STUDIES ON THE PRODUCTION OF BIOETHANOL FROM OIL PALM FROND JUICE AS A RENEWABLE AND SUSTAINABLE FERMENTATION SUBSTRATE

(KAJIAN PENGHASILAN BIOETANOL DARIPADA JUS PELEPAH KELAPA SAWIT SEBAGAI SUMBER SUBSTRAT FERMENTASI MAMPAN DAN BOLEH DIPERBAHARUI)

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2017

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious and the Most Merciful Alhamdulillah, all praises to Allah for the strength, His blessing and opportunity for me to step in the excellent world of science and completing this paper. I am grateful and would like to express my special profound gratitude to Research Management Centre (RMC) and Department of Research and Innovation (DR&I) for providing me this research grant RDU140326.

My gratitude goes to the community of Felda Lepar Hilir 3, Chini, Pahang for providing me raw material which is oil palm frond and to all my friends for their togetherness, kindness and moral support. Thanks for being with me through all the hardship during the project. Thank You. Appreciation also goes to all staff of laboratory Faculty of Chemical and Natural Resources Engineering (FKKSA) for helping me along to complete this study. I want to thank Universiti Malaysia Pahang (UMP) for providing me the financial support.

I am indebted to all of you from the bottom of my heart. I am truly grateful for having such good people around me who gave endless support throughout the study even though it takes very challenging time.

ABSTRAK

Kajian ini memfokuskan kepada penggunaan jus pelepah kelapa sawit (OPF) sebagai sumber mampan bagi menghasilkan produk melalui proses penapaian kerana ia mempunyai kandungan gula yang tinggi dan mudah diperolehi setiap hari di Malaysia. Jus OPF digunakan sebagai sumber karbon untuk pengeluaran bioetanol secara efektif dengan menggunakan yis *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 26622). Hasil daripada analisis dengan menggunakan Kromatografi Cecair Berprestasi Tinggi (HPLC) menunjukkan bahawa jumlah kepekatan gula di dalam jus OPF yang digunakan di sepanjang kajian ini adalah sebanyak 56.87 g/l. Seterusnya, untuk menentukan keadaan terbaik bagi parameter yang boleh mempengaruhi penghasilan bioetanol, kesan suhu, pH awal media dan kadar putaran kelalang goncang telah dikaji dengan menggunakan kaedah Satu Faktor Pada Satu Masa (OFAT). Ini diikuti dengan kajian pengoptimuman proses penapaian bagi penghasilan bioetanol dengan menggunakan kaedah gerak balas permukaan (RSM) untuk meneroka corak tindak balas dan nilai-nilai yang tepat mengenai parameter penapaian. Lima tahap-tiga faktor reka bentuk komposit berpusat (CCD) telahpun dikaji dan titik pusat setiap pemboleh ubah proses telah dipilih berdasarkan keadaan terbaik yang diperolehi daripada kaedah OFAT. Julat parameter yang telah ditetapkan adalah seperti berikut; pH awal media (5-9), suhu (27.5-37.5°C), kadar putaran kelalang goncang (80-120 rpm). Bioetanol dan sisa kepekatan gula telah dianalisa dengan menggunakan peralatan HPLC. Keadaan optimum untuk pengeluaran bioetanol adalah dicadangkan pada pH awal media (6.62), suhu (33.03°C) dan kadar putaran (96.51 rpm). Berdasarkan eksperimen untuk pengesahan, hasil bioetanol pada keadaan optimum adalah 0.50 ± 0.02 g/g gula dan nilai ini adalah hampir sama dengan ramalan model di mana perbezaannya hanyalah 4.17%. Dalam keadaan yang optimum, hasil bioetanol yang diperolehi adalah 47.06% lebih tinggi berbanding dengan keadaan yang tidak optimum. Penghasilan bioetanol dengan menggunakan kaedah berkelompok secara berulang pada skala besar telah dijalankan di dalam bioreaktor 2-L untuk mengkaji prestasi *S. cerevisiae* bagi jangka masa panjang untuk meniru proses penghasilan bioetanol secara jangka masa panjang pada skala industri. Bagi eksperimen berkelompok secara berulang, keputusan menunjukkan hasil bioetanol yang terhasil adalah paling tinggi pada kadar kelajuan pengaduk 50 rpm, isipadu pengeluaran (larutan penapaian) dan penambahan (jus OPF segar) pada kadar 75% dan sepuluh kitaran kumpulan berulang. Kepekatan bioetanol bagi sepuluh kitaran eksperimen berkelompok berulang pada kadar 75% isipadu pengeluaran (larutan penapaian) dan penambahan (jus OPF segar) adalah 0.41 g/g. Kesimpulannya, kajian ini menunjukkan bahawa jus OPF berpotensi untuk digunakan sebagai sumber bahan mentah penapaian yang boleh diperbaharui dan lengkap untuk penghasilan bioetanol untuk menyokong industri bioteknologi tersebut.

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ABSTRACT

This study focused on the utilization of oil palm frond (OPF) juice as sustainable source of fermentation products as it's have high sugar contents and easily obtained daily in Malaysia. OPF juice was used as the carbon source for the effective bioethanol production by the yeast *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 26622). Based on the sugar analysis using High Performance Liquid Chromatography (HPLC), it showed that the total sugars concentration in OPF juice used throughout the study was 56.87 g/l. In order to further evaluate the best condition of parameters which affecting the production of bioethanol, investigation on the effect of temperature, medium initial pH and rotation rate was carried out by using One-Factor-At-Time (OFAT) method. This was followed by optimizing the fermentation process of bioethanol production using response surface methodology (RSM) to explore the response pattern and accurate values of the fermentation parameters. A five-level-three-factor central composite design (CCD) was attempted in this study and the central point of each process variable was chosen based on the best condition obtained from the OFAT method. The parameters range were set as follows; medium initial pH (5-9), temperature (27.5-37.5°C), and rotation rate (80-120 rpm). Bioethanol and residual sugars concentration were determined by using HPLC analysis. The optimum conditions for bioethanol production were proposed to be medium initial pH (6.62), temperature (33.03 °C) and rotation rate (96.51 rpm). Based on the validation experiment, the optimum bioethanol yield was 0.50 ±0.02 g/ g sugars and this value was in close agreement with the model prediction where the difference was only 4.17%. Under the optimal conditions, the bioethanol yield obtained was 47.06% higher compared with non-optimized condition. Repeated batch of bioethanol production was carried out at larger scale by using 2-L bioreactor to study the performance of *S. cerevisiae* for long term experiment to mimic a long term industrial bioethanol production process. For the repeated batch experiment, results showed that highest bioethanol yield at rotation rate of 50 rpm, drain (fermentation broth) and fill (fresh OPF juice) volume at 75% and ten cycles of repeated batch. The average bioethanol yield of ten successive batches at 75% drain and fill volume were 0.41 g/ g sugars. As a conclusion, the present research has shown that OPF juice is promising to be used as a renewable and complete fermentation feedstock for bioethanol production to support the biotechnology industry.

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α	Alpha
DF	Z source inverter
P	Bioethanol Concentration
Qp	Bioethanol Production
Yp/s	Bioethanol Yield of the Ten Successive Cycle
k	Number of Factors
2^k	Axial Runs
Co	Central Points's Run

LIST OF ABBREVIATIONS

OPF	Oil Palm Frond
OPFj	Oil Palm Frond Juice
P (3HB)	Poly (3-hdroxybutyrate)
OFAT	One Factor At Time
CCD	Central Composite Design
C_2H_5OH	Ethanol
$C_2H_4+H_2O$	Ethylene
CO_2	Carbon Dioxide
OPT	Oil Palm Trunk
RSM	Response Surface Methodology
YPD	Yeast Peptone Dextrose
HPLC	High Performance Liquid Chromatography
ANOVA	Analysis of Variance

CHAPTER 1

INTRODUCTION

1.1 Background of Study

It is generally acknowledged that the world today faced an unprecedented set of problems relating to the environment which includes issues relating to ecological destruction, resource depletion and atmospheric change, *i.e.* global warming. We are already using the planets renewable resources faster than what the planet can replenish. The world population at an estimated 7 billion people today is projected to grow over 10 billion people as early as 2050. The planet is already struggling to cope with the demands placed upon it by the human race currently which then led to a steep rise in the demand of petroleum products which is limited and irreplaceable. Thus, world needs to find a reliable renewable source of energy urgently to ensure a sustainable source of energy demand.

Renewable energy has been identified globally as the perfect solution to this matter. Renewable energy could be a key driver to achieve economic growth while ensuring minimal environmental harm. Researcher attempts to investigate an alternate energy produce by microbial fermentation using renewable sources. Besides that, food price is likely continued to rise for many years to come, making poor farmers, consumers and countries more vulnerable to poverty and food insecurity. Demand from consumers in rapidly growing economies will increase, the population continues to grow, and further growth in biofuels will place additional demands on the food system and more people will continue suffer from lack of food.

On the other hand, feedstock for microbial fermentation today is currently taken from edible food source, such as soy bean, malt and glucose that were also consumed by

humans and animals. Competition on food consumption occurs between the needs for growth of human and animals and microbes may affect the food chain survival. Thus, studies on potential of oil palm biomass to be utilized as a source of fermentable sugars is carried out to reduce the production cost and the dependence on the food crops.

Oil palm frond (OPF) juice has been identified as a good candidate to replace commercial / technical grade sugars for the production of value-added products. Recently, it has been reported that OPF juice can be used as the sole renewable carbon source for the production of poly (3-hydroxybutyrate), P (3HB) (Zahari et al., 2012). Further evaluation on the potential of OPF juice as fermentation feedstock was tested for bioethanol production by Saccharomyces cerevisiae (Baker's yeast) (Zahari et al., 2014).

1.2 Problem Statement

The continuous use of the fossil fuel and increment of price with diminishing supply of fossil fuels have stimulated effort among researcher in finding alternative sources of energy. Fossil fuel plays tremendous role for fueling our transportation system. However, fossil fuels are a nonrenewable resource as they take millions of years to develop and can deplete in the future. In 2030, the demand for oil production is expected to rise yet become a concern whether the world beginning to run out of oil in the future. To mitigate the risks, production of new technologies from renewable resource such as production of ethanol is required for human demands in the next generation.

The ethanol production varied in different raw material containing simple sugars, starch or even the complex substrates such as lignocellulosics. Most common substrate used was first generation biofuels and yet those feedstocks contribute in human consumption and involving in animal food chain (Daniel et al., 2012). Competition with human consumption and increasing prices of food most likely will cause limitation feedstock and costly.

Main advantage of the utilization of sustainable sources contributes by the use of natural resources and production of bioenergy to reduce dependence on non-sustainable sources and for energy security. Nigam and Singh (2011) proposed, the utilization of inexpensive and eco-friendly raw material will reduce the competition between fuel demand and food requirement hence able to produce biofertilizer and biopesticides.

Utilization of edible food source such as potato, corn and sugar cane as a raw material for the production of bioethanol raise attention because of its importance as food source to humans. Therefore, a lot of research on the use of alternative sources and abundant waste as a raw material was investigated. Research conducted on the potential of biomass waste feedstock for bioethanol production (Sudiyani, 2013), utilization of oil palm as a sustainable energy source (Sumathi et al., 2008; Shuit et al., 2009; Yusoff, 2006), bioethanol production from agricultural wastes (Sarkar et al., 2012) and production of bioethanol from lignocellulosic materials (Balat, 2011; (Limayem & Ricke, 2012); Goh et al., 2010).

There were a lot of research on the use of oil palm frond such as for pulp (Wanrosli et al., 2007), α-Tocopherol antioxidants (Ofori-boateng & Lee, 2013), local beef and dairy production (Wan Zahari et al., 2003), feed for herbivores (Dahlan, 2000) and feed source for ruminants (Hassan et al., 1996; Islam, 1999) which limited research of OPF for the production of bioethanol with different treatment (Goh et al., 2010; Ofori-boateng & Lee, 2013). Preliminary studies done by Zahari et al. (2014) proposed, sugars derived from OPF juice can be potential fermentation feedstock for bioethanol production.

It was reported that the bioethanol yield obtained from OPF juice without nitrogen source supplementation was slightly lower compared with the fermentation supplemented with nitrogen source under non-optimized condition (Zahari et al., 2014). Even though, higher bioethanol yield could be obtained by nitrogen source supplementation, it was not recommended at industrial scale production since the addition of nitrogen source and other nutrient in fermentation medium could contribute to high production cost. In order to make the production of bioethanol feasible for industrial application, it is crucial to have high bioethanol yield without any nutrient and nitrogen supplementation. One of the alternatives is by optimizing the physical parameters including medium initial pH, temperature and rotation rate which could affect the bioethanol production. It was reported that the optimization study of the fermentation parameters; temperature, rotation rate and pH will contribute to high fermentation efficiency (Chin et al., 2010; Rodmui et al., 2008; Yan et al., 2009; Ho and Powel, 2014). Hence this study was done in order to further optimize the fermentation parameters using OPF juice as the sole renewable and sustainable promising sources for bioethanol

production in future.

1.3 Objectives

The purpose of this research study is to investigate the potential of OPF juice as alternative and renewable fermentation substrate for bioethanol production. The objectives are:

i. To determine the best condition of parameters affecting bioethanol production from OPF juice by *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 26622) using the One-Factor-At-Time (OFAT) method.

ii. To optimize the process parameters on the production of bioethanol from OPF juice as the sole renewable carbon source using Response Surface Methodology (RSM) with Central Composite Design (CCD).

iii. To study the performance of *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 26622) for long term experiment in 2L bioreactor by repeated batch fermentation process.

1.4 Scope of the Study

In this study, the fresh OPF as a resource for fermentation substrate for bioethanol production will be investigated by using yeast *Saccharomyces cerevisiae* Kyokai No. 7. OPF petioles (without leaves) were collected from the oil palm plantation at Felda Lepar Hilir, Gambang, Pahang, Malaysia. It was chopped and pressed through a pressing machine to produce OPF juice, and the juice was further characterized for sugars content and composition using HPLC analysis. For the initial part of fermentation study, OPF juice was use as a substrate for bioethanol production, whereby the effects of fermentation condition such as rotation rate (80-120 rpm), medium initial pH (5-9), temperature (27.5-37.5°C) and sterilization (autoclaved and non-autoclaved) will be screened out using One-Factor-At-Time (OFAT) method. This was followed by the selection of three best parameters from the OFAT experiment as a central point and further optimized by using Central Composite Design (CCD) with Response Surface Methodology (RSM). All experiments were carried out in shake flasks and the optimum point obtained from the model was validated further experimentally. The data reported in

the optimization study are mainly the average value at least two successful replicates and the statisctical analysis of the data was performed using analysis of variance. The least significant difference test was used to compare the means with a confidenceinterval of 95%. Finally, repeated batch of bioethanol production was carried out at larger scale by using a 2-L bioreactor to study the performance of *S. cerevisiae* for long term experiment to mimic a long term industrial bioethanol production process.

1.5 Significance of Study

This work is vital to provide sustainable environment for world in the future. In addition, to create waste to wealth by utilization of renewable resources for the production of value-added product. Other than that, producing bioethanol from OPF juice is an alternative to substitute the usage of edible food sources *i.e*. corn and soy bean and sorghum. Further investigation on the production of bioethanol by using OPF juice in 2L bioreactor by repeated batch fermentation process also has yet to be studied.

1.6 Structure of this Thesis

This thesis structured as follows. Chapter 2 represents the literature review about bioethanol production form OPF juice and equivalent sugar components. In addition, information on oil palm plantation and sugar from OPF juice also reviewed. Chapter 3 reviewed the materials and methods, source of raw materials and chemicals employed throughout the experiment. Types of equipment, experimental design and procedure of the experiment also reviewed. Further than that, information of calibration curve for the sugar content and ethanol concentration are also presented. Chapter 4 represents the results and discussion for the One- Factor-At-Time (OFAT) optimization study using response surface methodology and repeated batch experiment in 2-L bioreactor. Chapter 5 represents the concluding remarks and recommendations for future works.

CHAPTER 2

LITERATURE REVIEW

2.1 Bioethanol

Ethanol is an ethyl alcohol, grain alcohol, or chemically alcohol C_2H_5OH or EtOH is an alcohol which is soluble in water and has density of 789 g/l at 20ºC. Synthetic ethanol produced by the process catalytic hydration of petroleum products (ethylene) (Gnansounou & Dauriat, 2005).

$$\underset{\text{Ethylene}}{C_2H_4} + H_2O \rightarrow \underset{\text{Ethanol}}{CH_3CH_2OH} \qquad \text{Eq. 2.1}$$

Bioethanol is a liquid biofuel derived from alcoholic fermentation of sucrose or simple sugars which produced from several biomass feedstocks and conversion technologies. Balat (2011) proposed, different kinds of raw materials can be used to produce bioethanol via fermentation such as sucrose-containing feedstocks, starch materials and lignocellulosic materials. Sugars, starch and cellulose can be converted into ethanol directly, firstly hydrolyzed to fermentable sugars by action of enzymes from molds and can be converted into sugars via mineral acids action respectively (Liu & Shen, 2008). An oxygenated fuel like bioethanol has high octane number which provide good potential in lower carbon dioxide, CO_2 emission attract the most attention to be an alternative fuel to displace petroleum derived transport fuels. Ethanol produced from biomass promising great future for biofuel. Hence, research promoting the use of biomass in the development of bioethanol has a promising future to fulfill world demand.

Table 2.1 Different Raw material Used for Ethanol Production

Raw material	**Strains/microorganisms used**	**Keywords**	**References**
Soybean molasses	*Saccharomyces cerevisiae*	Soybean molasses, bioethanol, pilot scale, industrial scale, *Saccharomyces cerevisiae*	Siqueira *et al.*, 2008
Sweet sorghum juice	*Saccharomyces cerevisiae*	Repeated-batch fermentation, ethanol, low-cost nutrient, sweet sorghum juice, *Saccharomyces cerevisiae*	Ariyajarearnwong *et al*., 2011
Sweet sorghum juice	*Saccharomyces cerevisiae*	Batch, fed-batch fermentation, ethanol, *Saccharomyces*	Laopaiboon *et al.*, 2007
Cashew apple juice	Immobilized yeast cells by *Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*, immobilization cell, cashew apple juice, ethanol, continuous	Neelakandan and Usharani, 2009

		fermentation	
Sugar from dates	*Saccharomyces cerevisiae* ASN-3 and HA-4	Ethanol fermentation, yeast activated, dates, optimization	Noor *et al*., 2003
Oil palm trunk sap	*Saccharomyces cerevisiae*	Ethanol, yeast, nutrient addition, oil palm trunk	Nasir *et al*., 2014
Cull dates	*Candida kefir*	Cull date, *Candida kefir*, ethanol, fermentation	Chtourou *et al*., 2012
Sugar beet juice	*Saccharomyces cerevisiae* IR 2	Loofa sponge, carrier for cell immobilization, ethanol, sugar beet juice, scale up	Ogbonna *et al*., 2001
Stalk juice of sweet sorghum	Immobilized *Saccharomyces cerevisiae* CICC 1308	Suitable parameters, sweet sorghum, immobilized *Saccharomyces cerevisiae*	Liu and Shen, 2008
Oil palm trunk juice	*Saccharomyces cerevisiae* Kyokai No. 7	Oil palm trunk, *Saccharomyces cerevisiae*, *Scheffersomyces stipitis*,	Norhazimah and Faizal, 2013

		Zymomonas mobilis, Zymobacter palmae	
Oil palm trunk	*Saccharomyces cerevisiae* Kyokai No. 7	Oil palm, trunk sap, sugar, ethanol production, lactic acid production	Kosugi *et al*., 2010
Mango fruit juice	*Saccharomyces cerevisiae* 101	Mango juice, ethanol production, waste	Reddy and Reddy, 2007
Oil palm trunk, rubberwood, mixed hydrolysates	*Saccharomyces cerevisiae*	Lignocellulosic biomass, ethanol yield, fermentation time, fermentation efficiency	Chin *et al*., 2010
Sago starch	*Zymomonas mobilis* ZM4	Central composite design, ethanol, glucoamylase, response surface methodology, sago starch, simultaneous saccharification	Ratnam *et al*., 2003

2.2 Palm Oil and Oil Palm Biomass

Oil palm known as *Elaeis guianensis* is widely cultivated oil bearing tropical palm tree which originated from West Africa (Lee, K.T & Boateng, C.O., 2013). Zahari et al. (2012) proposed, oil palm has an economic life up to 20-25 years and bears 8 to 12 fruit bunches per annual. Each fruit bunch produces 1000-3000 fruits while each palm tree possesses about 40 kg of palm oil annually. Oil palm frond (OPF) and oil palm trunk (OPT) are two major by-products obtained from the plantation of palm oil.

Malaysia is the world's second largest palm oil producer and occupied huge plantations area up to 5.038 million hectares (MPOB, 2012). The oil palm industry has become the backbone of Malaysia's economic hence, generates a great amount of biomass every year yet only a small amount is utilized efficiently for value-added product like bioethanol. According to Zahari, et al. (2012), about 15.2 and 17.5 million tones (wet weight) of OPT and OPEFB were generated in Malaysia in year 2009. However, the most abundant biomass generated from oil palm plantation is oil palm frond (OPF). OECD FAO (2011) proposed, OPF form the largest group of oil palm wastes (OPW) in the form of solid residue which amounted to nearly 92.4 million tons (dry weight) annually for global generation capacity in 2011.

In Malaysia, oil palm industries generated 54.17 million and 54.24 million tons of OPF in 2010 and 2011 respectively (Wan Zahari et al., 2004). Generation of biomass from OPF showed increment about 19 million tons from 2004 to 2011 (Wan Zahari et al., 2004). Recently, Agensi Inovasi Malaysia (AIM, 2011) reported that about 100 million dry tonnes of solid biomass will be generate by Malaysia's palm oil industry in year 2020 in which OPF and OPT account for about 75 percent of the solid biomass volume.

According to AIM (2011), there are six types of oil palm biomass produced from palm oil industry as by-products which are oil palm fronds (OPF), oil palm trunk (OPT), empty fruit bunches (EFB), palm kernel shells (PKS), mesocarp fibre (MF) and oil palm mill effluent. However, this report studies on evaluation of OPF juice as a fermentation feedstock for the production of bioethanol.

2.3 Oil Palm Frond

In the plantations, OPF is available throughout the year as it can be obtained during pruning for harvesting of fresh fruit bunch (FFB). Oil palm fronds are the huge lignocellulosic biomass in Malaysia (Jung et al., 2012). Based on the report by MPOC (2010), fronds contributed about 83 million tonnes per year for oil palm biomass in year 2009. Due to huge amounts of OPF generated yearly, it has the great potential to be utilized to other value-added products such as bioethanol.

According to Lee (2013), OPF comprises three main components: a petiole (the stem) (about 6-8 m long), rachis (about 1-2 m long) and leaflets (250-350) at a time. In this research, petiole was used and divided into 1.0 m length at three different sections as shown in Figure 2.1. Petiole or basal rich in cellulosic materials and sugars which can be the key factor in the production of biofuels and bio-based chemicals. Leaves contain higher percentage of crude protein (CP) and ether extract (EE) than the petioles. Mostly, fronds are left rotting between the rows of palm trees which then functioning as soil conservation, erosion control and ultimately for the long-term benefit of nutrient recycling (Wan Zahari et al., 2002). Hence, pruned fronds are just left in the plantation. Study by Zahari et al. (2012) using OPF juice for renewable sugars shows OPF does not contain high metal contents yet contain high carbohydrates in the form of simple sugars. Hence, part of OPF benefits for other purpose without scarifying the nutrient recycling process.

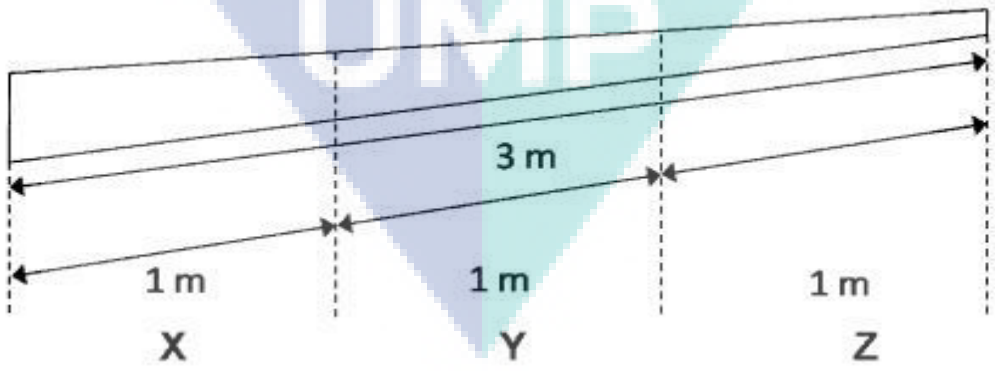


Figure 2.1 Schematic diagram of fresh oil palm frond (OPF) without leaves divided into three sections.

Source: Zahari et al. (2012)

OPF has the great potential as sustainable biomass resources due to the most

abundant lignocellulosic biomass yearly in Malaysia compared to other palm biomass. Recently, studies by Tan et al. (2011) shows study on bioethanol production from oil palm frond by using ionic liquid 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) as a pretreatment medium, potential of OPF as renewable biomass for ethanol production using aqueous ammonia pretreatment, saccharification and fermentation evaluation (Jung et al., 2012) and pretreatment of OPF using ethanolic hot compressed water (EHCW) to enhance the recovery of fermentable sugar (Goh et al., 2010).

2.4 Oil Palm Frond Juice

Oil palm frond juice are beneficial to be potential fermentation feedstock for production of bioethanol due to glucose content in OPF juice and availability during pruning and harvesting of fruit. OPF which are left rotting between the rows of palm trees contained an amount of fermentable sugar including glucose containing in juice. OPF juice has high carbon content thus contributes to be great renewable carbon source for the production of value-added product *i.e.* bioplastic, bioethanol, lactic acid and biobutanol (Zahari et al., 2012). Table 2.2 shows metal concentrations in the OPF and OPF juice. From the results (Zahari et al., 2012), revealed that OPF juice contained low heavy metals concentration (<100 ppm) and proven showed its potential as fermentation feedstock. Table 2.3 shows the sugars concentration, composition and distribution of renewable sugars in OPF juice at different section of OPF petiole.

Table 2.2 Nutrient and Metallic Elements in OPF and OPF Juice.

Analysis	**Fresh OPF**	**OPF juice**
N (%)	0.9	0.8
C (%)	49	39
C/N	56	50
*OC (%)	37	29
Composition of nutrients and metal elements		
S (%)	0.2	0.4
P (%)	0.02	0.02
K (%)	0.2	2.3
Ca (%)	1.4	2.9
Mg (%)	0.2	0.5
B (ppm)	4	2
Mn (ppm)	61	2
Cu (ppm)	2	2
Fe (ppm)	100	66
Zn (ppm)	3	9

*Organic carbon.

Source: Zahari et al (2012)

Table 2.3 Amount of Sugars Contained in the OPF Juice from Different Section of Fresh Oil Palm Frond

OPF section[a]	Fresh OPF weight (g)	OPF juice weight (g)	OPF juice (wt %)	Sugars (g/L)			Total sugar (g)
				Fructose	Glucose	Sucrose	
X^A	925.05	473.07	51.14	1.91	61.17	16.95	80.03
Y^A	595.13	306.79	51.55	0.78	57.48	19.89	78.15
Z^A	281.68	149.75	53.16	1.10	52.98	22.99	77.07
Total/average	1801.86	926.61	51.95	1.26	57.21	19.94	78.42
X^A	926.01	460.23	49.70	1.26	54.66	16.47	72.39
Y^A	596.06	317.94	53.34	1.42	51.94	20.18	73.54
Z^A	295.06	155.56	52.72	1.30	49.66	21.16	72.12
Total/average	1817.13	933.73	51.92	1.33	52.09	19.27	72.68
X^C	1038.12	526.33	50.70	1.97	54.51	19.60	76.08
Y^C	606.06	317.15	52.33	2.00	52.34	22.10	76.44
Z^C	391.38	198.23	50.65	3.35	50.83	24.77	78.95
Total/average	2035.56	1041.71	51.23	2.44	52.56	22.16	77.16
Average	1884.85 (±130.74)	968.35 (±63.57)	51.69 (±1.23)	1.68 (±0.75)	53.95 (±2.86)	20.46 (±1.56)	76.09 (±2.85)

[*] Values are means of triplicate samples.
[a] A, B, and C represents three (3) different OPF from different oil palm tree
[b] Determined by HPLC

Source: Zahari et al (2012)

2.5 *Saccharomyces cerevisiae*

Yeasts have been growing attention in the past years devoted as the most commonly used microorganisms for ethanol production. Yeast strains are generally chosen among *S. cerevisiae, S. ellypsoideuse, S. fragilis, S. carlsbergensis, Schizosaccharomyces pombe, Torula cremoris* and *Candida pseudotropicalis*. Among the ethanol-producing yeasts, the "industrial working horse" *S. cerevisiae* is the most attractive to be used in industry and research for ethanol fermentation (Ahindra, 2008). Yeast is a unicellular eukaryote classified in the kingdom 'Fungi' with about 1,500 species currently known. The yeast species *Saccharomyces cerevisiae* is widely used in baking industry to leavening bread and in brewing industry for production of alcoholic beverages for thousands of years (Dake et al., 2010).

S. cerevisiae species typically reproduces asexually by budding and sexually following the conjugation of cells of the opposite mating type. It is ellipsoid in shape with a large diameter of 5-10 µm and a small diameter of 1-7 µm. It is a eukaryotic

microorganism that portrays the ultrastructural features similar to that of higher eukaryotic cells and possesses a nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, vacuoles, microbodies and secretory vesicles.

Selecting the productive strain is beneficial to improve efficiency of ethanol production. Thus, ideal characteristic for productive ethanol-producing microorganism are identified (Walker, 2010):

i. High growth and fermentation rate.
ii. High ethanol yield.
iii. High ethanol and glucose tolerance.
iv. Osmotolerance.
v. Low optimum fermentation pH.
vi. High optimum temperature.
vii. General hardiness under physiological stress.
viii. Tolerance to potential inhibitors present in the substrate

Ethanol and sugar tolerance necessary for the conversion of concentrated feeds to concentrated products, reducing energy allows distillation and stillage handling whereas, osmotolerance enable handling of relatively dirty raw materials with their high salt content. Contamination can be prevented by low-pH fermentation meanwhile; high temperature tolerance helps reduction in cost for cooling fermentation units. General hardiness allows microorganisms to survive stress such as that of handling *i.e.* centrifugation. The microorganisms should also tolerate the inhibitors present in the medium. Among many microorganisms, *Saccharomyces cerevisiae* is the most widely used in industrial, environment and medical science hence, still remains the predominant industrial microorganism responsible for alcoholic fermentation. *S. cerevisiae* capable of fermenting the main sugars derived from first-generation feedstocks such as glucose and on disaccharide sucrose under large-scale industrial production. However, *S. cerevisiae* unable fermenting pentose (5-carbon) sugars such as xylose and arabinose to ethanol which derived from second generation lignocellulose feedstocks (Walker, 2010; Saha, 2003; Brandberg, 2005).

There are some ethanologenic bacterial non-Saccharomyces yeasts which also have the potential for bioethanol fermentation such as *Pichia stipitis, candida shehatae, Kluyveromyces marxianus, Pachysolen tannophilus* which capable fermenting pentose

sugars (Ahindra, 2008). Non-genetically modified strains like *Zymomonas mobilis* is an alternative microorganism to *S. cerevisiae* for production of bioethanol is able producing ethanol as the main fermentation product under anaerobic condition (Walker, 2010). However, *Zymomonas mobilis* performs less efficient than *S. cerevisiae* in terms of biomass formation and production of bioethanol due to this organism maintains its high glucose flux level through the Entner-Doudoroff (DE) glyceraldehyde-3-phosphate-to-pyruvate (GP) and pyruvate-to-ethanol (PE) pathways to overcome its low ATP yield than in *S. cerevisia* (Sootsuwan et al., 2007).

S. cerevisae still remains chosen as microorganisms for bioethanol production among others to be applied both in laboratory and industrial scales reviewed by (Bai et al., 2008; Siqueira et al., 2008). *S. cerevisiae* is widely used because its ability to produce and tolerate high loo (Bafrncova et al., 1999; Reddy & Reddy 2006; Bai et al., 2008). Study by Laopaiboon et al. (2007; 2009) also showed S. cerevisiae TISTR 5048 and S. cerevisiae NP 01 were high-ethanol-producing strains from sweet sorghum juice. Thus, *Saccharomyces cerevisiae* was used throughout the experiment and proved more productive for high concentration of ethanol and most desirable ethanol fermentations (Yan et al., 2006).

2.6 Fermentation Process

2.6.1 Overall Process of Bioethanol

Production of ethanol processes depends on the raw material used. For raw material from sugar substances such as molasses and sugar cane juice, process like milling, liquefaction, pretreatment, hydrolysis and detoxification are not required. Those processes necessary for production of fermentable sugar and ethanol production from starchy and lignocellulosic material respectively. A general simplified of overall process of bioethanol illustrated at Figure 2.2 (Taherzadeh & Karimi, 2008). Throughout the study, production of bioethanol use sugar substances.

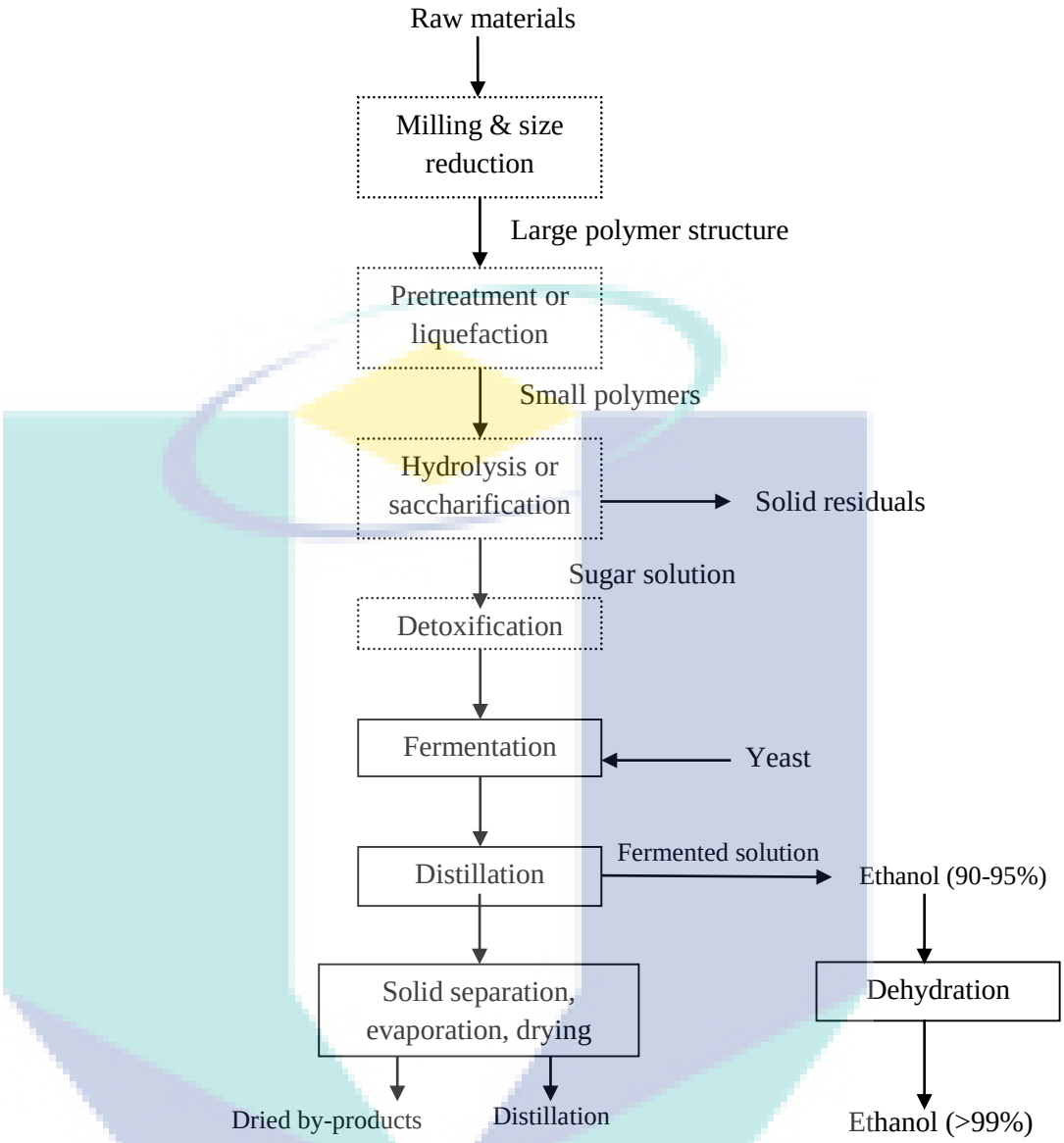


Figure 2.2 A general process scheme for overall process of ethanol production from different materials

2.6.2 Production of Bioethanol (sugar substances)

Ethanol can be obtained by two main processes which are chemical synthesis or by ethanol fermentation (biological pathway). Bioethanol produced by microbial fermentation processes which involving fermentation of biomass derived sugars as opposed to synthetically produced ethanol from ethylene. Dake et al. (2010) proposed, fermentation of ethanol is a biological process involving conversion of sugars to alcohol using yeast under anaerobic condition or in simple words, conversion of carbohydrate into alcohol or acids. Equation 2.2 shows general reaction for ethanol production during fermentation which microorganisms plays a role as a catalyst (Taherzadeh & Karimi,

2008).

$$Sugar(s) \xrightarrow[\text{microorganisms}]{} Ethanol + By\text{-}products \quad \text{Eq. 2.2}$$

The predominant microorganism responsible for ethanolic fermentations is organism such as yeast, *Saccharomyces cerevisiae*. *S.cerevisiae* known as ethanologenic which has the aptitude to convert sugars to ethanol by fermentation. The production cost of bioethanol depending on the source of biomass or price of raw materials and production method. Fuel prices rising from time to time due to increase demand by population in the world enhance the production of bioethanol with low cost production by using renewable resources to overcome diminishing supply of fuel in the future.

2.6.2.1 Factor affecting bioethanol production

The fermentation of bioethanol is affected by environment and surrounding. The influence of pH is the second important factor for ethanol yield and least important for CO2 weight loss rate (Liu & Shen, 2008). Raikar (2012) proposed, pH value has significant influence on alcoholic fermentation. In terms of temperature, lowering the temperature resulted in decrease of ethanol yield meanwhile increase of extremely high temperature resulted in decrease of ethanol yield and enzymes to be easily denatured (Chin et al., 2010). Rodmui et al. (2008) proposed, agitation is important in fermentation process to enhance cell mass and ethanol productivity. Faria et al. (2002) proposed, sterilization of fermentation medium resulted in decrease of nutrient concentration and yield products due to heat involvement. Tao et al. (2005) proposed, non-sterilized condition is energy productive and no potential of contamination. Table 2.4 shows different factor affecting production of bioethanol by other researchers.

Table 2.4 Factor Affecting Production of Bioethanol by Other Researcher

Factor	Range	Result	Keywords	References
Temperature	30-35ºC	31ºC	Carob pods, solid-state fermentation (SSF), *Z.mobilis*, The Plackett-Burman (P-B)	Mazaheri et al., 2012
	25-40ºC	32ºC	Palm-oil mill effluent (POME), bioethanol, direct bioconversion, *T. harzianum*, *S. cerevisiae*	Alam et al, 2009
	25-40ºC	35ºC	Grape waste, *S. cerevisiae*, Benzyl Penicillin	Raikar, 2012
	25-40ºC	31.73ºC	Oil palm trunk sap, CCD, FFD	Norhazimah, 2012
	28-35ºC	27ºC	*Saccharomyces cerevisiae*, ethanol production, Placket-Burman design, Response Surface Methodology (RSM)	Yingling et al., 2010
	28-36ºC	30ºC	Ethanol fermentation, activated yeast, dates, optimization	Noor et al., 2003
	25-40ºC	33.2ºC	Lignocellulosic biomass, ethanol yield, fermentation time, fermentation efficiency, Turkey-kramer multiple comparison test	Chin et al, 2010
	25-35ºC	32.5ºC	*Saccharomyces cerevisiae*, cashew apple juice, immobilization cell, continuous fermentation	Neelakandan et al., 2009

	25-40ºC	37ºC	Sweet sorghum, Immobilized *saccharomyces cerevisiae*, bioethanol	Liu et al., 2008
	30-38ºC	30ºC	Alcohol, fermentation, *saccharomyces cerevisiae*, toddy	Pramanik, K. 2003
	25-35ºC	30ºC	Mango juice, ethanol production, optimization, characterization	Veeranjaneya et al., 2007
pH/initial pH	3.00-9.00	6.00	Palm-oil mill effluent (POME), bioethanol, direct bioconversion, *T. harzianum, S. cerevisiae*	Alam et al, 2009
	4.00-5.50	5.00	Grape waste, *S. cerevisiae*, Benzyl Penicillin	Raikar, 2012
	3.00-7.00	5.50	Oil palm trunks sap, response surface methodology (RSM), CCD, FFD	Norhazimah, 2012
	4.40-5.60	5.30	Optimization, ethanol, pretreated molasses, *Saccharomyces cerevisiae*, fermentation, RSM	Shafaghat et al., 2010
	4.00-7.00	5.30	Lignocellulosic biomass, ethanol yield, fermentation time, fermentation efficiency, Turkey-kramer multiple comparison test	Chin et al, 2010
	3.00-5.50	5.00	Sweet sorghum, Immobilized *saccharomyces cerevisiae*, bioethanol fermentation, orthogonal experiments	Liu et al., 2008

	3.75-5.50	4.25	Alcohol, fermentation, *saccharomyces cerevisiae*, toddy	Pramanik, K. 2003
	3.50-6.00	5.00	Mango juice, ethanol production, optimization, characterization	Veeranjaneya et al., 2007
	4.50-5.50	5.50	*Saccharomyces cerevisiae*, ethanol production, Placket-Burman design, Response Surface Methodology (RSM)	Yingling et al., 2010
	4.00-8.00	6.00	*Saccharomyces cerevisiae*, cashew apple juice, immobilization cell, continuous fermentation	Neelakandan et al., 2009
Concentration	50-250 g/l	200 g/l	Alcohol, fermentation, *saccharomyces cerevisiae*, toddy	Pramanik, K. 2003
	10-18% (v/v)	16% (v/v)	Grape waste, initial sugar concentration, Ethanol	Raikar, R. 2012
Agitation	100-140	120	Ethanol fermentation, activated yeast, dates, optimization	Noor et al., 2003
	110-250	110	Oil palm trunks sap, response surface methodology (RSM), CCD, FFD	Norhazimah, 2012
	100-300	200	Agitation, aeration, orthogonal array design, *Saccharomyces cerevisiae*	Khongsay et al., 2012
	50-300	200	Sweet sorghum, Immobilized *saccharomyces cerevisiae*, bioethanol	Liu et al., 2008

	0-200	50	Ethanol, coculture, agitation	Rodmui et al, 2008
Sterilization	Autoclave or non-autoclave	Non-autoclave	Ethanol, *Zymomonas mobilis*, acid-tolerant strain	Tao et al., 2005

2.6.3 Batch Processes

There are various types of processes for ethanol fermentation such as batch fermentation, continuous fermentation, continuous fermentation with cell recycling, fed-batch and repeated batch culture (Yoshida et al., 1973). For a cultivation process under batch processes, all nutrients required for fermentation readily in the medium. For past research, batch process performed due to the ease of the operation, low cost of controlling and monitoring system, low requirements for complete sterilization, use of unskilled labor, low risk of financial problem and easy management of feedstock hence this process productivity is very low due to long turnaround times and initial lag phase (Kosaric et al., 1983). Therefore, cell recycling and application of several fermenters can be applied to overcome this disadvantages. Taherzadeh and Karimi (2008) proposed, reuse of produced cells helps increase productivity of process. Throughout the study, recycling volume (drain and fill), effect of rotation rate and successive cycle performed to increase yield of bioethanol production for longer time.

2.6.4 Scale Up to 2L Bioreactor by Repeated Batch Fermentation

Efficient ethanol production comes with great development of fermentation process (Alfenore et al., 2004; Ariyajarearnwong et al., 2011). Tang et al. (2010) proposed that industrial applied batch or continuous modes for fermentation processes. However, study by Najafpour et al. (2004) proposed that batch process has many disadvantages due to slow growing of microorganisms and affected by product inhibition. Meanwhile, continuous process requires a uniform substrate composition (Laopaiboon & Laopaiboon, 2012). Other lack of using continuous mode is it initiate.

In this research, repeated batch fermentation process will be conducted to increase the productivity. Repeated-batch fermentation has several advantages compared to conventional batch fermentation in which the medium of the fermentation broth will be withdrawn at the time intervals of operating fermentation and the residual part of the broth will be used as inoculum for the next batch (Ariyajarearnwong et al., 2012). Anastiassiadis and Rehm (2006) proposed, no new inoculum required for each batch, enhance productivity for long-term and enable cell adaptation to very high osmotic pressure take place during the repeated-batch fermentation. Repeated batch fermentation can be used due to operational control is easier compared to chemostat mode and

minimized time for cleaning and re-sterilization (Laopaiboon, 2012; Ariyajarearnwong et al., 2011).

Anastassiadis and Rehm (2006) proposed, continuous chemostat and repeated batch fermentation have many advantages than the traditional discontinuous industrial processes. In addition, by using repeated-batch fermentation, yeast can be used at least eight successive batches without marked reduction in ethanol production as it applied drain and fill technique (Ariyajarearnwong et al., 2011). However, Laopaiboon and Laopaiboon (2012) studied that method of drain fermented broth at appropriate amount and filled with fresh broth at the same volume for next cycle causing dilution of initial cell concentration in broth hence, decrement in ethanol production efficiency. To overcome this condition, immobilized cell can be used in repeated-batch fermentation. For efficient repeated-batch fermentation, factors such as cell concentration, fermentation time and recycling volume must be considered (Chen *et al*., 2008; Choi *et al*., 2009; Staniszewski *et al*., 2009). Table 2.5 shows ethanol production from different raw material using repeated-batch fermentation. Table 2.6 shows bio-product formation using repeated-batch process.

Table 2.5 Ethanol Production using Repeated-Batch Fermentation

Raw material	Working volume of each cycle (L)	Fill and drain volume (%)	Number of cycle	P* (g l^{-1})	Reference
Molasses medium (25% w/v)	3.0	75	6	106	Kida *et al.* (1991)
Molasses medium (22% w/v)	3.0	75	6	92	Morimura *et al.* (1997)
Cheese whey powder (125 g l^{-}	5.0	60	5	63	Ozmihci and Kargi (2007)
Cassava medium (185 g l^{-1} of sugar)	4.0	20	10	85	Choi *et al.* (2009)
Kitchen refuse (130 g l^{-1} of sugar)	< 1	60	>10	80	Ma *et al.* (2009)
Sweet sorghum juice (100 g l^{-1} of sugar)	0.01	100	16	44-48	Chohnan *et al.* (2011)
Sweet sorghum juice (230 g l^{-1} of sugar)	1.5	75	8	93	Ariyajarearnwong *et al.* (2011)
Sweet sorghum juice (240 g l^{-1} of sugar)	0.1		8	106	Laopaiboon and Laopaiboon (2012)

***P**, Ethanol concentration

Source: Ariyajarearnwong et al (2011)

Table 2.6 Bio-Product Formation using Repeated-Batch Process by Other Researcher

Bio-product	Keywords	References
Citric acid	Citric acid, *Candida oleophila* ATCC 20177, batch, continuous and repeated batch cultivation.	Anastassiadis and Rehm (2006).
Hydrogen	Semi-continuous biohydrogen production, solid substrate fermentation, organic wastes.	Poggi *et al*. (2009).
L-lactic acid	L-lactic acid, *Rhizopus oryzae*, semicontinuous fermentation.	Wu *et al*. (2011).
	Semi-continuous, L-lactate fermentation of garbage without sterile condition, analysis of the microbial structure.	Akao *et al*. (2007).
Ethanol	Repeated batch fermentation, flocculent hybrid, *Saccharomyces cerevisiae* CHFYO321.	Choi *et al*. (2009).
	Isolated mutant flocculent, *Saccharomyces cerevisiae* strain, kitchen refuse.	Ma *et al.* (2009).
	Semi-continuous, whey with co-immobilized enzyme, yeast cells, pervaporative recovery, kinetic model.	Staniszewski *et al.* (2009).

2.7 Experimental Design

2.7.1 One Factor at Time (OFAT)

Based on Table 2.3, researcher applied different analysis tools for the production of bioethanol. In this experiment, first screening will be employed OFAT method to select best parameter for the production of bioethanol from OPF juice. In OFAT, data analysis does not require advanced statistical knowledge and it is most common practice which holding other parameter constant (Sakkas et al., 2010). Studies by (Qu & Wu, 2005; Wahid & Nadir, 2013) used of OFAT is still popular in many organizations and enable

investigator to analyze the setting of factor rapidly. Wahid and Nadir (2013) proposed, OFAT procedure involving changing of one factor and holding other parameter fixed. The factor then fixed after varied to find out either it has any effect. Other factor then varied until reached the best setting and this analysis is repeated with another factor until achieve best parameter. Therefore, OFAT enable researcher to observe the response of experiment and from each run before entire experiment completed (Qu & Wu, 2005).

2.7.2 Response Surface Methodology

Experimental design is attained by performing the minimum number of experiments from data collection in order to obtained maximum information for the experiments. Information from experimental design involving the relevant factors simultaneously over a set of defined experiments thus relate results by a mathematical model which used for data interpretation, prediction and optimization. Response surface methodology (RSM) is one of the most efficient method for experimental design. It is widely used in fermentation technology to optimize parameters either laboratory scale or industrial level. RSM will be used in this experiment after OFAT. For RSM, design of experiment (DOE) which will be used is Central composite design (CCD). For CCD, it consists of 3 types of point which is cube point, axial point and center point. Three best parameters will be used in CCD to measure interaction effects and produces the optimum condition before scaling up to 2L-bioreactor. Equation 2.3 shows equation to determine total number of experiments (N) in CCD where *k* is the number of factors, 2^k is the cubic runs, *2k* is the axial runs and C_o is the center point's runs.

$$N = 2^k + 2k + C_o \qquad \text{Eq. 2.3}$$

RSM is applied by checking the significance of curvature to measure the presence of nonlinear behavior (Norhazimah & Faizal, 2013). So far, however, there has been no discussion of employing face-centered CCD for bioethanol production from OPF juice.

CHAPTER 3

METHODOLOGY

3.1 Raw Material and Sample Preparation

3.1.1 Extraction of Oil Palm Frond (OPF) Juice

In this study, fresh OPF (without leaves) which is the petiole part was collected at oil palm plantation, Felda Lepar Hilir 1, Gambang, Pahang, Malaysia during the dry season in the month of May. OPF was cut into 1.0 m length at three different sections; initial, middle and edge. The OPF juice then was extracted by pressing the frond using a conventional sugarcane press machine. The OPF juice was centrifuged at 15,000xg for 15 minutes at 4°C (Thermo Fisher Scientific, NC, USA) and the supernatant was filtered using a mixed cellulose ester membrane filter with the pore size between 3 to 5µ (Cole Parmer, Illinois, USA) and stored at -20°C for storage purposes before being used for fermentation. Procedure involved for the extraction of OPF juice were shown at Figure 3.1. Figure 3.2 shows flowchart of the research experiment.

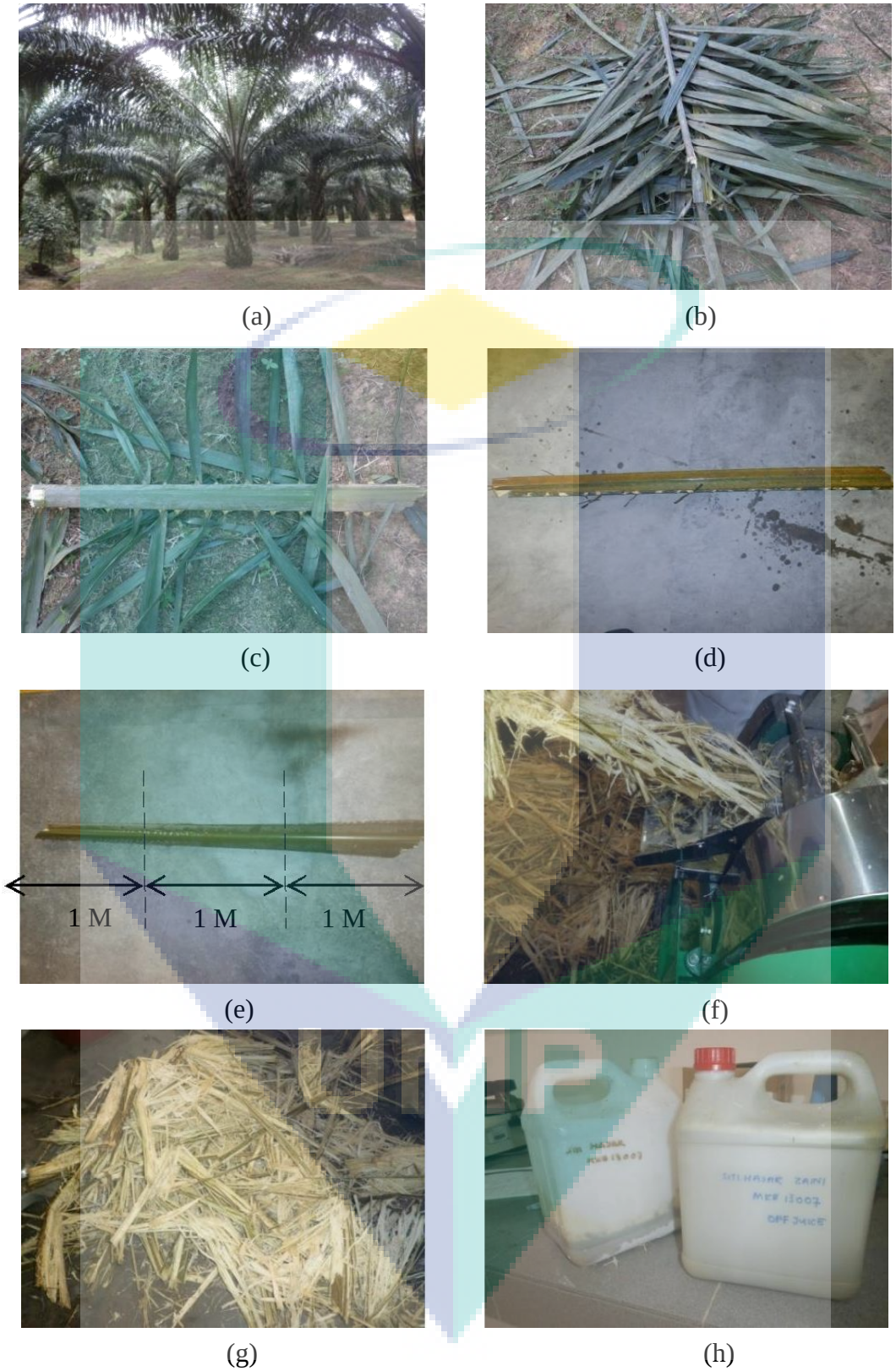


Figure 3.1 Preparation and extraction of OPF Juice (a) Oil palm tree (b) Collect frond (c) Leaves was removed (d) Frond with petiole part (e) Frond with three different section (f) Frond was extracted using sugarcane pressing machine (g) Fiber remains after pressing (h) OPF juice was obtained

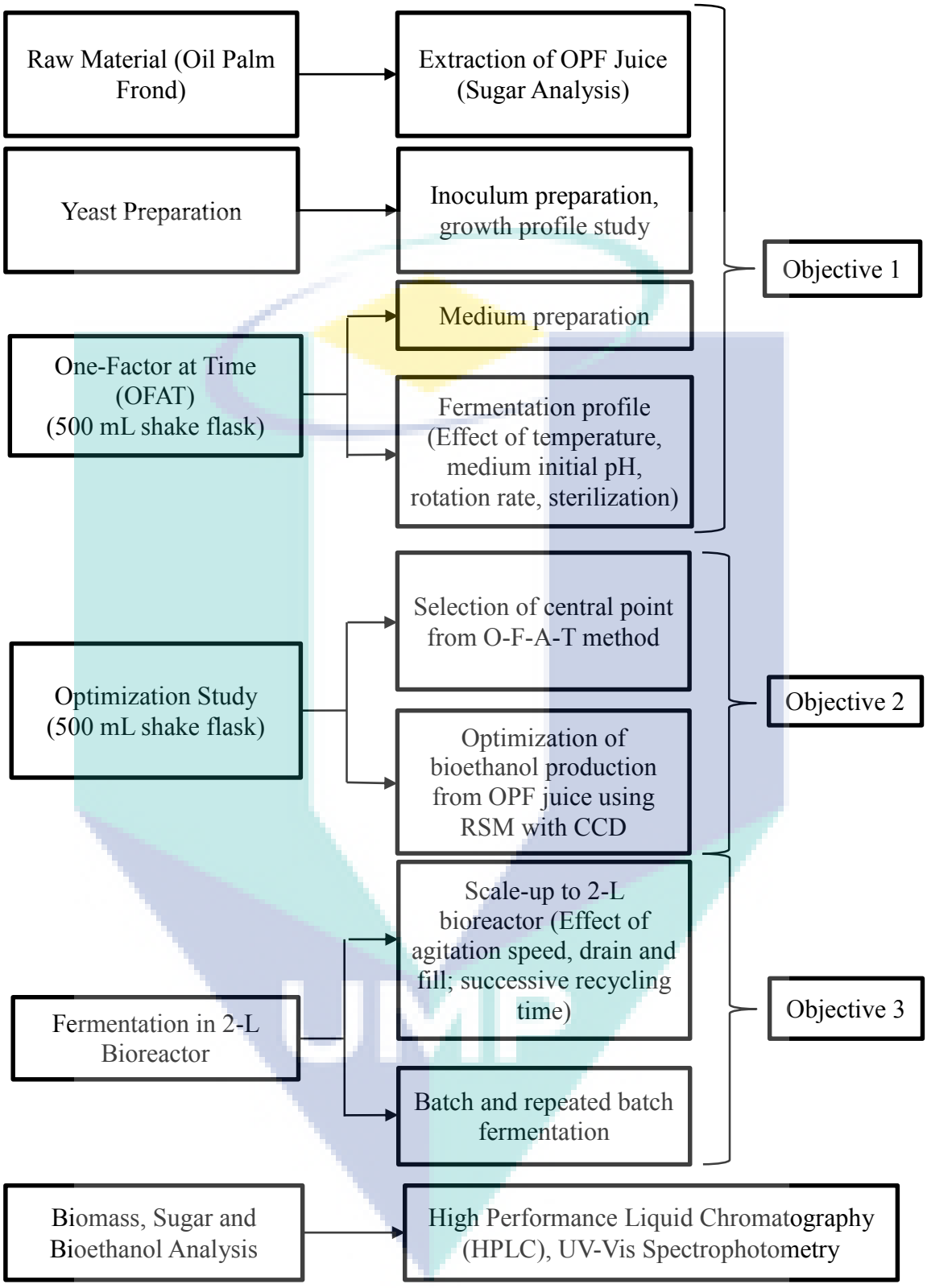


Figure 3.2 Flow diagram of the research procedure

3.2 Medium Preparation

3.2.1 Yeast Peptone Dextrose (YPD)

Yeast was grown and maintained on YPD agar following method described earlier by Kosugi et al, (2010) with some modification composed of 20 g/L technical agar, 20 g/L dextrose anhydrous, 20 g/L bacteriological peptone and 10 g/L yeast extract. After the media were prepared, the media were autoclaved at 121°C for 15 minutes. The YPD medium were used to culture yeast, *Saccharomyces cerevisiae* for 24 hours.

3.3 Pure Yeast Culture Establishment

Strain of yeast, *S. cerevisiae* Kyokai No 7 which were previously determined as potential ethanol producer were used throughout the study. The strain was subculture in 100 mL nutrient broth supplemented with yeast extract (1.5 g/L), peptone (1.5 g/L) and dextrose (20 g/L). Glucose solution which is dextrose was prepared and autoclaved separately from nutrient broth. The strain was cultured in a 250 mL conical flask and were incubated at 30°C using incubator shaker for 24 hours and agitated at 150 rpm. The strain was maintain for subcultured for every 5 weeks by subculture technique with the medium composition used as described previously with the addition of agar powder (20g/L). On the other hand, YPD agar also can be prepared by using 28 g/L of nutrient agar. After incubation, the culture was streaked and incubated for 2 days at 30°C. The grown colonies in the agar were sealed carefully using parafilm and stored in refrigerator at 4°C prior to use.

3.4 Yeast Screening

Inoculums for strain were prepared in 100 mL medium broth using 250 mL conical flask incubated at 30°C for 24 hours and agitated at 150 rpm. Optical density (OD) for each inoculums were using spectrophotometer (U-1900, Hitachi, Japan) and were standardized to an approximate value of OD 1.0. Standardized inoculums was incubated at 30°C for 24 hours at 150 rpm. After fermentation, yeast biomass, glucose consumption and ethanol production were analyzed in order to determine the best yeast strain.

3.5 Inoculum Preparation

A 3 loop full of microorganism (yeast strain *S. cerevisiae* Kyokai No. 7) from the

plate was transferred into the growth medium for a culture contained: 5 g/L glucose, 10 g/L peptone, 5 g/L yeast extract and 200 mL distilled water. The inoculum prepared vented with cotton wool to provide aeration and inhibit other microorganisms. The culture was grown in a 250 mL Erlenmeyer flask containing 100 mL growth medium on a shaker incubator at 30°C and 200 rpm for 24 h to reach the exponential phase following the method described earlier by Chin et al. (2010) and Bakri et al. (2011). The cell concentration was standardized to 0.2-0.4 g/L (OD = 1.5-2.0) determined using a calibrated UV-vis spectrophotometer U-1800 (Hitachi, Japan) at 600 nm. All of the procedure were carried out aseptically and analysis was run in duplicate.

3.6 Fermentation

3.6.1 Bioethanol Production Process

The OPF juice was thaw to ambient temperature and autoclave at 121°C with a retention time of 15 minutes.

3.6.2 Preparation of Fermentation

Fermentation is conducted in 250 ml Erlenmeyer flasks containing 100 ml of total working volume, including 10% v/v of inoculums. Temperature and agitation will be controlled using incubator shaker meanwhile, pH value will be set up with 2M NaOH or H_2SO_4. For sterilization, set with sterilization will be autoclaved first (121^0C, 15 minutes) while non-autoclaved will be directly used for fermentation. Samples will be taken aseptically at 6-hour interval for 48 hours to measure residual sugars, bioethanol content and biomass concentration. Initial sample will be taken at 0 hour to compare existence of bioethanol before and after fermentation. One factor at time (OFAT) will be used in this experiment. Table 3.1 shows design of experiment using OFAT.

Table 3.1 Screening of parameter affecting bioethanol production from OPF juice using OFAT method

Parameter	**Parameter range**				
Temperature (°C)	27.5	30	32.5	35	37.5
Medium initial pH	5	6	7	8	9
Rotation rate (rpm)	0	50	100	150	200

3.6.3 Screening of Process Variable by Shake Flask System using One Factor at Time (OFAT)

3.6.3.1 Preliminary Experiment

Preliminary experiment was carried out in the first place to study the effect of sterilization on bioethanol production by employing two set of experiments using autoclaved and non-autoclaved OPF juice as a fermentation substrate. In order to achieve this, yeast, *S. cerevisiae* Kyokai No. 7 (ATCC 26622) was cultured into autoclaved (sterile) and non-autoclaved (non-sterile) OPF juice. Result obtained from the study was used for subsequent experiment. Both of the experiments was conducted in a rotary shaker (150 rpm) under anaerobic condition at 30°C for 48 h without pH adjustment. Samples was withdrawn every 6 and 12 h from the broth for bioethanol and residual sugars determination.

3.6.3.2 Screening of Parameters Affecting Bioethanol Production from OPF Juice

For the first part of this study, the effect of different parameters on bioethanol production from OPF juice was screened using one-factor-at-time (OFAT) method. The OPF juice was filtered using 9.0 µm mixed cellulose ester membrane filter to remove unwanted particles as described by Norhazimah (2014). OPF juice (100% v/v) with total sugar concentration of 56.87 g/L which comprises of glucose, sucrose and fructose was used as the carbon source and nutrients throughout the study period. Pre-cultured yeast cells (10% v/v) were inoculated into a 250 mL Erlenmeyer flask containing 100 mL of autoclaved OPF juice following the previous method described by Kosugi et al. (2010) and Zahari et al. (2014) without any nutrient or nitrogen source supplementation.

In order to study the effect of medium initial pH on bioethanol production, the initial value of OPF juice was adjusted to pH 5.0-9.0 using 2 M NaOH prior to autoclave. Another set of experiments was conducted to study the effect of rotation rate on bioethanol production by investigating several rotation rates at 0, 50, 100, 150 and 200 rpm. Study on the effect of temperature was investigated by using various temperatures in the range of 27.5°C-37.5°C. Fermentation were run for 24 h under anaerobic condition and all experiments were conducted in duplicates. Samples was harvested at the end of the fermentation period for bioethanol and residual sugars determination. Table 3.2 to 3.4 shows screening using OFAT based on parameter (temperature, pH, rotation rate and

sterilization condition).

Table 3.2 Parameter set up for the effect of temperature on the bioethanol production using OPF juice at different temperature

Temperature (°C)	pH	Rotation rate (rpm)
27.5	7	150
30	7	150
32.5	7	150
35	7	150
37.5	7	150

Table 3.3 Parameter set up for the effect of pH on the bioethanol production using OPF juice at different pH

Medium initial pH	Temperature	Rotation rate (rpm)
5	32.5	150
6	32.5	150
7	32.5	150
8	32.5	150
9	32.5	150

Table 3.4 Parameter set up for the effect of rotation rate on the bioethanol production using OPF juice at different rotation rate

Rotation rate (rpm)	Temperature	Medium initial pH
0	32.5	7
50	32.5	7
100	32.5	7
150	32.5	7
200	32.5	7

3.6.4 Optimization of Process Variable using Response Surface Methodology (RSM)

3.6.4.1 Set up for Optimization Process

For the second part of this study, Response Surface Methodology (RSM) with central composite design (CCD) set-up was used in optimization process. There were three independent variables involved in optimization which are medium initial pH, rotation rate and temperature on the bioethanol surface optimized using a factorial Central Composite Design (CCD) of Response Surface Methodology (RSM). A five-level-three-factor CCD and the three most significant process variables namely the medium initial pH, temperature and rotation rate was employed in this study. Commercial software, Design Expert version 7.1.6 (Statease Inc., Minneapolis, Minn., U.S.A.) was attained to construct the experimental design table. A total design of 20 runs were set based on computer generated process variable including 6 replicate central points and $\alpha = 2$. The central point of each parameter studied in optimization experiment was selected based on the results obtained from OFAT experiment and the parameters ranges was set as follows; medium initial pH (5-9), temperature (27.5°C-37.5°C) and rotation rate (80-120 rpm) as shown in Table 3.5.

Table 3.5 Independent variable and their coded and actual values levels used in the RSM studies for optimizing the fermentation conditions of bioethanol production using OPF juice

Independent variable	**Unit**	**Symbol**	**Variation levels**				
			-α	**-1**	**0**	**1**	**+α**
Temperature	°C	A	20	25	32.5	40	45
Medium initial pH		B	3.5	5	7	9	10.5
Rotation rate	rpm	C	70	80	100	120	130

For the optimization study, fermentation was run for 24 h under anaerobic condition and all experiments was conducted in duplicates following the experimental plan which generated using the Design Expert Version 7.1.6 software as shown in Table 3.6.

Table 3.6 Twenty combinations of experimental runs using RSM, CCD

Std	Run	Temp (°C)	Medium initial pH	Rotation rate (rpm)
1	7	27.5	5.0	80.0
2	19	37.5	5.0	80.0
3	20	27.5	9.0	80.0
4	5	37.5	90	80.0
5	1	27.5	5.0	120.0
6	9	37.5	5.0	120.0
7	14	27.5	9.0	120.0
8	4	37.5	9.0	120.0
9	11	22.5	7.0	100.0
10	15	42.5	7.0	100.0
11	8	32.5	3.0	100.0
12	3	32.5	11.0	100.0
13	6	32.5	7.0	60.0
14	18	32.5	7.0	140.0
15	17	32.5	7.0	100.0
16	10	32.5	7.0	100.0
17	16	32.5	7.0	100.0
18	12	32.5	7.0	100.0
19	2	32.5	7.0	100.0
20	13	32.5	7.0	100.0

3.7 Statistical Analysis

Response surface methodology (RSM) was statistically analyzed using Design Expert version 7.1.6 software (Statease Inc., Minneapolis, Minn., U.S.A). The coefficients can be obtained through multiple regression analysis. Estimation of coefficients with levels higher than 95% ($p<0.05$) were included in the CCD models. The bioethanol yield can be expressed as a function of independent variables by a second order polynomial equation:

$$Y = \beta_0 + \sum \beta_0 x_j + \sum \beta_{jj} x_j^2 + \sum \beta_{jk} x_j x_k$$ Eq. 3.1

Where, Y is the response (bioethanol yield), represent the regression coefficient for intercept, linear, quadratic and interaction terms, respectively. The responses obtained were statistically evaluated by using analysis of variance (ANOVA) and the model was built based on the variables with confidence levels more than 95%.

3.8 Analytical Methods

3.8.1 Determination of Yeast Growth

The biomass concentration was determined by using UV-Vis spectrophotometer (OD = 600 nm) (Hitachi, Japan). Samples for quantitative analysis was centrifuged at 5,000 rpm for 20 minutes to obtain the supernatants. The supernatants were filtered through 0.22 µm membrane filter for the determination of residual sugars and bioethanol concentration.

3.8.2 Determination of Sugar and Bioethanol Concentration

Sugar and bioethanol were quantified through high performance liquid chromatography (HPLC) (Agilent 1200 series, U.S.A). A Rezex ROA organic acid H^+ (300 x 7.8 mm) column and RI detector were used for the separation. The chromatography grade 0.005 N H_2SO_4 was used as mobile phase and the flow rate was set at 0.6 mL/min. The column temperature was set at 60°C and RI detector temperature at 40°C. The injection volumes of 10 µl were applied. The components were identified by comparing their retention times with those of authentic standards under analytical conditions and quantified by external standard method (Zahari et al., 2012).

3.9 Scale Up to 2L Bioreactor by Repeated Batch Fermentation

3.9.1 Bioethanol Production using Bioreactor System

Bioethanol production process was further performed using 2.0 L bench-top Minifors Bioreactor. Refer Figure 3.3. Factors which affect the ethanol fermentation process such recycling volume, cycling time and agitation speed were investigated. The optimized condition such as temperature, initial pH obtained from shake flask system were used as initial conditions in the bioreactor system. Total working volume of the

fermentation process were kept constant at 1.5L and the process were carried out at 30°C with an air flow of 1 vvm. Each run was performed in duplicates according to the conditions. After the fermentation, sample were taken for analysis of sugar utilization and bioethanol production.

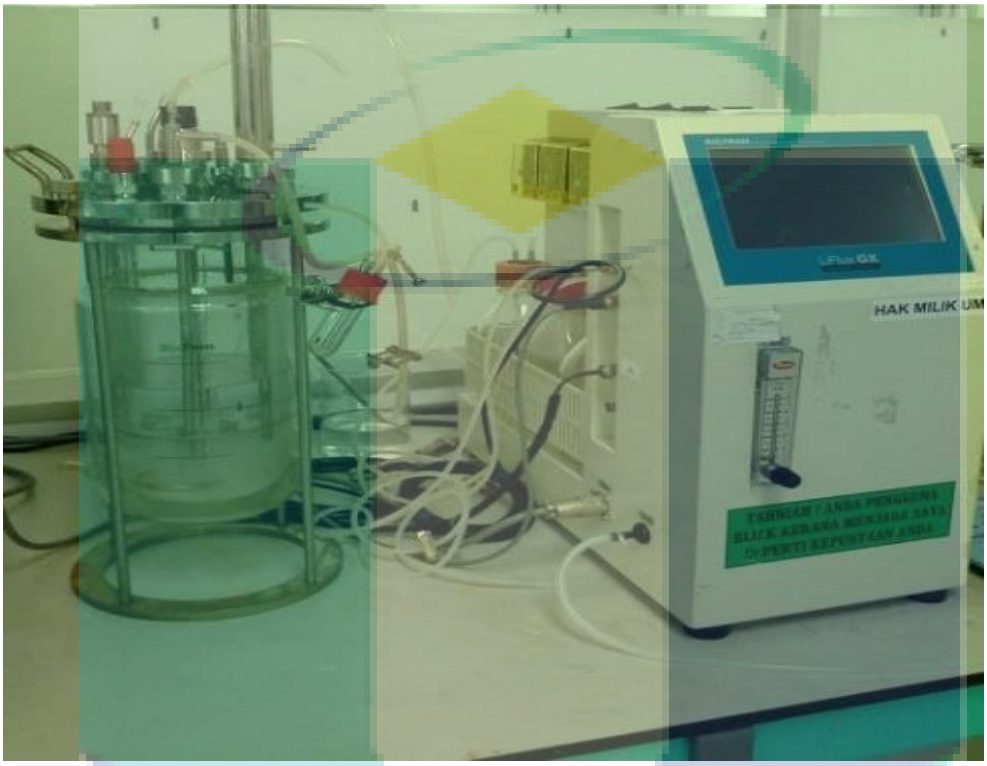


Figure 3.3 2L bench top stirred tank fermenter

3.9.2 Batch and Repeated Batch Fermentation System

The bioethanol production medium was transferred into a 2 L bioreactor (working volume of 1000 mL) and autoclaved at 121°C for 20 minutes. Repeated-batch operations were carried out as follows; agitation was stopped upon completion of the first batch fermentation, and the fermentation medium was allowed to sediment for 30 minutes. Then, the top phase of the fermentation medium was withdrawn and an equal volume of fresh OPF juice was initiated into the fermenter. This repetition step happened during every 24 hours, therefore defined as one series of the repeated-batch process. Prior to this, screening on factors affecting bioethanol production from OPF juice was run in batch mode of operation using the O-F-A-T method. In this study, evaluation on the effect of agitation speed (0 rpm and 50 rpm), drain (fermentation broth) and fill (fresh OPF juice)

volume (50% and 75%) were investigated in batch fermentation. This was followed by the effect of successive recycling time at 5, 10 and 15-cycles in repeated batch fermentation. During each cycle, the fermented broth was collected every 12-hour interval for the determination of sugar and bioethanol concentration using HPLC analysis.

3.9.2.1 Effect of Agitation Speed

Study on the effect of agitation speed was conducted by varied the agitator speed at two different value which is at 0 rpm and 50 rpm, respectively. This parameter was varied, meanwhile other parameters optimized in the previous study (medium initial pH and temperature) were remained constant throughout of the study period. Fermentation was carried out for 48 hours, meanwhile 5 mL sample was collected every 12 hours' interval for the determination of bioethanol concentration using HPLC. Results obtained from this study will be used for the subsequent experiment for the effect of drain and fill; and effect of successive recycling time.

3.9.2.2 Effect of Drain and Fill

After obtaining the best agitation speed for the cultivation of *S. cerevisiae* in 2-L bioreactor to produce bioethanol using OPF juice, the effect of drain and fill was conducted in batch mode of operation to study the effectiveness between drain and fill volume at 50% and 75% (v/v). In this study, the fermented broth was withdrawn at 50% and 75% (v/v) of the working volume and the same amount of fresh juice was immediately replaced after 24-h of fermentation period. After that, the fermentation was carried out for another 24 hour and samples were withdrawn at the end of fermentation period for the determination of bioethanol concentration. Results obtained from this experiment will be used for the subsequent experiment on the effect of successive recycling time.

3.9.2.3 Effect of Successive Recycling Time

In repeated batch fermentation, the numbers of successive recycling time (5, 10 and 15-cycles) were investigated. For five (5) cycle, it takes 120 hours to complete the 5-cycles. For 10-cycles, it required 240 hours to complete the cycle while, for 15-cycles,

360 hours were performed to complete all cycles. After complete each cycle in duration of 24 hours, sample were withdrawn about 5 mL for analysis. The sugar content and ethanol concentration were determined immediately.

For the effect of successive recycling time, 75% (v/v) (drain and fill) of recycling volume were investigated. When the total residual sugars in the broth drop slowly as found in the batch fermentation system, the fermented broth will be withdrawn at 75% (v/v) of the working volume and the same amount of fresh juice will immediately replace to initiate the next batch. The process will be repeated for 5 cycles, 10 cycles and 15 cycles. The fermentation time of incubation to replace with fresh juice will be observed by measuring the optical density (OD) value at 600 nm wavelength using UV-Vis spectrophotometry to observe growth profile or using HPLC to analyze the sugar content and bioethanol concentration.

3.9.3 Samples Collection

Samples aliquots of 2 ml was taken aseptically at various time: 0, 6, 12, 24, 30, 36, 42 and 48 hr. the samples were stored in the freezer at -20°C prior for analysis using HPLC. Samples were centrifuged for 5 min at 10, 000 rpm and filtered using 0.22 µm nylon syringe filter. Samples from 2L bioreactor will be taken of 75% and 50% at 6h interval. Sample then will be analyzes using HPLC for repeated-batch experiment.

3.9.4 Determination of Sugar and Bioethanol Concentration

Sugars and bioethanol concentration were determined by using HPLC according to the method described earlier in Section 3.8.2.

3.9.5 Statistical Analysis

Each experiment was repeated in duplicates. Analysis of variance (ANOVA) was performed by using the data analysis tools in Microsoft Excel 2010.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Calibration Curve

A calibration curve for sugar and ethanol content in the fermentation media was performed according to the method described previously. The peak of retention time for standard glucose (10.2-10.5 min), sucrose (9.0-9.5 min), fructose (11.1-11.3 min) and ethanol (21.0-22.4 min) as shown in Figure 4.1 to 4.4.

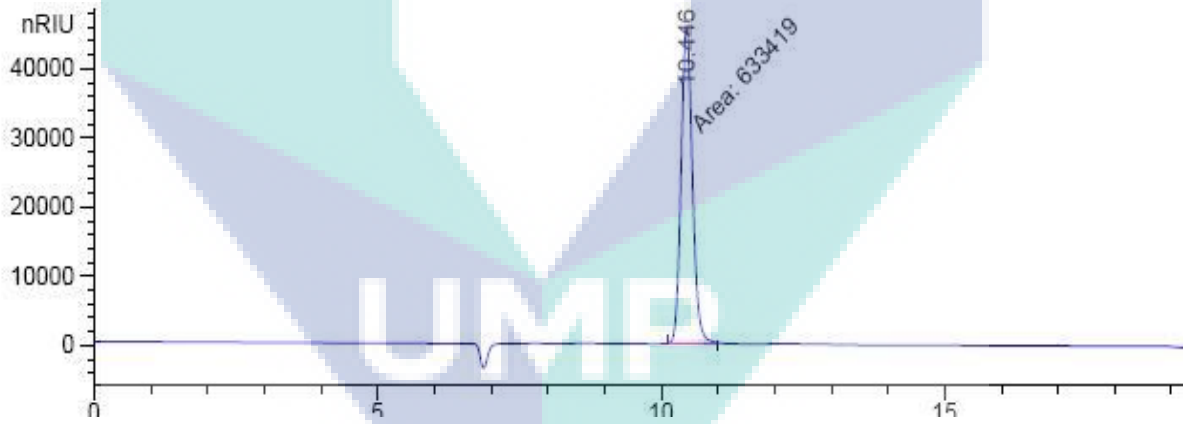


Figure 4.1 HPLC chromatogram of glucose standard solution at 5 g/L

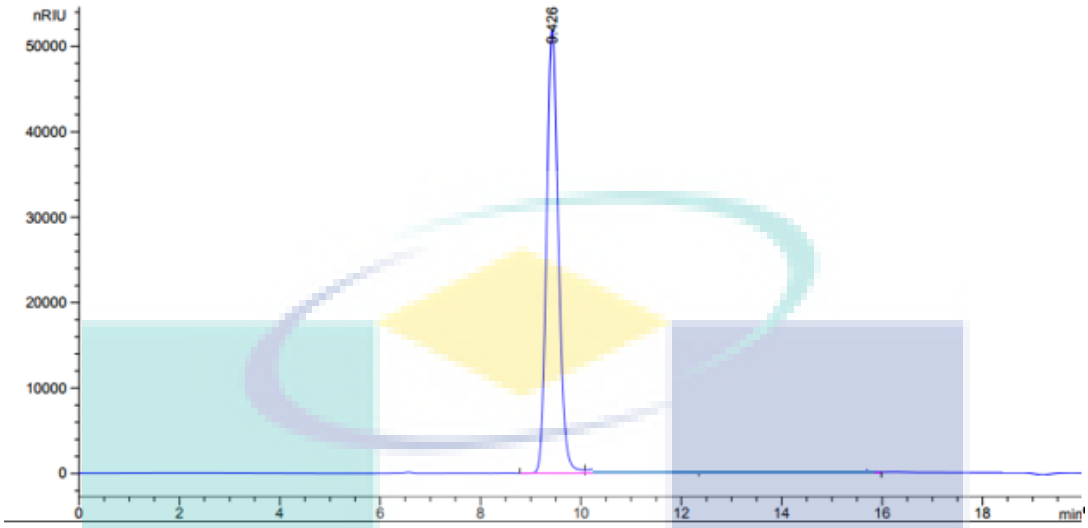


Figure 4.2 HPLC chromatogram, of sucrose standard solution at 5g/L

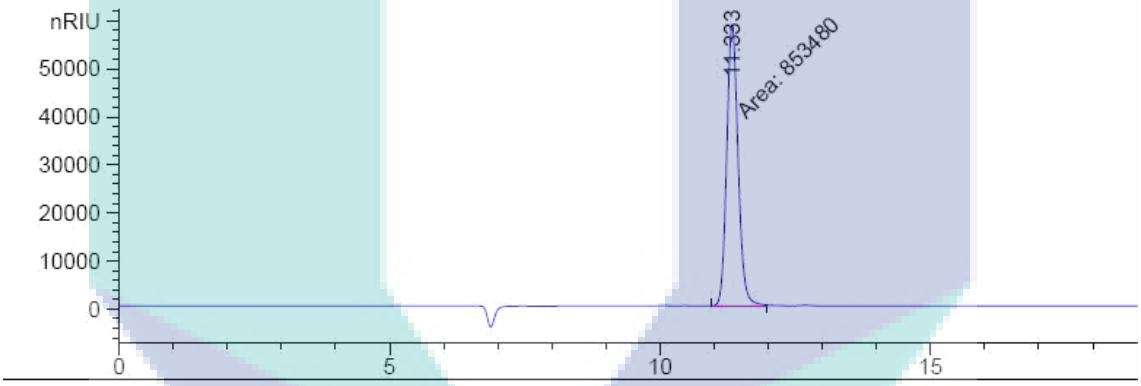


Figure 4.3 HPLC chromatogram of fructose standard solution at 5 g/L

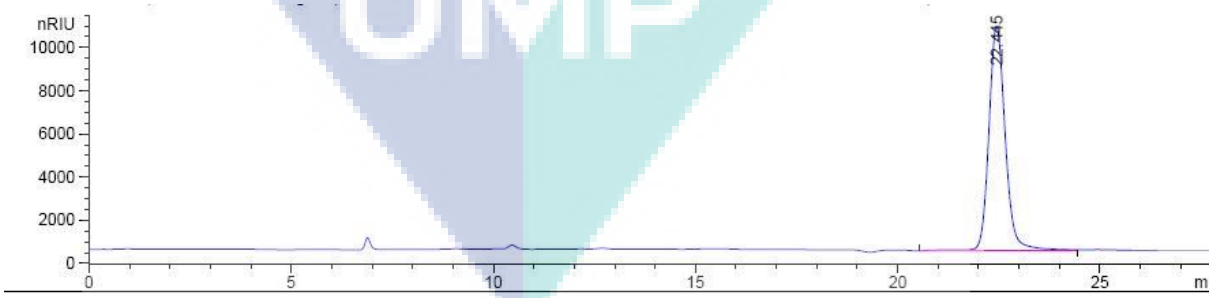


Figure 4.4 HPLC chromatogram of ethanol standard solution at 5 g/L

Calibration curve was plotted using Microsoft Excel^{TM} that depicts peak area (obtained from HPLC analysis) versus standard sugar and ethanol concentration. The peak area of the respective standard was used to calculate sugar and ethanol concentration, x (g/l) using standard curve as shown in Figure 4.5 to 4.8.

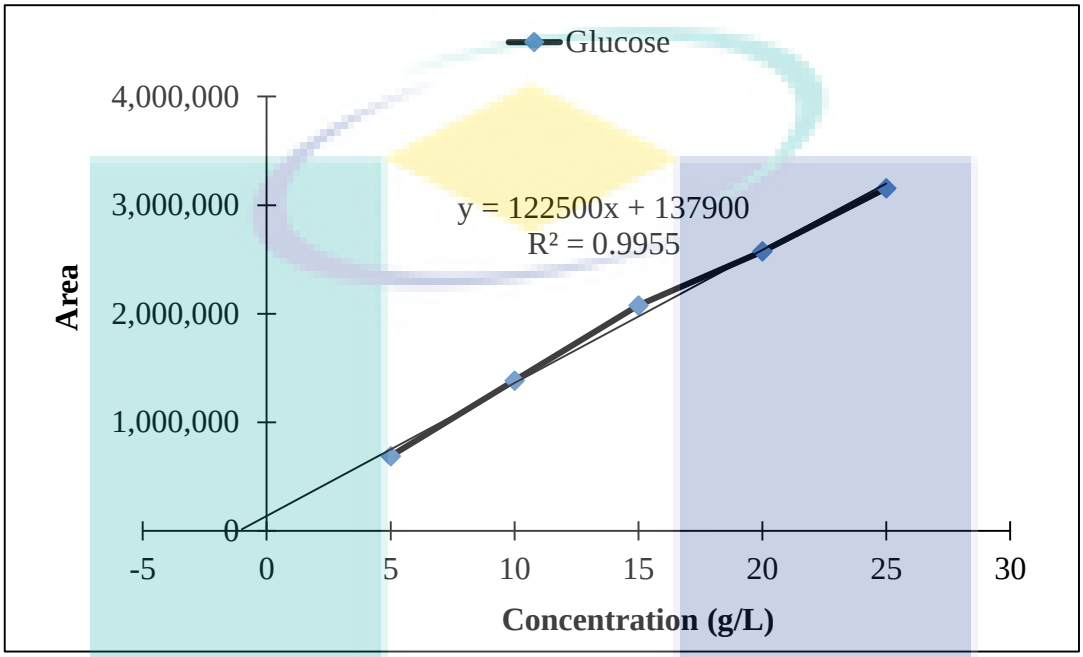


Figure 4.5 Glucose calibration curve

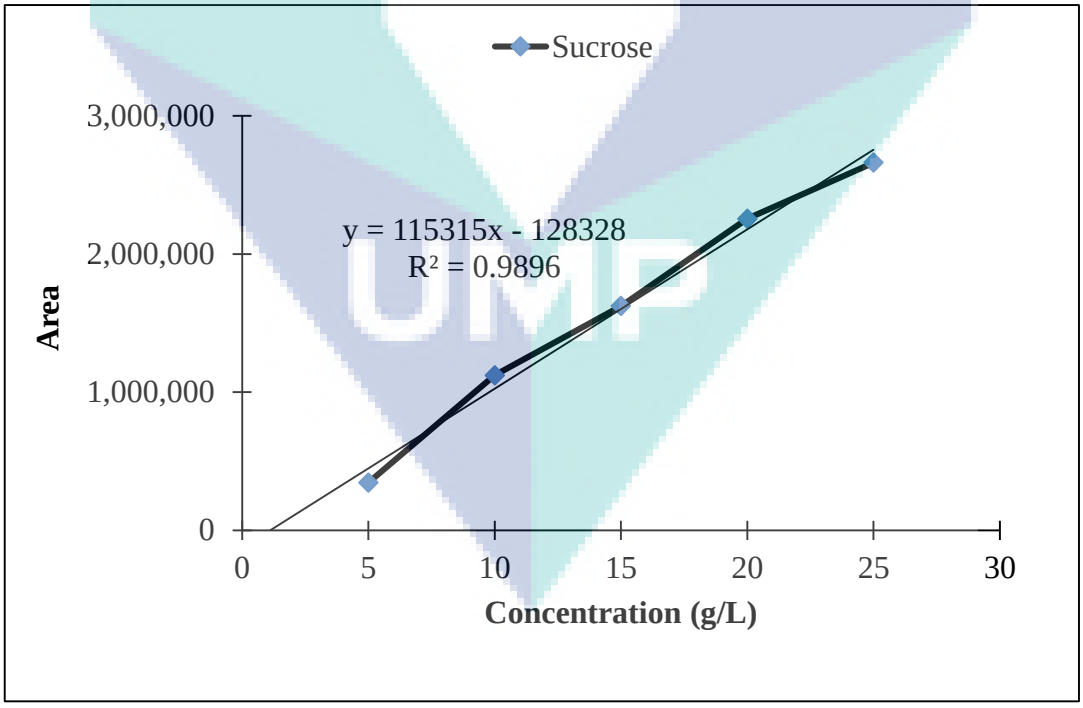


Figure 4.6 Sucrose calibration curve

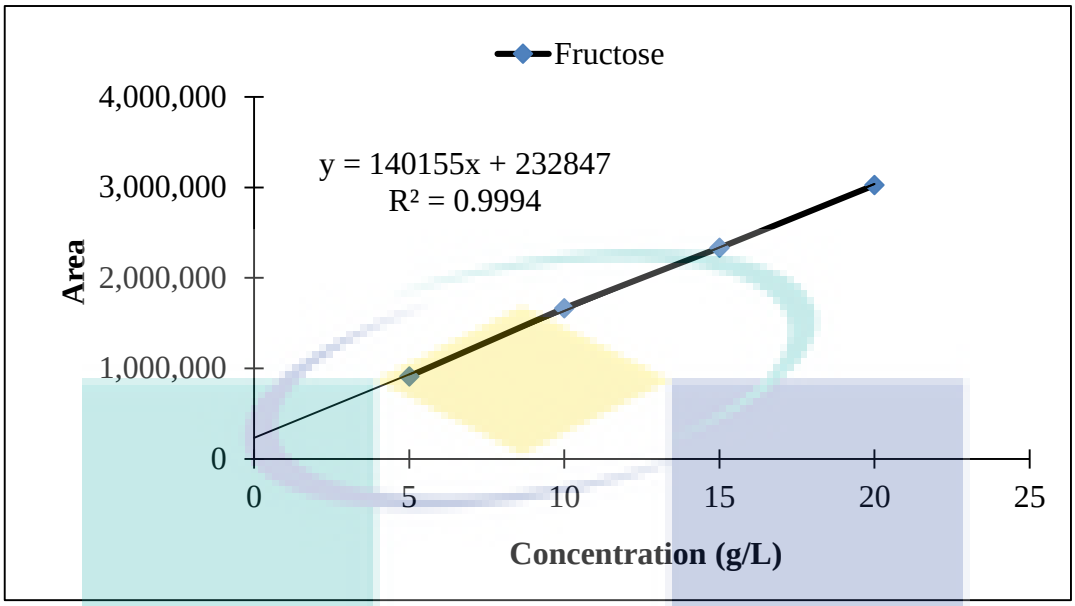


Figure 4.7 Fructose calibration curve

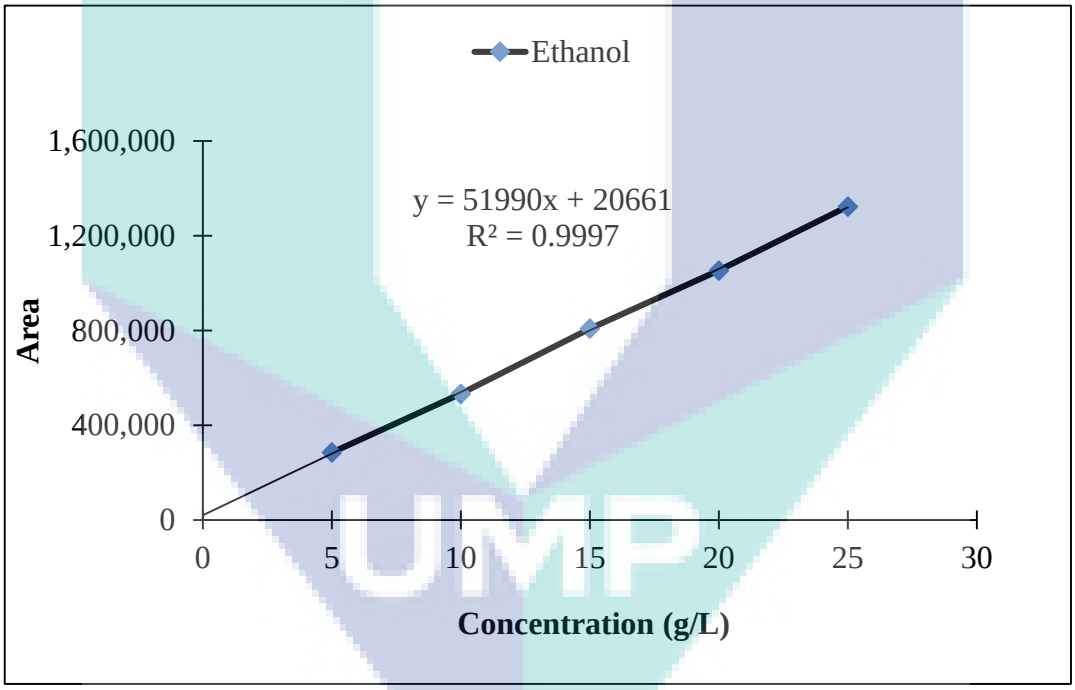


Figure 4.8 Ethanol calibration curve

4.2 Growth Profile of *Saccharomyces cerevisiae* Kyokai No.7 (ATCC 26622)

Bacterial growth is a complex process involving anabolic and catabolic reactions which resulted in cell division. Cell division or known as binary fission increase in the number of cells in a population. Growth curve of a microbial culture defined to study this population growth. Slow growth contributed by the poor nutrient and never achieving their metabolic actions. The growth of microorganisms is the result of controlled laboratory in which cultivated in liquid medium. There are two approaches to study the growth which is batch culture and continuous culture (Maier, 2009). In a batch culture, they are incubated in a closed vessel with a single batch of medium in which no fresh medium is added whereby a fixed amount of substrate. Maier (2009) proposed, continuous culture consists of steady influx of growth medium whereas the substrate remains constant. This information gives numerous benefits to the commercial production of a variety microbial products such as yeast, antibiotics, vitamins and amino acids. Several distinct growth phases can be observed by a growth curve.

Figure 4.9 (a) shows the growth profile of *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 26622) which can be divided into four distinct phases: lag phase, the exponential or log phase, the stationary phase and the death phase. Each phases represents different growth of *S. cerevisiae* Kyokai No. 7 (ATCC 26622) for the 48 hours of incubation under optimal growth condition. As shown in Figure 4.9 (a), at lag phase (0 h to 6 h), the growth of yeast is slow and near to zero. This might be contributed by the physiological adaptation state of the cell to the culture conditions (Maier, 2009). Maier (2009) also proposed, lag phase initially zero due to time requirement for induction of specific messenger RNA (mRNA) and protein synthesis. Slow growth at early stages also due to lack of nutrients from growing cells. Concentration of substrate might be low and diluted thus, low consumption of sugars and resulted in slower initiation of cell growth and division of yeast. The lag phase condition however, can be controlled depending on the type of medium and initial inoculum size. The lag phase was might due to the sugar was used as the sole carbon source thus, glucose catabolism required for synthesize the appropriate enzymes to the best condition.

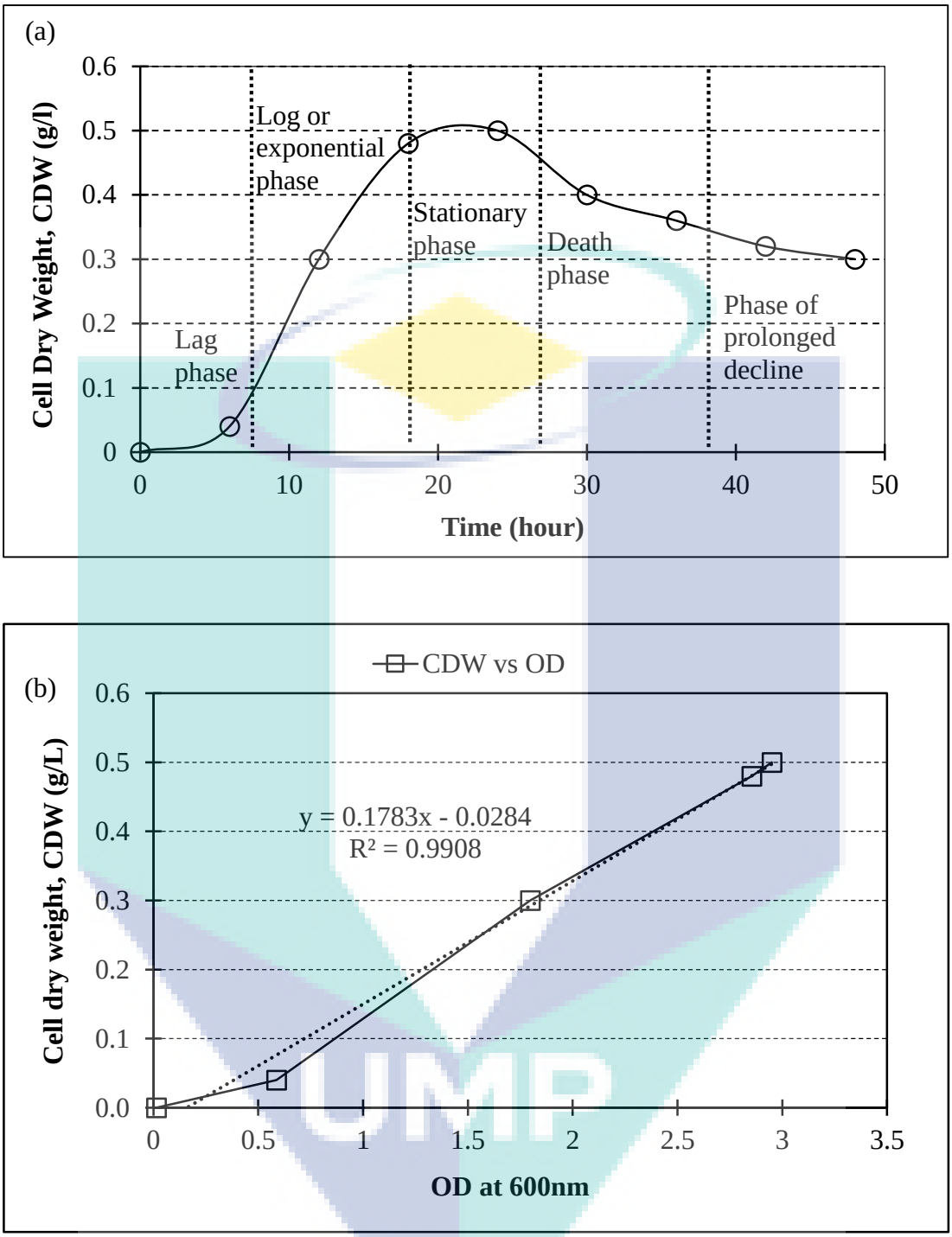


Figure 4.9 Growth profile of *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 26622); (a) cell dry weight, CDW (g/L) versus time (hour), (b) biomass standard curve of *S. cerevisiae* Kyokai No. 7.

The log phase (exponential) for this study was taking place from 8 h to 24 h of fermentation period. Bioethanol fermentation process normally performed at 16 to 24 h of incubation period. This might due to the increase of sugar consumption rate thus resulted to an increase of bioethanol production rate. Increase of sugar consumption rate produced high bioethanol concentration. This phenomenon was taken as a basis for drain and fill technique while conducting the repeated batch experiment in 2-L bioreactor. Throughout the repeated batch experiment, the addition of fresh OPF juice to replace the harvested fermentation broth was done at every 24 h of fermentation period.

As shown in Figure 4.9 (a), the stationary phase was taken place after 24 h of fermentation period might due to excessive of sugar consumption cause steady fermentation rate (Azhar et al., 2017). Azhar et al. (2017) reported that concentration of sugar exceeds the uptake capacity of the microbial cells hence, resulted a steady state. The growth of yeast appeared in death phase after 24 h as shown in Figure 4.9 (a) due to inhibition of microorganisms and viability. During this phase, yeast *S. cerevisiae* was unable to survive in medium with high concentration of bioethanol and thus, lead to low production of bioethanol concentration (Azhar et al., 2017). The plasma membrane of *S. cerevisiae* also affected with the presence of ethanol thus change the membrane organization and its permeability which then resulted in death phase of yeast growth (Alexandre and Charpentier, 1998). Therefore, it is important to study the optimal condition of yeast to avoid stress response hence produce biomass economically at great concentration of bioethanol.

4.3 Sugar Composition of Oil Palm Frond (OPF) Juice

Figure 4.10 shows the chromatogram analysis of OPF juice obtained from HPLC analysis. The total sugars concentration in OPF juice used in this study was 56.87 g/L. Glucose was found to be the dominant sugar (44.16 g/l) followed by sucrose (11.25 g/l) and fructose (1.46 g/l). This finding was almost similar as reported by Zahari et al. (2012) who reported glucose as dominant sugar in OPF juice followed by sucrose and fructose. However, Zahari et al. (2012) reported higher total sugars concentration (76.09 g/L) compared to this study where glucose, sucrose and fructose are 53.95 g/L, 20.46 g/L and 1.68 g/L respectively. This might be due to the different location of vegetation of the oil palm tree used in this study hence affect the sugars concentration in the OPF juice. The

OPF used in this study were obtained from the oil palm plantation in Felda Lepar, Gambang, Pahang whereby Zahari et al. (2012) obtained their sample from Serdang, Selangor. Another possible explanation might be contributed to the low sugars content in OPF juice in this study could be due to the time harvesting of fresh OPF during the dry season in the month of May. According to Yusof Basiron, chief executive officer of the Malaysian Palm Oil Council (MPOC), moderate amount of rain might provide a good condition to induce the growth of oil palm tree (Basiron, 2011) and thus influence the sugars content in the OPF as well. Table 4.1 shows initial sugar concentration contained in oil palm frond juice.

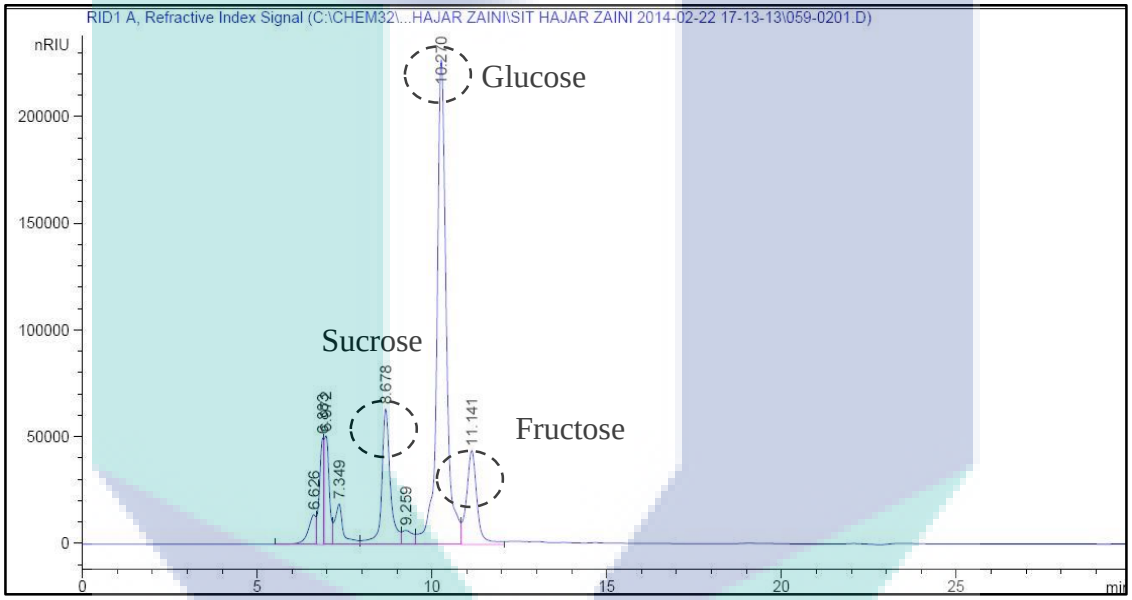


Figure 4.10 HPLC analysis of sugar compound in OPF juice

Table 4.1 Initial sugar concentration contained in oil palm frond juice

Types of sugar	Sugars concentration (g/L)	
	This work	Zahari et al. (2012)
Glucose	44.16	53.95
Sucrose	11.25	20.46
Fructose	1.46	1.68
Total	56.87	76.09

4.4 Preliminary Experiment

OPF juice is readily fermentable to produce bioethanol because it contains mixture of sugars and rich in minerals and nutrients which are essential for bacterial growth during fermentation. Bioethanol concentrations and total sugars consumed was analyzed using high performance liquid chromatography (HPLC) whereby the bioethanol yield (g g^{-1}) was calculated based on experimental bioethanol produced and expressed as g bioethanol per total g of sugar utilized (Equation 4.1):

$$Bioethanol\ yield\ \left(\frac{g\ bioethanol}{g\ sugars}\right) = \frac{Bioethanol\ concentration\ \left(\frac{g}{l}\right)}{Total\ sugars\ consumed\ \left(\frac{g}{l}\right)} \quad \text{Eq. 4.1}$$

4.4.1 Effect of Sterilization

In order to investigate the effect of sterilization on bioethanol production, two sets of experiment were conducted whereas autoclaved (sterile) and non-autoclaved (non-sterile) OPF juice was used as a substrate for fermentation. In order to achieve this, yeast, *S. cerevisiae* Kyokai No. 7 (ATCC 26622) was cultured into autoclaved and non-autoclaved OPF juice and the result was depicted in Figure 4.11.

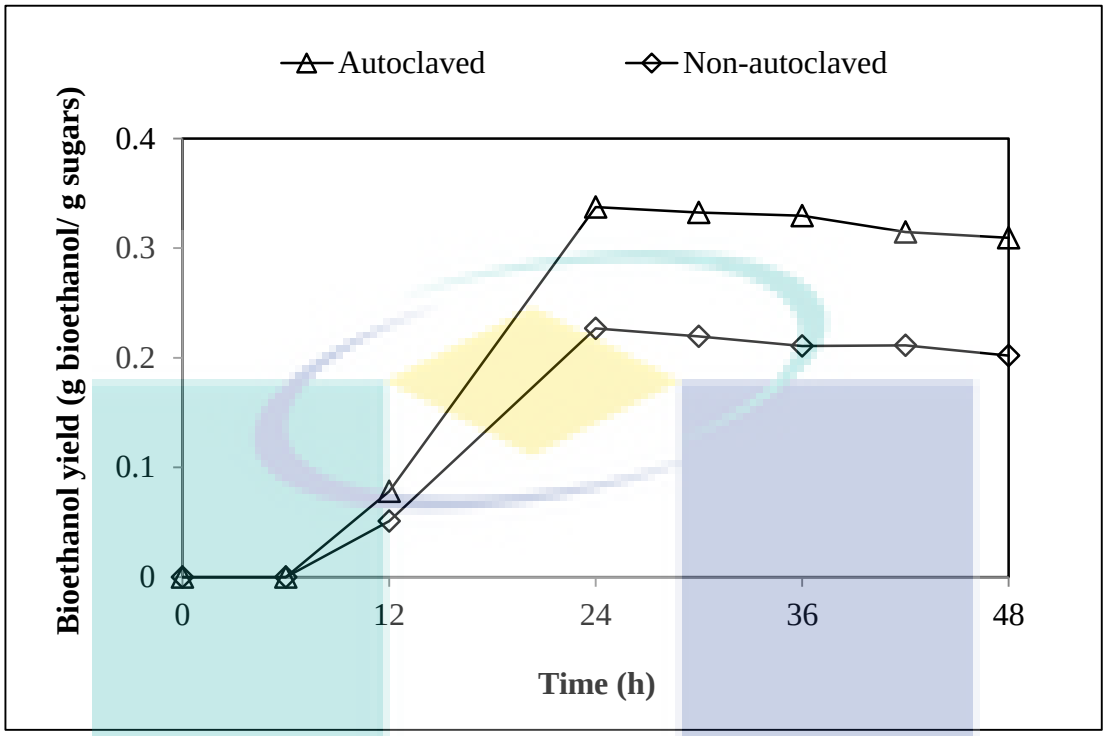


Figure 4.11 Comparison of bioethanol yield by *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 26622) supplemented with autoclaved and non-autoclaved OPF juice (Experiments were conducted in a rotary shaker (150 rpm) under anaerobic condition at 30°C for 48 h without pH adjustment).

Figure 4.11 shown the highest yield of bioethanol at 0.34 g bioethanol/ g sugars was obtained after 24 h of fermentation period when sterilized (autoclaved) OPF juice was used as fermentation substrate. Meanwhile, for the non-sterilized (non-autoclaved) OPF juice, only 0.23 g bioethanol/ g sugars were obtained within the same fermentation period. It was observed that the total bioethanol yield obtained was slightly higher using sterilized OPF juice as a fermentation feedstock. It is worth to mention that by autoclaving the OPF juice, any unwanted microorganisms which can cause contamination are inhibited or killed. This result is in agreement with other findings whereby heat sterilization may affect bioethanol production from oil palm trunk (OPT) sap by using similar yeast strain (Norhazimah, 2012). In the report it was mentioned that the maximum bioethanol concentration in heat sterilized sap was 29.96% higher than the fermentation in cold sterilized sap, and this was two times higher than the fermentation in non-sterile sap.

4.4.2 Sugars Consumption and Bioethanol Production

Fermentation profile for bioethanol production from sterilized (autoclaved) OPF juice using *S. cerevisiae* Kyokai No. 7 (ATCC 26622) was shown in Figure 4.12.

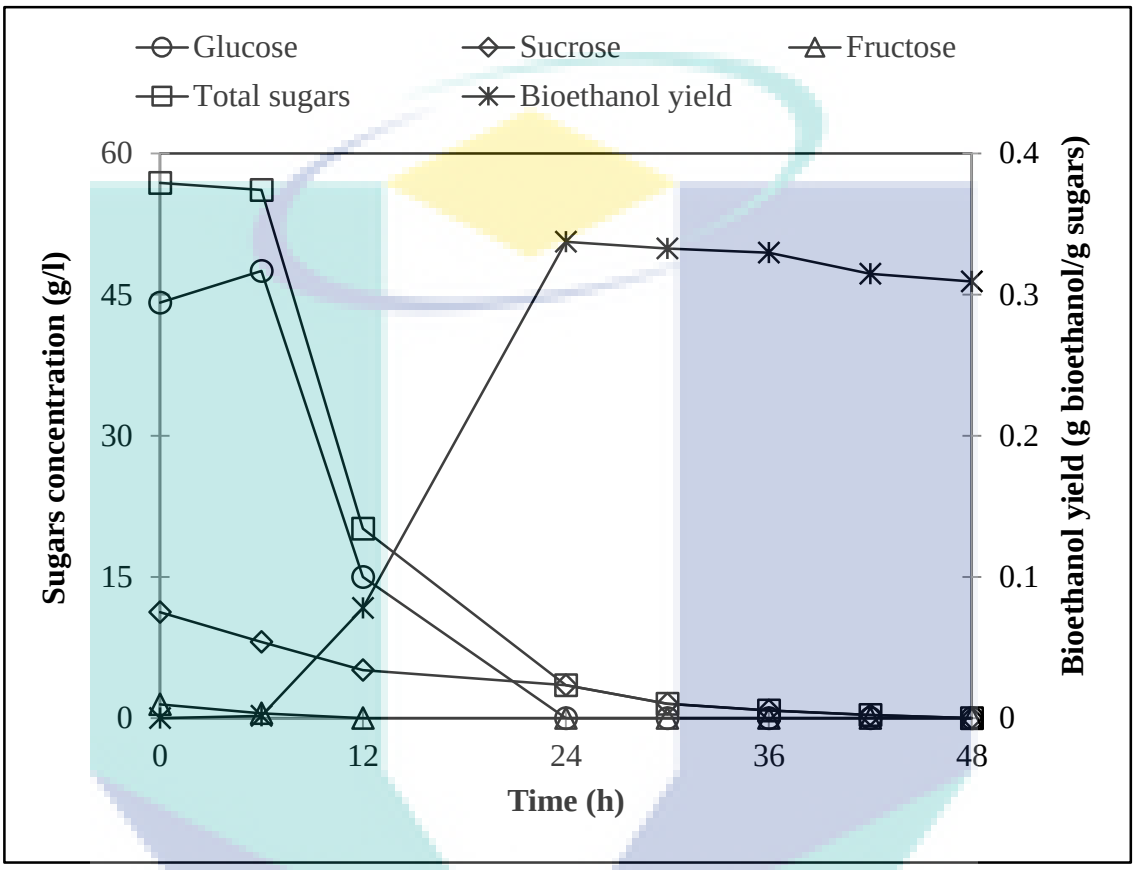


Figure 4.12 Sugars consumption and bioethanol production profile by *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 26622) supplemented with OPF juice

The bioethanol yield increased proportionally with reducing sugar content from fermentation broth. The highest bioethanol yield of 0.34 g bioethanol/ g sugars was obtained after 24 h of fermentation period. Prolonged time of incubation up to 48 h did not contribute to the increase in production of bioethanol. Whereby, it was slightly decreased to only 0.31 g/g sugars of bioethanol yield, which accounts for approximately 8.82% decrease. In Figure 4.12 there is a clear trend of decreasing in the production of bioethanol after 24 h of incubation might cause due to the decreased of sugars concentration level in the fermentation broth. During the fermentation, equal molarity of CO_2 and bioethanol was produced hence reduction of sugar consumption leads to weight lost in CO_2. This explained the reduction in bioethanol production in a longer period of time (Krishnamurthy et al., 2014). From this explanation, for the subsequent experiment,

the fermentation was conducted for 24 h of incubation period to evaluate the effects of several physical parameters on bioethanol production from OPF juice by *S. cerevisiae* Kyokai No. 7 (ATCC 26622).

Figure 4.12 also demonstrated the profile of sugars consumption by *S. cerevisiae* Kyokai No. 7 (ATCC 26622) in the fermentation broth throughout the incubation period. Overall, sugars in OPF juice was completely consumed by the yeast at the end of fermentation period including sucrose. During the first 6 h until 12 h, the sugars concentration decrease rapidly as bioethanol was produced. Our finding revealed that the concentration of fructose, sucrose and total sugar started to decrease during the first 6 h and then rapidly decreased afterward. This finding highlights the increase in glucose concentration can be attributed to the breakdown of sucrose to its monomer by the presence of invertase during the fermentation as sucrose is a disaccharide composed of glucose and fructose. The present finding also support Zahari et al. (2014) and Shahirah et al. (2014) study which concluded that *S. cerevisiae* has the ability to produce invertase enzyme.

Results obtained in this study was also in corroborates with the growth profile of *S. cerevisiae* Kyokai No. 7 (ATCC 26622) which has been discuss in Section 4.2. As shown in Figure 4.12, microbial growth is mainly associated with sugars consumption rate. For the first 6 h, lower consumption rate by *S. cerevisiae* was observed. On the other hand, at 24 h, sugars were fully utilized and drastically decreased after 25 h of cultivation period. From the growth profile study in Figure 4.9 (a), it can be observed that the detectable reduction of sugars concentration in the medium after 24 h was associated with bioethanol accumulation. It is interesting to note that, *S. cerevisiae* is completely utilized the sugars in OPF juice for the production of bioethanol.

4.5 Screening of Parameters Affecting Bioethanol Production from OPF Juice using OFAT

In the fermentation process, many operation parameters such as agitation, aeration, pH, temperature, effect of sterilization, dissolved oxygen and inoculum levels have to be investigated. However, only the effect of medium initial pH, effect of rotation rate and effect of temperature will be discussed throughout the study.

4.5.1 Effect of Medium Initial pH

The medium initial pH is a key factor which has significant influence of fermentation (Raikar, 2012; Lin et al., 2012). All organism and cellular processes are affected by pH; this is mainly due to the concentration of H^+ ions in the liquid environment. The cells grow and perform fermentation best within a certain pH range (Chin et al., 2010). In this study, the effect of medium initial pH on bioethanol production from OPF juice was conducted by adjusting the initial pH value of OPF juice prior to autoclaving between 5.0 and 9.0 with an increment of 1.0. Table 4.2 demonstrates that; bioethanol yield was found to be the highest when the medium initial pH was adjusted at pH 7.0 compared to others after 24 h of fermentation period. It appears from Table 4.2 that, the highest bioethanol yield obtained in this experiment was 0.39 g/g sugars. It is apparent from this table that pH may be an important factor to achieve maximum bioethanol yield. Optimum pH is essential for bioethanol yield to avoid maximum acidic or basic condition of medium hence retard the metabolic of yeast and cell growth (Willaert & Nedovic, 2006).

Table 4.2 Bioethanol yield at different medium initial pH with rotation rate and temperature were set at 150 rpm and 30°C, respectively.

Medium initial pH	Bioethanol concentration	Bioethanol yield (g/g sugars)
5	19.79	0.35
6	20.14	0.35
7	22.10	0.39
8	18.40	0.32
9	16.77	0.29

[a]Determination by HPLC from filtered supernatant after 24 h of incubation period

4.5.2 Effect of Rotation Rate

Rotation of incubator shaker is necessary for constant mixing of the medium components to provide uniform oxygen transfer rates. Rotation also played significant role in improving bioethanol concentration and yield (Yan et al., 2009). The effect of rotation rate is fundamental to obtain successful fermentation by providing adequate mixing, mass transfer and heat transfer (Rodmui et al., 2008). Besides assisting mass

transfer between two different phases of the medium, it also enables uniform suspension of microbial cells in homogenous nutrient medium. Table 4.3 illustrates bioethanol production by *S. cerevisiae* Kyokai No. 7 (ATCC 26622) using OPF juice as the fermentation feedstock at different rotation rate of 0, 50, 100, 150 and 200 rpm.

Table 4.3 Bioethanol yield at different rotation rate with medium initial pH and temperature were set at 7.0 and 30°C, respectively

Rotation rate (rpm)	Bioethanol concentration (g/L)	Bioethanol yield (g/g sugars)
0	19.00	0.31
50	19.47	0.34
100	23.00	0.40
150	22.10	0.39
200	19.86	0.35

[a]Determination by HPLC from filtered supernatant after 24 h of incubation period

As illustrated in Table 4.3, after 24 h of incubation period, rotation rate at 100 rpm gave the best bioethanol yield of 0.40 g/ g sugars compared to the other rotation rate. Thus, maximum productivity in microbial fermentation was achieved at optimum rotation rate. The result is in the lines of earlier literature (Mittal, 1992) that found rotation creates shear forces by causing morphological changes and disruption of cell structure. High speed agitation is not suitable for successful fermentation as it could contribute to the effect of hydrodynamic stress which can cause leakage of intracellular compounds (Bakri et al., 2011). However, rotation is needed to improve cell mass and bioethanol activity. Therefore, low rotation rate may contribute to low bioethanol production due to less nutrient consumption by yeast cells in static condition (Yan et al., 2009). The present findings also suggest that optimum rotation rate will enable symmetrical fermentation system hence accelerating nutrient consumption by yeast.

4.5.3 Effect of Temperature

In this study, the influence of different temperature on the bioethanol fermentation by *S. cerevisiae* Kyokai No. 7 using OPF juice was studied with regard to bioethanol production. Temperature is one of the most significant parameters that contribute to yeast growth and fermentation performance. *Saccharomyces* Kyokai no. 7 is a type of yeast which is mesophilic in nature, thus able to withstand temperature up to 48°C. However, Ho and Powel (2014) suggested the preferable temperature for *Saccharomyces* yeast is between 25 to 35°C and at a temperature up to 43°C, yeast cells began to lose their capability to be superior ethanologenic yeast strains. Table 4.4 shows the bioethanol yield at various temperatures from 27.5 to 47.5°C.

Table 4.4 Bioethanol yield at different temperature with medium initial pH and rotation rate were set at 7.0 and 150 rpm, respectively.

Temperature	Bioethanol concentration (g/L)[a]	Bioethanol yield (g/g sugars)
27.5	18.15	0.32
30	22.10	0.39
32.5	23.11	0.41
35	18.85	0.33
37.5	16.95	0.30

[a] Determination by HPLC from filtered supernatant after 24 h of incubation period

From the data in Table 4.4, it is apparent that highest bioethanol yield was obtained at temperature of 32.5°C (0.41 g bioethanol/ g sugars) and thus regarded as an optimum temperature for production of bioethanol using OPF juice by *S. cerevisiae* Kyokai No. 7. In Table 4.4 there is clear trend of decreasing in bioethanol yield at 37.5°C and this event was consistent with findings of past studies by Fakruddin et al. (2013) in which the production of bioethanol by strains *Saccharomyces unisporous* (P), *Saccharomyces cerevisiae* (C) and (T) gradually decreased.

4.6 Optimization of Bioethanol Production Employing Response Surface Methodology (RSM)

Based on the OFAT experiment, it was observed that the best condition for bioethanol production from OPF juice by *S. cerevisiae* Kyokai No. 7 was obtained at the following parameter's conditions; medium initial pH (7.0), temperature (32.5ºC) and rotation rate (100 rpm). These conditions were then selected as the central point for optimization study using CCD. Studies was carried out to establish the range of parameters such as medium initial pH, temperature and rotation rate to be optimized. A design matrix corresponding to the yield of bioethanol was subjected to regression analysis to study the effect of these parameters. The RSM experimental design matrix with three factors at five levels and the experimental results are presented in Table 4.5.

Table 4.5 The experimental results for bioethanol yield for the central composite design

Standard Run	Factor A Temp. (°C)	Factor B Medium initial pH	Factor C Rotation rate (rpm)	Response Bioethanol yield (g bioethanol/ g sugars)
1	27.5	5	80	0.35
2	37.5	5	80	0.37
3	27.5	9	80	0.30
4	37.5	9	80	0.30
5	27.5	5	120	0.27
6	37.5	5	120	0.35
7	27.5	9	120	0.27
8	37.5	9	120	0.30
9	22.5	7	100	0.22
10	42.5	7	100	0.26
11	32.5	3	100	0.31
12	32.5	11	100	0.26
13	32.5	7	60	0.35
14	32.5	7	140	0.31
15	32.5	7	100	0.49
16	32.5	7	100	0.46
17	32.5	7	100	0.49
18	32.5	7	100	0.48
19	32.5	7	100	0.46
20	32.5	7	100	0.49

Bioethanol yield was used as a response and was arranged into design expert experiment based on standard run. It appears from Table 4.5 that, the average value of all bioethanol yields was around 0.22-0.49 g bioethanol/ g sugars.

4.6.1 Analysis of Variance (ANOVA) and Model Development

ANOVA is known as analysis of variance which offers an excellent technique to determine the process variables that gives significant impact on process quality and their possible interaction. ANOVA which includes *F*-value, p-value, R^2 and lack of fit was applied to determine suggested model that fit with experimental data. R^2 is known as coefficients of determination to ensure the quality of fit for the model. The p-values of less than 0.05 were indicated as statistically significant. The significant terms showed whether the parameters studied affects fermentation process. The model and individual coefficient will be more significant if the results show a larger magnitude of *F*-value and a smaller p-value. The relationship between independent variables and response can be performed from analysis of quadratic model as shown in Table 4.6.

Table 4.6 Analysis of Quadratic Model

Source	**Sum of Squares**	***DF**	**Mean Square**	***F*-Value**	***p*-value Prob>F**	
Process order: Quadratic						
Model	0.1530	9	0.0170	48.74	< 0.0001	Significant
A-Temp	0.0027	1	0.0027	7.90	0.0184	
B-pH	0.0045	1	0.0045	13.06	0.0047	
C-Speed	0.0027	1	0.0027	7.90	0.0184	
AB	0.0006	1	0.0006	1.76	0.2146	
AC	0.0010	1	0.0010	2.90	0.1192	
BC	0.0006	1	0.0006	1.76	0.2146	
A^2	0.0946	1	0.0946	271.43	< 0.0001	
B^2	0.0631	1	0.0631	181.03	< 0.0001	
C^2	0.0379	1	0.0379	108.87	< 0.0001	
Residual	0.00348	10	0.00034			
Lack of Fit	0.00240	5	0.00048	2.22	0.2010	**Not significant
Pure Error	0.00108	5	0.00021			
Cor. Total	0.15649	19				
Standard Deviation		0.019	R^2		0.9777	
Mean		0.35	Adjusted R^2		0.9577	
C.V. %		5.27	Predicted R^2		0.8679	
PRESS		0.021	Adequate Precision		20.574	

*DF = Degree of freedom

**Lack of Fit is not significant relative to the pure error.

The mathematical model derived from the experimental results for bioethanol yield (Y) was shown in Equation (4.2):

$$Y = +0.47+0.013\times A-0.017\times B -0.013\times C -0.0088\times A\times B +0.011\times A\times C+0.0088\times B\times C-0.061\times A^2-0.05\times B^2 -0.039\times C^2 \quad \text{Eq. 4.2}$$

Where Y is bioethanol yield, A is temperature, B is medium initial pH and C is rotation rate. The quadratic model was selected to provide the best fit with the experimental results.

The model presented in Table 4.6 exhibits a high determination coefficient (R^2 = 0.9777), explaining 97.77% of the variability in the response, as well as a high value of the adjusted determination coefficient (adjusted R^2 = 0.9577), suggesting a high significance of the model. A very low probability ($p < 0.0001$) obtained from the regression analysis of variance (ANOVA) demonstrated that the model was significant. In this study, all the linear model terms including temperature (A), medium initial pH (B) and rotation rate (C) have significant effect, as the p-values calculated for this factor was less than 0.05. Therefore, changes in this parameter could significantly impact the bioethanol production from OPF juice fermentation. The most significant effect is the linear term of medium initial pH (B), followed by rotation rate (C) and temperature (A). All the two level interactions including temperature and rotation rate (AC), temperature and medium initial pH (AB) as well as medium initial pH and rotation rate (BC) were indicated as significant. In a similar manner, all the second order effects showed the significant results including A^2, B^2 and C^2. Generally, the lack of fit p-value of 0.201 implied that the lack of fit is not significant relative to the pure error. The non-significant lack of fit is positive because it demonstrates a good fit of the model to the data. A good fit means that the generated models adequately explained the variation of data.

4.6.2 Response Surface Plot

Residual is known as the difference between actual response and predicted response. Distribution of residuals was analyzed to clarify the adequacy of the model. Figure 4.13 (a) demonstrates the normal probability plot of the residuals and Figure 4.13 (b) illustrates the plot of residuals versus predicted.

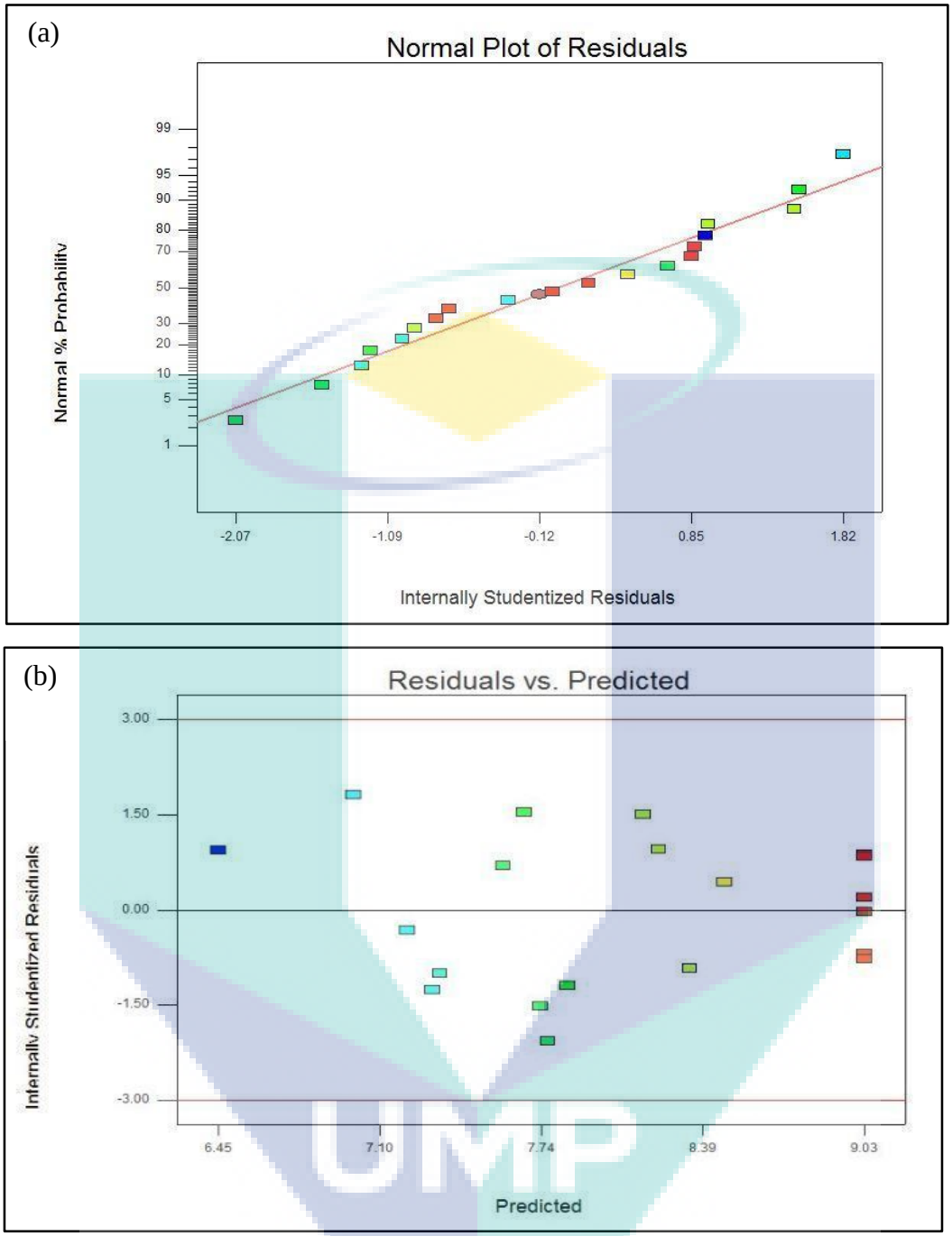
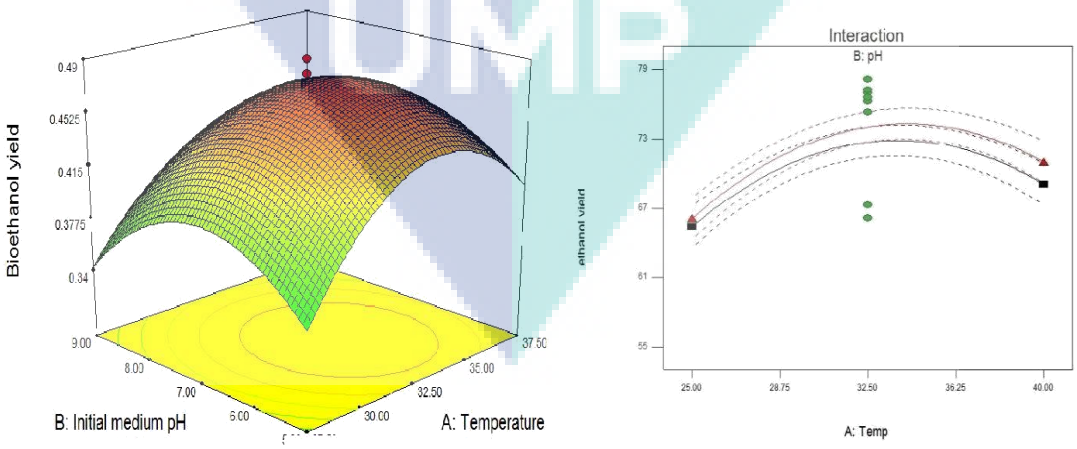


Figure 4.13 (a) Normal percentage probability plot for bioethanol yield and (b) plot of residual versus predicted response

Response surface plots based on Equation (4.2), with the relationships between the response and variables, are presented in Figure 4.14, 4.15 and 4.16. The plots were constructed by plotting the response (bioethanol yield) on the Z-axis against any two dependent variables while maintaining the other variables at their optimal values. Figure 4.14 depicts the interaction between temperature and the medium initial pH (AB) while holding factor C (rotation rate) at 100 rpm. Response surface plotted in Figure 4.14

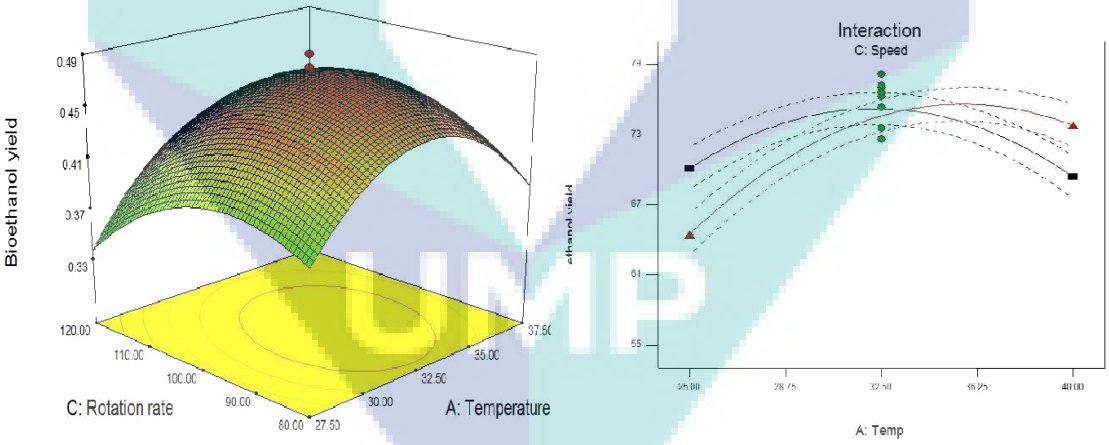
clearly show that bioethanol yield increased when the temperature was changed from 27.5 to 37.5°C as medium initial pH increased from 5.0 to 9.0. From this data it can be observed that the bioethanol yield decreased by lowering the temperature to 27.5ºC with similar effects by increasing the temperature to 37.5ºC. Report by Chin et al. (2010) found that, temperature affected the enzyme activity which explained the facilitation of chemical reactions within the yeast which is in good agreement with the results of the present study. Despite prior evidence, high bioethanol yield was observed at moderate temperature and medium initial pH ranges. Based on the optimum result suggested by the Design-Expert, Version 7.1.6 software, bioethanol yield was relatively high at temperature of 33.03°C and medium initial pH of 6.62. According to an investigation by Adnan et al. (2009), bioethanol production from glycerol by *Escherichia coli* SS1 was greatly influenced by pH and an optimum pH value of 7.61 was identified. Further increases in the pH resulted in lower bioethanol production. The initial pH is an important factor that influences the NADH to NAD^+ ratio, which greatly affects the metabolic flux under anaerobic conditions (Adnan et al., 2014). The findings suggest that to obtain optimal bioethanol production, it is necessary to control the medium initial pH under optimum conditions. In general, yeast is able to grow and efficiently ferment substrates into bioethanol at pH values of 3.5 – 6.0 and temperatures of 28 – 35ºC (Alam et al., 2009). The optimum medium initial pH (6.62) and temperature (33.03ºC) obtained in this work is within the range of those reported in the literature especially for *Saccharomyces cerevisiae*.



(a) (b)

Figure 4.14 Response surface plots depicting the interaction of medium initial pH and temperature (AB) in the production of bioethanol from OPF juice (a) 3D response surface plot and (b) interaction of medium initial pH and temperature.

The interaction between temperature and rotation rate (AC) while holding medium initial pH (B) at 7.0 towards bioethanol yield in terms of 3D is shown in Figure 4.15. It was observed that the bioethanol yield decreased at higher rotation rate (120 rpm) as the temperature was increased from 27.5 to 37.5ºC. In contrast with lower rotation rate (80 rpm), it shows an increasing trend when temperature was increased. The lower bioethanol yield (0.31 g/g sugars) showed at higher rotation rate (140 rpm) compared to central point rotation rate value (100 rpm) which has much higher bioethanol yield (0.49 g/ g sugars). Rotation is known to have an important role in ensuring uniform adequate mixing, mass transfer and heat transfer within the fermenter in medium components Shahirah (2014). The effects of rotation rate are required for successful fermentation process to improve product yields. The advantages of agitation toward performance and growth of microorganism cells could improve the mass transfer on substrates, products or byproducts and oxygen. In addition, according to Shahirah (2014) better mixing process has the capability to maintain adequate supply of sugars and nutrients to the cells as well as to maintain the concentration gradient between interior and exterior cells in fermentation broth.

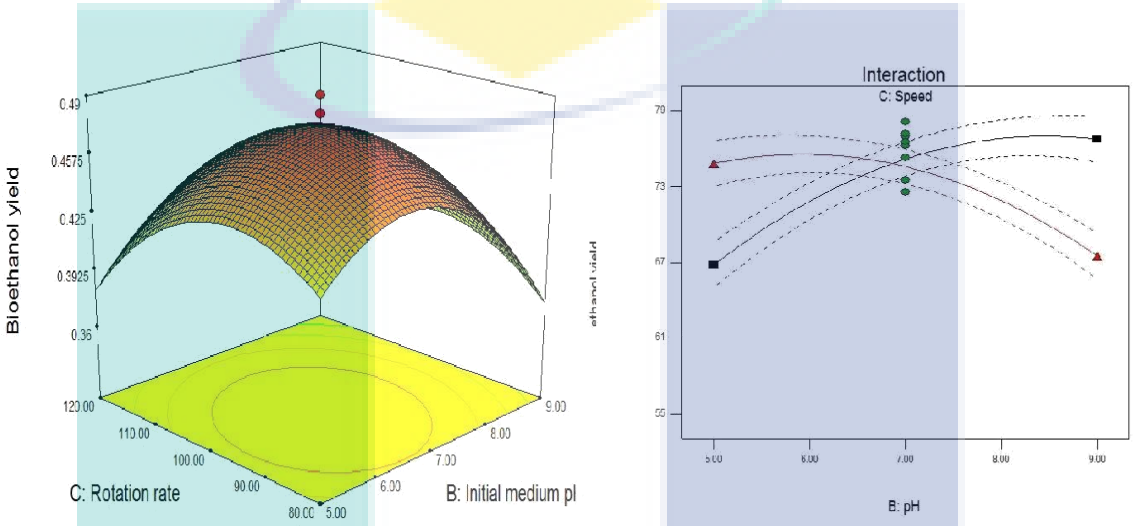


(a) (b)

Figure 4.15 Response surface plots depicting the interaction of temperature and rotation rate (AC) in the production of bioethanol from OPF juice (a) 3D response surface plot and (b) interaction of temperature and rotation rate

Meanwhile, Figure 4.15 depicts the interaction between the medium initial pH and rotation rate (BC) while holding factor A (temperature) at 32.5ºC. The interaction between medium initial pH and rotation rate (BC) plotted in Figure 4.16 clearly show bioethanol yield decreased when rotation rate changed from 80 to 120 rpm as medium

initial pH increased from 5.0 to 9.0. At higher rotation rate (120 rpm), the response yield indicates a linear decrease with increasing pH value. In contrast, lower agitation (80 rpm) showed a pattern of increasing slope. The medium with pH variations may lead to the changes in enzyme activity as well as changes in reaction rate. pH plays a significant role in bioethanol fermentation by *Saccharomyces cerevisiae* as pH affects the growth of yeast, by-product formation and fermentation rate due to the concentration of H^+ ions in the liquid environment (Shahirah et al. 2014; Chin et al. 2010; Pramanik 2003).



(a) (b)

Figure 4.16 Response surface plots depicting the interaction of medium initial pH and rotation rate (BC) in the production of bioethanol from OPF juice (a) 3D response surface plot and (b) interaction of medium initial pH and rotation rate

In summary, many interesting results indicating the potential of OPF juice as fermentation feedstock for the production of bioethanol. Among the plausible explanations for these finding is that high temperature still showed the production of bioethanol, however, slightly decreased with time of incubation. Changes in the medium initial pH might lead to the changes in the fermentation pathway. The most obvious findings to emerge from this study is that medium initial pH showed the highest significant effect towards the production of bioethanol. It was also shown that minimal rotation rate was required to produce maximum bioethanol yield to ensure uniform mixing and consumption of nutrition. Therefore, it is necessary to enhance optimal temperature, medium initial pH and rotation rate to accelerate cell activities, thus achieve high bioethanol yield.

4.6.3 Optimization and Validation

The reproducibility of the model was tested by performing the fermentation under the optimal conditions obtained from the CCD in Design Expert 7.1.6 software. This validation was also used to verify the accuracy of the model by determination on correlation between factors level with responses and factor. According to Norhazimah et al. (2011), maximum desirability was obtained by selecting within the range for temperature, medium initial pH and rotation rate whereby bioethanol yield were set at maximize goal. As shown in Figure 4.17, analysis of the computed results suggested that optimum bioethanol production could be achieved at medium initial pH of 6.62, temperature of 33.03^{O}C and rotation rate of 96.51 rpm.

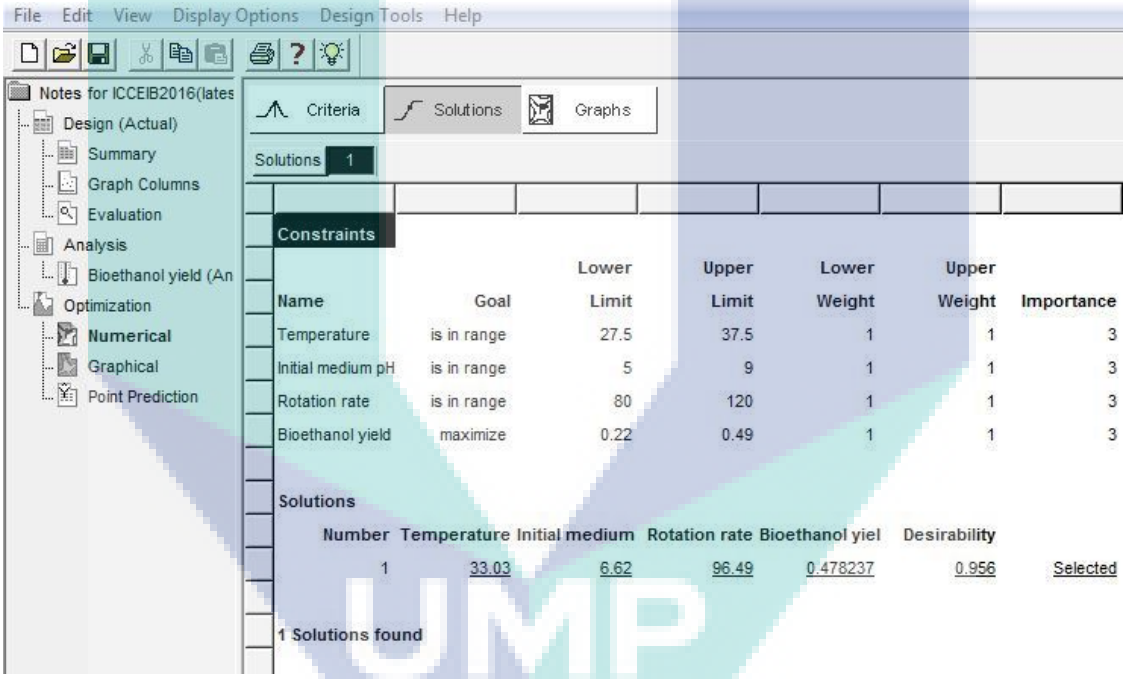


Figure 4.17 Simulation results suggested by Design Expert 7.1.6

Figure 4.17 indicates the predicted bioethanol yield under these optimum conditions was 0.48 g bioethanol/ g sugars. It was also demonstrated that the desirability equal to 0.956 which is near to "1.00" hence good to be used (Norhazimah, 2011; Shahirah, 2014). Three replicates of the batch fermentation using OPF juice without nutrient supplementation under the optimized conditions was conducted in 250 mL shake flask with a working volume of 100 mL to confirm the model validity. Maximum bioethanol yield of 0.50 ± 0.02 g/ g sugars was obtained from the confirmation test. In order to determine the percentage error analysis, actual result and predicted result was

calculated based on Equation 4.3.

$$Error\ (\%) = \frac{(Actual\ value - Predicted\ value)}{(Predicted\ value)}\ x\ 100 \qquad \text{Eq. 4.3}$$

Table 4.7 Results for validation experiment

	Bioethanol yield (g/L)	Percentage error (%)
Predicted	0.48	4.17
Actual	0.50	

As illustrated in Table 4.7, these experimental findings were in close agreement with the model prediction, with a difference of only 4.17%. Hence, we confirmed that the model developed from the response surface methodology could reliably predict bioethanol yields. As highlighted by Adnan et al. (2014) differences between experimental and predicted values of less than 10% confirm the validity of a model. The yield obtained in this study was 47.06% higher compared with the bioethanol produce under non-optimized condition (0.33 g bioethanol/ g sugars). These findings suggest that in general, medium initial pH, temperature and rotation rate may indeed play an important role in the bioethanol production by *S. cerevisiae* Kyokai No. 7 (ATCC 2662) utilizing OPF juice as a complete medium. In addition to that, even without the supplementation of nitrogen source into the fermentation medium, the bioethanol yield obtained in this study was almost comparable to those reported by Zahari et al. (2014). They have reported that 0.49 g/g sugars of bioethanol yield were obtained from OPF juice supplemented with 4 g/L of peptone and yeast extract (nitrogen source). The evidence from this study suggests that OPF juice can be used directly as the fermentation medium for bioethanol production at industrial scale.

4.7 Batch and Repeated Batch Experiment in 2L Bioreactor

In order to further investigate the potential of OPF juice as complete fermentation feedstock for bioethanol production, batch and repeated batch of bioethanol production was carried out at larger scale by using a 2-L bioreactor to study the performance of *S.*

cerevisiae for long term experiment to mimic a long term industrial bioethanol production process.

4.7.1 Effect of Agitation Speed in Batch Fermentation

Effect of agitation speed on the production of bioethanol by *S. cerevisiae* in 2-L bioreactor using OPF juice was conducted at two different agitation speed which is at 0 rpm and 50 rpm, respectively. Agitation will create mass transfer capability hence important for an adequate rotation rate to enhance productivity. High agitation speed in small scale will cause splashing thus creates foam which can overflow the flask or bioreactor which can lead to contamination. Fenice et al (2012) claims, from scale-up perspective, low agitation speed is necessary to save an energy. As shown in Figure 4.18, bioethanol concentration run in experiment with agitation speed at 50 rpm was higher compared to the bioethanol concentration for the experiment conducted at agitation speed of 0 rpm. From this study, agitation speed at 50 rpm was considered as the best conditions for the cultivation of *S. cerevisiae* in 2-L bioreactor to produce bioethanol using OPF juice. Results obtained from this study will be used in the subsequent experiment whereby; agitation speed at 50 rpm will be set throughout of the study period on the effect of drain and fill volume and effect of successive cycle.

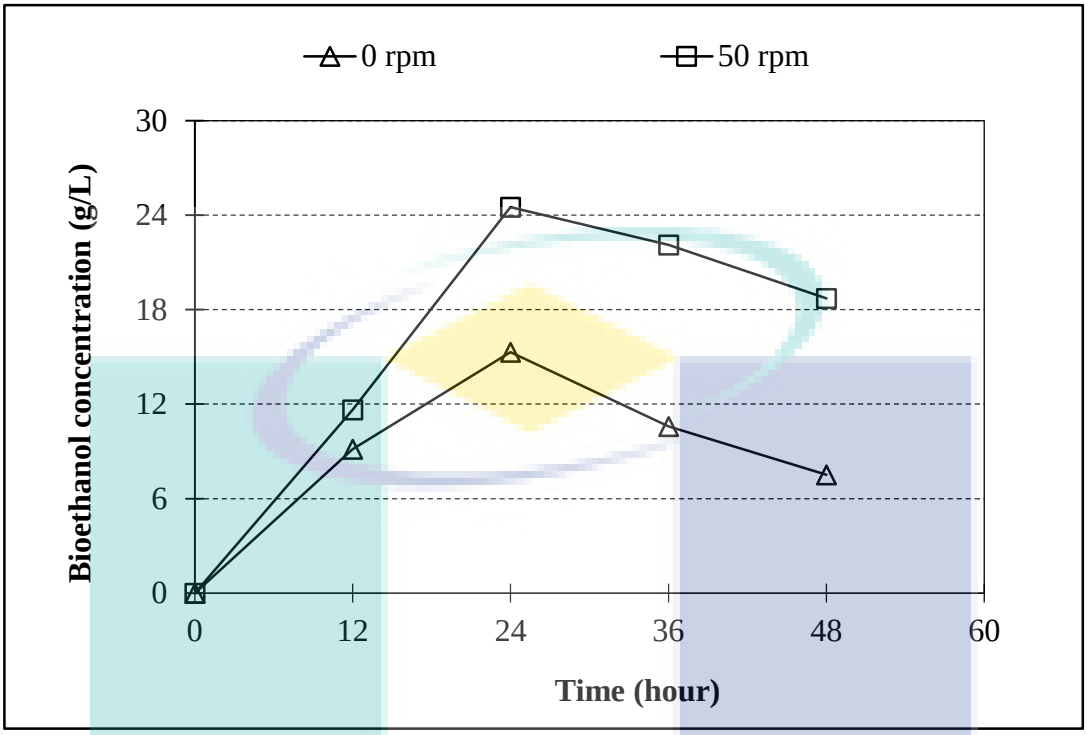


Figure 4.18 Effect of agitation speed at 0 RPM (open triangle) and 50 RPM (open square) on bioethanol yield by using 2-L bioreactor

4.7.2 Effect of Recycling Volume (Drain and Fill) Technique on Bioethanol Production in Repeated Batch Fermentation System

In the repeated batch fermentation system in 2L bioreactor, study was conducted at 50% and 75% recycling volume. At hour-24, the fermentation substrate which is consists of OPF juice and 10% inoculum was withdrawn at 50% and 75% hence the same volume of fresh OPF juice was immediately replaced. This process was obtained after rotation speed was stopped to enable sedimentation occurred before the process of recycling volume takes place. Table 4.8 indicates the result obtained from the study. It appears from Table 4.8 that the 75% drain and fill gave higher yield compared to 50%. This were similar to those reported by Ariyajarearnwong et al. (2011). They proposed that total rates of ethanol production at 75% drain and fill volume was 0.51 g h^{-1} whereby at 50% drain and fill volume yielded only 0.39 g h^{-1}. As highlighted by the author, 75% drain and fill volume gave higher yield due to amount of fermented broth being discarded was more than 50% drain and fill volume. This could be explained might due to bioethanol in the beginning of each broth to be more diluted and thus prevent product

inhibition effect. By replacing 75% drain and fill volume, it increases effectiveness and enhance productivity by replacing of fresh medium at a constant period. The finding is consistent with finding of past studies by Choi et al. (2009), which indicate higher drain and fill volume gave higher concentration than low drain and fill volume. Figure 4.19 clearly showed that the bioethanol concentration with drain and fill volume at 75% yielded 22.25 g/L, which was higher compared to 50% (drain and fill volume) yielded 14.30 g/L after 48 h of fermentation period. In both studies, the bioethanol production rate was 0.30 g/L/h for 50% (drain and fill volume) and 0.46 g/L/h for 75% (drain and fill volume), respectively.

Table 4.8 Bioethanol (g/L) at 50% and 75% drain and fill reycling volume

Time (hour)	**Bioethanol concentration (g/L)**	
	50% (v/v)	**75% (v/v)**
0	0.00	0.00
12	17.35	17.60
24	23.76 (before drain and fill)	24.13 (before drain and fill)
24	10.12 (after drain and fill)	5.55 (after drain and fill)
36	13.50	17.52
48	14.30	22.25

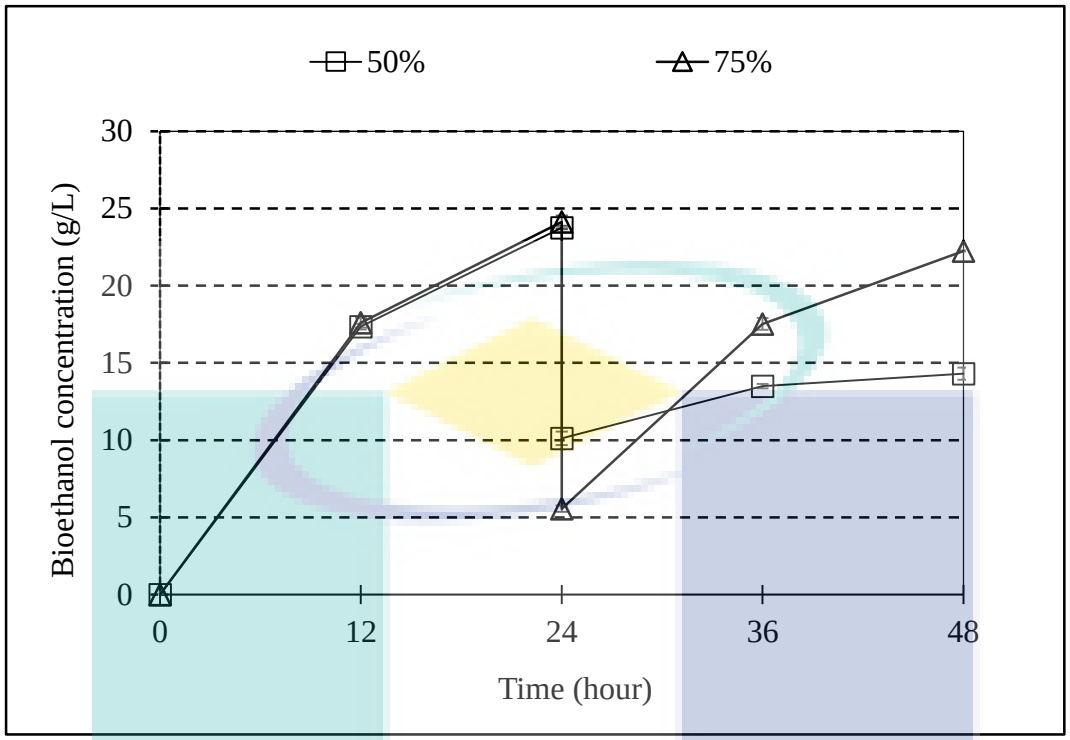


Figure 4.19 Bioethanol production profile with drain and fill volume at 50% (open square) and 75% (open triangle) of fresh OPF juice by Saccharomyces cerevisiae Kyokai No. 7 (ATCC 2662) takes place at 24 h of fermentation period.

4.7.3 Effect of Successive Cycle in the Production of Bioethanol by Repeated-batch Fermentation System

Effect of successive cycle was studied throughout the experiment to approach in which cycle yield good bioethanol production. Repeated batch enable the fermenter retain high cell concentration without separation process thus enhancing the production of bioethanol from OPF juice using lab scale bioreactor. Repeated batch were performed to observe long term stability of bioethanol production. After each cycle, results were analyzed to observe the pattern in terms of sugar consumption and production of bioethanol. Figure 4.20 (a), (b) and (c) shows the repeated batch fermentation under 5-cycles (120 hours), 10-cycles (240 hours) and 15-cycles (360 hours) of bioethanol fermentation at 75% drain and fill volume from OPF juice containing 56.87 g/L of total sugars by *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 2662) in 2-L bioreactor, respectively.

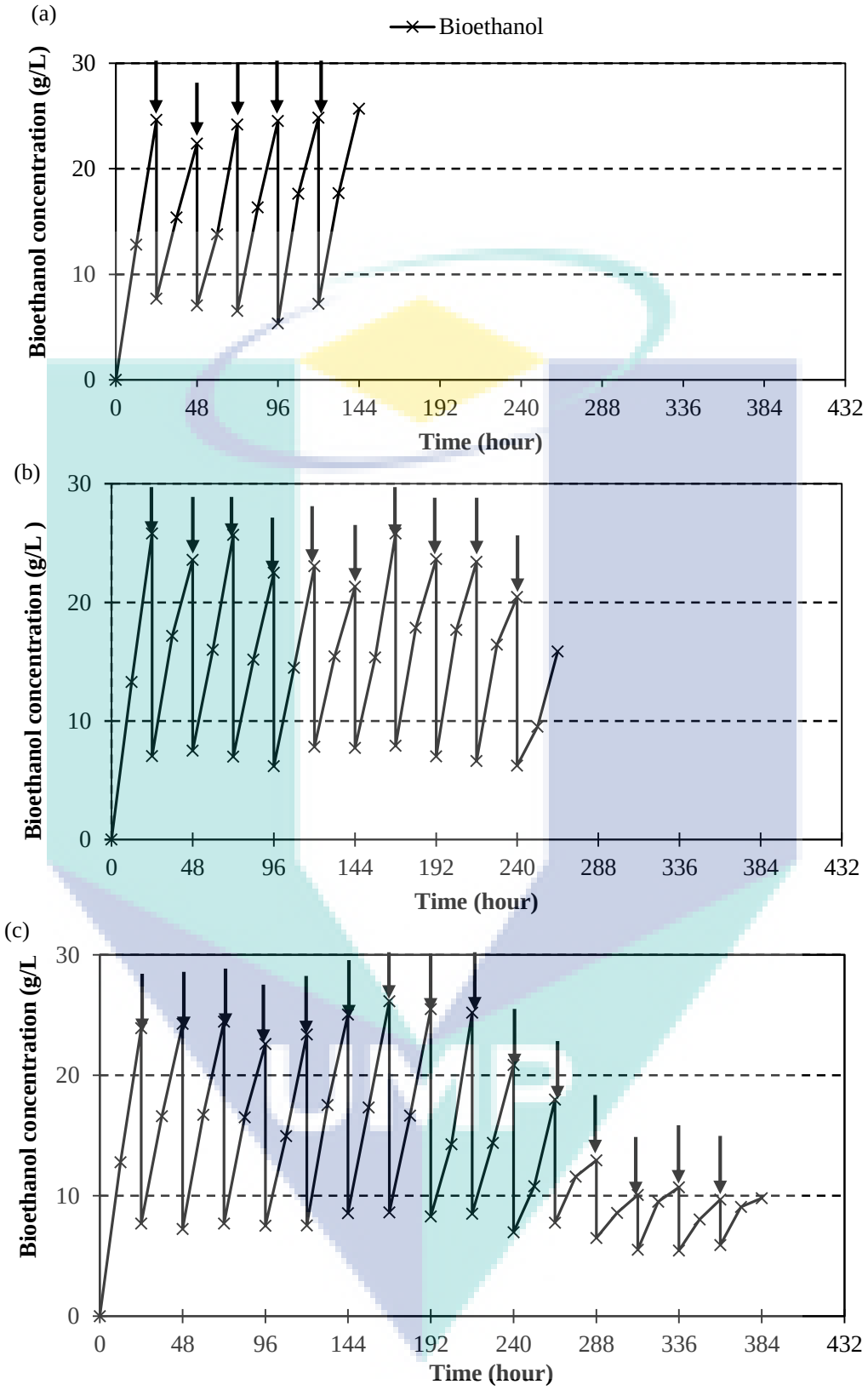


Figure 4.20 Repeated-batch bioethanol fermentation (a) 5-cycles, (b) 10-cycles, and (c) 15-cycles at 75% (v/v) drain and fill volume from OPF juice containing 56.87 g/L of total sugars by *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 2662) in 2-L bioreactor. The arrows indicate the start time of each cycle.

Table 4.9 Kinetic parameters of bioethanol production from OPF juice by *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 26622) using 10-cycles repeated-batch fermentation at 75% (v/v) drain and fill volume in the 2 L bioreactor.

Cycle number	Parameter		
	***P* (g/L)**	**Q_P (g/L/h)**	**$Y_{P/S}$ (g bioethanol/ g sugars)**
1	25.80	1.08	0.45
2	23.57	0.98	0.41
3	25.68	1.07	0.45
4	22.50	0.94	0.40
5	23.05	0.96	0.41
6	21.32	0.89	0.37
7	25.81	1.08	0.45
8	23.64	0.99	0.42
9	23.41	0.98	0.41
10	20.45	0.85	0.36
Average	**23.52**	**0.98**	**0.41**

**P*, Q_P and $Y_{P/S}$; bioethanol concentration, bioethanol productivity and bioethanol yield of the ten successive cycles, respectively.

As seen in Figure 4.20 (a) – (c), it was observed that there was a fast consumption of sugars for the first 12 hours with high production of bioethanol. This might be due to the yeast cells were in the log phase of growth conditions. The consumption of sugars continued to increase at fermentation time from 12 to 24 hours of fermentation period in which yeast cells reached an exponential growth phase with a maximum activity. Bioethanol concentration and yield at 24 hours of fermentation period for 5-cycles, 10-cycles and 15-cycles experiment were as follows; 24.63 g/L and 0.43 g bioethanol/g sugars; 23.57 g/L and 0.41 g bioethanol/ g sugars; 23.90 g/L and 0.42 g bioethanol/ g sugars; respectively. When the yeast cell viability decreased, production become slow with a poor ability to ferment normally after 24 hours of fermentation period which reached a saturation phase. Samples were analyzed using HPLC for bioethanol concentration at 24 hours before and after the addition of fresh OPF juice. Results showed that after the addition of fresh OPF juice, the concentration of bioethanol was drastically decreased. This was due to the dilution factor was occurred when the addition of fresh OPF juice into the bioreactor to replace the fermentation broth. To increase the

fermentation activity, fresh OPF juice were added continuously until 120 hours at every 24-hour cycle for the experiment with 5-cycles, 240 hours for the experiment with 10-cycles and 360 hours for the experiment with 15-cycles. This might increase the bioethanol concentration and thus ensure high yeast growth. However, from Figure 4.18 (c), bioethanol production was gradually decreased as it reaches death phase after 240 hours of fermentation period. In the current set of experiment, 15-cycles showed gradual decrease in bioethanol production after the 10^{th} cycle (batch). As a summary, it showed that the OPF juice containing 56.87 g/L of total sugars without any nutrient supplement could be used directly as a low-cost medium instead of the typical yeast extract rich medium as previously reported by Zahari et al. (2014). Bioethanol production from OPF juice by *S. cerevisiae* Kyokai No. 7 (ATCC 2662) using repeated-batch fermentation could be carried out at least ten successive batches (cycles). As shown in Table 4.9, the average bioethanol concentration, productivity and yield for the ten successive cycles were as follows; 23.52 g/L, 0.98 g/L/h and 0.41 g bioethanol/ g sugars; respectively. This experiment shows that the drain and fill volume in the repeated-batch system did not affect the bioethanol production efficiencies until ten successive cycles. After that, the bioethanol production started to decrease might be due to the yeast cell reaches the death phase

CHAPTER 5

CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

Huge oil palm plantation in Malaysia generates huge amount of waste especially oil palm frond (OPF). The abundance waste will pose environmental challenge if there is no initiative and non-appropriate management on the waste. The utilization of renewable biomass such as OPF as fermentation feedstock could reduce the huge volume of biomass generated from the oil palm plantation, which indirectly reduced the negative impact of oil palm biomass to the environment. Currently, at industrial scale, the production of bioethanol tends to focus on utilization of edible food sources such as corn starch and sugarcane juice, which were also consumed by humans and animals. Competition on food consumption occurs between the needs for growth of human and animals; and microbes may affect the food chain survival. Therefore, the use of OPF juice as a source of fermentation feedstock for the production of bioethanol is urgently needed to reduce the dependence on food crops and avoiding the food versus fuel issue. The current findings add substantially to our understanding of OPF juice can be a potential complete fermentation feedstock for the production of bioethanol by *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 26622). The best fermentation conditions for bioethanol production was screen by using the method of One-Factor-at-Time (OFAT); followed by the determination of optimal condition for bioethanol production using response surface methodology (RSM) which employed central composite design (CCD). The RSM is used to determine the optimum conditions for medium initial pH, temperature and rotation rate. Further investigation on the viability and reliability of OPF juice as a sole renewable carbon source for the production of bioethanol at larger scale was conducted in 2-L bioreactor with batch and repeated batch experiment.

To summarize the achievements of the study, the following are the conclusions or findings from this project:

1- The characterization of OPF juice in this research study was performed in order to determine the sugars composition and concentration for the production of bioethanol. Based on the HPLC analysis, it was indicated that the total sugars concentration in OPF juice used in this study was 56.87 g/L; with glucose was found to be the dominant sugar (44.16 g/l) followed by sucrose (11.25 g/l) and fructose (1.46 g/l).

2- Screening and optimization of bioethanol production was then carried out in 250 mL shake flasks experiment using OPF juice as the sole renewable feedstock to increase the bioethanol production. Several parameters such as medium initial pH, temperature and rotation rate were found affecting the production of bioethanol from OPF juice. The maximum bioethanol yield of 0.50 g/ g sugars was obtained under the following optimum condition; medium initial pH (6.62), rotation rate (96.51 rpm) and temperature (33.03°C). Experimental results indicated that the temperature exert significant effects on bioethanol yield. However, validation experiment shows that only a small error exists between the predicted value which is 0.48 g/ g sugars compared to the actual experimental value. By culturing *Saccharomyces cerevisiae* Kyokai No. 7 (ATCC 26622) under the aforementioned optimized condition using OPF juice as the sole renewable carbon source in shake flask, higher bioethanol production was obtained compared to the non-optimized condition. Under the optimal conditions, the bioethanol yield obtained was 47.06% higher compared to non-optimized condition.

3- The production of bioethanol from OPF juice was then scaled up to further investigate its potential as the sole renewable carbon source for fermentation process at larger scale. Repeated batch of bioethanol production was carried out in 2-L bioreactor to study the performance of *S. cerevisiae* for long term experiment to mimic a long term industrial bioethanol production process. Bioethanol production from OPF juice by *S. cerevisiae* Kyokai No. 7 (ATCC 2662) using repeated-batch fermentation was successfully run at least ten successive batches (cycles). The average bioethanol concentration, productivity and yield for the ten successive cycles were as follows; 23.52 g/L, 0.98 g/L/h and 0.41 g bioethanol/ g sugars; respectively. As a summary, it showed that the OPF juice containing 56.87 g/L of total sugars without any nutrient supplement

could be used directly as a low-cost medium instead of the typical yeast extract rich medium as previously reported by Zahari et al. (2014). In a nutshell, these research findings will serve as a basis for future studies and make several contributions to the current literature.

5.2 Recommendations for Future Work

Further investigation and experimentation on the use of OPF juice as fermentation feedstock for bioethanol production is strongly recommended. It is recommended that for future research study, fresh OPF juice should be kept in -20°C freezer rather than in the chest freezer to avoid microbial contamination, avoid thawing OPF juice at high temperature and for longer period of keeping time to avoid sugar degradation and low quality of raw material. In addition, yield of bioethanol can be increased by using OPF obtained from high potential oil palm plantation which was given good nutrition and intensive care. Fresh OPF is suggested to be harvested within 1-2 days after felling from oil palm tree for pruning or harvesting of fresh fruit bunch, to ensure that only the fresh OPF juice could be used; which subsequently increased the bioethanol yield. More broadly, research is also needed to determine various strains of microorganisms which may affects the production of bioethanol. In addition, the experiment may also be performed by using different strain of *Saccharomyces cerevisiae* to obtain higher yield of bioethanol. The development and modified yeast strains was successfully done by previous work to improve ethanol and multiple-inhibitor tolerance including thermal tolerance (Zhao and Bai, 2009; Shahirah et al., 2014).

Moreover, it would be interesting to assess the effects of inorganic nutrient addition on OPF juice for the bioethanol production. It has been reported that bioethanol production using oil palm trunk sap (OPTs) with inorganic nutrient addition was improved gradually compared to without nutrient addition (Shahirah et al., 2014). Similar to OPTs, it was postulated that bioethanol production from OPF juice supplemented with inorganic nutrient will be improve compared to without nutrient addition. It is recommended that the investigation on the production of bioethanol from OPF juice using repeated batch experiment with the addition of inorganic nutrient under optimized condition could be conducted in future study to establish a greater degree of accuracy and understanding regarding to this matter.

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APPENDIX A
CENTRAL COMPOSITE DESIGN

Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	2.51	1	2.51			
Linear vs Mean	0.010	3	3.356E-003	0.37	0.7780	
2FI vs Linear	2.237E-003	3	7.458E-004	0.067	0.9763	
Quadratic vs 2FI	0.14	3	0.047	134.46	< 0.0001	Suggested
Cubic vs Quadra	7.313E-004	4	1.828E-004	0.40	0.8042	Aliased
Residual	2.757E-003	6	4.595E-004			
Total	2.67	20	0.13			

*"Sequential Model Sum of Squares [Type I]": Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Figure A1 Fit summary of optimization study

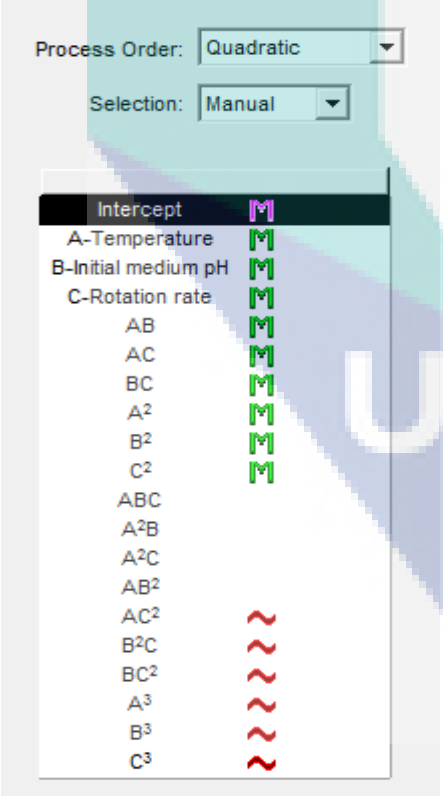

Figure A2 CCD model

Response 1 Bioethanol yield

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.15	9	0.017	48.74	< 0.0001	significant

The Model F-value of 48.74 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A, B, C, A^2, B^2, C^2 are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.
If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The "Lack of Fit F-value" of 2.22 implies the Lack of Fit is not significant relative to the pure error. There is a 20.10% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.019	R-Squared	0.9777
Mean	0.35	Adj R-Squared	0.9577
C.V. %	5.27	Pred R-Squared	0.8679
PRESS	0.021	Adeq Precision	20.574

The "Pred R-Squared" of 0.8679 is in reasonable agreement with the "Adj R-Squared" of 0.9577.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 20.574 indicates an adequate signal. This model can be used to navigate the design space.

Figure A3 ANOVA for optimization

Factor	Estimate	df	Error	Low	High	VIF
Intercept	0.47	1	7.449E-003	0.46	0.49	
A-Temperature	0.013	1	4.669E-003	2.722E-003	0.024	1.00
B-Initial medium	-0.017	1	4.669E-003	-0.027	-6.472E-003	1.00
C-Rotation rate	-0.013	1	4.669E-003	-0.024	-2.722E-003	1.00

Final Equation in Terms of Actual Factors:

Bioethanol yield =

-3.34426

+0.15705 * Temperature

+0.17352 * Initial medium pH

+0.013588 * Rotation rate

-8.75000E-004 * Temperature * Initial medium pH

+1.12500E-004 * Temperature * Rotation rate

+2.18750E-004 * Initial medium pH * Rotation rate

-2.45455E-003 * Temperature^2

-0.012528 * Initial medium pH^2

-9.71591E-005 * Rotation rate^2

Figure A3 ANOVA for optimization (continued)

APPENDIX B
EXPERIMENTAL DATA OF FERMENTATION CHARACTERISTICS OF THE REPEATED BATCH FERMENTATION WITH THE *S. CEREVISAE* ON OPF JUICE

Table B-1 Data for concentration of bioethanol, glucose, fructose and sucrose on sugar utilization in repeated batch fermentation at cycle 5.

Cycle	Time (h)	Ethanol (g/L)	Glucose (g/L)	Fructose (g/L)	Sucrose (g/L)
5 Cycle					
	0	0	44.16	1.46	11.25
	12	12.79	10.99	0.55	3.86
1	24	24.63	1.25	0.00	0.00
	24	7.70	38.16	0.86	9.35
	36	15.39	9.57	0.44	3.58
2	48	22.39	0.76	0.00	0.00
	48	7.05	36.51	1.02	9.17
	60	13.79	12.93	0.17	3.56
3	72	24.19	1.07	0.00	0.00
	72	6.53	34.55	0.91	8.12
	84	16.33	11.83	0.43	3.17
4	96	24.51	1.12	0.00	0.00
	96	5.35	31.79	0.83	8.13
	108	17.62	12.54	0.86	3.34
5	120	24.82	1.37	0.00	0.18
	120	7.21	33.22	0.94	8.20
	132	17.68	12.41	0.85	3.82
6	144	25.69	1.76	0.00	0.00

Table B-2 Data for concentration of bioethanol, glucose, fructose and sucrose on sugar utilization in repeated batch fermentation at cycle 10

Cycle	Time (h)	Ethanol (g/L)	Glucose (g/L)	Fructose (g/L)	Sucrose (g/L)
10 Cycle					
	0	0.00	44.16	1.46	11.25
	12	13.27	15.20	0.37	4.61
1	24	25.80	9.34	0.34	0.00
	24	7.04	34.40	0.87	7.36
	36	17.16	11.60	0.30	4.06
2	48	23.57	6.06	0.10	0.28
	48	7.50	36.60	1.04	8.15
	60	16.00	9.66	0.60	2.98
3	72	25.68	4.86	0.80	2.67
	72	6.99	38.60	0.93	10.50
	84	15.17	18.90	0.89	3.40
4	96	22.50	13.30	0.07	1.20
	96	6.17	36.04	1.28	9.01
	108	14.47	9.69	0.42	6.25
5	120	23.05	3.82	0.91	0.83
	120	7.81	37.60	0.94	9.81
	132	15.44	13.40	0.29	0.40
6	144	21.32	6.85	0.88	0.94
	144	7.71	31.00	0.77	8.28
	156	15.35	8.85	0.17	0.41
7	168	25.81	3.13	0.63	0.96
	168	7.90	30.90	1.09	11.00
	180	17.86	38.50	0.00	0.54
8	192	23.64	12.10	0.40	4.30
	192	7.00	30.70	0.73	8.40
	204	17.67	30.50	0.34	0.56
9	216	23.41	11.40	0.00	0.80
	216	6.62	35.00	9.60	9.45
	228	16.43	12.30	9.32	0.74

10	240	20.45	5.02	7.15	3.05
	240	6.23	38.29	6.37	7.43
	252	9.51	4.56	8.06	3.29
	264	15.85	1.62	0.00	2.81

Table B-3 Data for concentration of bioethanol, glucose, fructose and sucrose on sugar utilization in repeated batch fermentation at cycle 15

Cycle	Time (h)	Ethanol (g/L)	Glucose (g/L)	Fructose (g/L)	Sucrose (g/L)
15 Cycle					
	0	0.0	44.16	1.46	11.25
	12	12.78	6.03	0.49	1.37
1	24	23.90	1.42	0.00	1.11
	24	7.68	37.90	0.72	9.25
	36	16.61	16.70	0.18	1.68
2	48	24.27	7.87	0.20	1.74
	48	7.23	36.70	0.89	7.34
	60	16.72	15.10	1.02	4.15
3	72	24.45	4.22	0.02	0.48
	72	7.70	30.70	1.30	8.61
	84	16.52	18.90	0.10	5.21
4	96	22.60	10.10	0.40	6.79
	96	7.50	31.10	0.93	8.39
	108	14.96	9.66	0.70	4.93
5	120	23.39	3.66	0.69	3.51
	120	7.53	30.50	1.02	6.90
	132	17.53	20.90	0.13	6.33
6	144	25.03	16.90	0.24	6.24
	144	8.56	36.00	1.04	8.02
	156	17.32	8.67	0.69	2.76
7	168	26.14	5.66	0.08	3.77
	168	8.62	38.80	1.26	8.77
	180	16.64	18.20	0.40	5.26
8	192	25.49	11.70	0.96	5.72
	192	8.28	36.10	0.97	8.26
	204	14.27	17.30	0.30	6.11
9	216	25.20	10.90	0.26	5.20
	216	8.51	35.90	0.95	7.95
	228	14.39	13.40	0.60	4.16

10	240	20.86	8.17	0.70	4.12
	240	6.97	29.60	1.04	8.78
	252	10.79	15.40	0.90	4.76
11	264	17.98	6.55	0.85	4.45
	264	7.76	33.70	1.12	7.44
	276	11.59	19.30	0.12	6.00
12	288	12.93	9.15	0.29	6.52
	288	6.50	38.54	0.80	7.34
	300	8.57	3.89	0.23	0.00
13	312	10.07	1.17	0.25	4.70
	312	5.51	30.00	1.00	7.4
	324	9.50	8.84	0.90	7.63
14	336	10.69	2.57	0.96	8.40
	336	5.44	31.60	0.80	10.60
	348	8.03	8.34	0.42	2.14
15	360	9.69	2.43	0.72	1.03
	360	5.90	34.00	0.73	10.05
	372	9.07	9.90	0.46	0.18
	384	9.79	2.77	0.00	0.00

APPENDIX C
LIST OF PUBLICATIONS

1. Siti Hajar Mat Zani, Mior Ahmad Khushairi Mohd Zahari, Nina Suhaity Azmi (2017). Evaluation of factors affecting bioethanol production from oil palm frond juice by *Saccharomyces cerevisiae* using central composite design. Research Journal of Biotechnology (**Under Review**)

2. Siti Hajar Mat Zani, Mior Ahmad Khushairi Mohd Zahari, Nina Suhaity Azmi (2016). Evaluation of factors affecting bioethanol production from oil palm frond juice by *Saccharomyces cerevisiae* using central composite design. International Conference of Chemical Engineering and Industrial Biotechnology, Melaka 28-30 October 2016 (ICCEIB2016). **(Oral Presentation)**

3. Siti Hajar Mat Zani, Mior Ahmad Khushairi Mohd Zahari, Nina Suhaity Azmi (2015). Repeated-batch bioethanol fermentation from oil palm frond juice by *Saccharomyces cerevisiae*. International Conference on Fluids and Chemical Engineering (FluidsChE 2015), 25 – 27 Nov. 2015, Langkawi, Kedah, Malaysia. (**Oral Presentation**)

4. **Siti Hajar Mat Zani**, Mior Ahmad Khushairi Mohd Zahari, Nina Suhaity Azmi (2015). Optimization of parameters affecting bioethanol production from oil palm frond juice by *Saccharomyces cerevisiae.* Asian Congress on Biotechnology 2015 (ACB2015). 15 – 19 Nov. 2015, Kuala Lumpur, Malaysia. (**Oral Presentation**)

5. **Siti Hajar binti Mat Zani**, Mior Ahmad Khushairi Mohd Zahari, Nina Suhaity Azmi (2015). Effect of Temperature, Initial pH and Agitation Speed on Bioethanol Production from Oil Palm Frond Juice by *Saccharomyces cerevisiae*. Conference on Nanotechnology and Bioresource Technology (NBT2015). 28 – 29thMarch 2015, Universiti Kebangsaan Malaysia, Bangi, Malaysia. (**Oral Presentation**)

6. **Siti Hajar binti Mat Zani**, Mior Ahmad Khushairi Mohd Zahari, Nina Suhaity Azmi (2015). Utilization of oil palm frond (OPF) juice for the production of bioethanol. National Postgraduate Conference (NCON-PGR 2015). January 2015, Universiti Malaysia Pahang, Kuantan, Malaysia. (**Oral Presentation**)

7. **Siti Hajar binti Mat Zani**, Mior Ahmad Khushairi Mohd Zahari, Nina Suhaity Azmi (2014). Factors affecting bioethanol production from oil palm frond juice by *Saccharomyces cerevisiae*. AFOB Regional Symposium 2014 (ARS2014) February 9–11, 2014, Kuala Lumpur, Malaysia. (**Poster Presentation**)