FINAL REPORT RDU 140340: UTILIZATION OF NYPA (NYPA FURTICANS) PALM FROND AS A POTENTIAL FOR BIO-ETHANOL

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ABSTRACT

Bioethanol has been gaining much interest recently in terms of research and development. Since there are various factors such as rising oil price, environmental issues and high rate consumption of fossil fuel, the global demand for bioethanol has shown a remarkable increase. This aim of this study was to discover the potential of Nipa palm frond juice as a sustainable potential feedstock for production of bioethanol. Nipa palm frond is known to contain high sugar for production of bioethanol. NPF was obtained from Kg Miang, Pekan and the juices were extracted by pressing the fresh NPF using the conventional sugarcane pressing machine. The NPF juice contains higher glucose content, which is about 70% of the total free sugar. Hence, it has a high potential to be the carbon source for producing bioethanol. In this study, bioethanol was produced by fermentation using *Sacchromyces cerevisae.* The parameters investigated in this research work are incubation temperature, pH of NPF juice, concentration of NPF juice, incubation time and agitation speed. The experimental design was planned using 2-level factorial with the aid of Design Expert Software 7.1.6. The sugar concentration was analysed by HPLC while ethanol concentration from the fermentation sample was analysed using GC-FID. Using the ethanol concentration obtained from GC-FID analysis, the main factors affecting ethanol fermentation were screened using factorial analysis and best condition for the production of bioethanol was suggested. The validation experiments were conducted based on one suggested best condition from Design Expert 7.1.6 in triplicate. Based on the Pareto chart, the best main parameters influencing ethanol yield were incubation temperature, concentration of NPF juice and incubation time. Highest ethanol yield produced was found to be 1.541 g/L with the condition of 12 hours fermentation, 32 °C, pH 4, and 160 rpm with 100 % juice concentration. The lowest ethanol yield was 0.562 g/L with the condition of 24 hours incubation time, 25 °C, pH 7, and 80 rpm with 50 % juice concentration. The models with the selected effects were analyzed using analysis of variance (ANOVA) and found significant with high correlation (R^2 = 0.9944) between the experimental data and model data was obtained. The best condition for ethanol production suggested at 12 hours, 32 °C with pH 4, 160 rpm and 100 % juice concentration. The validation experiments were conducted at the suggested best conditions and the error from these runs were 3.2 %, 1.7 %, and 3.7 %. Based on the predicted and experimental results presented, the experimental values were in good agreement with the predicted values proposed by the model with an error less than 10 % and proved to be an adequate model.

ABSTRAK

Bioetanol telah mendapat perhatian yang lebih baru-baru ini dari segi penyelidikan dan pembangunan. Oleh kerana terdapat pelbagai faktor seperti harga minyak yang semakin meningkat, isu-isu alam sekitar dan penggunaan kadar yang tinggi bahan api fosil, permintaan global untuk bioetanol telah menunjukkan peningkatan yang luar biasa. Tujuan penyelidikan in adalah untuk mengenalpasti potensi pelepah Nipah sebagai bahan mentah bagi pengeluaran bioethanol. Nipa pelepah sawit diketahui mengandungi gula yang tinggi untuk pengeluaran bioetanol. NPF diperolehi dari Kg. Miang, Pekan jus diperah menggunakan mesin konvensional tebu. Jus NPF mengandungi kandungan glukosa yang lebih tinggi, iaitu kira-kira 70%. Oleh itu, ia mempunyai potensi yang tinggi untuk menjadi sumber karbon untuk menghasilkan bioetanol. Bioetanol dihasilkan daripada jus Nipa pelepah sawit oleh penapaian dengan Saccharomyces cerevisiae. Faktor – faktor yang diselidiki dalam kajian ini ialah suhu fermentasi, pH jus pelepah Nipah, kepekatan jus pelepah Nipah, tempoh fermentasi dan kelajuan pergolakan. Rangka experiementasi dibuat menggunakan 2 -level factorial dalam perisian Design Expert 7.1.6. Kepekatan gula dianalisi menggunakan HPLC manakala etanol daripada sampel fermentasi dianalisis menggunakan GC-FID. Menggunakan kepekatan etanol yang diperolehi daripada analisis GC-FID, faktor- faktor utama mempengaruhi fermetasi etanol ditapis dan keadaan terbaik untuk pengeluaran bioethanol dicadangkan. Pengesahan experimentasi dibuat secara 3 kali berdasarkan keaadan terbaik yang dicadangkan daripada Design Expert 7.1.6. Berdasarkan rajah Pareto, paramater utama yang terbaik dalam mempengaruhi penghasilan etanol adalah suhu fermentasi, kepekatan jus pelepah Nipah dan tempoh fermentasi. Kepekatan etanol tertinggi dicatat ialah 1.541 g/L dengan kondisi 12 jam fermentasi, 32 °C, pH 4, dan 160 rpm dengan 100 % kepekatan jus. Kepekatan etanol terendah dicatat sebanyak 0.562 g/L dengan kondisi fermentasi selama 24 jam, sushu 25 °C, pH 7, dan kelajuan 80 rpm dengan kepekatan jus sebanyak 50 %. Modal dengan kesan – kesan terpilih dianalisis mengunakan ANOVA dan didapati signifikant dengan hubungan tinggi (R^2= 0.9944) antara data experimentas dan data modal diperolehi. Kondisi terbaik yang disarankan untuk penghasilan etanol ialah pada 12 jam fermentasi dengans ushu 32 °C , ph 4, kelajuan 160 rpm dengan kepekatan jus 100%. Pengesahan experiment dijalankan dengan menggunakan kondisi yang diarankan dan ralat daripada ketiga – tiga experimentasi tersebut adalah 3.2 %, 1.7% dan 3.7%. Berdasarkan data ramalan dan experimentasi yang dibentangkan, nilai data experimentasi adalah dalam penaakulan yang baik dengan nilai ramalan yang dicadangkan oleh modal dengan ralat kurang daripada 10 % dan dibuktikan sebagai modal yang sesuai.

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LIST OF ABBREVIATIONS

CCD	Central composite design
FAO	Food and Agricultural Organization of the United Nations
GC FID	Gas Chromatography Flam Ionization Detection
HPLC	High performance liquid chromatography
NPF	Nipa Palm Frond
OPF	Oil Palm Frond
RSM	Response surface methodology

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

During the recent years, demands for alternative fuel resources has increased tremendously, driven by factors involving rising fuel prices due to overdependence on fossil-derived fuels across the globe, unstable political landscapes in major oil-producing countries, and a rapidly increasing demand for energy due to emerging economies such as India and China. 87 percent of the world's primary energy consumption in the year 2012 alone consisted of use of fossil fuels (Daniel, 2013), which undoubtedly has contributed towards the increase in the atmospheric carbon dioxide, quickly propelling towards an alarming global warming threat (Chin and H'ng, 2013) and climate change across the globe. This will threaten the global diversity of flora and fauna, leading towards thousands of species becoming extinct in the next 100 years (Bellard et al., 2012). Hence, it is imperative that an energy source that is sustainable, affordable, and environmentally clean in nature is developed in order to facilitate the reduction of use of fossil fuels (Soetaert and Vandamme, 2009).

As a natural response to this, several renewable energy sources are explored to be utilized as biofuels. Biofuel is defined as fuel energy derived from agricultural-based materials, and is divided into two groups according to technology of production. Utilising plants as raw materials, first generation biofuels have been produced and

commercialized successfully, but the process might threaten the food chain and biodiversity in the long run. Meanwhile, second generation biofuels are mostly still in R&D, pilot, or lab phase, and lignocellulosic biomass are utilised as raw materials. A promising example of such biofuel is bioethanol, which is heavily commercialized on the global market by countries such as Brazil and the United States in the form of sugarcane and corn raw materials. Production of bioethanol from renewable resources such as biomass is garnering a lot of attention as a viable solution (Vohra et al., 2014) due to an extended petroleum shelf life and reduction in terms of dependence on imports of oil as notable advantages (Goldenberg, 2007). It is also worth emphasizing that cleaner combustion is made possible due to the increased oxygen content in comparison to gasoline (Cot et al., 2007), reducing emission of carbon dioxide into the atmosphere (Balat et al., 2008), and its common availability from biomass source ensures that it is sustainable in the long run (Demirbas, 2008).

As noted in recent researches, Nipa palm frond juice (NPF) juice is a good candidate for the production of biofuel due to the high concentration of α – cellulose and hemicellulose (Tamunaidu and Saka, 2011). The α – cellulose and hemicellulose are required in the production of ethanol by direct microbial fermentation. Yeast, particularly Saccharomyces cerevisiae is commonly used as fermenting microorganism (Ingledew, 1999) due to its excellent fermenting capacity, high tolerance to ethanol, relatively tolerant to low pH values and capacity to grow rapidly under anaerobic conditions (Visser et al., 1990). In order to maximize the growth and the bioethanol production of microorganisms, suitable environmental and nutritional conditions are the key factors (Thomas et al., 1996; Bafrncova et al., 1999).

Previously, before conducting any optimization, screening experiments was executed. A screening experimentation typically includes only two levels of each factor and can also be called characterization testing or sensitivity analysis (Telford, 2007) using variables tools such as two-level full factorial design can be used to check the initial significance (Martendal et al., 2007).

1.2 PROBLEM STATEMENT

In today's world, fossil fuels and nuclear power are the main suppliers of energy for industrial, commercial and residential purposes, electricity generation and transportation. There are strong indications that climate change is intertwined with greenhouse gases in the atmosphere, and a major factor contributing towards this is due to human activities, especially the combustion of fossil fuels (Smith, 2009). The production of raw biomass material and its subsequent conversion to fuels provide jobs for the local economy, contributes towards the development of the regional economy, and an leads to higher farm and forestry incomes (Mulchandani, 2004). However, Columnist George Monbiot pointed out that widespread hunger might take place if biomass is used to produce fuels (Garza and Gale, 2007).

At present, maize (corn) and sugarcane are some examples of food crops commonly used as raw materials for biofuel productions. If this current trend continues, food scarcity might become a reality, threatening the world's food security. In light of these findings, Food and Agricultural Organization of the United Nations (FAO) identified that Nipa palm is a non – threatened and underutilized palm in South Asia (FAO, 1998), making it a more sustainable choice. Hence, Nipa palm is selected instead of feed sources due to it originating and growing in the mangrove environment, since there will be no competition in terms of land use for the food crops (Tsuji et al., 2011).

Several advantages of use of ethanol are net reduction in the emissions of carbon dioxide, and improved waste utilization. However, costs incurred in manufacturing process of ethanol are still relatively high (Mulchandani, 2004), where the costs of batch processing for the production of bioethanol is higher compared to the costs of continuous process. It is because we continuously withdraw the product without needing to run over the whole process in a batch processing plant.

Bioethanol (or ethyl alcohol; chemical formula C2H5OH) is produced from simple sugars derived from plant sources by utilising microbes via fermentation process. Bioethanol is a promising solution due to its biodegradable nature, low toxicity and fewer effects on the environment. Some beneficial properties of bioethanol as fuel energy are higher octane number (108), evaporation enthalpy, and flame speed and wider range of flammability.At the same time, it gives higher compression ratio (CR) with shorter burning time (Zabed et al., 2014). Bioethanol gives out carbon dioxide (CO2) and water when combusted. This CO2 is used up by the plants and at the same time, oxygen is released in the same volume. This indicates that it is a more beneficial candidate over fossil fuels which gives out CO2 and other toxic gases. Some bioprocesses have recommended possible routes to produce bioethanol in large volumes using low cost substrates (Gunasekaran and Raj, 1999). Bioethanol is obtained through the batch fermentation process using *Saccharomyces cerevisae*. Research by Tumainadu et al., 2013 suggests that Nipa palm frond, which contains renewable sugars, could be a potential carbon source for bioethanol production using S. cerevisea. Thus, the optimum condition to produce bioethanol using NPF juice needs to be determined.

1.3 RESEARCH OBJECTIVE

This study is aim to discover the potential of Nipa palm frond (NPF) juice as a sustainable potential feedstock for production of bioethanol. The objectives are:

i. To determine process parameters involve in the production of bioethanol from the fermentation of NPF juice.
ii. To identify the optimum condition for production of bioethanol from the fermentation of NPF juice.
iii. To suggest the optimum parameter condition for scale-up the fermentation of NPF juice.

1.4 RESEARCH SCOPE

In order to achieve the objectives, the following scopes have been identified:

i. Determination of sugar composition in NPF juice

   High Performance Liquid Chromatography (HPLC) with Refractive Index (RI) detector was used to determine the concentration, composition and renewable sugars in Nipa palm frond juice.

ii. Fermentation experimental design

   Fermentation profile was designed using Design Expert Software with 5 parameters to be studied such as incubation time, pH, juice concentration, incubation temperature, and agitation speed.

iii. Factorial design analysis

   The 5 main parameters were screened using 2 – level factorial design to determine the best parameters for optimum ethanol yield and validating the conditions suggested by Design Expert.

CHAPTER 2

LITERATURE REVIEW

2.1 OVERVIEW OF ETHANOL

Nowadays, ethanol is one of the most promising renewable fuels leading towards the reduction of negative environmental effects caused by the dependence of fossil fuels seen across the globe (Cardona and Sanchez, 2007). Research and development initiatives aimed towards commercial production of ethanol from renewable resources have shown increase due to limited fossil fuel reserves (Mojovic et al., 2006). The global ethanol production industry, accounting together for 70% of the world's production and nearly 90% of ethanol is used for fuel, is currently being led by Brazil and the United States. In the year 2006 alone, the manufacturing of 16.3 billion liters of ethanol in Brazil comprises 33.3% of the world's total ethanol production. 3.6 million hectares of Brazilian soil was occupied by sugarcane plantation for ethanol production, and this was responsible for producing 7,500 liters of ethanol per hectare. Meanwhile, 3,000 liters per hectare of maize ethanol was produced in the United States (Cardona and Sanchez, 2007).

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Ethanol is commonly utilised as fuels (92%), industrial solvents and chemicals (4%) and beverages (4%) (Logsdon, 2006). A key point often emphasized in ethanol production is whether it is economical, and current research initiatives are targeted towards designing and improving the processes to produce transportation fuel that are more sustainable and economical. It is worth taking note that a very important factor in

establishing cost-effective technology is low cost of feedstock (Mojovic et al., 2006). Therefore, there is a lucrative demand for efficient ethanol production with low cost raw material and production process (Liu et al., 2007).

2.1.1 ETHANOL CHARACTERISTIC

Ethanol or C2H5OH (empirically C2H6O) is a type of the aliphatic alcohol series with molecules contain a hydroxyl group, –OH, attached to a carbon atom. Known as ethyl alcohol, ethyl hydroxide and etc. ethanol is a transparent, colorless, and unstable liquid with a characteristic of pleasant odor (Pradyot Patnaink, 2007). Table 2.1 shows the physical properties of ethanol. In a dilute aqueous solution, ethanol has a slightly sweet flavor, but in undiluted solutions it has a burning taste. Other than that, ethanol also can be miscible (mixable) in all proportions with water and most organic solvents, with moderately an affinity for moisture absorption, even from the air.

Table 2.1
Physical properties of Ethanol

Component	Value
Formula weight	46.06 g/mol
Boiling point	78.5 °C
Melting point	-114.1 °C
Density	0.789 g/mL at 20 °C
Vapor pressure	43 torr at 20 °C

Table 2.1 continued

Component	Value
Latent heat of evaporation	396 BTU/lbm
Gravimetric lower heating value	11,604 BTU/lbm
Auto ignition temperature	362 °C
Flash point	13 – 14 °C

Source: CRC Handbook of Chemistry and Physics, (1993)

The high combustion energy of ethanol enables it to be used as energy sources. Complete combustion of ethanol yields only carbon dioxide and water; not destructive to the environment and it becomes a motivation for research on utilizing ethanol as alternative energy. It can be used as transportation fuel individually or blend with petrol to formulate an ethanol-petrol mixture, raise octane levels and prolong the supply of gasoline. With those advantages, ethanol is extensively used by main oil companies and distributors (Okamoto et al., 2011; Trummer, 2006). Apart from that, it is also used as indicator in thermometers, as an industrial solvent, and for sterilization purposes in hospital and laboratory. Ethanol also can be consumed as alcoholic beverages.

2.1.2 ETHANOL PRODUCTION

There are two main processes that can produce ethanol; synthetic process such as hydration of ethane, and via biological pathway such as fermentation. From ethane, ethanol is manufactured by reacting ethane with steam as in Eq.2.1.

$$C_2H_4 + H_2O \rightarrow CH_3CH_2OH \qquad \text{(Eq. 2.1)}$$

(ethane)

Ethanol is generally produced by fermentation of hexose sugar by using organism such as yeast *Saccharomyces cerevisiae*. Under certain condition, yeast will converted existing sugar to the ethanol. The equation for the fermentation of glucose is as Eq.2.2.

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad \text{(Eq. 2.2)}$$

(in the presence of yeast)

The economical concern between both processes is dependent on the raw material cost. As an example, petroleum price will influence ethanol manufactured via synthetic pathway while fermented ethanol will be dependence on 70 % cost of the raw material (Shuler and Kargi, 2002). Fermentation of conventional ethanol is based on sugar (glucose). For example, fruits, sugar cane, or grains such as corn and wheat, potatoes and soy starches have been utilised as feedstock for the ethanol production in several plants, globally (Trummer, 2006).

In general, ethanol production is divided into two groups, where first generation production refers to ethanol derived from edible sources, while second generation ethanol derived from non-edible sources such as lignocellulosic biomass. Ethanol produced using renewable feedstock is called as bioethanol. In western Asia, wine-making can be traced back as early as 5400-5000 BC at a site in today's northern Iran and, further south in Iran, at a site from 3500 to 3000 BC (Kamm et al., 2007). Ripe grapes provide sugar and other nutrients required for prompt microbial fermentations as well as the causative yeasts (Mousdale, 2008). By crushing the grapes, ethanol will be produced at 5-10 %v/v by fermentation in the unstirred vessel. Grape wines, cereals beers and alcoholic drinks prepared from honey, dates, and other fruits developed in the Fertile Crescent are expected to have ethanol concentrations lower than 10 %v/v (Mousdale, 2008).

2.1.3 ETHANOL AS FUEL

Primary biofuels (untreated and natural), and the secondary biofuels (usually used for combustion, heating, cooking fire, and power consumption) are two parts of biofuel. Ethanol, biodiesel and methanol were included in the secondary biofuels (Larson, 2008). Most of the feedstock for secondary biofuels or second-generation fuels was obtained from agricultural waste (residue), wood and grass.

The use of ethanol as fuel started from the dawn of the use of vehicles itself, and even Henry Ford‘s Model T. built in 1908 ran on ethanol. This trend continued until the availability of cheap petrol effectively rendered ethanol irrelevant as a major transport fuel in the early part of the 20th century. Several countries have conducted initiatives to replace some of their gasoline consumption with ethanol by mixing certain percentage of ethanol into gasoline to achieve the resulting product called gasohol, with the largest national fuel ethanol industries existing in Brazil. Hence, there is ample growth potential for this sector in the global market.

At present, bioethanol is an alternative for fuels and gasoline for automobile, with most ethanol used for fuel is being blended into gasoline at concentrations of 5 to 10 %. The antiknock performance is a key advantage for ethanol when compared to gasoline, since this allows its use in higher compression ratio engines. At the same time, there is less pollution emission produced, where it is noted that the reduction yielded was 50 % less in terms of smog forming emissions. Based on Figure 2.1, ethanol used as a fuel has a remarkable reduction on greenhouse gases (GHG) emissions rather than gasoline. Study conducted by U.S. Department of Energy's Clean in 2013 showed that, ethanol based on cellulosic biomass provided a greater advantage in reducing greenhouse (GHG) emissions by up to 86% and next up to 78 % by using sugarcane. Current researches have shown the significance of bioethanol in substituting the gasoline for automobiles fuels.

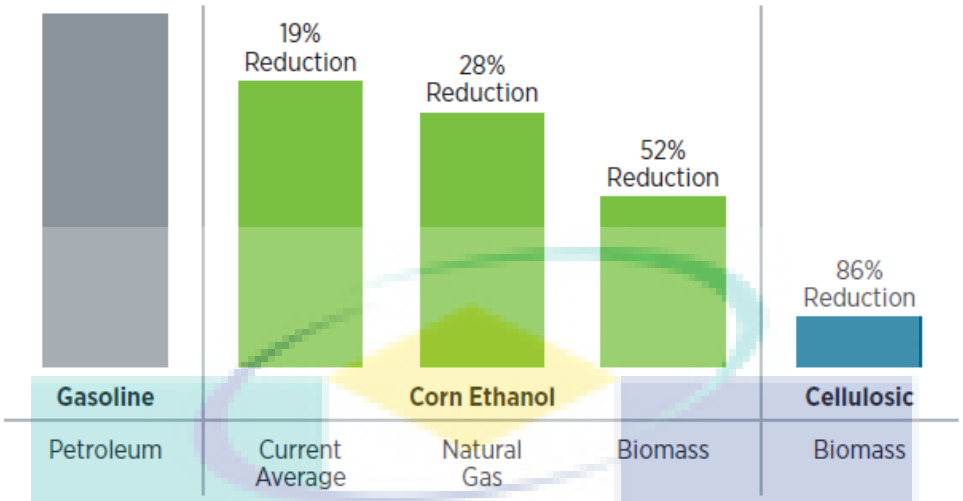

Figure 2.1. Life cycle Energy and Greenhouse Gas Emission Impacts

Source: U.S. Department of Energy's Clean, 2013

Ethanol-powered cars have garnered widespread acceptance in the green car market due to its ease of manufacture, its eco-friendly nature, and good fuel efficiency without compromising the delivery power. Table 2.2 shows a brief comparison in terms of properties of both ethanol and gasoline. Due to its higher heat of vaporization, ethanol produces superior thermal efficiency at high temperature. Compared to gasoline, ethanol can burn richer fuel/air mixtures; permits higher engine power output. Nevertheless, the usage of ethanol cause higher fuel consumption due to its lower heating value (Rodrigo & Jose, 2010).

Table 2.2
Comparsion between some properties of ethanol and gasoline (Hasan, 2013)

Properties	**Gasoline**	**Ethanol**
Chemical formula	C_4—C_{12}	C_2H_5OH
Molecular weight	100-105	46

Oxygen (mass %)	0-4	34.7
Net lower heating value (MJ/kg)	43.5	27
Latent heat (kJ/L)	223.2	725.4
Stoichiometric air/fuel ratio	14.6	9
Vapor pressure at 23.5 °C (kPa)	60-90	17
MON	82-92	92
RON	91-100	111

Bioethanol as a neutral carbon comprises no harmful sulphur and aromatics (Yin et al., 2011). Ethanol produces only carbon dioxide and water as the product of complete combustion and does not contain any dangerous substances either. Ethanol also does not damage any seals or valves and does not have corroded effect. Figure 2.2 represents closed carbon dioxide cycle showing that after ethanol burnt, released carbon dioxide is recycled back as source for plant photosynthesis and in those terms, environmental friendly ethanol will become an interest in alternative energy research area. Prasad et al. (2007) stated that study on optimizing ethanol production has been a highlight for both ecological and economic reasons, predominantly for its usage as a substitute to petroleum based fuels. By utilizing bioethanol, air pollution, CO_2 buildup, and petrol consumption can be reduced.

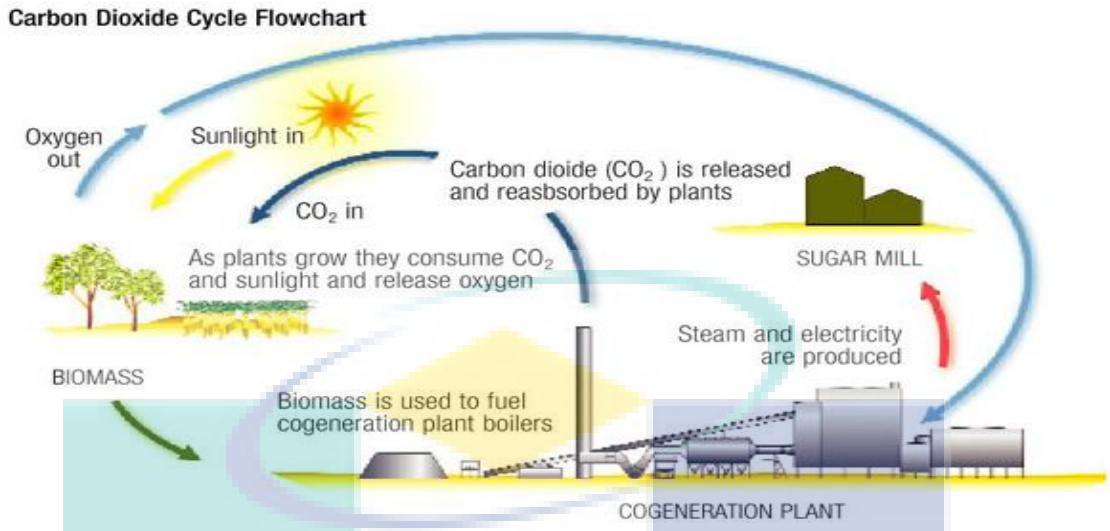

Figure 2.2. Bioethanol presents closed CO_2 cycle (Department of Environment, Australia Government).

Feedstock for bioethanol can also be obtained from Mahula flowers, Madhuca latfolia L. by Saccharomyces cerevisiae in solid-state fermentation (S-SF) (Mohanty et al., 2009). Still, this feedstock needed additional agricultural land for crop growing and affected other plants cultivation. Furthermore, human food and animal feed also comes from that same cultivation and as a result, these plants are not adequate to fulfill the increasing demand for biofuels. Based on the fact above, lignocellulose biomass was used to replace the crops as it is cheaper and has high availability than sugars and starch. Lignocellulose waste materials attained from energy crops, wood and agricultural deposits signify the most plentiful global source of renewable biomass. Olokayode (2012) stated that this type of feedstock can provide clean energy and stable national food security for future generations. The technology should also adapt recycling of agricultural feed stocks and increase soil fertility as well considering human health (Sivakumar et al., 2010).

Wheat straw is the major biomass feedstock among the agricultural wastes in Europe and the second largest in the world after rice straw. Approximately 21 % of the world's food depends on the wheat crop and the worldwide production of this crop needs to be increased to fulfill the rising demand of human consumption. Thus, in 21st century, wheat straw is considered as a good potential raw material for bioethanol

production. Based on the wheat straw pretreatment method proposed by Tablenia (2010), a sugar concentration of ethanol production achieved was in the range of 74-99.6 %. Instead, rice straw also has possibly produced 205 billion liters of bioethanol per year and it such a potentially lignocellulosic material for bioethanol production in India. High range of bioethanol has been produce by applying a Separate Hydrolysis and Fermentation (SHF) method using yeast cells, *Saccharomyces cerevisiae* and Pachysolen tannophilus to ferment rice straw (Balasubramanian, 2013). The percentage of bioethanol manufactured was 24.50 % (v/w) in which 100 g of rice straw produced 19.10 g of bioethanol. The optimum pH for *S. cerevisiae* and *Pachysolen tannophilus* was determined as 4.5 and 5.5 respectively and both organisms was found to be very active at 30°C.

In Malaysia, most of the cars are still running on petroleum, and there is currently no large-scale production of bioethanol and biomethanol due to the low demand for this alternative fuel source (Shuit et al., 2009) Moreover, lignocellulosic biomass feedstock for bioethanol is quite a new idea in Malaysia as the improvement of lignocellulose – related technologies are still not very well-established in the world (Goh et al., 2009). Malaysia Fuel Diversification Policy has been continuously revised to elude the over dependency on a single source of energy. As a participant to the UN Convention on Climate Change and the Kyoto Protocol committing to take action to reduce greenhouse gaseous emissions, Malaysia is responsible to vary the energy blend with more sources of renewable energy (Goh et al., 2009). The worldwide fuel orders for Malaysia by 2020 are 10-15 % of bioethanol. In 2007, around 11 billion liters of petrol was used and from this amount, it was expected that 10 to 15 % of bioethanol mixtures needs 1.10-1.65 billion liters of bioethanol per year (Rashid and Ibrahim, 2009).

As the production of of oil palm in Malaysia ie relatively high, bioethanol production could be produced by sap squeezed from old oil palm trunks felled with S. cerevisiae Kyokai no. 7. Akihiko et al. (2010) found that the total of ethanol produced

corresponded to 94.2 % of the theoretical yield calculated grounded on consumption of glucose, sucrose, fructose, and galactose in oil palm trunks felled.

2.2 NIPA PALM FROND

Nipa (*Nypa fruticans* Wurmb) is one of the most frequent distributed and beneficial palms in the mangrove forests of Southeast Asia. Nipa (*Nypa fruticans*) goes to the family Palmae (Burkill 1935; Corner 1966; Gee 2001) or Arecaceae (Gee 2001; Hamilton & Murphy, 1988). The genus *Nypa* has been positioned in its own subfamily, the Nypoideae (Moore 1973; Uhl & Dransfeld 1987) and is the only species in genus *Nypa* (Jian et al., 2010). Nipa is used for several traditional purposes in this area and is identified by different dialect names such as "chak" and "at-ta" in Thailand, "dua la" and "dua muoc" in Vietnam, "dani" in Myanmar and "atap palm" in Singapore (Baja-Lapis et al., 2004). Nipa is also a valuable feedstock of biofuel because has the high amount of sap to be converted into alcohol. Compared to sugar cane (3,350-6,700 L/hectare per year), nipa can produce more ethanol (6,480-10,224 L/hectare per year) (Hamilton & Murphy, 1988). Besides, Nipa is beneficial agriculturally and ecologically because Nipa habitat does not compete with food crops and the growth of this plant is sustainable. Lately, Malaysian scientists recognized the worth of Nipa as potential feedstock and proposed a plan for effective management of Nipa (Latiff, 2008).

Despite such usefulness, there is still a lack of scientific reports generally on Nipa compared with other benefical tropical palms such as coconut and oil palm. Countless years has passed since publication of useful scientific reviews on Nipa (Hamilton & Murphy 1988; Päiväke 1996) and this review described the biological characters, geographic circulation, traditional use, economic aspects and the advantages of Nipa as a crop by citing recent reports in addition to Hamilton and Murphy (1988) and Päiväke (1996).

2.2.1 FUEL – ALCOHOL PRODUCTION

Compared with other crops, Nipa is capable of producing higher yields of alcohol: Nipa by traditional management 6,480-10,224 L/hectare per year, sugarcane 3, 3506,700 L/hectare per year, cassava 3,240-8,640 L/hectare per year, sweet potato 6,750-18,000 L/hectare per year, coconut sap 5,000 L/hectare per year (Hamilton & Murphy, 1988). The quantity of alcohol is likely to rise up to 18,165 L by modern management (Halos, 1981), making Nipa a potentially source of biofuel.

Previously, before the World War II, Malaysia dealt with the production of alcohol out of Nipa, which was utilized as fuel for vehicles (Baja-Lapis et al., 2004). Malaysian plantation as the lone real commercial plantation in the world before World War II, produced close to 15,600 L/hectare per year (Dennett, 1927). In the early decades of 20 century, industrial alcohol from produced from Nipa was also an important industry in the Philippines (Whitmore, 1973). Nevertheless, the manufacturing was momentary due to political problems and competitive price of gasoline conquered during that era (Fong 1984; Whitmore 1973).

The use and research on alcohol form Nipa continuous discreetly until the middle of 20th century. According to Chai and Lai, 1984, there have been two factories produced alcohol from Nipa in Sarawak, Malaysia up to the 1980's. During the same period, similar research were carried out in the Philippines (Halos, 1981) and in Papua New Guinea (Newcombe et al. 1980). Currently, since the focus to use biofuel as future energy source become the prime concern, it is predictable the usage and research of alcohol from Nipa become a center concern once again.

2.3 FACTOR AFFECTING BIOETHANOL PRODUCTION

2.3.1 TEMPERATURE

Temperature increases the rate of a reaction. According to (Hashem et al., 2013), fermentation of ethanol at high temperature is described to be significant for effective production in tropical countries as the regular day-time temperatures are usually high through the year. The benefits of prompt fermentation at high temperatures are not only to lessen contamination, but also to decrease the cooling cost. Yet, yeast is greatly affected by temperature and high temperature affects yeast metabolism.

2.3.2 pH

The pH of a solution can have numerous effects of the enzymatic structure and activity. An alteration in pH affects the shape of an enzyme, structure of an enzyme, and properties of the substrate, and in that way either the substrate cannot bind to the active site or it cannot undergo catalysis. This will affect the fermentation activity as substrate and active cite more likely will not attached and this will slow the catalytic activity

2.3.3 AGIGATION SPEED

Agitation speed will affect the quantity of dissolved oxygen in the cultivation medium along the fermentation process. Agitation rate is essential for ample mixing, mass transfer and heat transfer because it helps mass transfer activity between different phases present in the culture.

2.4 MICROORGANISM RELATED TO BIOETHANOL PRODUCTION

Bioethanol can be produce by either using traditional yeast or new developed bacterial, *Zymomonasmobilis* isolated from tropical fruits bioethanol can be produced (Suraini, 2002). Based on research by Bailey and Ollis (1986), yeast is the lone important microbes in alcoholic beverages production industries for the supply of consumer market. The yeasts that generally used in alcohol production industries were *Saccharomyces cerevisiae* (glucose, fructose, maltose, maltoriose), *Saccharomyces uvarum* (carlbergensis), *Saccharomyces diataticus* (dextrins), *Kluyveromyces fragilis* and *Kluyveromyces lactus* (lactose) (Kun, 2003).

Kluyveromyces fragilis or *Candida* sp. can be used when the availability of lactose and pentoses are high. Alternative pentose and hexose fermenting organism such as *Clostridium hermosaccharolyticum* and *Thermoanaerobacter ethanolicus* are the thermophilic organisms that grant noteworthy advantages for ethanol fermentation and separation. Regrettably, it can gain unwanted end product and produce dilute ethanol (Shuler and Kargi, 2002). In addition to that, a Gram-negative bacterium, *Zymomonas mobilis*, is also deliberated as an alternate organism for the large scale ethanol production fuel due to higher ethanol yield, higher sugar uptake and lower biomass production (Maiti et al., 2011). This bacterium is capable to use glucose, fructose and sucrose as the substrates for the ethanol production. Improvement of genetic engineering also has magnificently converted bacterium *E. Coli* to an ethanol producer where it was able to reach 43 %v/v of the ethanol concentration (Shuler and Kargi, 2002).

On the other hand, synchronized saccharification and fermentation (SSF) as one of the direct bioconversion was promising for the fermentation of lignocellulosic biomass as to reduce reliance on chemical pretreatment. Okamoto et al. (2011) have utilized a white rot basidiomycetes, *Trametes hirsuta*, a fungi that fit for biological pretreatment of the lignocellulosic biomass to directly ferment wheat bran and rice

straw for bioethanol production. Correspondingly, mixed cultures including *Phanerochaete chrysoporium*, *Thrichoderma harzianum*, *Mucor hiemalis* and *S. cerevisiae* has been used for direct ethanol bioconversion from POME (Alam et al. (2009).

In this study, Yeast cells, named *Saccharomyces cerevisiae* was used. It is facultative anaerobes, chosen due to its high growth rates, efficient glucose repression, efficient ethanol production, and tolerance for environmental stresses. Moreover, it is said to be able to produce more than 20% (v/v) bioethanol (NurulAin, 2010; Blanch and Clark, 1996).

2.5 BIOETHANOL FERMENTATION

Fermentation process can be conducted under aerobic and anaerobic condition. Fermentation process by yeast to produce alcohol required a lesser amount of oxygen for cell to grow, but then, no air is necessary (Rao D.G.et al., 2010). In anaerobic fermentation process, less heat is produced per unit of glucose consumed, and it can be removed externally. Additionally, *S. cerevisiae* is facultative anaerobes and it can ferment glucose to ethanol under anaerobic conditions (Bakker, Lee, & Charles, 2007).

In addition, fermentation of bioethanol is a kind of immersed fermentation, where the microorganisms and substrate are cultivate in the liquid form. The effectiveness of mass and heat transfer is high in this type of fermentation. Furthermore, it is amenable for process modeling and easy in kinetics field study (Rao D.G.et al., 2010).

2.6 PREVIOUS WORK ON BIOETHANOL PRODUCTION

Currently, researchers have been motivated on the probability of new claim on converting biomass to alternative energy carriers, as an example, ethanol, butanol, and acetone based fuel produced by utilizing different types of biomass such as sugar cane molasses, sunflower hulls, cassava mash, soybean molasses and etc. (Kaylen et. al., 2000). Variety of biomass had been selected in the research of bioethanol production. Still, certain of the biomass required pre-treatment or extraction to gain the sugar for bioethanol fermentation. The earlier works that had been conducted in current year were shown in the Table 2.3 and Table 2.4

Table 2.3
Screening on previous works.

Biomass	**Microorganism**	**Parameters**	**References**
Corn Stover	*S. cerevisiae*	Agitation Temperature	(Liu and Shen, 2008)
Mango fruit juice	*S. cerevisiae*	-	(Veeranjaneya , 2007)
Soybean molasses	*S. cerevisiae*	pH Agitation	(Yingling et al., 2010)
OPT sap	*S. cerevisiae*	- Temperature	(Kosugi, 2010) (Chin et al., 2010)
Cassava mash	*S. cerevisiae* *S. cerevisiae*	pH Temperature	(Sanjeev et al., 2004)
Sunflower hulls	*S. cerevisiae var. ellipsoideus*	pH Temperature	(Sanjeev et al., 2004)
Banana peel waste	*S. cerevisiae*	Kinetic Parameters	(Miguel et al., 2013)
Nipa Palm sap	*S. cerevisiae*	-	(Tamunaidu et al., 2013)
Soft drink waste water	*S. cerevisiae* *S. cerevisiae*	pH	(Yu et al., (2009)
Sugarcane molasses	*S. bayanus* *Z. mobilis* *Z. mobilis* *Z. mobilis*	Substrate Concentration pH Agitation Temperature	(Pradeep and Reddy, 2008) (Maiti et al., 2011) (Cazetta et al., 2007)

Table 2.4
Research on bioethanol from biomass

Authors	Biomass	Pretreatment*	Initial raw material	Maximum yield of ethanol (g/l)	Time (h)
(Turhan et al., 2010)	Carob pod extract	Five nutrients added	115.3 g/l sugar	42.60	48
(Han et al., 2011)	Cassava stem	Dilute acid pretreatment and enzymatic hydrolysis	9.5 g/l wheat bran (15.5 g/l glucose)	7.55	24
(Rocha et al., 2010)	Cashew apple bagasse	Dilute acid pretreatment and pH adjustment	25.1 g/l of glucose	12.44	48
(Ferreira et al., 2011)	*Acacia dealbata*	Dilute acid pretreatment and enzymatic saccharification	31.1 g/l of glucose	10.31	24
(Cheng et al., 2011)	Corncob residues	Sulfite pretreatment and enzymatic hydrolysis	15 % w/v of glucan substrate loading	60.08	72
(Arslan and Eken Saracoglu, 2010)	Hazelnut shell	Partial synthetic xylose supplementation	50.0 g/l of TRS	16.79	90
(Onsoy et al., 2007)	Jerusalem artichoke juices	Acid and enzymatic hydrolysis	-	104.20	36 – 48
(Ferreira et al., 2010)	Forest residue *Pterospartum tridentum*	Dilute acid pretreatment	9.8 g/l sugar	3.20	24

Table 2.4. continued.

(Murai and Kondo, 2010)	OPT sap	Hot water extract, saccharification and liquefaction	-	25.25	-
(Swain et al., 2007)	Mahula flowers	Immobilized cells. Steam cooked and pH adjustment	9 ^{0}Brix	33.99	96
(Sharma et al., 2007)	Kinnow waste and banana peels	Steam exploded, enzymatic saccharification and SSF	63.0 g/l reducing sugar	26.84	24
(Hashem and Darwish, 2010)	Potato starch residue	$ZnCl_2$ added	10.0-20.0 g/l starch	5.52	36
(Kosugi et al., 2010)	OPT sap	No	55 g/l	30.00	48

*Comminuted, milling and sterilization was excluded from the table (basic pre-treatment)

There are varies type of biomass for bioethanol production research such as hazelnut shell (Arslan and Eken-Saracoglu, 2010), bagasses (Rocha et al., 2010), soybean molasses at the different scale (Siqueira et al., 2008), sorghum (Liu and Shen, 2008; Salvi et al., 2010; Yu et al., 2009), mahula *Madhuca latifolia L*. flowers (Swain et al., 2007) and carob pod (Turhan et al., 2010). Despite the fact that the research on using lignocellulosic biomass for conversion of sugar to bioethanol became a concern among researchers, it was found that production has numerous technical and economic challenges thus delayed the commercialization (Sindhu et al., 2011).

Particular biomass required to be treated depending on its nature. A pretreatment stage is essential to break the lignin and to expose cellulose and hemicelluloses for enzymatic saccharification, for the conversion of biomass into fermentable sugars if there are no ready sugar was available. Steam explosion and dilute acid hydrolysis is the most common techniques of pretreatment in commercial use (as in Table 2.4), but both techniques having the disadvantage due to the development of inhibitor that affects fermentation process (Sindhu et al., 2011). Instead, efficiency of enzymatic hydrolysis still needs enhancement even though it has numerous benefits over acid hydrolysis such as less environmental impact and reduce by-product yield. In reality, costs of the enzymatic hydrolysis of the biomass become a main setback in the biomass-to-ethanol conversion (Okamoto et al., 2011).

The economical aspects of a hydrolysis process depend on the yield of the functional component such as glucose (Chin et al., 2011). Xu et al. (2009) clear up that some alternatives recommended reducing costs of conversion of lignocellulosic to ethanol such as increasing cellulose hydrolysis yield, removing pretreatment, improving enzyme activity and enhancing the fermentation yield. However, each pretreatment technique can be considered to be conducted for large scale commercial production as this technique has its own advantages and disadvantages. It is more advantageous if the feedstock already comprise a lot of fermentable sugar such as OPT sap.

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

This chapter presents the methodology used in this study. Several methods and experimental designs were used to achieve the objective of this study. Figure 3.1 provided the experimental workflow for this study.

3.2 MATERIALS

In this study, 23 kg fresh Nipa palm frond was attained from the mangrove swamp area of Kg. Miang, Pekan, Pahang. All the leaves were removed from the Nipa palm fronds and squeezed through conventional sugarcane press machine to collect the juice. The NPF juice was filtered using coffee filter and collected into a big container. It was mixed well before allocated into 5 L bottles and stored in a – 20 °C freezer.

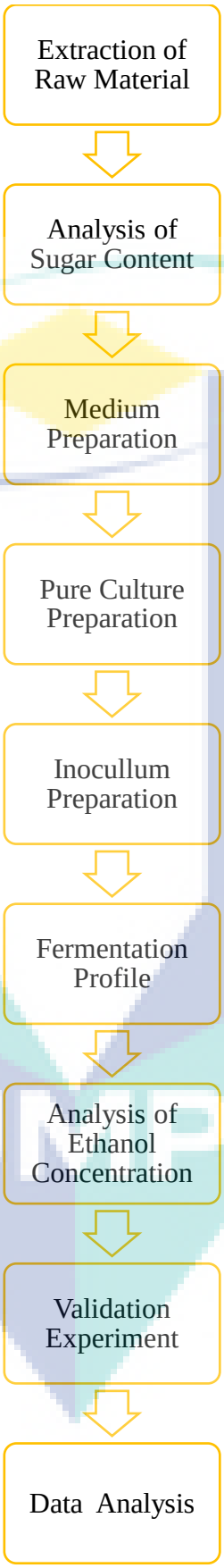

Figure 3.1. Experimental workflow

3.3 CHEMICAL REAGENTS

Medium component such as yeast extract were purchased from Fisher Scientific, peptone and agar powder from Sigma – Aldrich. Acid and base such as sulphuric acid and sodium hydroxide were brought from Fisher Scientific. Sucrose HPLC grade were brought from Sigma – Aldrich, glucose and fructose from Merck (Germany), while methanol and ethanol GC-FID grade were purchased from Fisher Scientific.

3.4 MEDIUM PREPARATION

Two types of medium were used in this research; nutrient agar (NA) and nutrient broth (NB). For this work is the Yeast Extract Peptone Dextrose (YDP) agar is chosen as nutrient agar. YPD agar was prepared by mixing 20 g of agar, 20 g of peptone and 10 g of yeast extract in 900 mL of distilled water in 1 L Schott bottle. 20 g of dextrose was mixed with 100 mL of distilled water in a 250 mL Erlenmeyer flask. Then, both bottle and flask were covered with aluminum foil and was sterilized in an autoclave (Hirayama HVE-50, Japan) for 20 minutes at 121 °C. After autoclave, 100 mL of 20% w/v glucose was added in the agar to avoid Maillard reaction. Agar was poured into sterilized Petri dish after the temperature has dropped to 60 °C and left to solidify. All plates were sealed before being kept in the refrigerator at 4 °C until advance use. The procedure was the same to make YDP broth except no agar powder was added.

3.5 MICROORGANISM AND MEDIUM

3.5.1 MICROORGANISM PREPARATION

Baker's yeast (*Saccharomyces cerevisiae)* was used in this research. 5 g of yeast was dissolved in 10 m of sterilized distilled water and swabbed on the agar plate and kept in incubator at 37 °C for 24 hours. After 24 hours, the yeast was store at 4°C until further use.

3.5.2 INOCULUM PREPARATION

About 3-4 loops were taken from the agar plate and inoculated into YPD broth in shake flask. The flask was incubated at 30 °C under aerobic condition with agitation speed at 160 rpm for 24 hours.

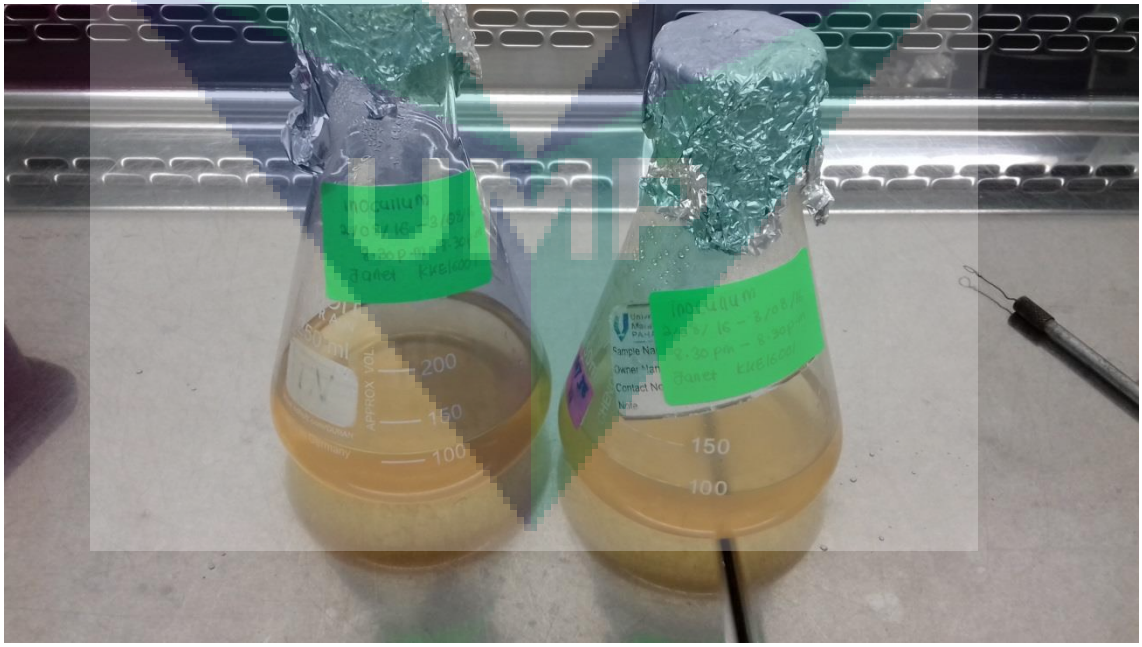

Figure 3.2. Preparation of inoculum for fermentation

3.6 CHEMICAL ANALYSIS OF SUGAR CONTENT IN NPF JUICE

The sugar content in the NPF juice supernatant was analyzed using a High Performance Liquid Chromatography (HPLC) (Agilent Series 1200, USA) with a Refractive Index (RI) detector. The temperature was kept at 30 °C. The individual standard sugar (sucrose, glucose and fructose) were prepared in concentration of 5, 10 and 20 g/L each before HPLC analysis. Each of sugars (sucrose, glucose and fructose) will produced a peak at different retention times. The sugar components in NPF juice were identified by comparing their retention times with the peak and standard curves of the prepared sugar standards.

3.7 FERMENTATION

3.7.1 PREPARATION OF NPF JUICE FOR FERMENTATION

NPF juice was filtered and centrifuged for 10 minutes at 15,000 g and 4 °C. The supernatant was filtered using a nylon filter with the pore size of 0.2 µm before used in the fermentation. The NPF juice was then transferred to a 250 mL with working volume of 100 mL. NPF juice was prepared in according to experimental plan in section 3.8. The two concentrations of the juice were obtained by diluting the juice with distilled water. The pH value of the medium was adjusted with the addition of 2M H_2SO_4 and 2M NaOH.

3.7.2 PREPARATION OF FERMENTATION PROFILE

10% (v/v) of inoculums suspension from activated yeast flask was transferred into the sterilized NPF juice. Total working volume for each flask was kept constant at

100 mL for every run. Then, the shake flask was purged with nitrogen gas, before being placed in incubator shaker at preferred setting. Each of the conical flasks was set as shown in Figure 3.3.

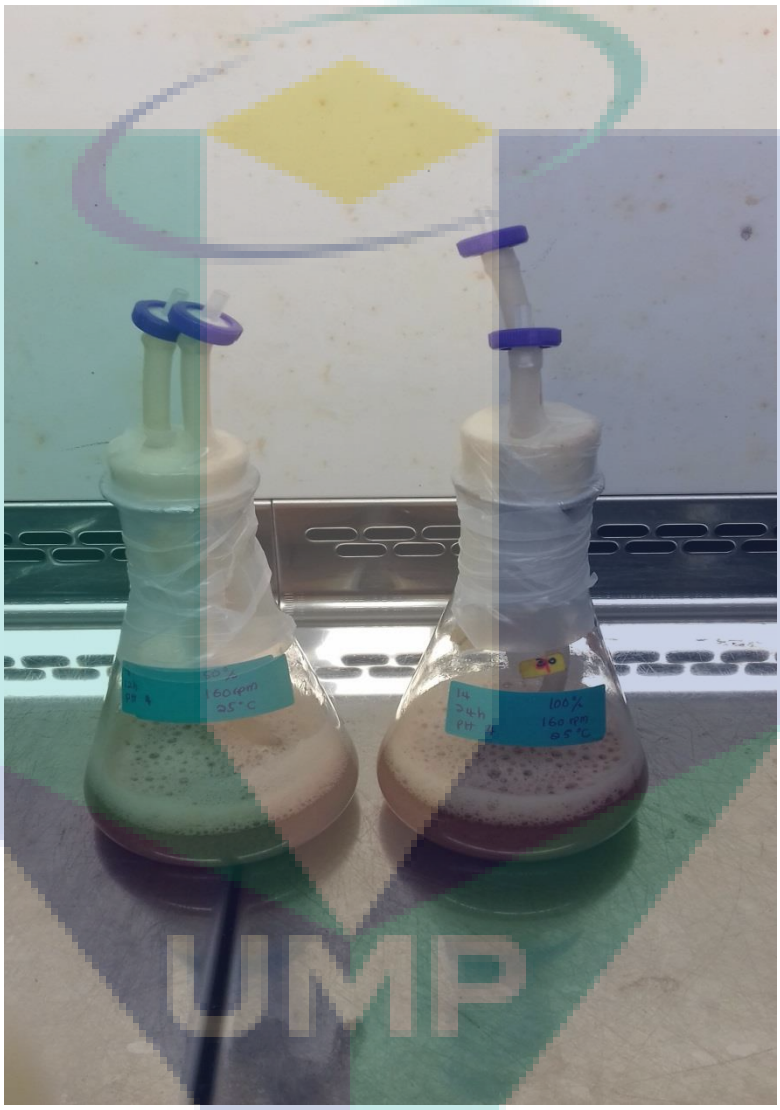

Figure 3.3. Fermentation set – up

3.7.3 ETHANOL CONTENT ANALYSIS

After 24 hours, 5 mL sample was collected and centrifuged at 15,000 g for 5 minutes at 4 ◦C (Thermo Fisher Scientific, NC, USA). The supernatant was then diluted

with methanol GC- FID grade and filtered using 0.20 µm syringe filters into Agilent 2 mL GC vials before analyzed by Gas Chromatography Flame Ionization Detection (GC-FID). The individual standard ethanol was prepared in concentration of 2, 4, 6, 8 and 10 g/L each before GC-FID analysis by diluting ethanol GC-FID grade with ultra-pure water. Ethanol will produced a peak at certain retention time. The ethanol components in NPF fermentation sample were identified by comparing their retention times with the peak and standard curves of the prepared ethanol standards.

3.8 FACTORIAL ANALYSIS OF MAIN PARAMETERS OF ETHANOL PRODUCTION

The experimental design was generated using Design Expert version 7.1.6. Fractional factorial designs were used in this study to reserve some resources for unforeseen contingencies and follow-up runs. Some experts endorse using only 25% of the resources in the first experiment (Telford, 2007). The half fractional factorial design of 2^{5-1} was chosen in this study to decrease the number of experiments without losing a lot of information on the possible effect of factors on ethanol yield from fermentation of NPF juice. The 5 factors studied were the concentration of NPF juice, the incubation time, incubation temperature, pH of the NPF juice, and agigation speed. Table 3.1 listed the factors for fermentation profile and the low and high value were chosen accordingly to past studies (Tamunaidu et al., 2013; Farhana, 2010) as discussed in Section 2.3.

Table 3.1
Factors and their designated low and high value

Factor	**Units**	**Low value (-1)**	**High value (+1)**
A: Incubation Time	Hour	12	24
B: pH of juice		4	7
C: Concentration of juice	v/v%	50	100
D: Agitation Speed	rpm	80	160
E: Incubation Temperature	°C	25	32

The fermentation plan was prepared according to procedure section 3.7 with conditions according to Table 3.2. The best combination condition suggested by the design program was validated by performing fermentation in triplicate according to the suggested parameters.

Table 3.2
Experimental design matrix for screening

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Std	**A: Incubation Time**	**B:pH of juice**	**C: Concentration of Juice**	**D:Agitation speed**	**E:Incubation Temperature**
	Hour		**v/v%**	**rpm**	°C
1	12	4	50	80	32
2	24	4	50	80	25
3	12	7	50	80	25
4	24	7	50	80	32
5	12	4	100	80	25
6	24	4	100	80	32
7	12	7	100	80	32
8	24	7	100	80	25
9	12	4	50	160	25
10	24	4	50	160	32
11	12	7	50	160	32
12	24	7	50	160	25
13	12	4	100	160	32
14	24	4	100	160	25
15	12	7	100	160	25
16	24	7	100	160	32

3.9 METHOD OF ANALYSIS

In this experiment two method of analysis were used such as HPLC for determination of sugar components in NPF juice and GC – FID for ethanol concentration analysis.

3.9.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC (Agilent Series 1200, USA) was used to analyze the concentration of sugar in NPF juice. The mobile phase was acetonitrile: water (75%:25%) at a flow rate of 1.0 mL/min. The loop of injection was optimized for 10 µL injection volume. The specification for HPLC analysis for sugar content is shown in Table 3.3.

Table 3.3
Specifications for HPLC for sugar analysis

Column	Supelcosil LC-NH2
Mobile phase	75% acetonitrile: 25% water
Standard preparation	5 g/l, 10 g/l, 15 g/l, 20 g/l and 25 g/l for each sample
Flow rate	1.0 mL/min
Injection volume	10 µL

3.9.2 GAS CHROMATOGRAPHY FLAME IONIZATION DETECTION (GC-FID)

The experiment were performed using a GC system (Agilent 7890, Santa Clara, CA) equipped with an FID and the method for GC – FID was set according to Lin et al. (2013). This method have been improved from the previous existing method which reducing the analysis time per sample to less than 10 minutes compared to those of a conventional GC – FID (more than 20 minutes). The specification for GC – FID analysis for ethanol concentration is shown in Table 3.4.

Table 3.4

Specifications for GC – FID for ethanol analysis

Column	60 m HP – INNOWAX (0.25 mm i.d, coated polyethylene glycol (0.25 µm film thickness)
Carrier gas	Nitrogen
Standard preparation	2 g/l, 4 g/l, 6 g/l, 8 g/l and 10 g/l
Flow rate	2.0 mL/min
Injection volume	1 µL
Injector temperature	180 °C
FID temperature	220 °C

The column was held initially at 70 °C for 0.5 min, then increased to 190 °C at 20 °C/min and held for 4 min. Chromatographic data were documented and combined using Agilent Chemstation software.

3.10 EXPERIMENT VALIDATION

Following the design and analysis of the 5 main factors, the best and optimized condition was suggested by the use of fitted model to predict the highest possible ethanol concentration that can be achieved within the range of factor studied. Experiments were run according to the suggested experimental conditions and results of the experiment were compared with the suggested results to verify the significance of the factorial model. An error below 10 % was desired between the predicted and experimental factor, calculated using the following equation:

$$Error(\%) = \frac{\left| \text{predicted value - experimental value} \right|}{\text{experimental value}} \times 100\% \qquad (3.1)$$

CHAPTER 4

RESULT AND DISCUSSION

4.1 CHEMICAL COMPOSITION OF NPF JUICE

High Performance Liquid Chromatography (HPLC) with Refractive Index (RI) detector was used to determine the concentration, composition and distribution of renewable sugars in Nipa palm frond juice. The sugar content in NPF juice is mainly consists of sucrose, glucose and fructose. As shown in Table 4.1, the NPF juice contain high amount of sugar with the total amount of sugar is 105.37 g/L.

Table 4.1

Composition of sugar content in Nipa palm frond juice

Sugar	Concentration (g/L)	Percentage (%)
Sucrose	43.26	41.06
Glucose	35.88	34.05
Fructose	25.23	24.89
Total sugar (g/L)	105.37	100

The amount of sugar content in NPF are much higher compared to the overall sugar content in oil palm frond (OPF) juice which accounted about 76.09 g/L (Zahari

et al., 2012). Oppositely, the total sugar in NPF juice is lesser than the total sugar in Nipa palm sap (Tamunaidu et al. 2013). Sucrose is the main component of sugar in NPF juice with the concentration of 43.26 g/L which accounting for 41.06% of total sugar. Other sugar such as glucose (35.88 g/L) and fructose (26.23 g/L) are also present. The compositions of sugar in NPF juice are nearly same with sugar composition in Nipa palm sap where sucrose becomes the major component of sugars in the sap (Tumainaidu et al. 2013).

4.2 GC – FID RESULT ANALYSIS

At the initial stage, 5 ethanol standards were analyzed using GC – FID to get the ethanol retention time and then the standard curve of ethanol standard were then plotted to obtain the equation needed to calculate the ethanol concentration in the fermented samples. Figure 4.1 shows the standard curve for ethanol where the R2 is 0.9971 and the equation obtained is:

$$y = 882.55x \quad (4.1)$$

Where y = area of the peak (pA*s)

x = concentration (g/L)

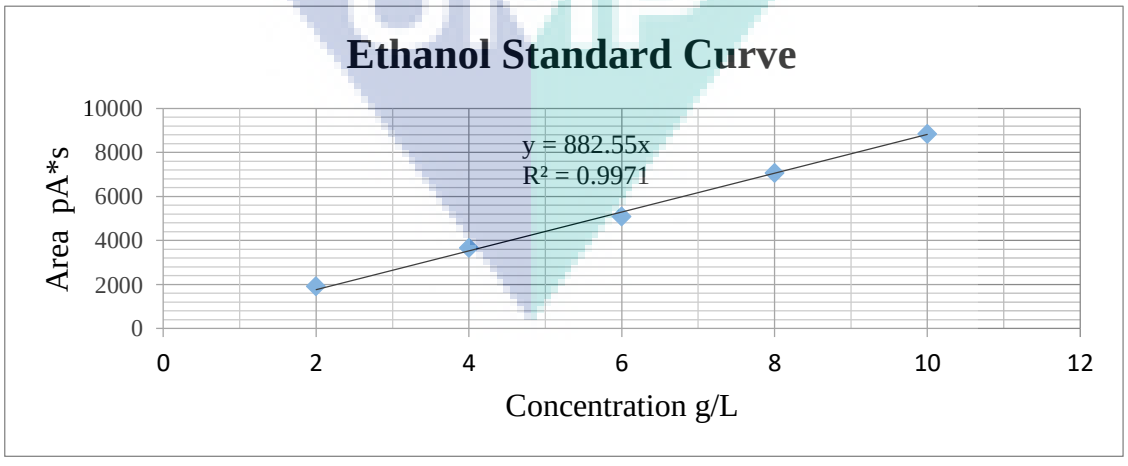

Figure 4.1. Ethanol standard curve

The ethanol components in NPF fermentation sample were determined by comparing their retention times with the peak and standard curves of the prepared ethanol standards. Figure 4.2(a) and 4.1(b) show the GC chromatograms obtained from the analysis of standard and fermentation samples. For ethanol standard, the peak occur at retention time of 2.418 minutes and comparing with the peak obtained from the NPF fermented sample, we can conclude that there is ethanol content in the juice as there is peak obtained at 2.439 minutes.

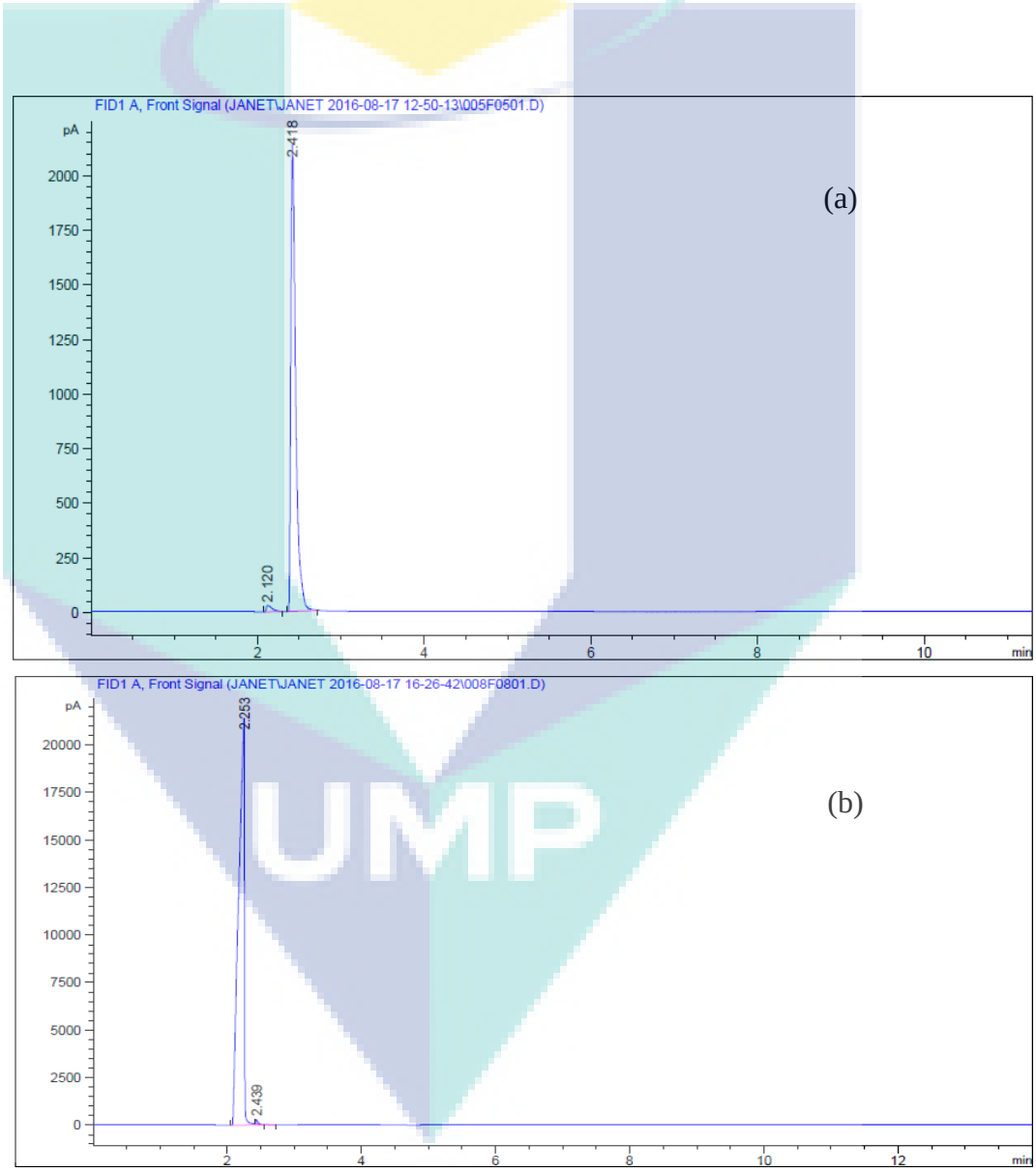

Figure 4.2. GC chromatograms obtained from the analysis of ethanol standard (a) and NPF fermented juice sample (b)

4.3 FACTORIAL ANALYSIS PARAMETERS FOR ETHANOL PRODUCTION

2^{5-1} factorial designs with total of 16 experiments were executed. The ethanol concentration for each of the fermented sample is calculated using equation 4.1. The fractional factorial experimental design and the resulted response are shown in Table 4.2. Response was analyzed by observing fitting a model, understanding the model graphically, finding the best parameter, and validating the model.

Table 4.2

Experimental design and response for factorial analysis

Std. Order	**A: Incubation Time**	**B: pH of juice**	**C: Conc. of juice**	**D: Agitation speed**	**E: Incubation Temperature**	**Response: Ethanol Concentration**
	Hour		**v/v%**	**rpm**	**°C**	**g/L**
1	12	4	50	80	32	1.392
2	24	4	50	80	25	0.638
3	12	7	50	80	25	0.733
4	24	7	50	80	32	0.841
5	12	4	100	80	25	0.947
6	24	4	100	80	32	1.528
7	12	7	100	80	32	1.357
8	24	7	100	80	25	0.562
9	12	4	50	160	25	1.104
10	24	4	50	160	32	0.875
11	12	7	50	160	32	1.309
12	24	7	50	160	25	0.671
13	12	4	100	160	32	1.541
14	24	4	100	160	25	1.277
15	12	7	100	160	25	0.813
16	24	7	100	160	32	1.226

As observed in Table 4.2, the highest ethanol concentration produced is 1.541 g/L while the lowest ethanol yield is 0.562 g/L. For the 12 hours fermentation, the ethanol yield ranging from 0.733 g/L to 1.541 g/L and for 24 hours fermentation, ethanol concentration produced at the range of 0.562 g/L to 1.277 g/L.

4.3.1 MODEL FITTING AND EFFECT ANALYSIS

Complete 16 fractional factorial designs was systematically performed using Design Expert 7.1.6, to calculate effect estimates using Yates algorithms. The percent input comes from the addition of the total sum of squares and then taking each term's sum of squares and dividing by the total to get a percentage (Anderson et al., 2009). The effect estimate and percent input was calculated and tabulated in Table 4.3. The interaction terms for the model were chosen based on percent contribution; however major effects sidestep this process due to model hierarchy. Interaction terms with percent contribution more than 1 % were chosen for the regression model. The interaction terms were AC, AE, BC, BE, CE, and DE. The fitted model for the factorial analysis in coded form was shown in Equation (4.2).

$$Ethanol\ concentration\ factor = 1.05 - 0.099 * A - 0.11 * B + 0.11 * C + 0.051 * D + 0.21 * E + 0.090 * A * E - 0.043 * A * E - 0.055 * B * C + 0.037 * B * E + 0.049 * C * E - 0.072 * D * E \quad (4.2)$$

Table 4.3

Effect list for factorial analysis

Term	Effect Estimate	Sum of Squares	% Contribution
A-Incubation time	-0.20	0.16	9.58
B-pH of juice	-0.22	0.20	12.27
C-Conc. of juice	0.21	0.18	10.98
D-Agitation speed	0.10	0.042	2.58
E-Incubation temperature	0.42	0.69	42.47
AB	-0.030	0.003	0.23
AC	0.18	0.13	8.04
AD	-0.018	0.001	0.079
AE	-0.085	0.029	1.79
BC	-0.11	0.049	2.98
BD	0.029	0.003	0.21
BE	0.073	0.021	1.31
CD	0.014	0.000	0.046
CE	0.097	0.038	2.33
DE	-0.14	0.083	5.11

The relative size of effects is visually validated as Pareto chart in Figure 4.3, where the bar length are proportional to the absolute value of estimated effect. For main effects, an effect is assumed to be positive when an increase to its high level will cause the response to increase while the negative effect is when an increase in its high level will affect the response to decrease. For interactions, when both factors were a chance to the same level (low or high), the response will increase and this considered as positive effect. The response will increase for the negative effect when both factors were altered to the opposite level (one at its low and the other at its high) (Martendal et al., 2007). In the Pareto chart, the positive effect is in orange shaded and the negative one is in blue.

Effects of t-value limit (black line) are reflected as significant at 95% confidence level while effects below t-value limit are not expected to be significant. For every model with a small global p-value, Bonferroni's corrected t-test were executed on the individual terms in the model to validate individual terms in models selected by forward selection (Mee, 2009). Effect exceeding Bonferroni's corrected t-value limit (red line) is almost undoubtedly significant (Anderson et al., 2009). A prompt analysis was conducted on the selected effects using Pareto chart to check the significance of the selected effects at 95% confidence level. At both t-value limit and Bonferroni's corrected t-value limit, all the selected effects (A, B, C, D, E, AC, AE, BC, BE, CE, and DE) shown to be significant.

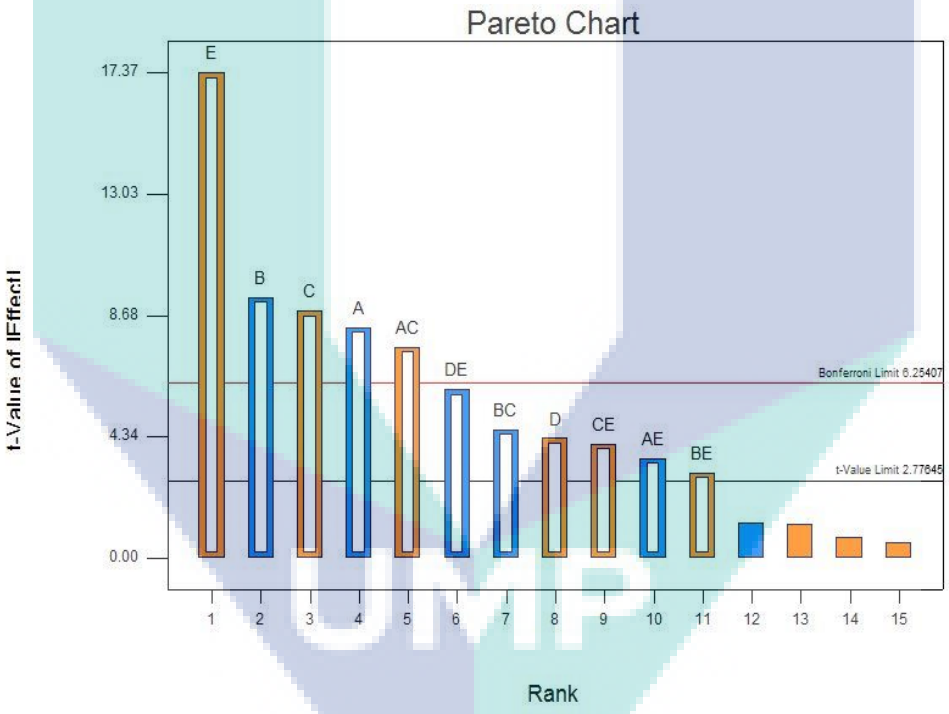

Figure 4.3. Pareto chart of effects ethanol concentration factor

4.3.2 ANOVA

The models with the selected effects were studied using analysis of variance (ANOVA) and found significant as shown in Table 4.5. The coefficient of determination (R^2) is the proportion of variation in the response accredited to the model. High correlation (R^2 = 0.9944) between the experimental data and model data was gained. From the study, the regression coefficient for all the designated model terms is lower than the interception, which specified the existent of the design plateau. Therefore, this plateau indicated that the design had an optimum point, where advance optimization experiment can be implemented (Box et al., 1978). The best experimental condition for fermentation of NPF juice for ethanol production was shown in Table 4.4.

Table 4.4

Suggested best condition for factors in NPF juice fermentation for maximizing ethanol production

Factors	**Best condition**
A-Incubation time	12 hours
B-pH of juice	4
C-Concentration of juice	100 %v/v
D-Agitation speed	160 rpm
E-Incubation temperature	32 ° C

Table 4.5

ANOVA analysis for the factorial model

Source	SS[a]	Df[b]	MS[c]	F-Value	p-value	
Model	1.62	11	0.15	64.21	0.0006	**significant**
A-Incubation time	0.16	1	0.16	68.03	0.0012	
B-pH of juice	0.20	1	0.20	87.13	0.0007	
C-Conc. of juice	0.18	1	0.18	78.02	0.0009	
D-Agitation speed	0.042	1	0.042	18.03	0.0129	
E-Incubation Temperature	0.69	1	0.69	301.65	<0.0001	
AC	0.13	1	0.13	57.13	0.0016	
AE	0.029	1	0.029	12.73	0.0234	
BC	0.049	1	0.049	21.18	0.0100	
BE	0.021	1	0.021	9.34	0.0378	
CE	0.038	1	0.038	16.56	0.0152	
DE	0.083	1	0.083	36.27	0.0038	
Residual	9.16E-03	4	2.29E-03			
Cor Total	1.63	15				
C.V. =4.56%; R^2=0.9944; Adjusted R^2=0.9789; Adeq. Precision=23.291						

[a]Sum of squares.
[b]Degree of freedom.
[c]Mean Square

4.3.3 EFFECT OF MAIN FACTORS ON ETHANOL CONCENTRATION

All the main factors studied were statistically significant at 95 % confidence level toward ethanol concentration as shown in Figure 4.3. Factor A (incubation time) and B (pH of juice) were found to have negative effect and factor C (NPF juice concentration), D (agitation speed), and E (incubation temperature) having a positive effect. First, we look into the main factor for higher ethanol yield, factor E which is temperature. According to Hashem et al. (2013), fermentation for bioethanol will be more effective in higher temperature compare to lower temperature. The recommended temperature in this study is 32 °C which more effective in producing higher ethanol yield. Factor C (concentration of NPF juice) became the second most influence factor in this study. Higher concentration of NPF juice contributes to the higher ethanol concentration in the fermentation process due to the abundance of carbon source for the *Saccharomyces Cerevisae* (Dun, 1985). Factor A (incubation time) and factor B (pH of juice) were found to be significantly inverse effect for ethanol yield, in other words ethanol concentration decreases with the increase of initial pH and incubation time (Martendal et al., 2007). Factor D (agitation speed) was also be found to be positively effective but less influence to ethanol yield compare to other factors. The interaction of factor A and factor C were found to give the highest impact for ethanol yield while interaction of factor B and factor E reported to was found to have fewer influence on ethanol concentration. Factor C and E were found to be independent which each other because their interaction effect has a smaller influence on ethanol concentration compared to their individual main effects.

4.3.4 VALIDATION OF MODEL

The validation of experiments was performed based on recommended best condition in from Design Expert 7.1.6 in 3 runs. The experiments were executed based on the suggested best condition in Table 4.5 and the result is shown in Table 4.6. The error from these runs were 3.2 %, 1.7 %, and 3.7 % after validation has been executed.

Grounded on the predicted and experimental results tabulated, the experimental values were in good agreement with the predicted values suggested by the model with an error less than 10 % and verified to be an adequate model.

Table 4.6

Comparison between predicted and experimental value for best condition for ethanol production

Description	**Ethanol concentration**		
	Run 1	**Run 2**	**Run 3**
Predicted Value	1.573	1.573	1.573
Experimental Value	1.524	1.601	1.513
Error	**3.2 %**	**1.7 %**	**3.7 %**

4.4 EFFECT OF TEMPERATURE ON ETHANOL CONCENTRATION

Temperature has affectly influenced ethanol fermentation. Ethanol production was optimum at 32 °C compare to fermentation at 25 °C as shown in Figure 4.4. The average of ethanol yield at 32 °C is 1.258 g/L and it is higher than average ethanol yield at 25 °C which is 0.843 g/L. This is in agreement with work testified by other workers (Rainess-Casselman, 2005; Strand, 1998). Tamuinaidu et al., 2013 and Farhana, 2010 also recommended temperature range from 28 °C to 32 °C which based on the fermentation of ethanol in Nipa sap and oil palm trunk sap and use S. cerevisae as microorganism. Temperature showed obvious influence on ethanol production by the strain consuming sugar as carbon source and influenced the microorganism growth for the rapid conversion of the sugar into ethanol.

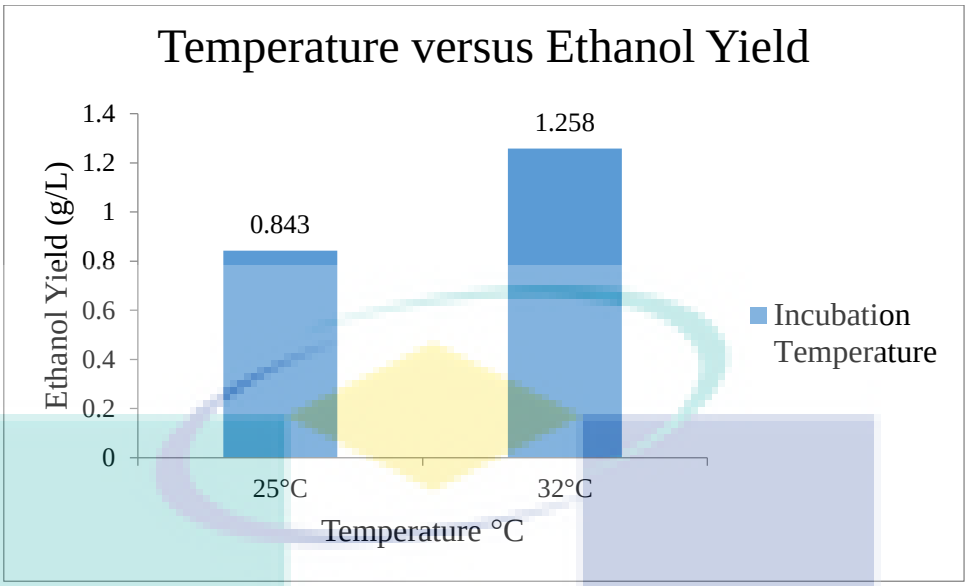

Figure 4.4. Influence of incubation temperature on ethanol yield

4.5 EFFECT OF PH ON ETHANOL CONCENTRATION

Initial pH of the fermentation media was kept in the range of 4 - 7. The maximum average ethanol production (1.163 g/L) was achieved at pH 4. With a further increase in pH ethanol production was declined (Figure 4.5). Control of pH during ethanol fermentation is essential for two reasons: 1) acidic solution will retard the growth of harmful bacteria. 2) Yeast cultivates well in acidic conditions (Mathewson, 1980). *Saccharomyces cerevisiae* can be influenced by pH value and the optimum pH range for this yeast is at pH 4 to 5 (Buzas et al., 1988). There are the possibilities that *S. cerevisae* cannot adapt at pH 7 which causing lower ethanol concentration produced in this experiment.

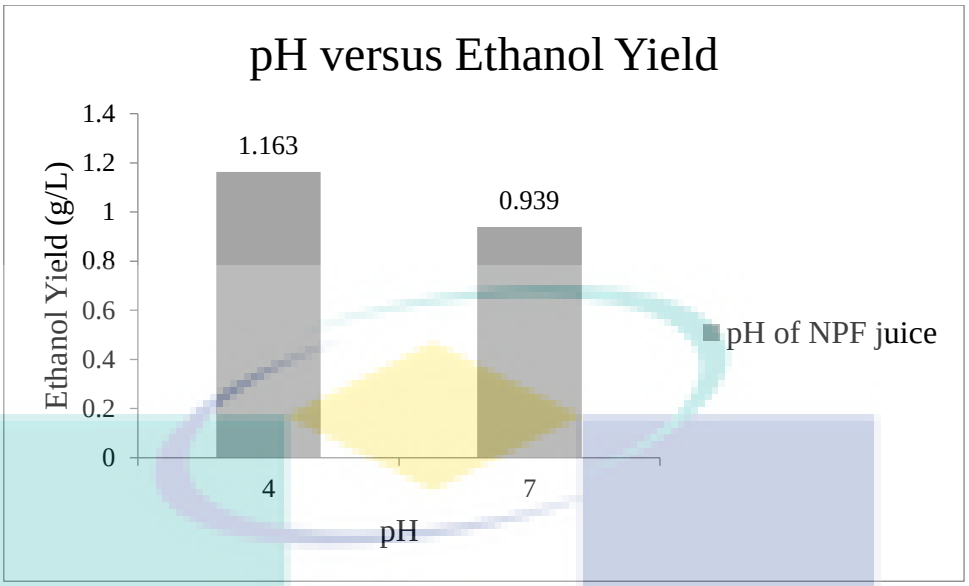

Figure 4.5. Influence of pH value on ethanol yield

4.6 EFFECT OF INCUBATION TIME ON ETHANOL CONCENTRATION

The fermentation was conducted at different time period 12 and 24 under designated conditions. As shown in Figure 4.6, maximum ethanol production was observed at 12 h (1.149 g/L) compared to ethanol concentration at 24 h (0.952 g/L). The ethanol production rate is the product of specific (per cell) productivity and concentration of cells. Primarily, the rate of alcohol production is relatively low, but as the number of yeast cells rises the overall production rate increases. The reason for this situation is the NPF juice may undergoes rapid fermentation. It has been proved in the research done by Tamunaidu et al. 2013 on the Nipa sap for production of ethanol. The research reported that the fermentation of Nipa sap were rapid in 12 hours and were almost complete after 30 hours. There is a chance the Nipa palm frond juice has similar characteristic with Nipa sap as both of these are part of Nipa palm.

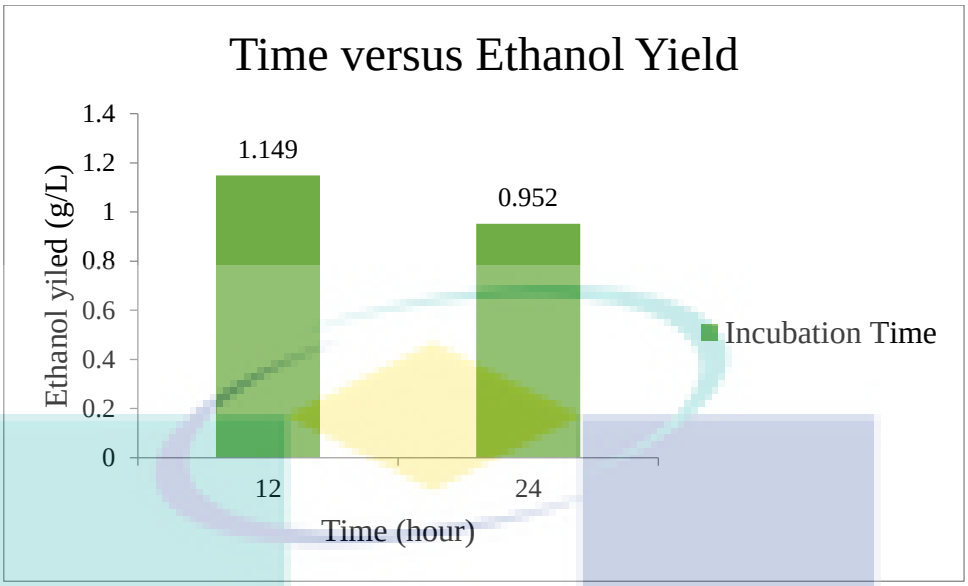

Figure 4.6. Influence of incubation time on ethanol yield

4.7 EFFECT OF CONCENTRATION OF NPF JUICE ON ETHANOL CONCENTRATION

During microbial fermentation, the carbon source not only fits as a major essential for building of cellular material, but it is also utilized for polysaccharides synthesis and as energy source (Dhake et al., 2007). In order to study the feasibility of NPF juice concentrate as a carbon source for bioethanol production by *Saccharomyces cerevisiae*, different juice concentration 50 % and 100 % were used. Figure 4.7 shows the profiles of bioethanol production at different initial concentration of NPF juice concentrates. It is found that ethanol yield for 100 % juice concentrated is higher (1.156 g/L) than 50 % juice (0.945 g/L). NPF juice with 100 % concentrated has higher sugar content than 50 % juice and this show that an optimal concentration of sugar used in fermentation process is capable to increase the ethanol yield efficiently (Siqueira et al., 2008).

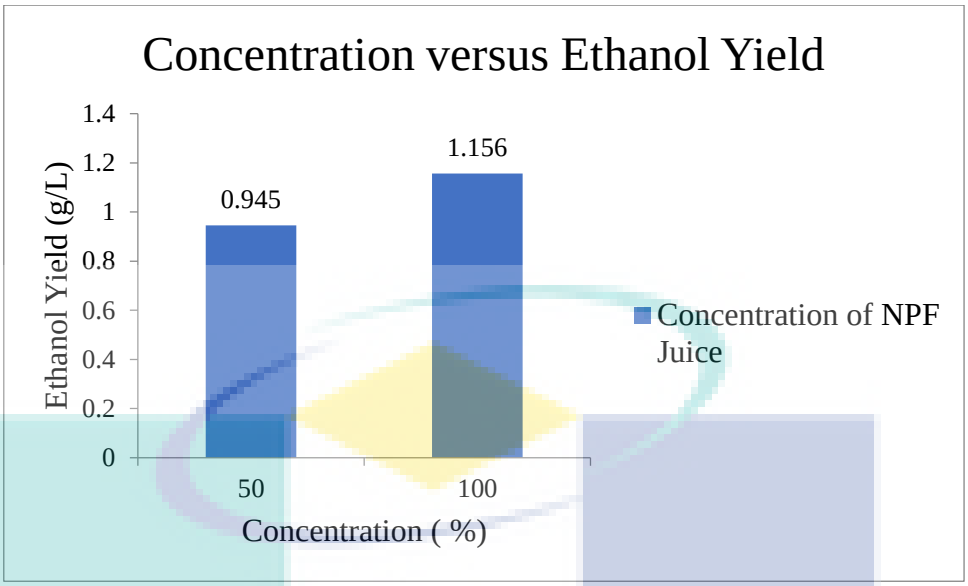

Figure 4.7. Influence of concentration of NPF juice on ethanol yield

4.8 EFFECT OF AGITATION SPEED ON ETHANOL CONCENTRATION

Agitation could be useful to the development and performance of the microorganism cells by refining the mass transfer characteristics with respect to substrates, products/byproducts and oxygen. Hence, agitation results in a better mixing of the fermentation broth, assisting to keep a concentration gradient between the interior and the exterior of the cells. Figure 4.8 shows that sample that agitated at 160 rpm (1.102 g/L) produce higher concentration of ethanol rather than sample that undergo fermentation at 80 rpm (0.999 g/L). Agitation also favors nitrogen supply to the cells that is important for high ethanol concentration.

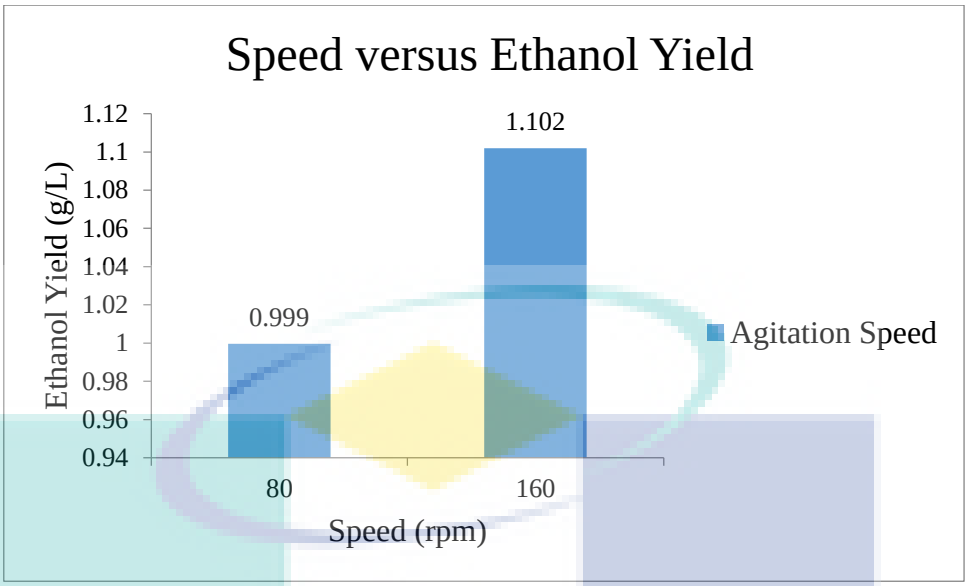

Figure 4.8. Influence of agitation speed on ethanol yield

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

Nipa palm frond juices were found to contain high amount of sugars which were composed of sucrose, glucose and fructose. The composition of sugar in Nipa palm frond juice was similar to Nipa sap. The previous research by Tamunaidu et al., 2013 has successfully showed the ability of Nipa sap in the ethanol fermentation. Research utilizing oil palm frond (OPF) juice as a fermentation feedstock for ethanol production also shows a positive results (Farhana, 2010). Based on these both results, this experiment was initiated to discover the potential of Nipa palm frond juice as a renewable feedstock for ethanol. It is found that the Nipa palm frond juice has the ability to produce ethanol but on low amount. The highest ethanol yield for this study is 1.541 g/L while the lowest concentration is 0.562 g/L.

Fractional factorial designs are mostly conducted in screening experiments for determination of significant factors, to propose the best condition for ethanol production. In this study, the fractional factorial designs by Design Expert software 7.1.6 were used to evaluate the significant factors in production of bioethanol from NPF juice. Five factors include incubation time, initial pH of NPF juice, concentration of NPF juice, agitation speed and incubation temperature were investigated as design variables and ethanol concentration (g/L) was considered as experimental design

response. The response was fitted with a multiple linear regression equation as shown in Equation (4.2). High correlation (R^2 = 0.9944) between the experimental data and model data was achieved. The ranking of factors in preparation of fermentation profile of NPF juice to produce high ethanol yield were incubation temperature > pH of NPF juice > concentration of NPF juice > incubation time > agitation speed. From the three factors, incubation temperature, concentration of NPF juice and agitation speed has positive effect on ethanol concentration produced. Though all three were significant at 95 % confidence interval, only incubation temperature and concentration of NPF juice above Bonferroni's corrected t-value limit shown in Figure 4.3. This comes to the conclusion only incubation temperature and NPF juice concentration were the significant factors for ethanol production.

The best fermentation conditions for ethanol production were calculated to be 12 hours, pH 4, 100 % juice concentration, 160 rpm and 32 °C for incubation time, pH of juice, concentration of NPF juice, agitation speed and incubation temperature, respectively. Under these optimum conditions, triplicate experiment was conducted to validate the suggested condition to verify the factorial model. Maximum ethanol concentration produced in this was 1.601 g/L, which was close to the predicted ethanol concentration 1.573 g/L with 1.573 % error. Thus, this study presents a promising potential of utilization of NPF juice as feedstock for ethanol alternative to the existing feedstock.

5.2 RECOMMENDATIONS

There are some recommendations for future research based on this study.

1. The present study has clearly shown that fermentation of Nypa palm frond juice can produced bioethanol at certain yield. The insights gained from this study provide a strong basis for continued development of Nipa palm frond fermentation with a crucial objective of obtaining higher ethanol yield. Future works on NPF may further improve fermentation factor such as incorporating the effect of inoculum size, utilizing another type of microorganism such as *Zymomonas mobilis*, variation of fermentation time, removal of fermentation inhibitor and use anaerobic chamber to create fully anaerobic condition. The size of inoculum may affect the production of ethanol as there will be more organism cells for conversion of sugar in NPF juice to ethanol.

2. Optimization of the fermentation profile by Response Surface Method (RSM) should be executed for future work as we still need to optimize the correlation between suggested factor and condition propose in this study. Application of statistical methods in bioprocess optimization such as response surface methodology (RSM) is used in current years in the documentation of the effects of individual variables and the determination of the optimum conditions of a multivariable system (Chongkhong et al., 2012). RSM can calculate the main effects and factor interactions, hence making its use a important part in developing large scale biotechnological processes such as bioethanol production from various substrates (Karuppaiya et al., 2010; Uncu and Cekmecelioglu, 2011). The main advantage of RSM is the reduced quantity of experimental trials necessary to estimate numerous parameters and also their relations (Karacan et al., 2007). Furthermore, even in the occurrence of complex relations, RSM can be used to observe

the comparative importance of multiple affecting factors. Optimization of the fermentation process using RSM has been utilized to improve productivity without increasing cost.

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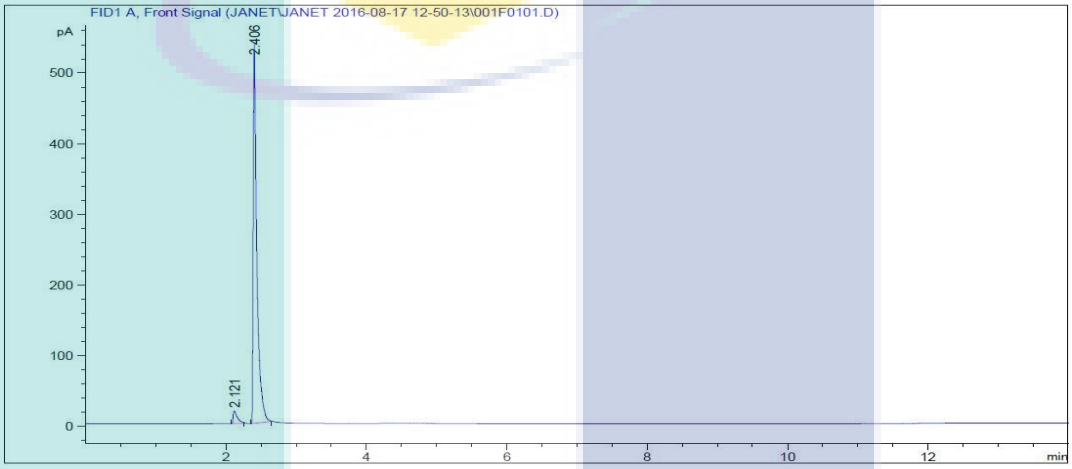
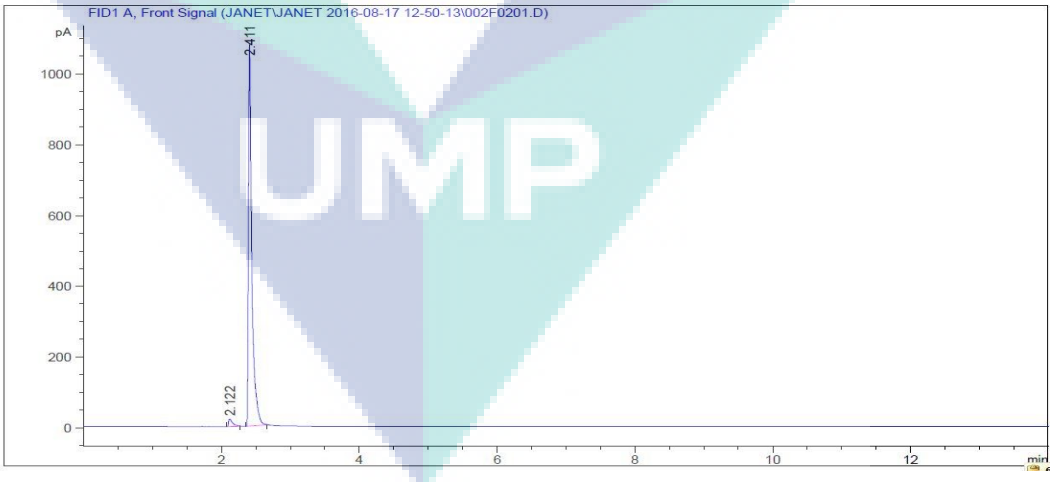
APPENDIX A

GC FID CHROMATOGRAPHY

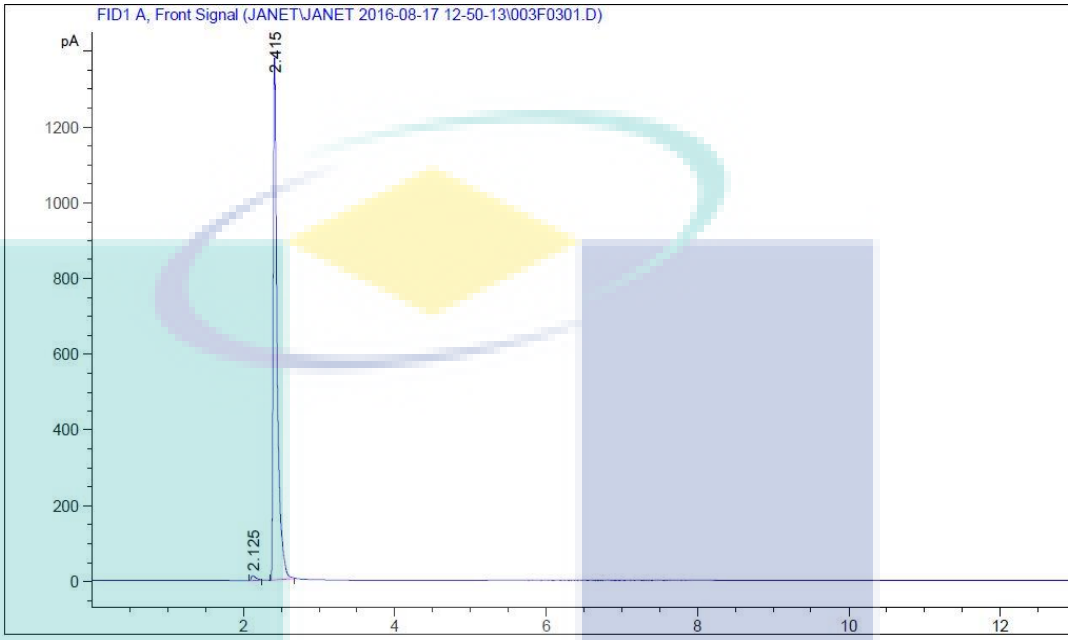
A-1 Ethanol standard 2 g/L



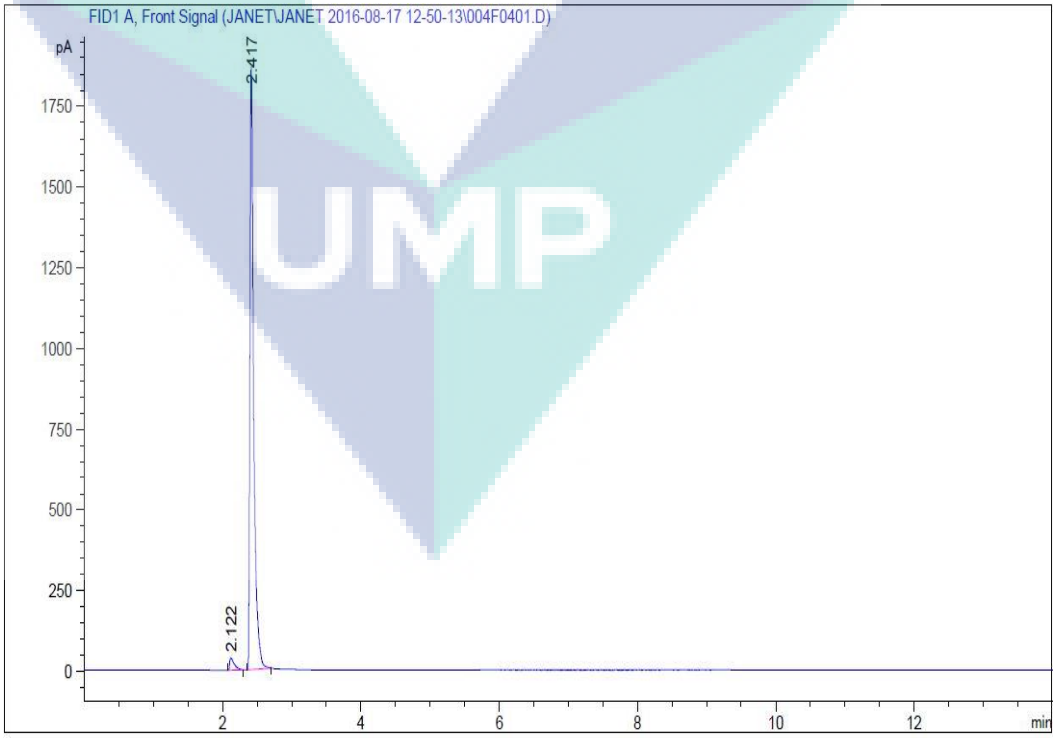
A-2 Ethanol standard 4 g/L



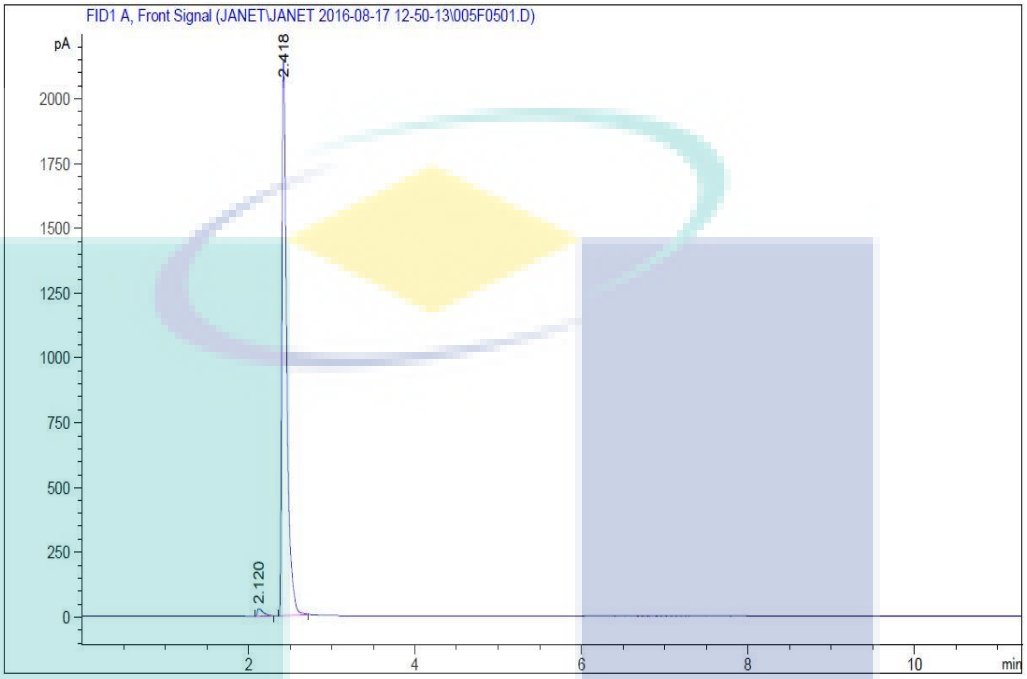
A-3 Ethanol standard 6 g/L



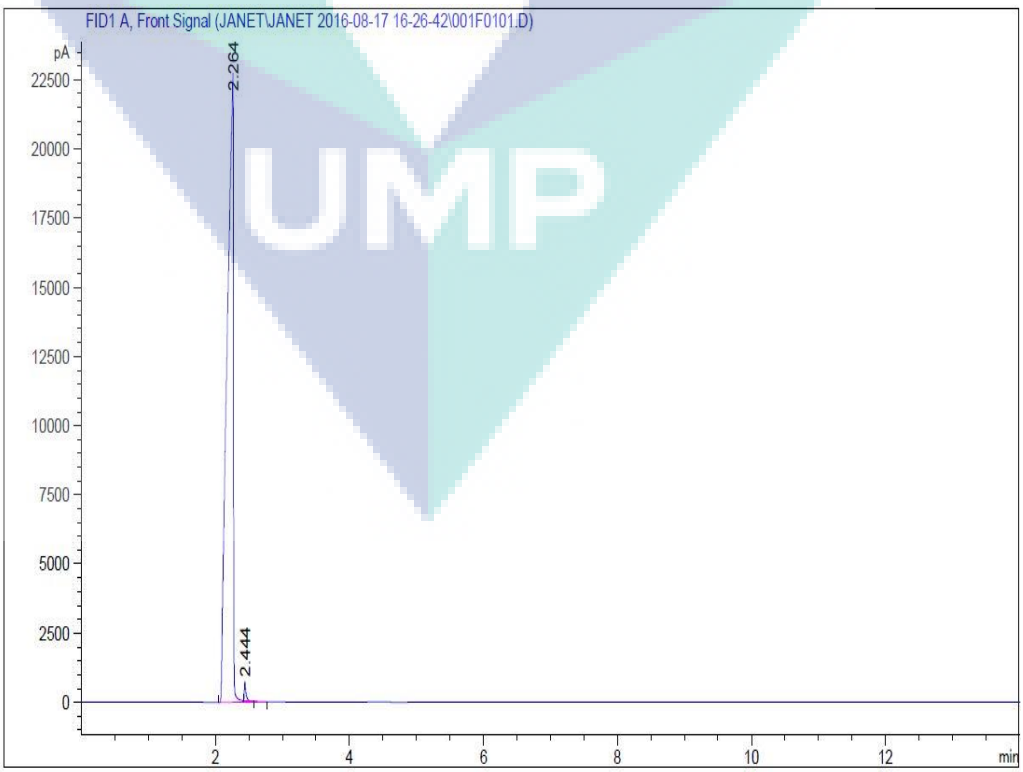
A-4 Ethanol standard 8 g/L



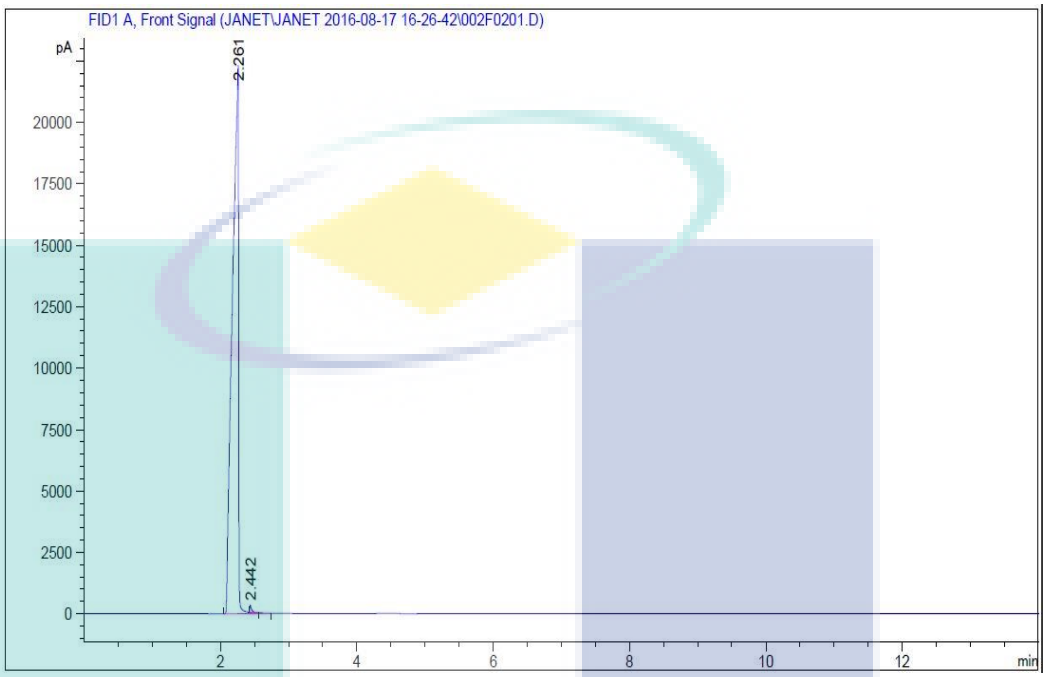
A-5 Ethanol standard 10 g/L



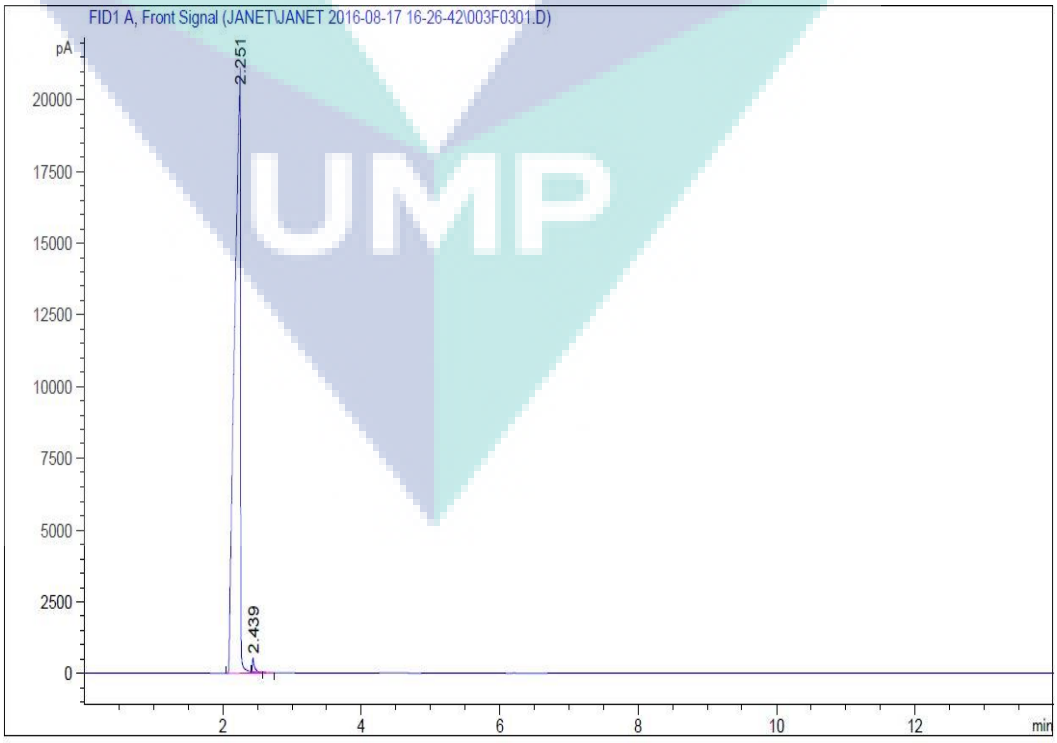
A-6 Sample Fermentation 1



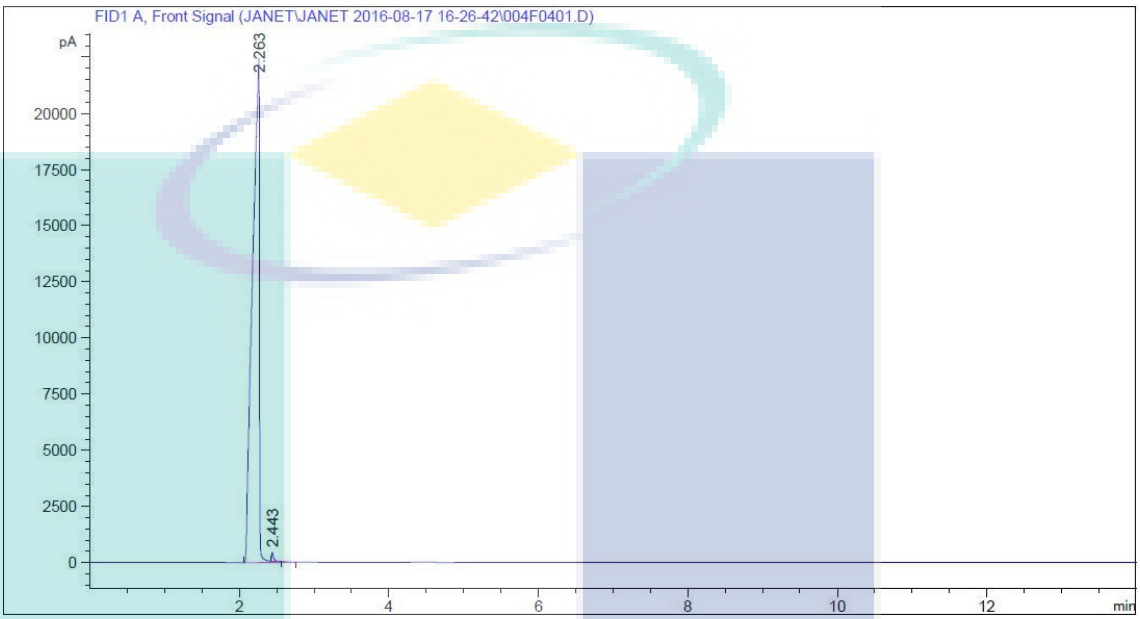
A-7 Sample Fermentation 2



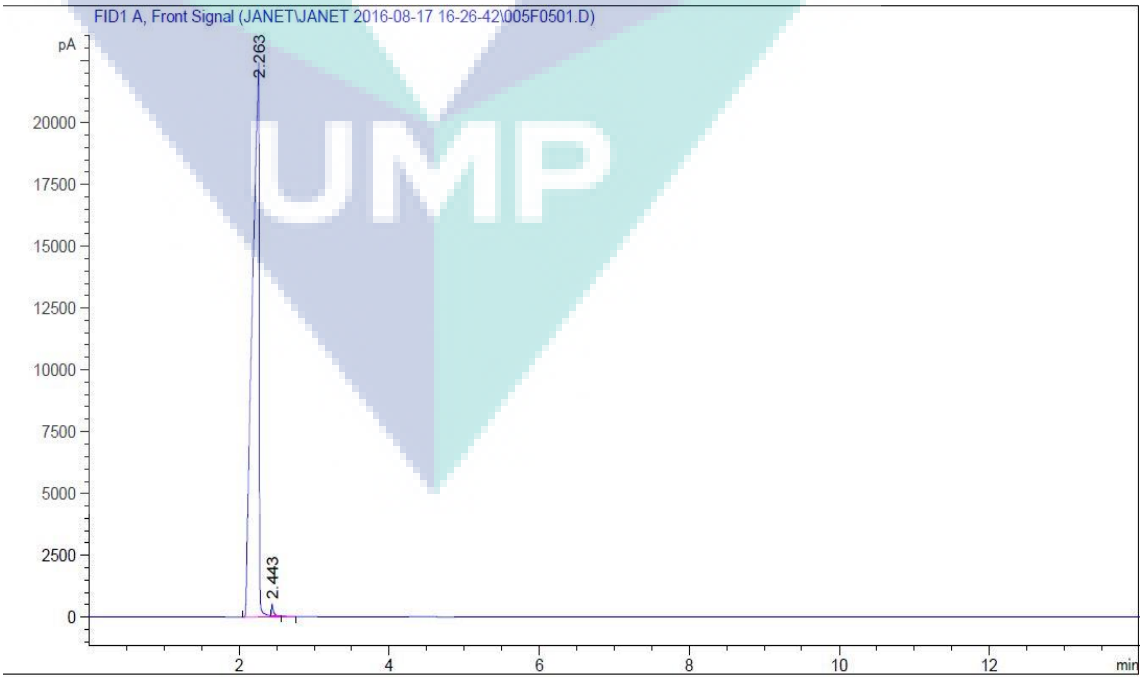
A-8 Sample Fermentation 3



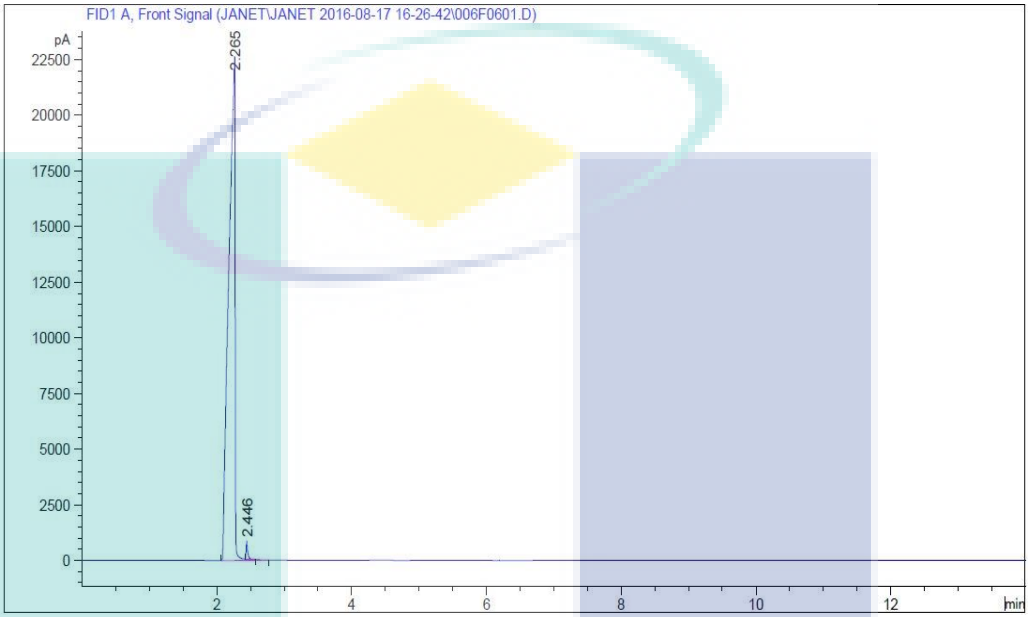
A-9 Sample Fermentation 4



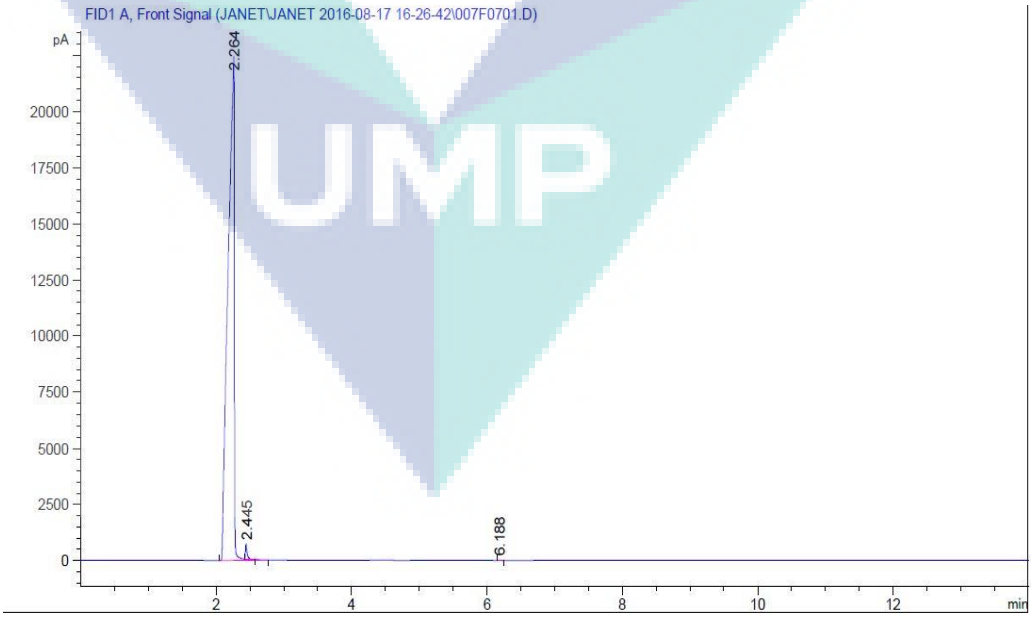
A-10 Sample Fermentation 5



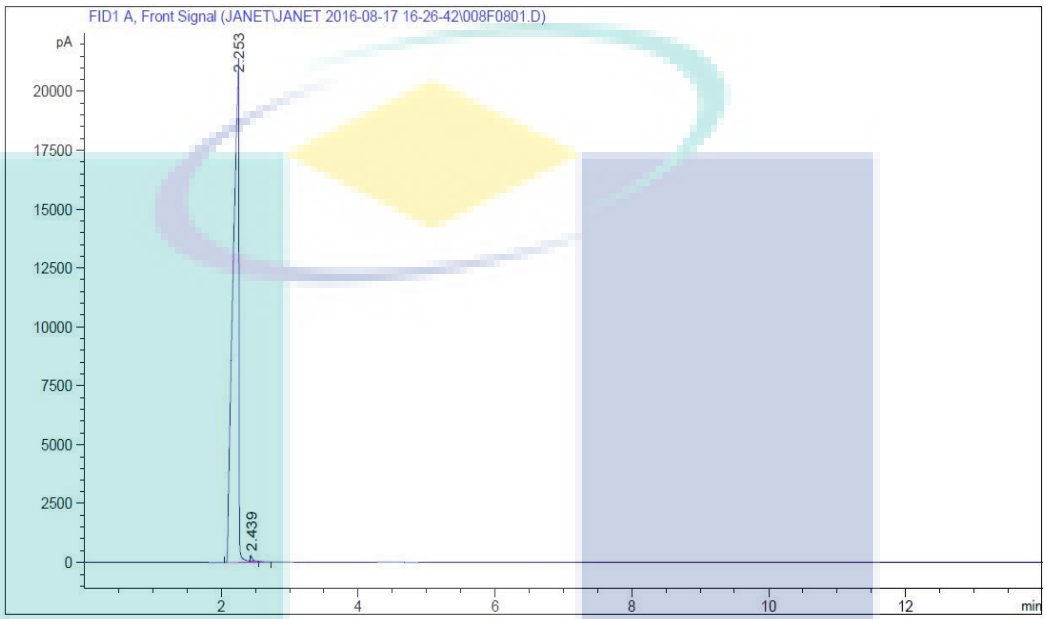
A-11 Sample Fermentation 6



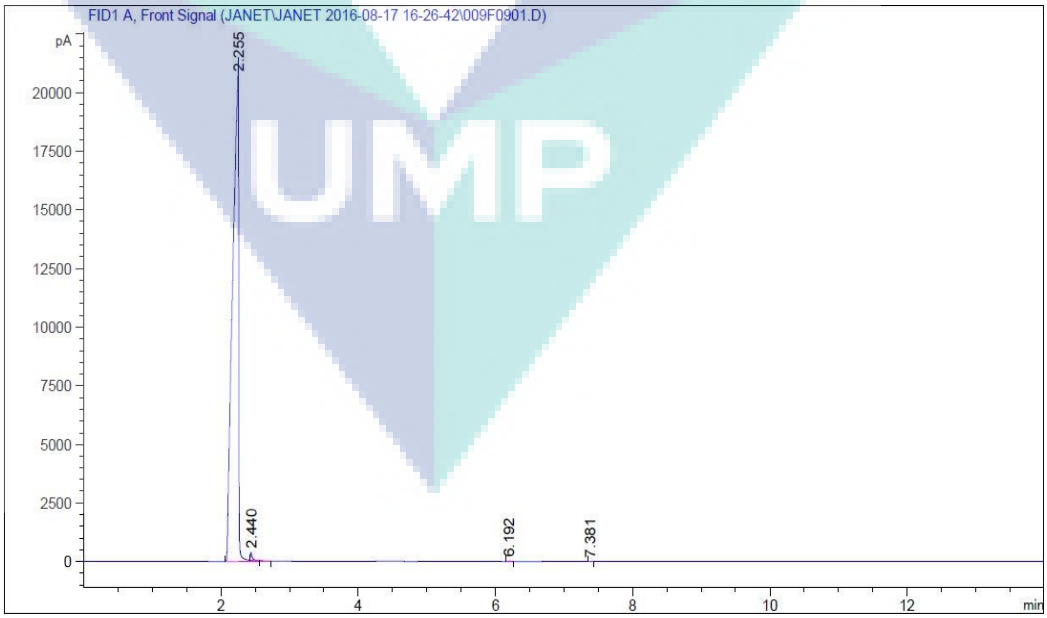
A-12 Sample Fermentation 7



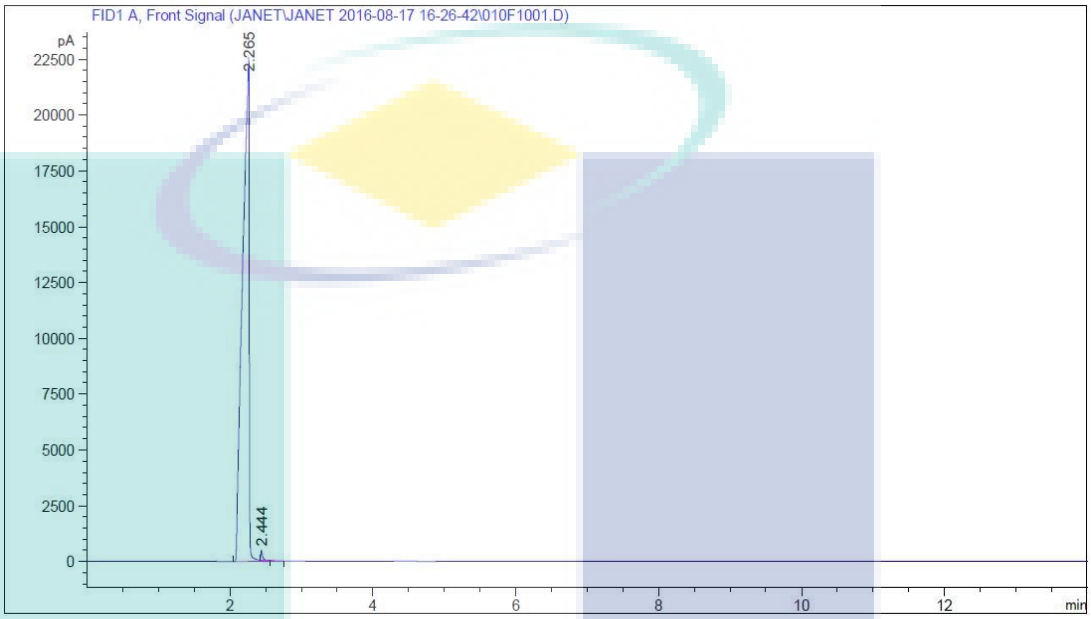
A-13 Sample Fermentation 8



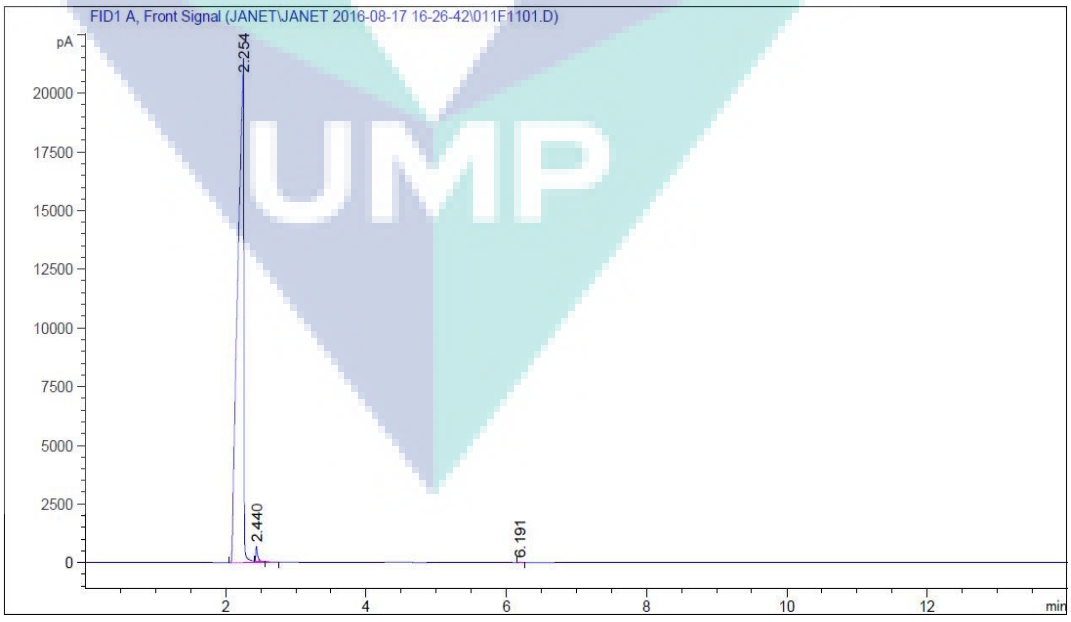
A-14 Sample Fermentation 9



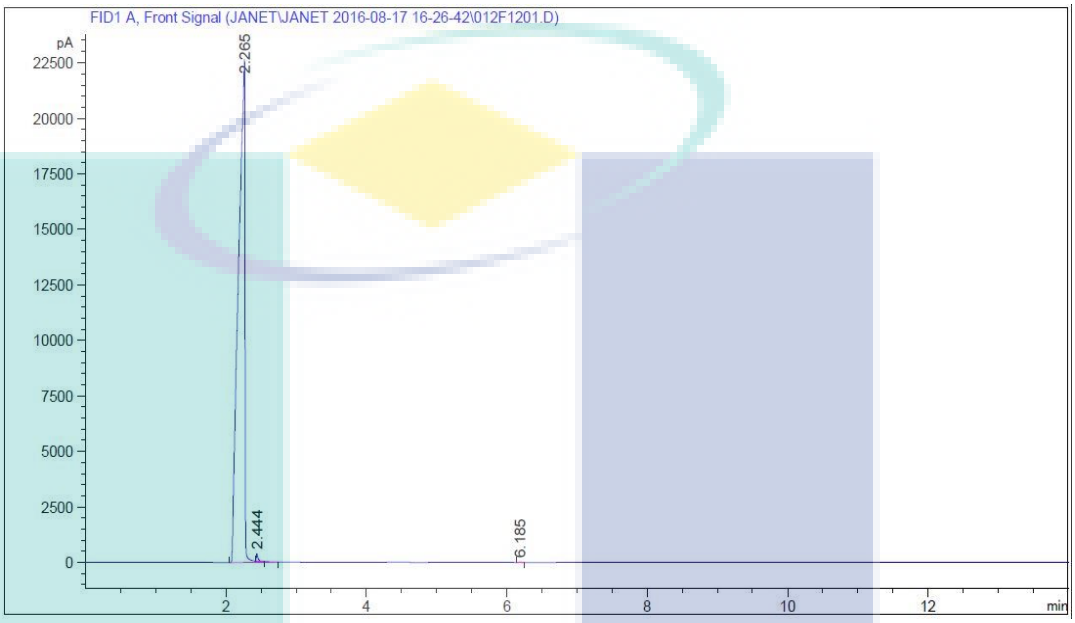
A-15 Sample Fermentation 10



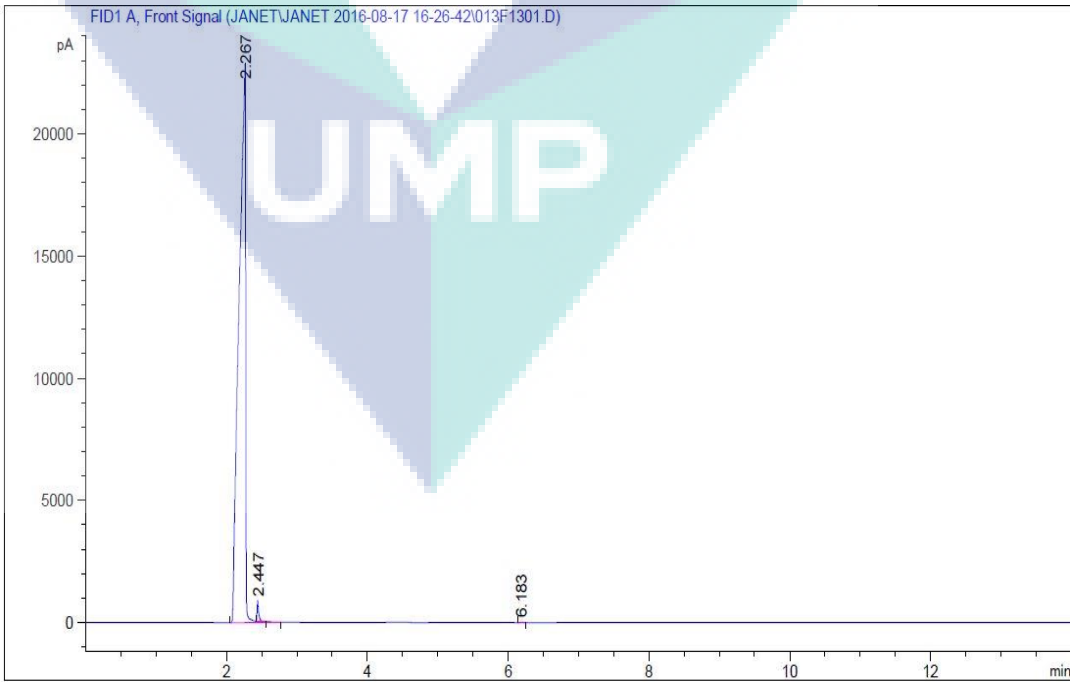
A-16 Sample Fermentation 11



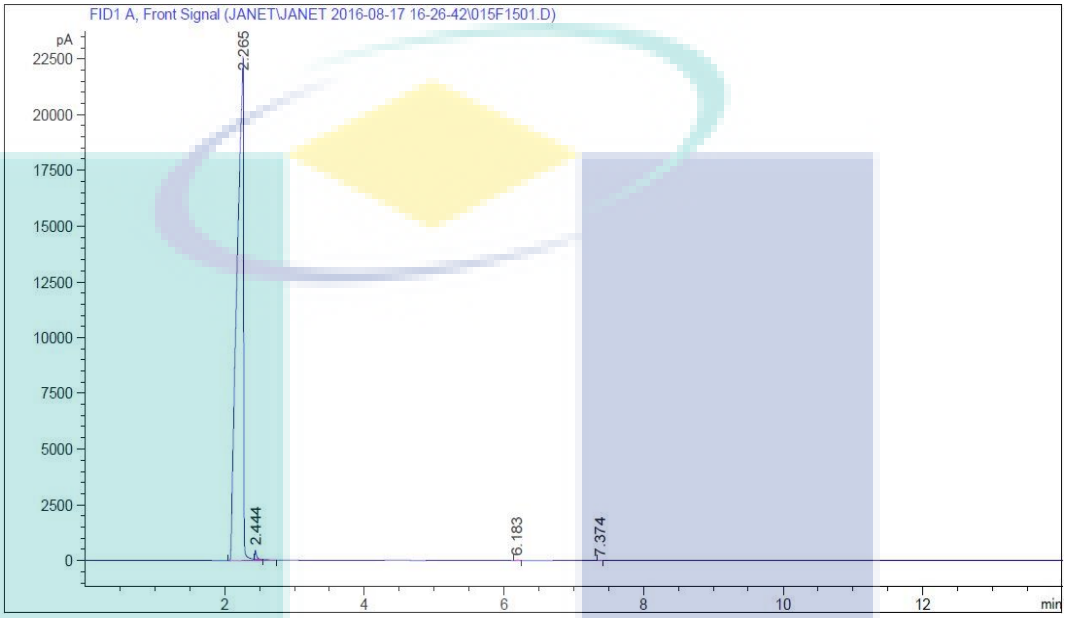
A-17 Sample Fermentation 12



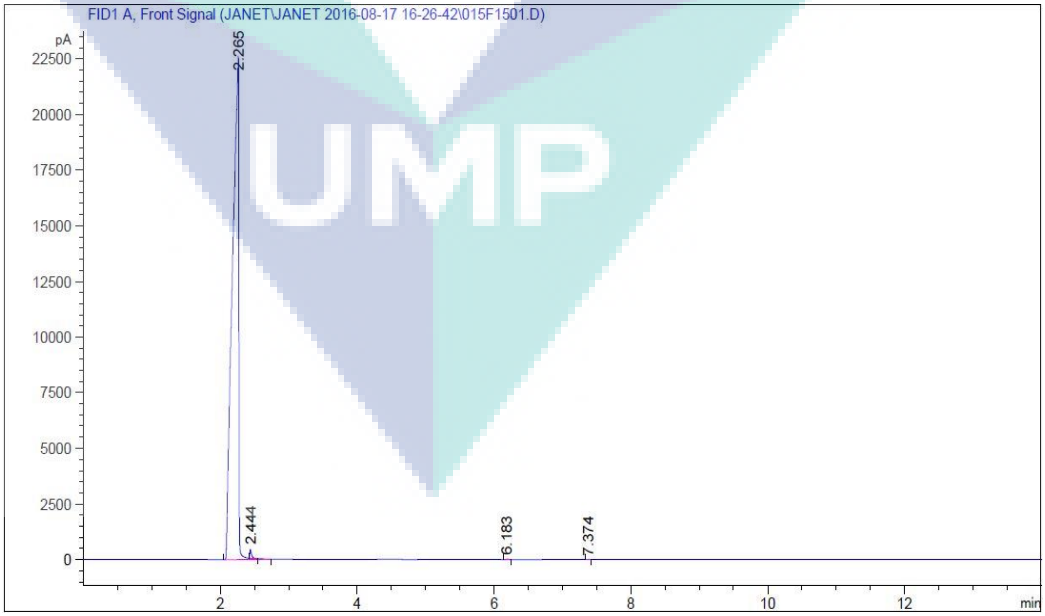
A-18 Sample Fermentation 13



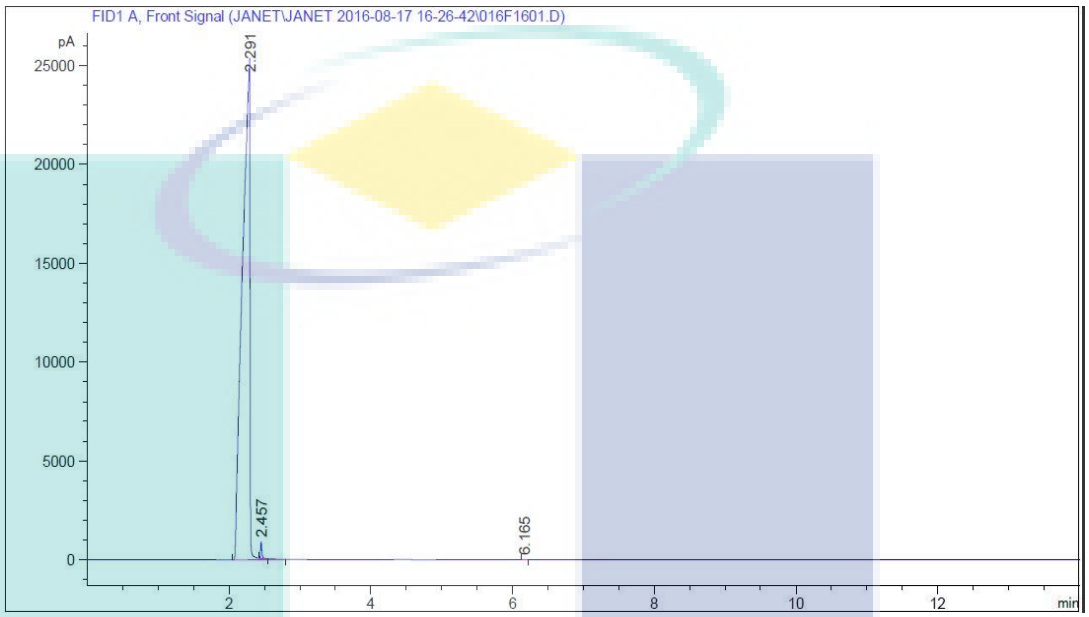
A-19 Sample Fermentation 14



A-20 Sample Fermentation 15



A-21 Sample Fermentation 16



APPENDIX B

ETHANOL CONCENTRATION TABLE

B-1 Area under the curve for ethanol standard

Area	Concentration (g/L)
1925.963	2
3671.441	4
5093.693	6
7076.156	8
8845.24	10

B-2 Area under the curve for fermentation sample

Sample	Area (pA*s)	g/L
1	1228.627	1.392133
2	562.8832	0.637792
3	646.4802	0.732514
4	742.4284	0.841231
5	835.7284	0.946947
6	1348.209	1.527628
7	1197.226	1.356553
8	496.4152	0.562478
9	974.1338	1.103772
10	771.8092	0.874522
11	1155.798	1.309612
12	592.6234	0.67149
13	1359.929	1.540909
14	1126.714	1.276658
15	717.4683	0.812949
16	1082.229	1.226252

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