o}C and the mixture of ethanol-water boils at 78.3-100^{o}C. When the fermentation process is operated under vacuum pressure the boiling point temperature of this mixture decreases. To overcome these limitations, the study of Nguyen *et al*. proposed an alternative operation condition for fermentation under vacuum pressure. This study showed that the yeast cells can survive and grow with very little amount of oxygen when the fermentation process is operated under vacuum pressure. In addition, the amount of sub-saturated carbon dioxide can be trapped almost by liquid nitrogen, so that vacuum condition can be maintained in the bioreactor.

2.2.5 Ethanol distillation

- **Basic principle of distillation**

The classical thermodynamic models commonly used in the literature to treat these mixtures at low pressure required a great amount of binary parameters to be determined from experimental data. The fundamental equation of Vapor–Liquid Equilibrium can be expressed as the equality of fugacities of each component in the mixture of both phases (Perry, 1998);

$$\overline{f}_i^L = \overline{f}_i^V \qquad (5)$$

The fugacity of a component i in the vapor phase ($\overline{f}_i^V$) is usually expressed through the vapor fugacity coefficient ($\overline{\phi}_i^V$),

$$\overline{f}_i^V = y_i \overline{\phi}_i^V P \qquad (6)$$

At low pressure (less than 2 bars), it is supposed that the vapor phase is ideal gas which means $\overline{\phi}_i^V = 1$, therefore,

$$\overline{f}_i^V = y_i P \qquad (7)$$

In addition, the fugacity of a component in the liquid phase is expressed through either the liquid fugacity coefficient ($\overline{\phi}_i^L$), or the activity coefficient (γ_i). The standard state fugacity (f_i^0) can be replaced by the saturate vapor pressure (P_i^{sat}) at the temperature of the system. As a result,

$$\overline{f}_i^L = x_i \gamma_i P_i^{sat} \qquad (8)$$

Therefore,

$$y_i P = x_i \gamma_i P_i^{sat} \qquad (9)$$

In Equation (9), P is the pressure of the system, x_i is the liquid phase mole fraction of component i, γ_i is the activity coefficient calculated by using the UNIQUAC model, and P_i^{sat} is the saturation vapor pressure of component i which can be expressed through Antonie's equation, respectively. The values of pure

components parameters of van der Waals properties, and Antoine's equation constants are given in Table 2.6.

Table 2.6 Pure components parameters: van der Waals properties of r_i and q_i, and Antoine's equation constants A_i, B_i, and C_i (Perry, 1998).

Components	r_i	q_i	**Antoine's constants**		
			A_i	B_i	C_i
Ethanol	2.1055	1.9720	7.1688	1552.60	222.42
Water	0.9200	1.4000	7.0436	1636.91	224.92

When a mixture of two or more liquids is heated and boiled, the vapor has a different composition than the liquid. For example, if a 10% mixture of ethanol in water is boiled, the vapor will contain over 50% ethanol. The vapor can be condensed and boiled again, which will result in an even higher concentration of ethanol. So, distillation operates on this principle. Clearly, repeated boiling and condensing is a clumsy process, however, this can be done as a continuous process in a distillation column. In the column, rising vapors will strip out the more volatile component, which will be gradually concentrated as the vapor climbs up the column. The vapor/liquid equilibrium (VLE) relationship between ethanol and water is shown in Figure 2.3. At 95.6% by weight of ethanol the composition of the liquid and the gaseous phase are equal. It seen that there is an azeotrope and the mixture cannot be separated after this point, the boiling point at 1 atm of mixture at azeotropic is 78.1°C.

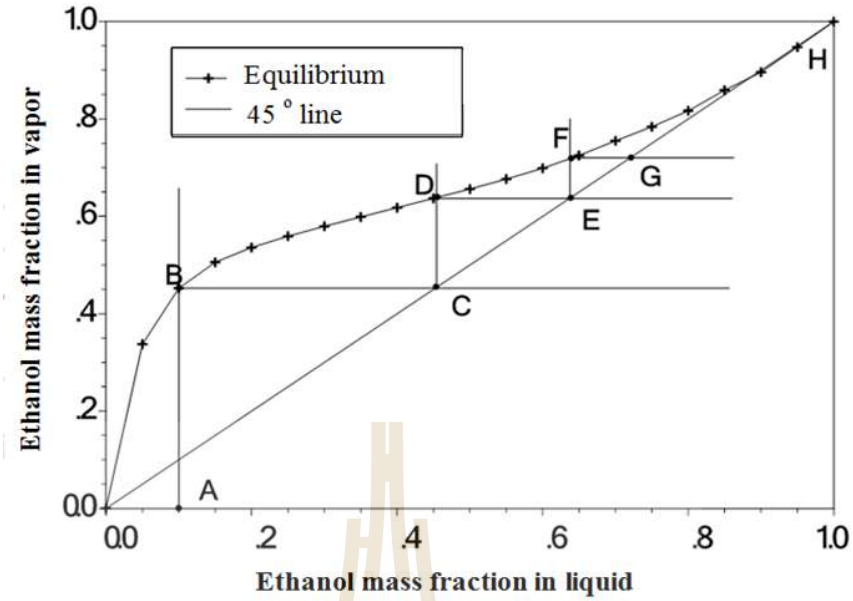

Figure 2.3 Shows the Vapor-Liquid-Equilibrium (VLE) diagram of EtOH/H_2O system at 1 atm (ACS, 2006).

In general, the process of ethanol purification starts when the fermentation process finish, and the initial concentration of the ethanol in broth is approximately 10-12%wt. The fermentation broth will be distilled in a fractionating column using heat until the concentration of ethanol eventually reach 95.6%wt, and will not be able to make ethanol concentration higher than this value. The ethanol/water mixture at this concentration is called azeotrope (azeotropic solution). Hence, in order to produce ethanol for use as fuel, it must use an additional process to break this concentration. In general, there are 3 processes involved namely azeotropic distillation, membrane process (pervaporation and vapor permeation), and molecular sieve adsorption processes.

Although the fermentation of ethanol from cassava or molasses is not difficult, the main problem in the production of fuel ethanol is the distillation to have the highest concentration of 95%wt before water removal in the last step. Because water and ethanol have close boiling points (100°C and 78.32°C, respectively), the

vapor mixture has ethanol and water at different concentrations depending on the ethanol concentration in the liquid phase. By nature, aqueous ethanol solution at different concentrations (x-axis) results in an ethanol concentration that different concentrations in the vapor phase (y-axis) called vapor-liquid equilibrium (VLE). The balance of vapor-liquid equilibrium of binary water and ethanol system at different concentrations is shown in Figure 2.3. For example, the typical fermentation broth contains ethanol at concentration approximately 10%wt (point A). The concentration of ethanol in the vapor phase as it occurs in the first distillation can be obtained by dragging a line to meet the VLE curve at point B which equal to 45%wt. If this vapor condenses, it becomes a liquid of the same concentration (point C). A repeated distillation once again will have an ethanol concentration at approximately 64%wt (point D), and condenses to become a liquid at the same concentration (point E). For 3 distillation cycles, the concentration of distillate ethanol is approximately 70% (point F). In order to increase its concentration, a series of further distillation steps is required. However, the magnitude of the increasing concentration will be reduced in comparison to the previous distillation cycle (AB>CD>EF). At 95%wt (point H), distillation will not be able to increase the concentration of the ethanol due to the formation of azeotropic mixture. From Figure 2.3, it can be seen that multiple distillation steps is required in order to obtain the highest purity of distillate ethanol.

In practical, more than 70 distillation cycles is conducted for this purity of the distillate ethanol. In order to increase the efficiency of refining system, a special system had been designed to be able to distill the fermentation broth using multiple distillation cycle at the same time called fractional distillation.

Fractional distillation is the separation of a mixture into its component parts, or fractions, such as in separating chemical compounds by their boiling point by heating them to a temperature at which one or more fractions of the compound will vaporize. For fractional distillation, the liquid mixture boil at less than 25°C from each other under a pressure of one atmosphere (atm). If the difference in boiling points is greater than 25°C, a simple distillation is used. Fractionating columns are widely used in the separation of ethanol from fermentation broth. Industrial distillation is typically performed in large, vertical cylindrical columns known as "distillation towers" or "distillation columns". Inside the column, the down-flowing reflux liquid provides cooling and condensation of the up-flowing vapors thereby increasing the efficacy of the distillation tower. The more reflux and/or more trays provided, the better is the separation of ethanol from higher boiling point of water. Bubble-cap "trays" or "plates" are one of the types of physical devices, which are used to provide good contact between the up-flowing vapor and the down-coming liquid inside an industrial fractionating column (shown in Figure 2.4). Each tray consists of the channel brought the rising vapor up through a slot for caps, and bubble mixture condensation downward (downcomer). In addition, each tray also has a small wire to detain some liquid on the tray. Hot vapor coming up from the lower tray makes the liquid to boil on the tray, and removes the ethanol that has a lower boiling point, rising up to the upper tray. By this manner, the volume in the tray increase due to partial condensation of the vapor before flow down to the lower tray via a weir to the downcomer channel.

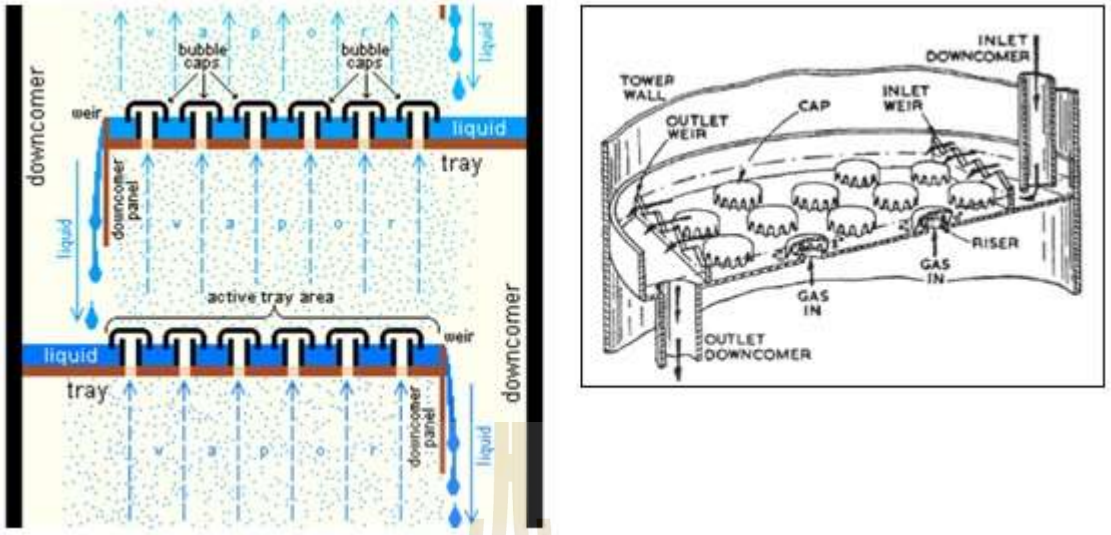

Figure 2.4 Internal structure of cap tray fractionating column (ACS, 2006).

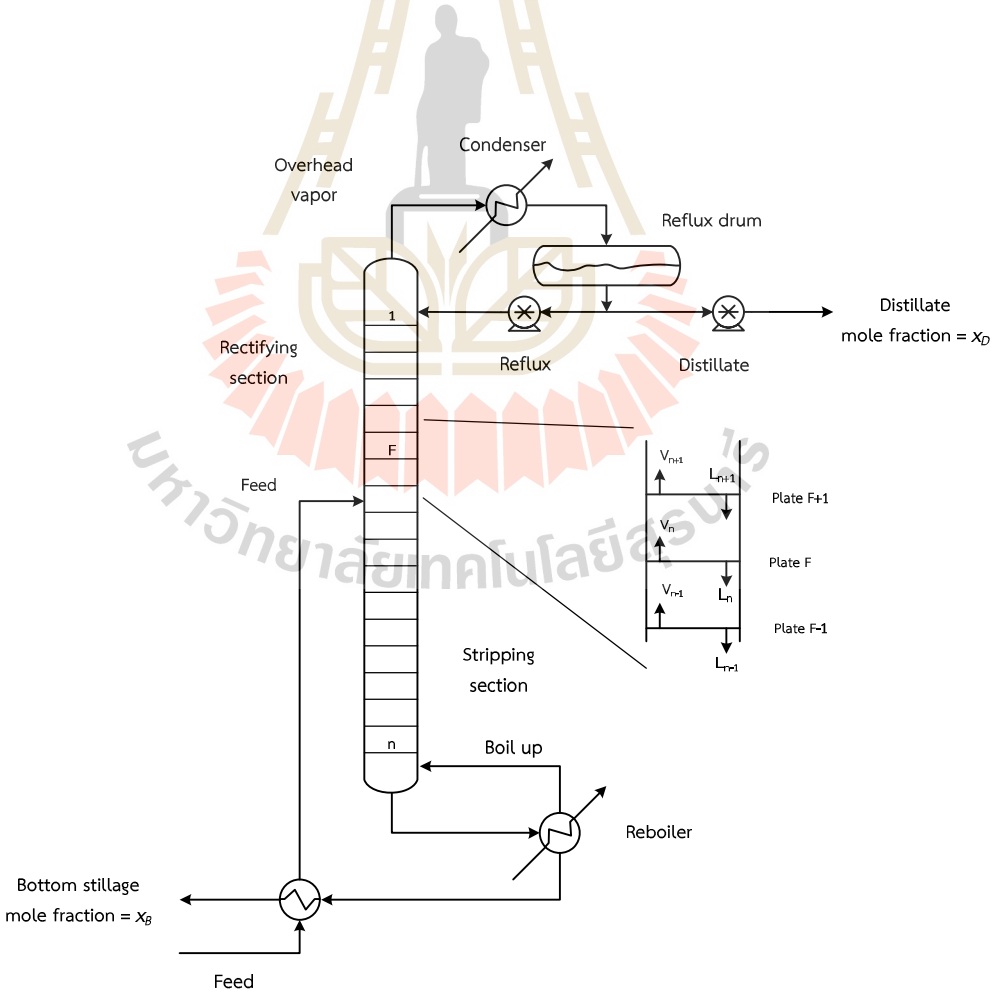

Figure 2.5 Working principle of the continuous distillation column (ACS, 2006).

Figure 2.5 depicts an industrial fractionating column separating a feed stream into one distillate fraction and one bottoms fraction. However, many industrial fractionating columns have outlets at intervals up the column so that multiple products having different boiling ranges may be withdrawn from a column distilling a multi-component feed stream. The "lightest" products with the lowest boiling points exit from the top of the columns and the "heaviest" products with the highest boiling points exit from the bottom.

Figure 2.5 shows a continuous distillation system where fermentation broth is fed at the middle point of the fractionating column. Trays are installed inside the column and the column is divided into two main parts. The first part is called a stripping section which is the lower part of the feeding point. The rectifying section refers to the portion of the column located above the feeding point. When the fermentation broth enters the column, ethanol will be vaporized from the fermentation broth, rising up to the upper tray. Consequently, the concentration of ethanol will be increasingly in the tray above. The fermentation broths with a reduced ethanol concentration will flowdown to the lower tray via the downcomer. The main heating system (reboiler) will cause the thin stillage to boil and the boiling vapor (boil up) depletes ethanol from fermentation broth prior to flow out of the column with high temperature. However, it can go into a heat exchanger to warm or preheat the fermentation broth before entering the fractionating column as the feed. This make the fermentation broth enters the column at a higher temperature resulting in a reduction of the production cost. For the rectifying section, the distillate ethanol exits the distillation column at the top (overhead vapor) before condenses through a heat exchanger (condenser) and goes into a tank (reflux drum). However, the problems that

arise in the development of distillation is the number of trays required to get the highest concentration of ethanol. For example, it requires more than 70 plates in order to obtain 95%wt distillate ethanol which makes the construction and maintenance cost expensive. Figure 2.6 shows an industrial scale fractionating column for continuous ethanol distillation using molasses as the substrate.

Figure 2.6 Shows an industrial scale fractionating column for continuous ethanol distillation using molasses as the substrate.

2.2.6 High efficiency distillation based on partial condensation concept

The economic competitiveness of the ethanol production process strongly depends on the amount of used heat and power. To increase the economic efficiency of anhydrous ethanol, a number of optimization steps have been proposed concerning the purification of ethanol, because especially concentration and dewatering of ethanol by distillation requires a high amount of thermal energy. One of the most difficult tasks for separation of ethanol from fermentation broth is distillation. Although ethanol is highly volatile, separation becomes more difficult especially at lower percentage of water in the vapor phase. Distillation is usually the method of choice for primary separation. However, water cannot be completely removed due to the presence of azeotrope at 95.6% by weight. Continuous fractionating distillation is usually employed; however, the number of stages required to achieve the desired degree of separation is very high. In general, industrial distillation column possesses more than 70 plates which results in very high investment cost. Smaller scale distillation units are not technically and economically feasible. Recently, a high efficiency lab scale distillation based on partial condensation concept was successfully developed in SUT. The design was very unique in terms of the column internal, and distillation performance.

- **Equipment design**

Figure 2.7 shows the schematic diagram of the continuous distillation unit based on forced-mixing concept. The feeding solution was pre-heated by entering the condenser, and heat exchanger prior to enter the column through the feeding point located at the middle of the column by using a peristaltic pump. The column was

equipped with a set of internal impellers. The middle impeller located at the feeding point served as a dispenser whereas the lower set of impellers had a function of stripping ethanol from fermentation broth. In addition, the upper set of impellers pushed the rising vapor to partially condense along the side of the column. The high agitation rate resulted in close contact between the vapor and liquid components resulting in a great number of condensation-vaporization cycles. As a result, high efficiency separation was achieved in a short distance of distillation column. At the top of column was connected with a variable speed motor (120 watts), which can be adjusted the stirrer speed by the controller machine. A set of internal impellers was fixed on a central shaft driven by a variable speed motor. The lower set of impellers had 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 was installed below the exit point of the column where distillate temperature T_1 can be precisely controlled by re-circulation of a cooling liquid (thermostat). As a result, high efficiency separation was obtained within a short distance of rectifying column, and reflux is not necessary.

During distillation process, the ethanol vapor was leaved to the top of column by controlling of a ball valve and then ethanol vapor entered to the condenser became to the ethanol-rich product. An oil bath is used as the main heating device. This process can operate continuously where the stillage was continuously removed from the bottom of the system. This system was designed for the energy saving where

2 heat exchangers were used to warm the feed solution as well as to cool down the distillate vapor and thin stillage. The design of this distillation column based on partial condensation technique is illustrated in Figure 2.7.

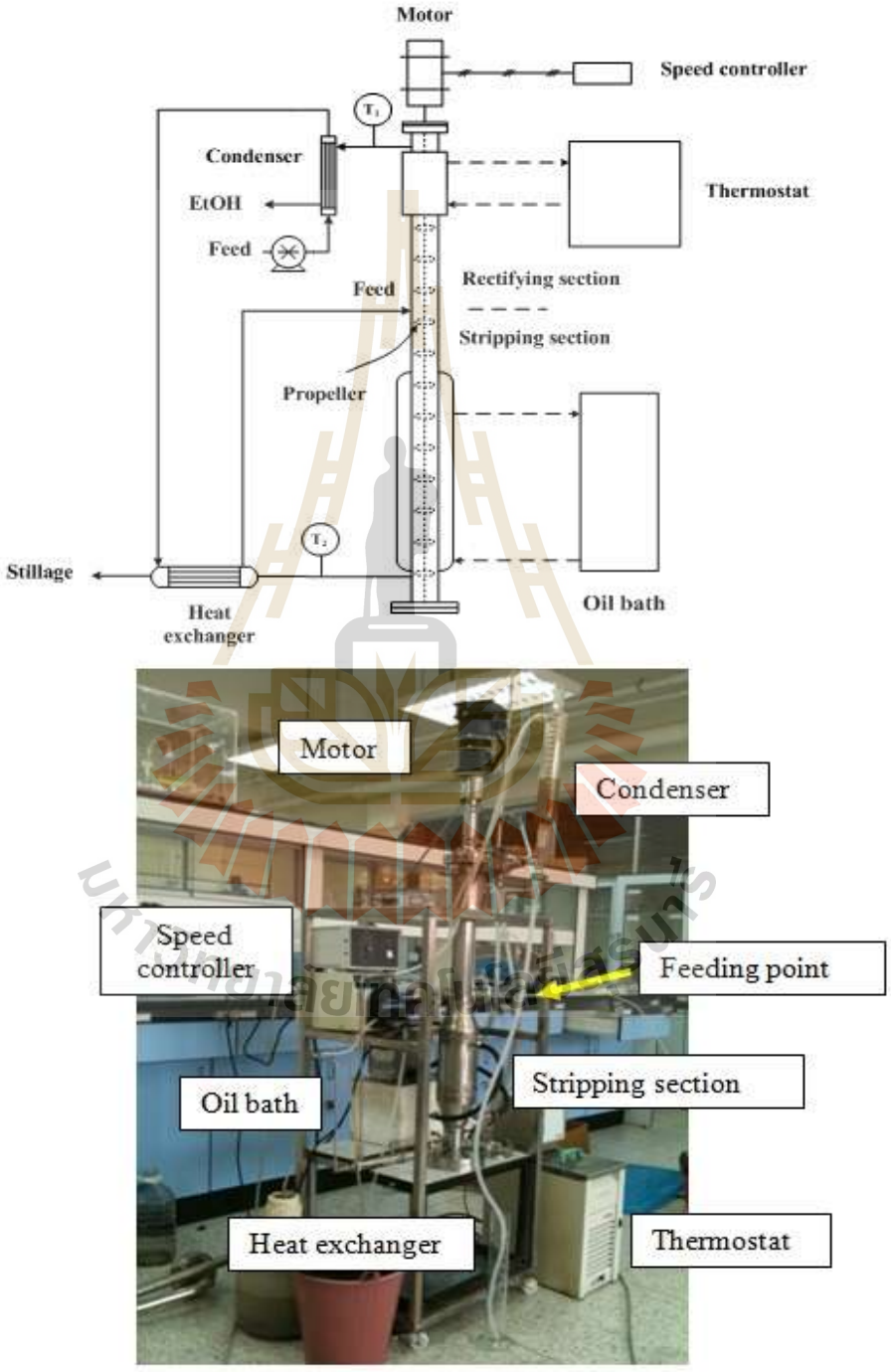

Figure 2.7 Schematic diagram and the working principle of the continuous fractionating column based on partial condensation technique.

CHAPTER III

METERIALS AND METHODS

3.1 Chemicals and fermentation process

All chemicals used in this study were of analytical grade. HYDRANAL® reagents for determination of water content were purchased from Sigma (Singapore). A commercially available dry distillery yeast (*S. cerevisiae*) manufactured in Denmark was used as the ethanol producer.

3.2 Experimental set up

The schematic diagram of the system and the experimental set up are illustrated in Figure 3.1 and Figure 3.2. A stainless steel column with a height of 40 cm was placed on top of 2.5 L glass bioreactor. In order to generate a well-mixed condition inside the column, a series of propellers was fixed on a central shaft driven by a variable speed motor. A mechanical seal was installed internally in order to create a leak-free condition. In addition, a water jacket was equipped below the exit port where a temperature controller was used to control the temperature of the exit vapor (T_1).

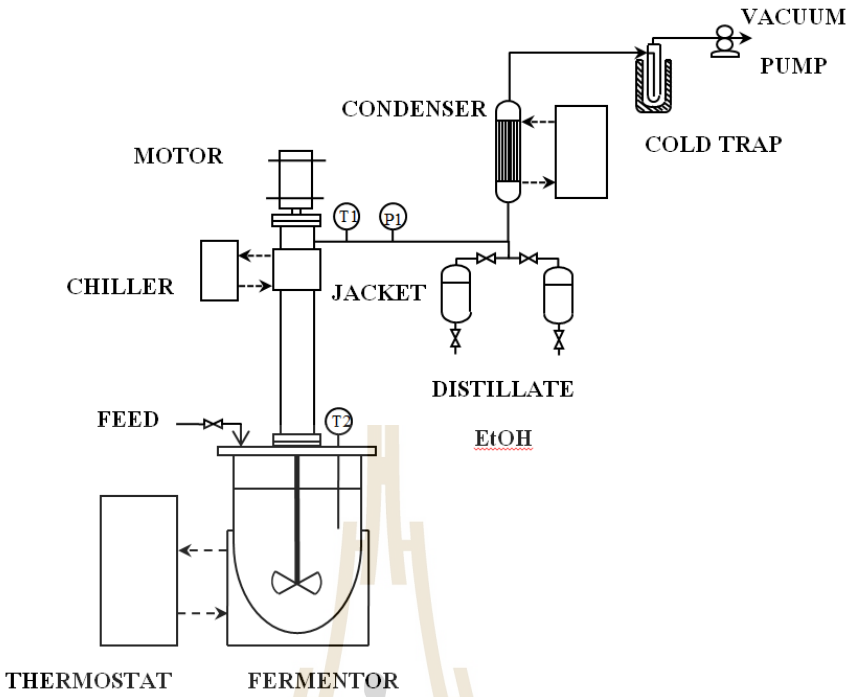

Figure 3.1 Schematic diagram and experimental set up of extractive fermentation of ethanol using vacuum fractionation technique.

Figure 3.2 Experimental setup for extractive fermentation of ethanol using vacuum fractionation technique.

3.3 Fermentation processes

The modified YM medium comprises of (per litre); 1.5 g of $(NH_4)_2SO_4$, 3.0 g of yeast extract, 3.0 g of malt extract, and 5.0 g of peptone. The pH was adjusted to 4.5 with citric acid prior to sterilization. Subsequently, glucose powder was added and the yeast was introduced into the fermentation medium at the concentration of 50 g/L. Fermentation was carried out at 35^{o}C without aeration. For extractive fermentation using vacuum fractionation technique, the experiment started after 1 h of inoculation by lowering the pressure to 65 mBar by using a vacuum pump (ChemStar®, Welch USA). The fermentation broth began to boil and the rising vapor of ethanol/water mixture was fractionated before leaving the column. Rotational speed of the propellers was fixed at 1000 rpm in order to ensure the well mixed condition inside the column. Temperature of the exiting vapor (T_1) was maintained at 19.4^{o}C where excessive water of the rising vapor condensed back into the bioreactor allowing approximately 93% by weight of ethanol to leave the column. The distillate ethanol was condensed using a series of a condenser (-30^{o}C) in connection with a glass cold trap containing liquid nitrogen. Two receiving units were installed so that the distillate ethanol can be removed without disturbing the vacuum condition inside the bioreactor. For repeated batch extractive fermentation, addition of glucose powder was carried out when the concentration in fermentation broth was lower than 0.5 g/L.

3.4 Analysis

Water content of the distillate ethanol was determined by using an automatic Karl Fisher's titration (Titro Line Plus, Schott, Germany) as shown in Figure 3.3. Glucose concentrations were determined by using a glucose analyzer (YSI, USA).

Cell viability analysis was carried out by using the methylene blue test. 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 + 9% 20 mM Na_2HPO_4 (pH 2) at a flow rate of 1 mL/min. The HPLC column was ZORBAX SB-Aq (4.6 mm x 150 mm). Ethanol concentration in the fermentation broth was analyzed by using a gas chromatography (SRI Instrument, USA) equipped with a FID Detector. The GC column was a 30 m x 0.32 mm fused silica capillary column (Carbowax®, Restek, USA). The injector and detector were set at 200, and 250^{o}C, respectively. The oven was operated at programmed temperature from 40-90^{o}C with the rate of 10^{o}C/min.

Figure 3.3 Setting of apparatus for an automatic Karl Fisher's titration (Titro Line Plus, Schott, Germany).

- **Determination of moisture content in samples using Karl Fischer titration**

The determination of water percent in samples is realized through a rapid test kit: HYDRANAL® - Moisture Test Kit (Sigma-Aldrich). The principle applied for the determination of water is based on the Karl Fischer method. It involves the oxidation of sulphur dioxide by iodine with consumption of water. The test kit uses ethanol-based reagents. The solvent, HYDRANAL®-Solvent E, is placed in the titration vessel and is titrated to dryness using HYDRANAL®-Titrant Component. The end point of the titration is indicated by a sharp color change from near colorless to yellow. The sample is introduced by means of a syringe and the titrated again. Karl's Fischer Titration reaction can be defined as follow;

$$CH_3OH + SO_2 + RN \longrightarrow [RNH]SO_3CH_3 \quad (9)$$

$$H_2O + I_2 + [RNH]SO_3CH_3 + 2RN \longrightarrow [RNH]SO_4CH_3 + 2\,[RNH]I \quad (10)$$

First, the titre of the HYDRANAL®-Titrant component must be determined. The titre is standardized during production, but in case of frequent use of the solution, it is however possible that a drop in titre is brought about by outside influences (introduction of moisture). For this reason it is recommended that a determination of titre is carried out at regular intervals, prior to use. An exact volume of 0.50 mL of the HYDRANAL®-Standard 5.00 included in the test kit is used in place of the sample. The titre (b) is then calculated from the consumption (a), using the following equation:

$B = (5.0 * 0.50)/A$

$$\text{Titre [mg water/mL]} = \frac{\text{Water content of standard [mg water/mL]} \times \text{Volume of standard [mL]}}{\text{Consumption [mL]}}$$

The water content, in percent by volume, is calculated from the consumption (a), the titre of the titrating solution (b), and the sample volume (V, in µL).

C = A*B*100 / V

$$\text{Concentration, in \% by volume} = \frac{\text{Consumption [mL]x Titre [mg water/mL] x100}}{\text{Sample volume[µL]}}$$

Remark: The density of ethanol at 20°C is 0.7893 g/mL and the water density at 20°C is 0.9982 g/mL.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Substrate and product inhibitions characteristic

Substrate and product inhibition kinetics were independently investigated in order to understand the effect of each compound on fermentation performance. For the application in the bio-ethanol production, it can be useful or necessary to apply fermentation media that have very high sugar concentrations. Such fermentations of high gravity media can increase downstream efficiency. However, high sugar concentrations may lead to growth inhibition, or increased formation of fermentation by-products such as glycerol, acetic acid, and higher alcohol leading to a reduction in ethanol yield. For the substrate inhibition kinetic, the experimental results and mathematical modeling of specific ethanol productivity are presented in Figure 4.1A as a function of initial glucose concentration ranging from 0-400 g/L. The values were determined by plotting ethanol concentration versus time at various glucose concentrations (data not shown). The period for rapid increasing in ethanol concentration was considered. All calculations were carried out by Marrquardt-Levenberg algorithm using the Sigma Plot program (Systat Software, USA). The mathematical modeling for substrate inhibition was in a good agreement with the experimental data at the r^2 value of 0.9462. 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 in this work. The high value of K_i' implying that fermentation can be carried out at high substrate concentration. 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.

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 concentration was shown in Figure 4.1B. Experimental data confirm that ethanol plays an important role on fermentation performance event 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_m' in the equation (1). The correlation was best fitted with an *ai* 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.

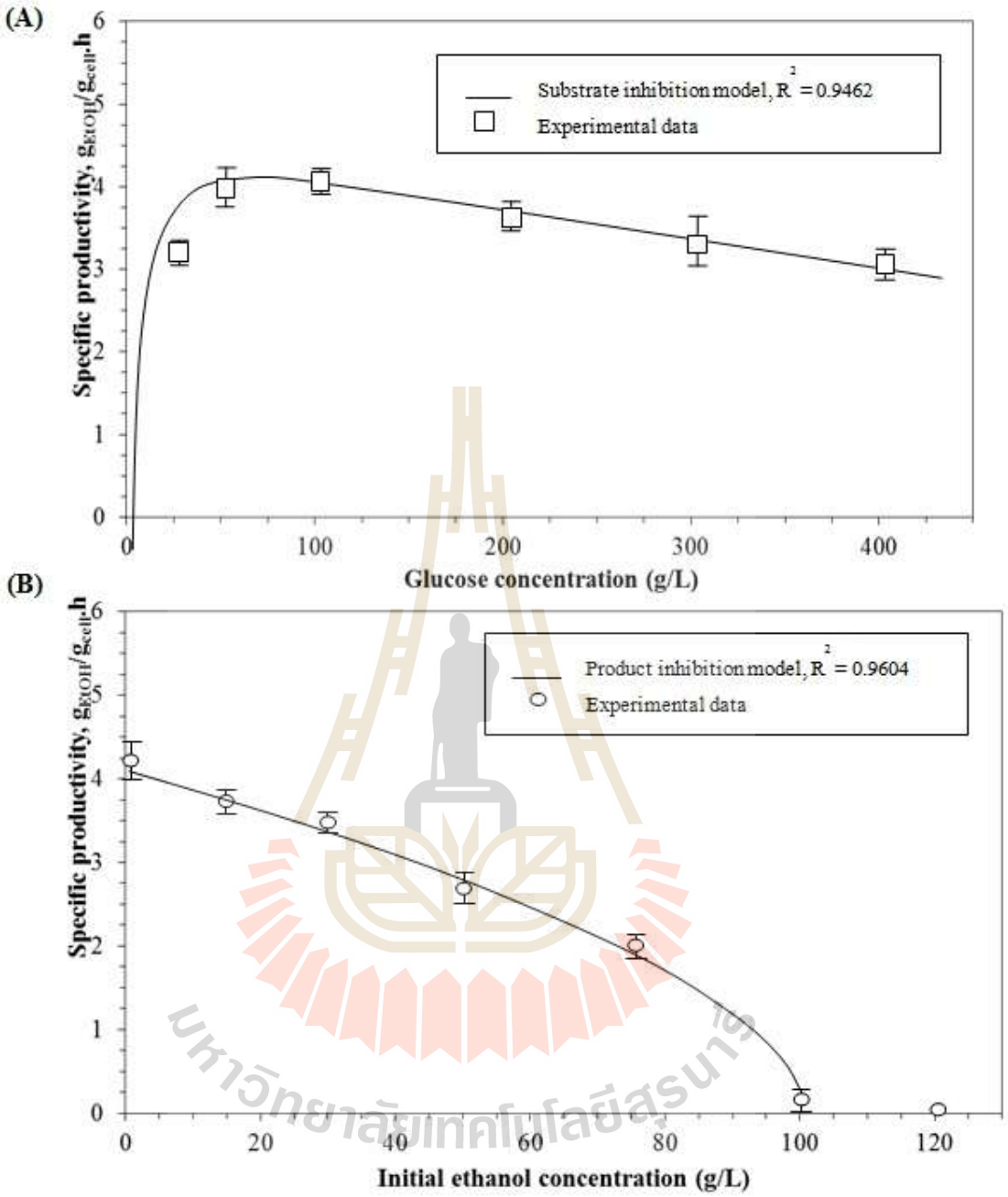

Figure 4.1 Variation of the specific productivity as a function of the initial substrate concentration (A), and initial ethanol concentration addition (B).

4.2 Characterization of distillate ethanol

4.2.1 Effect of feed concentration

The fractionating data for the ethanol–water binary system in comparison with vapor liquid equilibrium (VLE) are shown in Figure 4.2 as an *x*-*y* diagram. Two distinct experiments were carried out by taking sample at the interface and at the top of the column. The main objective for studying the VLE was to check for the practical feasibility of our novel distillation using the partial-condensation concept, and to compare distillation performance of the two systems. Experimental results for simple distillation showed a very good agreement with the UNIQUAC and Antonie's correlation of equation 9. For our distillation system, the vapor phase concentration has been altered reaching the azeotropic point (y_{EtOH} 0.894) at the mole fraction of ethanol in the liquid phase (x_{EtOH}) as low as 0.05. Even x_{EtOH} of 0.004, the ethanol mole fraction in the vapor phase (y_{EtOH}) was 0.735. Complete experimental results at various molar feed concentrations were given in Table 4.1. The values of relative volatility (α) obtained from $(y_{EtOH}/x_{EtOH})/(y_{water}/x_{water})$ showed a high value exceed 690 at the lowest feed mole fraction of 0.004. These excellent experimental results of fractionating data could pave a way to develop this new system of distillation to replace the existing fractionating columns. In addition, this system could promote the development of cellulosic ethanol production process where initial feed concentration is usually less than 5%wt (Hamelinck *et al*., 2005). For an industrial scale, the column internal requires more than 70 active plates resulting in a high investment cost, and high maintenance. In addition, distillation of ethanol is only achieved in an industrial scale making the small scale production of anhydrous of fuel grade ethanol extremely difficult. In this work, the successful fractionation was due to the effect of the

homogeneity of the raising vapor inside the column in combination with a precise temperature control causing condensation of the excessive water.

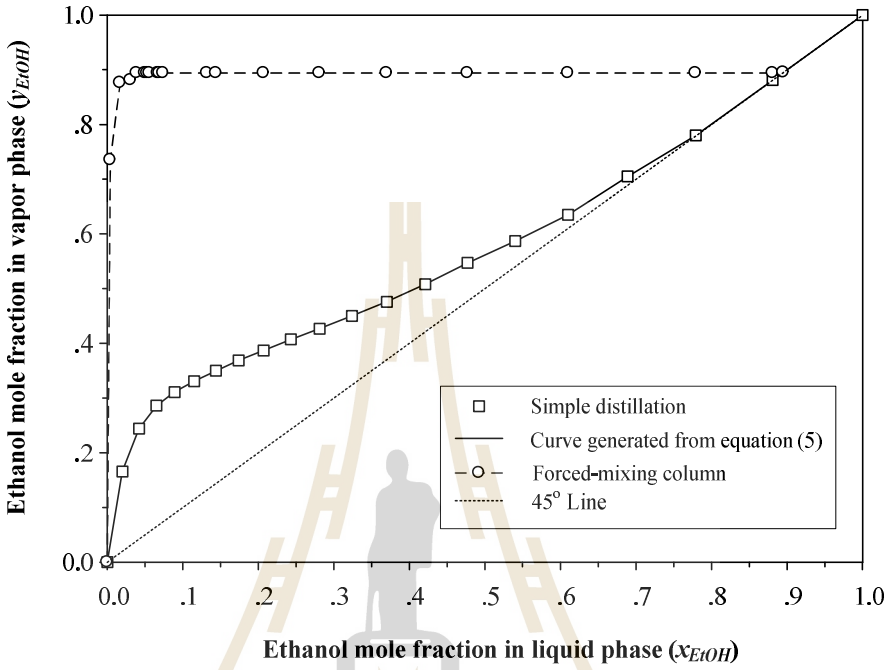

Figure 4.2 Experimental data for EtOH/H_2O system; open square was the simple distillation whilst the curve was generated from equation 9, open circle was the result obtained from our distillation system.

Table 4.1 Distillation performance of the binary EtOH/H_2O mixtures; liquid phase mole fraction (x), vapor phase mole fraction (y), liquid temperature (°C), and relative volatility (α).

Liquid temperature (°C)	x_{EtOH}	x_{water}	y_{EtOH}	y_{water}	A
100.0	0	1	0	1	-
99.0	0.004	0.996	0.735	0.265	690.62
98.3	0.017	0.983	0.876	0.124	408.50
97.5	0.031	0.969	0.881	0.119	231.41
95.3	0.039	0.961	0.894	0.106	207.82
94.6	0.050	0.950	0.894	0.106	160.25
93.2	0.056	0.944	0.894	0.106	142.17
88.4	0.074	0.926	0.894	0.106	105.54
83.7	0.207	0.793	0.894	0.106	32.31
82.7	0.281	0.719	0.894	0.106	21.58
81.0	0.370	0.630	0.894	0.106	14.36
80.2	0.477	0.523	0.894	0.106	9.25
78.1	0.779	0.221	0.894	0.106	2.39
78.1	0.895	0.105	0.894	0.105	1.00

4.2.2 Effect of partial reflux temperature

After batch fermentation, the fermentation broth was also subjected to test for the vacuum fractionation experiment. The initial ethanol concentration in the feed was approximately 10%wt. The purpose of this study was to determine the

characteristic of distillate ethanol as the function of partial reflux temperature (T_1 of Figure 3.1) using a vacuum fractionation technique. The boiling point of the fermentation broth was reduced to 35°C by decreasing the vacuum pressure to 70 mBar. The distillate ethanol was trapped with liquid nitrogen prior to analyze for its components. It is the fact that fermentation broth also contains several volatile impurities which can be presented in the distillate stream. This volatile impurity can be the group of aldehyde, higher alcohol, ester, and volatile organic acid, respectively. Changing in partial reflux temperature would affect the concentration of these compounds in the distillate ethanol product. The effect of partial reflux temperature on purity of the distillate ethanol was shown in Figure 4.3. It was found that the purity of the distillate ethanol was inversely proportional to the partial reflux temperature. The lower the temperature, the higher the purity obtained. At partial reflux temperature of 35°C, the chromatogram showed various impurities especially acetic acid at retention time 16min. This temperature was the same as in the fermentation broth; therefore, this distillate ethanol was not fractionated and represented the vapor liquid equilibrium between the liquid and gas phase. For the partial reflux temperature of 0°C, the chromatogram showed less impurity in comparison to the higher reflux temperature.

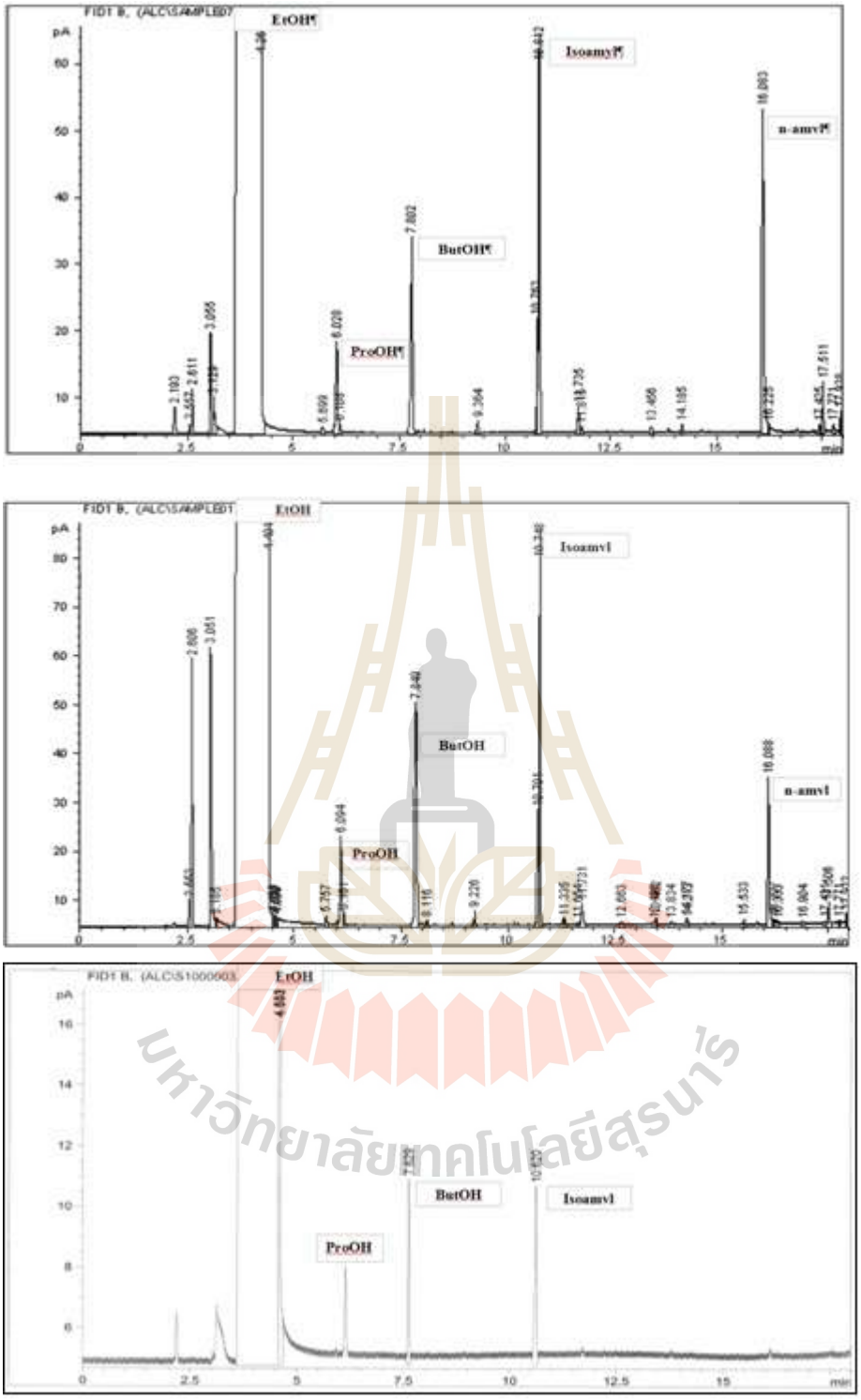

Figure 4.3 Chromatogram of the distillate ethanol at various partial flux temperatures at (A) 35°C, (B) 10°C, and (C) 0°C during vacuum fractionation experiments.

Table 4.2 Mass flow and purity of the distillate ethanol as a function of partial reflux temperature.

Temperature of water chiller (°C)	Flow rate (kg/h)	EtOH (%wt)	H_2O (%wt)	Impurities (mg/L)			
				ProOH	ButOH	Isoamyl	n-amyl
0	0.048	89.9	6.4	66.28	9.71	270.13	17.73
4	0.090	87.0	9.3	76.87	10.65	310.21	21.43
7.5	0.187	83.8	12.3	77.04	12.25	314.08	26.79
10	0.200	80.9	15.7	85.81	12.55	321.32	28.18
15	0.267	74.2	22.1	92.97	14.07	334.48	27.91
35	0.296	44.7	51.4	106.94	14.68	379.97	30.22

Table 4.2 shows the detailed experimental results for mass flow rate and chemical composition of the distillate ethanol at various partial reflux temperature of water chiller ranging from 0-35°C. It also showed that reducing the partial reflux temperature resulted in a decreasing in water concentration of the distillate ethanol as well as a reduction in mass flow rate. The mass flow rate of the distillate ethanol was inversely proportional to the ethanol concentration. For the temperature of 0°C, the flow rate of 0.048 kg/h, and ethanol concentration of 89.9%wt were obtained. For the temperature of 35°C, the flow rate of 0.296 kg/h, and ethanol concentration of only 44.7%wt were obtained. In addition, the amount of other impurities namely propanol (ProOH), butanol (ButOH), is o-amyl alcohol (Isoamyl), and n-amyl alcohol (n-amyl) were also significantly reduced at a reducing partial reflux temperature. In conclusion, fractionation of the distillate ethanol was effectively controlled with an optimal

removal of vapor temperature resulting in a partial condensation of less volatile components. As a result, a high concentration of ethanol was obtained.

4.3 Conventional batch fermentation and batch extractive fermentation

From the experimental data of the previous section, 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 4.4 illustrates the time course for glucose consumption, ethanol formation, and relative viability of the yeast cells. The concentration of glucose was rapidly decreased at the first 12h of fermentation before the consumption rate gradually decreased. However, approximately 30 g/L of glucose still remained at the end of fermentation indicating that it was not completely consumed by the yeast cells. The decrease rate of glucose consumption was associated with the increasing ethanol concentration. The ethanol concentration rapidly increased at the first 15h with a volumetric productivity of approximately 6.20 g/L.h. Subsequently, the value gradually increased until the maximum concentration of 119.7 g/L was reach corresponding to 93.6% of the theoretical yield. A decrease in volumetric productivity was observed at the ethanol concentration higher than 70 g/L. The main reason for a reduced product formation and substrate consumption rate was clearly due to the product inhibition effect as evidenced by the viability test. Since the ethanol was accumulated in the fermentation broth, the value of relative viability decreased since the fermentation was started. However, a sharp decrease was observed after 18h where ethanol concentration higher than 100 g/L. At the end of fermentation process,

there was no glucose consumption, ethanol formation, and most of the yeast cells lost their viability.

In contrary to batch fermentation, the extractive fermentation using vacuum fractionation technique yield a much better result as illustrated in Figure 4.4B. After 1h of inoculation, vacuum pressure was gradually applied to the system at the rate of 200 mBar/min until the value reached 65 mBar. Glucose concentration reduced constantly with the consumption rate of 26.6 g/L.h before it was completely consumed at 21h of operation. This high consumption was attributed to continuous removal of the ethanol as the distillate. The average concentration of the distillate ethanol was 93% by weight and can be dehydrated without further distillation. The volumetric production was calculated at approximately 12.5 g/L.h and the value gradually reduced at the end of the process. This reduced rate of productivity was not a result of product inhibition, but it came from low glucose concentration in the system. In addition, the ethanol concentration in the fermentation broth was constantly low and never reached 25 g/L. When glucose was depleted, no more ethanol was produced and the ethanol concentration in the fermentation broth was still reduced to approximately 2.7 g/L. In conclusion, a recovery ratio of nearly 100% was obtained at the end of fermentation process. The total ethanol produced in this experiment was 268 g from 560 g of glucose (2 L of fermentation broth) which corresponds to 93.63% of theoretical yield. Although the value was in the same magnitude of batch fermentation, this system has advantages over conventional batch fermentation particularly the high relative viability of the yeast cells as evidenced by methylene blue test (Figure 4.5B). More than 90% of the relative viability was observed through 30h of operation. Some yeast cells were stained with the dye;

however, the majority was still very active and some cells were in budding stage. This consequence indicated that more glucose can be added to produce more ethanol.

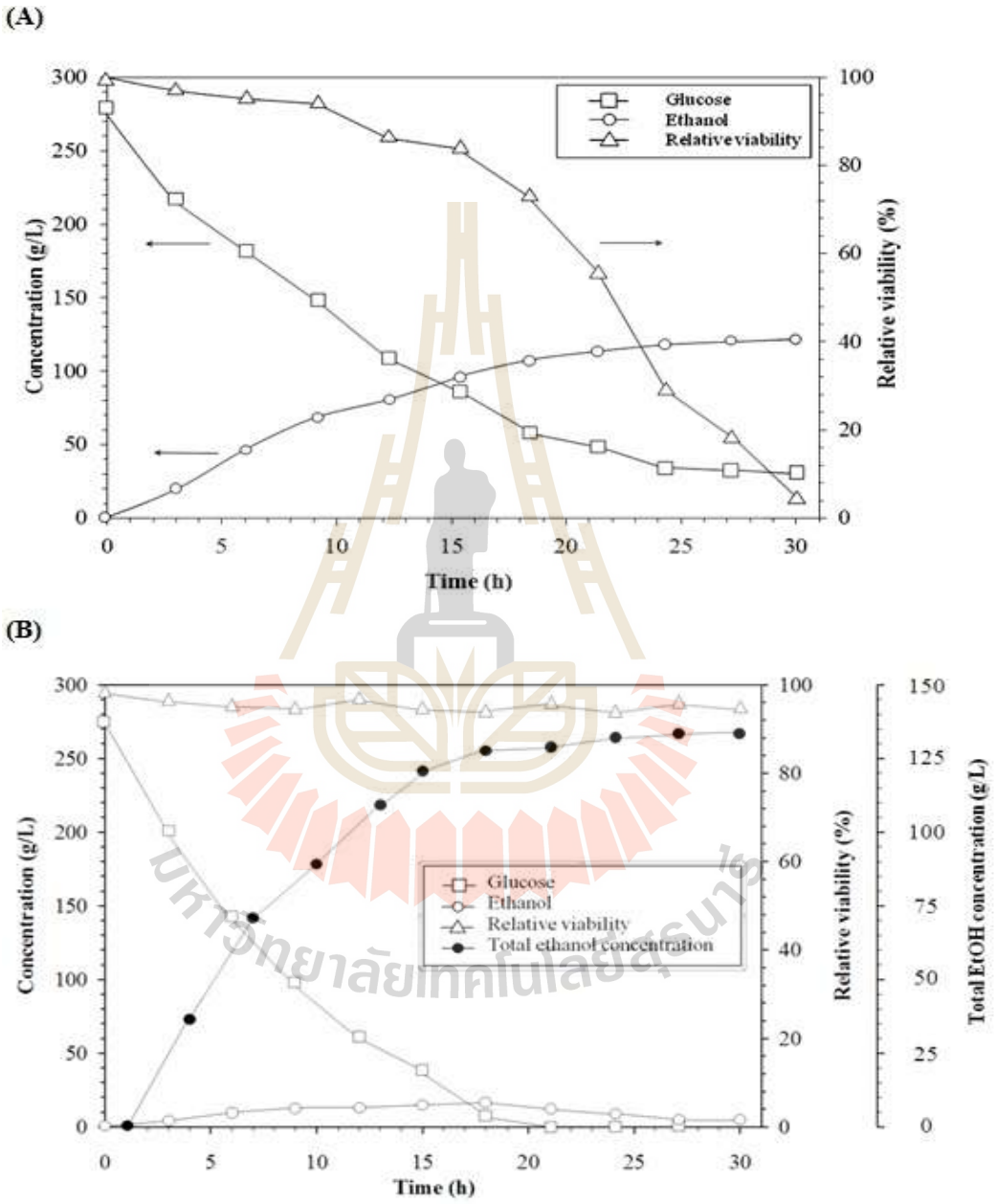

Figure 4.4 Time course for glucose concentration (□), ethanol concentration (○), relative viability of yeast cells (Δ), distillate ethanol (●) of batch fermentation (A), and batch extractive fermentation using vacuum fractionation technique (B).

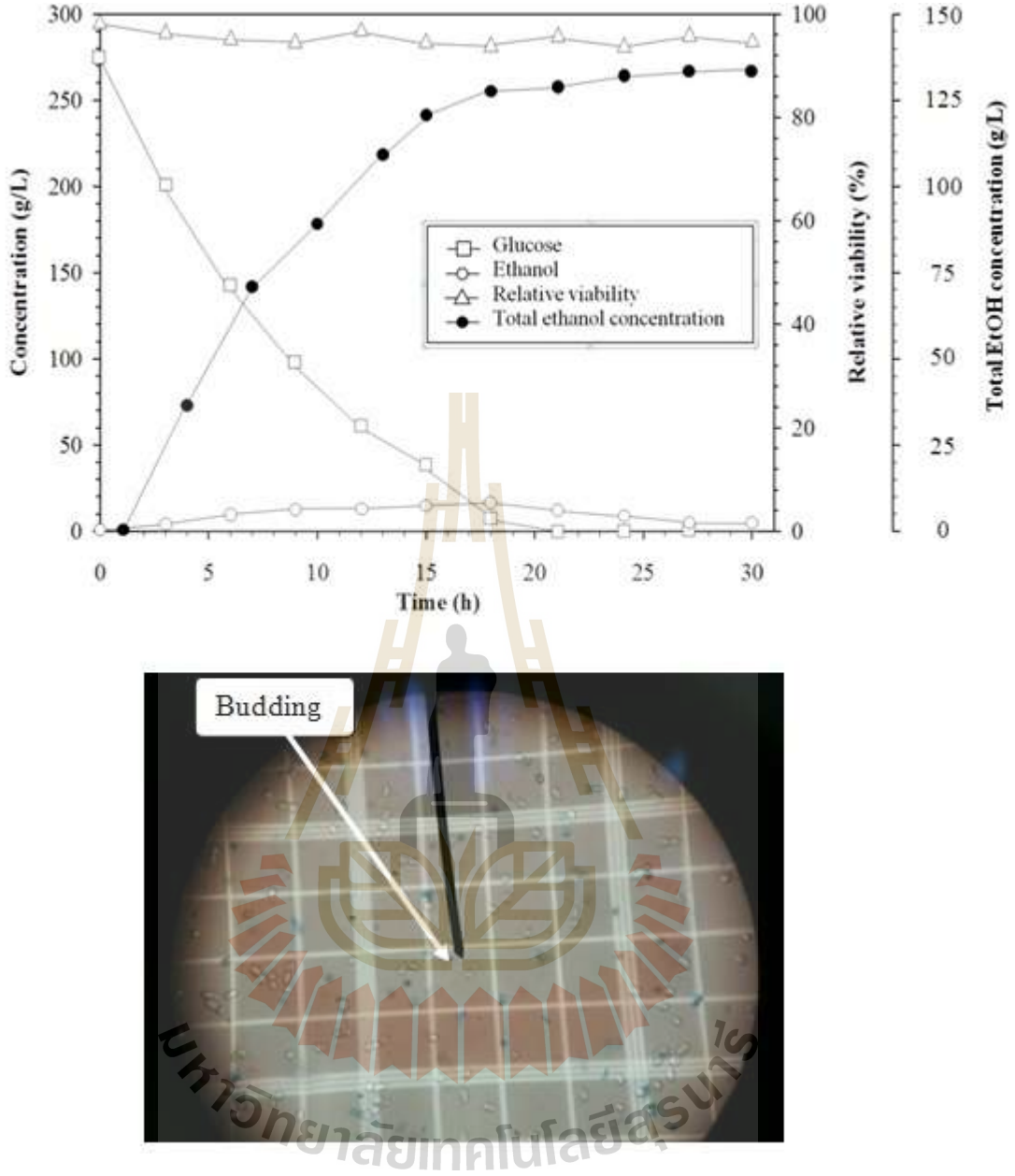

Figure 4.5 Time course for glucose concentration (□), ethanol concentration (○), relative viability of yeast cells (Δ), distillate ethanol (●), and methylene blue test for batch extractive fermentation using vacuum fractionation technique.

In addition, the typical extractive fermentation using vacuum fractionation experiment was shown in supplementary material 1. The boiling point of broth at the

temperature of 35°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 fractionation of the vapor mixture where excessive water was condensed before flowing back into the reactor. As a result, the volume of the fermentation broth was relatively constant and substantial amount of water can be conserved for subsequent fermentation process. The concentration of the distillate ethanol can be controlled solely by the controlling of the exit vapor temperature (T_1 of Figure 3.1). 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°C resulting in a substantial low permeation flux of ethanol, some other fermentation by-product especially organic acids reduced the separation factor (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 system, the separation performance of this particular system 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.

4.4 Extractive fermentation of ethanol in repeated-batch mode

The overall benefit from this extractive bioreactor was extremely positive in that it was a one stage integrated process. Separation of the target product could be obtained in a concentrated form, and could results in an increase of the product formation. From the previous experiment, more than 90% relative viability at the end of the batch extractive fermentation suggesting that more glucose can be added into the system. In order to avoid substrate inhibition effect, the initial glucose concentration of 250 g/L was used for each cycle. Figure 4.6 shows the time courses of glucose concentration, ethanol concentration, mass of distillate ethanol and relative viability during repeated-batch extractive fermentation. After 0.5h for the addition of glucose and inoculation of yeast cells, the extractive fermentation was started. Experimental data showed that glucose concentration decreased for the first 15h, and the consumption rate gradually decreased until glucose was completely consumed after 24h. The produced ethanol was continuously fractionated from the system at the initial rate of 25 g/h with the concentration of approximately 93% by weight. Experimental results also revealed a constant ethanol concentration in the fermentation broth below 20 g/L. When the glucose concentration was low, the ethanol removal rate exceeded the production rate resulting in a decreasing of the ethanol concentration. The second addition of glucose was carried out when the glucose concentration depleted. The vacuum condition of the system was stopped and the glucose powder was introduced through the feeding port. This time interval took approximately 5 minute before a vacuum condition was applied to the system again. Since glucose was added in the form of solid powder, volume change of the fermentation broth was negligible. The addition of glucose was repeated for another

8 times, and system was very stable for 230h. The total ethanol concentration was obtained at 995.2 g/L. After the ninth time of addition; however, the fermentation performance was significantly reduced since glucose consumption was poor. The experiment ceased after 250h when glucose concentration was constant and no ethanol was produced. The consequence was accompanied by a substantial decrease of relative viability of the yeast cells. Finally, the sample was centrifuged, filtered and analyzed using HPLC. The experimental result showed that approximately 70 g/L of lactic acid was found and this could be the reason to the death of the yeast cells. It is evidenced that lactic acid is among by-products generated during ethanol fermentation (Chovau *et al*., 2011).

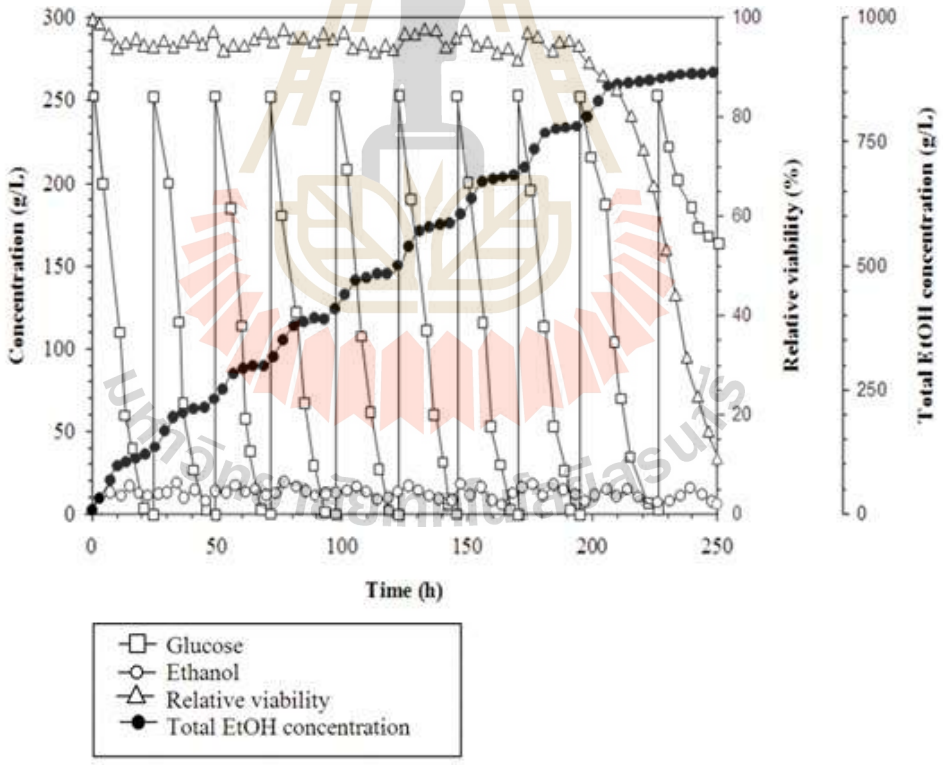

Figure 4.6 Time course for glucose concentration (□), ethanol concentration (○), relative viability of yeast cells (Δ), distillate ethanol (●) during repeated batch extractive fermentation experiment.

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. A high purity of ethanol was produced more than 8-fold in comparison to the conventional batch fermentation.

4.5 Comparison of extractive fermentation technique

Cysewski *et al.* (1977) investigated vacuum fermentation with cell recycling for continuous ethanol reduction. 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% by weight and approximately 30% by weight, respectively. Ghose *et al*. (1984) 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% by weight was collected. Lee *et al*. (1981) 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. Condensate ethanol concentration was obtained for up to 40% by weight. Nguyen *et al*. (2011) studied a continuous vacuum fermentation integrated with separation process. A fermentation-separation column was filled with yeast cells immobilized on bioreactors. During a quasi-steady state, volumetric productivity of 4.8 g/L.h and the average distillated ethanol concentration of 33.2% by weight were obtained. For the repeated batch extractive fermentation presented in this work, an average productivity of 10.97 g/L.h and the average ethanol concentration of 93% by weight were obtained

throughout the 230h 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. For the Comparisons of extractive fermentation technique are shown as Table 4.3.

Table 4.3 The comparisons of extractive fermentation technique.

References	**Methods**	**Productivity (g/L.h)**	**Distillate Ethanol Concentration**
Cysewski *et al*.	Vacuum fermentation with cell recycling for continuous ethanol reduction.	82	30% by weight
Ghose *et al*.	Simultaneous saccharification and fermentation of lignocellulosic materials to ethanol under vacuum cycling and step feeding.	4.5	13.6% by weight
Lee *et al*.	Vacuum fermentation of ethanol by using Zymomonasmobilis.	85	Up to 40% by weight
Nguyen *et al*.	Continuous vacuum fermentation integrated with separation process.	4.8	33.2% by weight
This work	Repeated batch extractive fermentation.	10.97	93% by weight

4.6 Recommendation for future work

Although this work showed a significant improvement on ethanol production 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 obtained from the system using a low vacuum pressure of 70 mBar. 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 venturi is highly encouraged.

- Glucose is an expensive carbon source. Therefore, a cheap and readily available substrate should be investigated. In addition, different substrate feeding strategies should be tested such as fed-batch at constant sugar concentration in order to maintain a high volumetric productivity.

- Pilot scale experiment is highly recommended so that the experimental data can be used for process simulation. Plant design is another interesting topic in order that the comparison for economic aspect can be made between the proposed design and conventional bio-ethanol production plants.

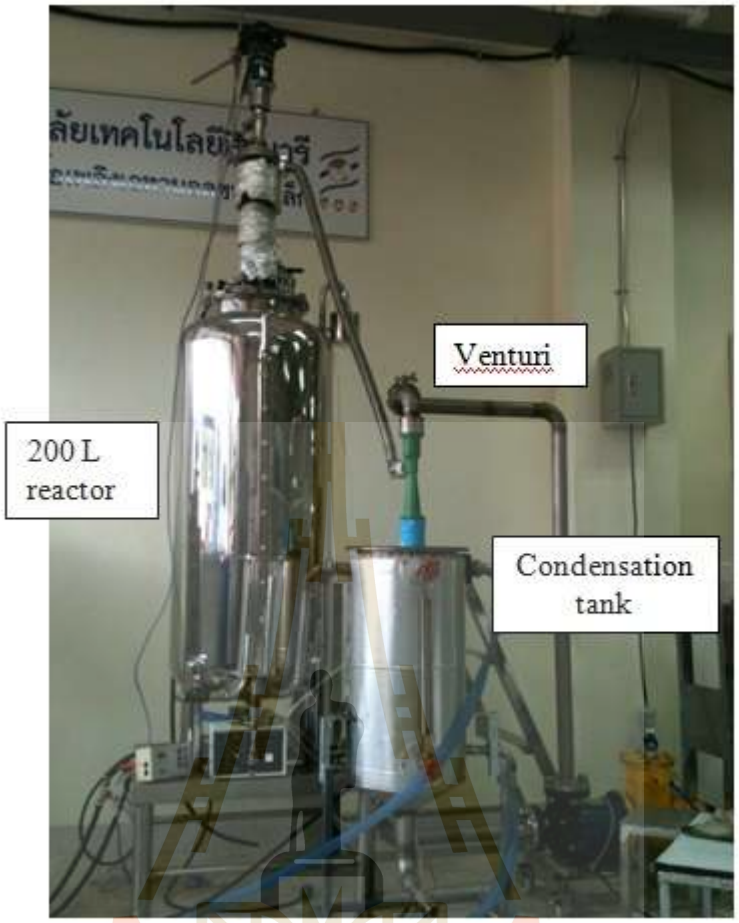

Figure 4.7 Experimental setup for extractive fermentation of ethanol from molasses using vacuum fractionation technique. A venture was used to generate vacuum condition and the vapor distillate ethanol was condensed.

CHAPTER V

CONCLUSIONS

The extractive fermentation using a vacuum fractionation technique was successfully developed to have a great potential in enhancing the productivity of ethanol production process. The removal of 93% by weight ethanol from fermentation broth is the key to successful application of this approach. The integration of fermentation and separation process has a positive impact on the ethanol productivity. The high concentrate of ethanol removal was achieved by the controlling of the exiting vapor temperature (T_1) at 19.4^{o}C under pressure approximately 70 mbar for condensed excessive water of the rising vapor back into the bioreactor. In repeated-batch mode of extractive fermentation, a long operation time and a high ethanol yield were attributed to minimized product inhibition effect to the yeast cells. This particular system has advantages over conventional fuel ethanol process in term of simpler system design, longer life of the yeast, and lower water discharge. Still, cheaper raw materials feeding and more steady process operation should be further improved for a larger scale experiment.

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APPENDICES

APPENDIX A

BOILING POINT OF WATER AND ETHANOL AT DIFFERENT PRESSURES

Table 6.1 Boiling points of water and ethanol at different pressures.

Pressure (mmHg)	Boiling point (^{o}C)	
	Water	Ethanol
1.3	-22.5	-33.6
3.3	-11.7	-23.6
6.7	-2.7	-15.3
13.3	7.0	-6.4
33.3	21.1	6.5
66.7	32.9	17.3
133.3	45.8	29.1
333.3	65.0	46.5
666.7	81.4	61.4
1333.3	99.7	78.0
2666.7	120.3	96.0
6666.7	151.9	125.4
13333.3	180.0	150.9
26666.6	212.4	180.7

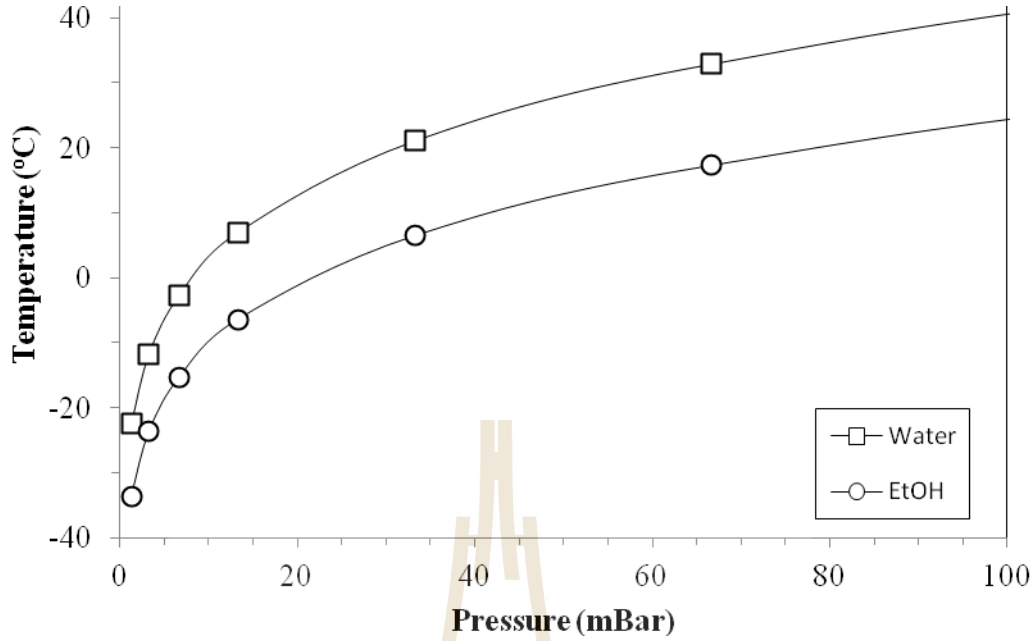

Figure 6.1 Boiling points of water and ethanol at different pressures.

Under batch extractive fermentation, the operating pressure must be maintained at approximately 65 mBar in order to keep temperature of fermentation broth at 35°C. Moreover, in order to control high concentrate ethanol leave the column at 93% by weight the excessive water must be condensed back into the bioreactor. So, if we control the temperature of exiting vapor (T_1) at 19.4°C the ethanol will rising to the vapor phase cause of the ethanol boiling point and also can be condensed excess water back to the bioreactor. These operating points are described by the graph relationship between temperature and pressure of water and ethanol as in Figure 6.1.

APPENDIX B

THE PERFORMANCE OF FRACTIONATION COLUMN

Table 6.2 Comparison between fractionation of ethanol obtained from this work and from vapor liquid equilibrium; liquid phase ethanol concentration (L_{EtOH}), distillate ethanol concentration (V_{EtOH}), and relative volatility (α).

Temperature (°C)		L_{EtOH}	V_{EtOH} **(wt%)**		A	
Liquid	**Vapor (T_1)**	**(wt%)**	**Distillate**	**VLE**	**Distillate**	**VLE**
100.0	100.0	0.00	0.00	0.00	-	-
99.8	94.8	0.24	52.61	1.95	461	8.27
99.5	80.4	0.52	90.11	4.86	1743	9.77
99.0	78.5	0.98	94.41	8.11	1706	8.92
98.7	78.0	1.20	95.10	10.05	1612	9.28
98.0	78.0	2.54	95.21	18.21	763	8.54
96.7	78.0	5.03	95.24	33.40	378	9.47
95.6	78.0	7.61	95.34	41.23	248	8.52
94.4	78.0	10.21	95.40	45.23	182	7.26
93.7	78.0	12.67	95.13	48.52	135	6.50
91.6	78.0	15.00	95.17	50.02	112	5.67

The data from the previous study as per Table B-1 used to describe the performance of the fractionation column. This table showed the values of relative volatility (α), at the L_{EtOH} of 0.52%wt the highest value of α is 1,743 whilst the average value of lower than 10 was obtained for most of the tested concentrations. At the high value of α, it was indicated that easy of using this fractionation column to separate the ethanol from water in a mixture (Perry and Green, 1998).

The relative volatility (α) was obtained from the Eq. 10.

$$\alpha = (y_{EtOH}/x_{EtOH})/(y_{water}/x_{water}) \quad (10)$$

Where; y_{EtOH} is the mole fraction of ethanol in vapor phase, x_{EtOH} is the mole fraction of ethanol in liquid phase, y_{water} is the mole fraction of water in vapor phase, and x_{water} is the mole fraction of water in liquid phase, respectively.

APPENDIX C

SUBSTRATE AND PRODUCT INHIBITIONS CHARACTERISTIC

Table 6.3 The specific productivity at various initial glucose concentrations for 30h.

Initial glucose conc. (g/L)	EtOH conc. (g/L)		Cell (g/L)		Specific productivity (g EtOH/g cell.h)		
	1	2	1	2	1	2	Average
25	11.88	11.85	0.122	0.113	3.25	3.51	3.38
50	22.75	23.75	0.200	0.185	3.80	4.28	4.04
100	46.50	45.50	0.395	0.358	3.92	4.24	4.08
200	97.00	95.00	0.903	0.808	3.58	3.92	3.75
300	142.50	139.50	1.484	1.224	3.20	3.80	3.50
400	186.00	182.00	2.033	1.758	3.05	3.45	3.25

Table 6.4 The specific productivity at various initial ethanol concentrations for 30h.

Initial ethanol conc. (g/L)	EtOH form (g/L)		Cell (g/L)		Specific productivity (g EtOH/g cell.h)		
	1	2	1	2	1	2	Average
0	46.50	45.50	0.395	0.358	3.92	4.24	4.08
15	42.65	38.66	0.381	0.345	3.73	3.73	3.73
30	39.59	36.29	0.379	0.344	3.48	3.52	3.50
50	29.60	27.22	0.365	0.331	2.70	2.74	2.72
75	23.44	18.28	0.363	0.329	2.15	1.85	2.00
100	2.59	2.35	0.332	0.301	0.26	0.26	0.26
120	0.02	0.02	0.213	0.193	0.00	0.00	0.00

Table 6.5 The calculation results of substrate inhibition model.

Initial glucose concentration (g/L)	Substrate inhibition model	K_s' (g/L)	K_i' (g/L)
25	3.837	8.92	620.71
50	4.199		
100	4.099		
200	3.841		
300	3.507		
400	3.237		

Table 6.6 The calculation results of product inhibition model.

Initial ethanol concentration (g/L)	Product inhibition model	P'_m (g/L)	*Ai*
0	4.080		
15	3.725		
30	3.341		
50	2.767	8.92	620.71
75	1.877		
100	0.000		
120	0.000		

APPENDIX D

CONVENTIONAL BATCH FERMENTATION AND BATCH EXTRACTIVE FERMENTATION

Table 6.7 The results of batch fermentation.

Time (h)	Ethanol concentration (g/L)	Glucose concentration (g/L)	Relative viability (%)
0	0	280	100
3	20.00	218.20	98
6	46.21	185.30	94
9	70.50	148.60	92
12	79.40	105.10	86
15	93.07	80.50	84
18	102.85	53.20	72
21	111.10	44.30	54
24	115.40	32.70	28
27	118.80	31.40	18
30	119.70	30.20	4

Table 6.8 The results of batch extractive fermentation using vacuum fractionation technique.

Time (h)	Ethanol concentration (g/L)	Glucose concentration (g/L)	Relative viability (%)
0	0	280.0	100
3	6.20	200.2	96
6	10.80	140.3	94
9	19.80	98.0	93
12	20.10	62.8	96
15	20.30	40.5	94
18	23.00	12.8	95
21	10.20	0.0	96
24	7.80	0.0	95
27	3.40	0.0	96
30	2.70	0.0	94

Table 6.9 The value of distillated ethanol of batch extractive fermentation using vacuum fractionation.

Time (h)	Distillate ethanol (g/L)	Error
1	0	-
4	30	± 0.92
7	63	± 0.87
10	87	± 0.98
13	110	± 0.95
15	118	± 0.85
18	125	± 0.98
21	128	± 0.92
24	130	± 0.96
27	133	± 0.84
30	134	± 0.95

BIOGRAPHY

Miss Weeraya Samnuknit was born on October 20, 1985 in Surin province. She obtained her Bachelor of Engineering degree in Chemical Engineering from School of Chemical Engineering, Institute of Engineering, Suranaree University of Technology in 2009. After that, she decided to further study master degree in the field of biotechnology. During study, she received financial support from the Suranaree University of Technology. After she finished coursework, she worked in the project title of "Extractive fermentation of ethanol using vacuum fractionation technique", in Biofuel Production from Biomass Research Unit, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Thailand. During this time she had practiced skills in the field of fermentation technology, distillation, and adsorption technique, and she had an experience oral presentation in the title of "A small scale production of motor fuel ethanol using distillation/vapor permeation technique" at the 3rd SUT Graduate Conference, 2010.