IMPROVED BIOETHANOL PRODUCTION FROM OIL PALM TRUNK SAP

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ABSTRACT

Renewable energy such as bioethanol produced from biomass waste is gaining a lot of interest worldwide due to depletion of fossil fuel reserve. The OPT felled for replanting is a suitable raw material to produce bioethanol because it is an abundant waste material produced by the palm oil industry in Malaysia. In order to utilize the felled OPT, fermentation by suitable microorganism can be performed to obtain bioethanol. Hence, this study was carried out to select the best microorganisms for bioethanol fermentation using OPT sap and to optimise nutrient supplementation in OPT sap for bioethanol fermentation. In this study, four microorganisms were tested for ethanol production at fixed temperature, pH, agitation and inoculum size which are *Saccharomyces cerevisiae* ATCC 9763, *Saccharomyces cerevisiae* CCT 0762, dry baker's yeast and *Kluyveromyces marxianus* ATCC 46537. The parameters were set at inoculum size of 10 % (v/v), agitation speed of 150 rpm, incubation temperature of 30 ºC and fermentation time of 72 hours. Two types of analytical method were performed to analyse the data which are cell dry weight measurement and High Performance Liquid Chromatography (HPLC). The results showed that *K. marxianus* ATCC 46537 produced the highest ethanol yield (0.39 g/g) at a shorter fermentation time (16 h) compared to the other strains. Then, response surface methodology (RSM) was employed to optimise the nutrient supplementation in OPT sap. Using Two-level Factorial Design, six nutrients, namely ammonium sulphate, di-ammonium hydrogen phosphate, magnesium sulphate, β-alanine, calcium chloride and potassium dihydrogen phosphate were screened using *K. marxianus* ATCC 46537 and the selected significant nutrients were magnesium sulphate and β-alanine. Subsequently, the optimisation study using Central Composite Design found the optimum value of magnesium sulphate was 7.93 g/L and 0.90 g/L for β-alanine. Under optimum conditions, the predicted ethanol concentration was 0.46 g/g while the experimental value (0.47 g/g) was in agreement with the predicted value with 2.13 % error. As a conclusion, *K. marxianus* ATCC 46537 were able to improve ethanol production from 0.39 g/g without nutrient supplementation into 0.47 g/g with nutrient optimisation using RSM.

ABSTRAK

Tenaga yang boleh diperbaharui seperti bioetanol yang dihasilkan daripada sisa biojisim semakin mendapat perhatian di seluruh dunia disebabkan oleh pengurangan rizab bahan api fosil. Batang pokok kelapa sawit yang ditebang untuk penanaman semula merupakan bahan mentah yang sesuai bagi menghasilkan bioetanol kerana ia adalah bahan buangan yang banyak dihasilkan oleh industri minyak kelapa sawit di Malaysia. Dalam usaha untuk menggunakan batang pokok kelapa sawit yang ditebang ini, penapaian oleh mikroorganisma yang sesuai boleh dilaksanakan untuk mendapat bioetanol. Oleh itu, kajian ini dilakukan untuk memilih mikroorganisma yang terbaik bagi menghasilkan bioetanol menggunakan jus batang kelapa sawit dan untuk mengoptimakan penambahan nutrien dalam jus kelapa sawit semasa penapaian bioetanol. Dalam kajian ini, empat mikroorganisma telah diuji untuk pengeluaran etanol pada suhu, pH, agitasi dan saiz inokulum yang tetap, iaitu *Saccharomyces cerevisiae* ATCC 9763, *Saccharomyces cerevisiae* CCT 0762, serbuk penaik yis dan *Kluyveromyces marxianus* ATCC 46537. Parameter telah ditetapkan pada saiz inokulum 10 % (v/v), kelajuan agitasi 150 rpm, suhu inkubasi 30 °C dan masa penapaian selama 72 jam. Dua jenis kaedah analisis telah dijalankan untuk menganalisis data iaitu pengukuran berat kering sel dan *High Performance Liquid Chromatography* (HPLC). Hasil kajian menunjukkan bahawa *K. marxianus* ATCC 46537 menghasilkan jumlah etanol tertinggi (0.39 g/g) pada tempoh penapaian yang lebih pendek (16 h) berbanding dengan jenis lain. Kemudian, *response surface methodology* (RSM) telah digunakan untuk mengoptimumkan penambahan nutrien dalam jus batang kelapa sawit. Dengan menggunakan *Two-level Factorial Design*, enam nutrien iaitu ammonium sulfat, di-ammonium hidrogen fosfat, magnesium sulfat, β-alanina, kalsium klorida and kalium fosfat dihydrogen telah disaring menggunakan *K. marxianus* ATCC 46537 dan nutrien penting yang terpilih adalah magnesium sulfat dan β-alanina. Selepas itu, kajian pengoptimuman menggunakan *central composite design* mendapati nilai optima magnesium sulfat adalah 7.93 g/L and 0.90 g/L bagi β-alanina. Di bawah keadaan optimum, kepekatan etanol yang diramalkan adalah 0.46 g/g sementara nilai sebenar eksperimen yang diperoleh (0.47 g/g) adalah selari dengan nilai yang diramalkan dengan ralat sebanyak 2.13 %. Kesimpulannya, *K. marxianus* ATCC 46537 dapat meningkatkan pengeluaran etanol daripada 0.39 g/g tanpa penambahan nutrien kepada 0.47 g/g dengan penambahan nutrien menggunakan RSM.

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B	Beta
°C	Degree Celsius
%	Percentage

LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ANOVA	Analysis of variance
APEC	Asia Pacific Economic Cooperation
ATCC	American Type Culture Collection
C	Carbon
Ca^{2+}	Calcium ion
CCD	Central composite design
Cm	Centimetre
CV	Coefficient of variation
ENO	Enolase
Eq.	Equation
FBPA	Fructose biphosphate aldolase
GADPH	Glyceraldehydes-3-phosphate dehydrogenase
GC	Gas Chromatography
GNTK	Gluconate kinase
h	Hour
H	Hydrogen
HK	Hexokinase
HPLC	High Performance (Pressure) Liquid Chromatography
K	Potassium
K^{+}	Potassium ion
ktoe	Kilo tonne of oil equivalent
MEIH	Malaysia Energy Information Hub

Mg	Magnesium
Mg^{2+}	Magnesium ion
Min	Minute
mM	Millimolar
Mn^{2+}	Manganese ion
MJ/Kg	Mega Joules per Kilogram
Mtoe	Million tonne of oil equivalent
MPOB	Malaysian Palm Oil Board
N	Nitrogen
N/A	Not available
Ni^{2+}	Nickel ion
NH^{4+}	Ammonium ion
Nm	Nanometre
O	Oxygen
OD	Optical Density
OPT	Oil palm trunk
P	Phosphate
PDC	Pyruvate decarboxylase
PFK	Phosphofructokinase
PGI	Phosphoglucoisomerase
PGK	Phosphoglycerate kinase
PGM	Phosphoglyceromutase
PYK	Pyruvate kinase
S	Sulphur
T	Temperature

TPI	Triose phosphate isomerase
YPD	Yeast extract-Peptone-Dextrose
Zn^{2+}	Zinc ion

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND STUDY

Energy demand and supply in Malaysia are facing many issues like fuel supply and pricing, energy security, energy efficiency and conservation (Tan *et al*., 2013). Crude oil, natural gas, coal and hydropower as the primary energy sources have increased by 9.07% from 77,041 ktoe to 84,733 ktoe in 2011 and 2012 respectively (MEIH, 2014). Globally, the energy projection has shown a continued increase in natural oil reserves exploitation for the years to come. Therefore, deployment of renewable energy such as biomass fuels will cause less dependency towards fossil fuels and ensure energy security in the future.

Biothanol production from renewable resources such as biomass is currently gaining a lot of interest worldwide as the most promising biofuels (Vohra *et al*., 2014). Many advantages can be gained by using bioethanol as a fuel since it extends the shelf life of petroleum and reduces the reliance on oil imports (Goldenberg, 2007). Not only that, bioethanol has higher oxygen content compared to gasoline which can results in cleaner combustion (Cot *et al*., 2007) and reduce carbon dioxide emission into the atmosphere (Balat *et al*., 2008). Most importantly, bioethanol is readily available from common biomass source (Demirbas, 2008) which can contribute to sustainability as biomass is a renewable resource.

In order to maintain oil productivity, oil palm trees for palm oil production at an interval of 20 to 30 years need to be replanted (Lim *et al*., 1997; Yamada *et al*., 2010) and this will generate about 15.2 million tonnes of oil palm trunks annually (Fazilah *et*

al., 2009). As a result, the felled palm trunks become one of the most important biomass resources in Malaysia. Although there is an attempt to manufacture plywood using the felled palm trunks, only the outer part is partially used while the inner part is removed in huge amounts due to its fragile properties (Kosugi *et al*., 2010). According to Kosugi *et al*. (2010), old oil palm trunk (OPT) that has been felled for replanting contains high glucose content in the sap. Due to the high concentration of glucose present in the OPT sap, it is very suitable to be converted into bioethanol.

The conversion into bioethanol from OPT sap is possible using direct microbial fermentation. The usual fermenting microorganism used in this process is yeast, particularly *Saccharomyces cerevisiae* (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 maximise 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).

Over the years, statistical methods have been applied in bioprocess optimisation. Statistical design, particularly response surface methodology (RSM) is used mainly because it can identify the effects of individual variables and efficiently determine the optimum conditions of a multivariable system (Chongkhong *et al*., 2012). RSM can predict the main effects and factor interactions, thus making its use a vital part in developing large scale biotechnological processes such as bioethanol production from various substrates (Karuppaiya *et al*., 2010; Uncu and Cekmecelioglu, 2011).

1.2 PROBLEM STATEMENT

The OPT felled for replanting is a suitable raw material because it is an abundant waste material produced by the palm oil industry in Malaysia. In order to utilize the felled OPT, especially the inner part, fermentation appears to be the simplest and most viable way to obtain suitable bioethanol concentrations in the broths for distillation. This is because OPT sap contain high amount of sugar which can be a potential feedstock for bioethanol production using direct microbial conversions. Out of number of microorganisms available for direct conversions of sugar into bioethanol,

only a few may be considered as a good fermenting agent. Thus, selecting the best microorganism is crucial provided the feedstock is utilizable by the microorganisms which in this study is OPT sap. Microorganisms used in the fermentation can ferment high amount of sugars in the medium when all essential nutrients are available in sufficient amounts (Bafrncová *et al.*, 1999; Liu and Qureshi, 2009; Watanabe *et al*., 2007). Particular nutrients such as nitrogen, trace elements or vitamins are desired to obtain rapid fermentation with high ethanol yield. Therefore, it is crucial to exploit these nutrient sources to supply the nutritional requirements for microorganism growth and fermentation.

1.3 OBJECTIVE

The objectives of this study are outlined below.

1. To select the best microorganisms for bioethanol fermentation using OPT sap.
2. To optimise selected nutrient supplementation in OPT sap for bioethanol fermentation.

1.4 SCOPE

This research focus on optimising bioethanol production from OPT sap to produce bioethanol from renewable resources. First, the sugar composition in OPT sap was determined using HPLC. Then, the kinetic study of four microorganisms were carried out in their standard medium at fixed temperature, initial pH, agitation and inoculum size which are *Saccharomyces cerevisiae* ATCC 9763, *Saccharomyces cerevisiae* CCT 0762, baker's yeast and *Kluyveromyces marxianus* ATCC 46537. Subsequently, these four strains were tested for bioethanol fermentation using OPT sap at fixed temperature, initial pH, agitation and inoculum size. Next, the best strain selected was utilized in Two-level Factorial Design which includes ammonium sulphate, di-ammonium hydrogen phosphate, magnesium sulphate, β-alanine, calcium chloride and potassium dihydrogen phosphate at fixed temperature, pH, agitation and inoculum size. One Factor at a Time (OFAT) was applied to determine the concentration range of significant factors obtained from the Two-level Factorial Design for optimisation study. Finally, the range obtained from OFAT was used in Central

Composite Design to get the concentration of the factors that will give the optimum yield.

1.5 SIGNIFICANCE OF STUDY

From this study, the microorganism that produced the best yield from OPT sap can be found and the optimum nutrient can be acquired for bioethanol production from OPT sap.

1.6 OVERVIEW OF THE THESIS

The remainder of this thesis is overviewed as follows. Chapter 2 reviews on the bioethanol production from various feedstocks. Oil palm plantation and the composition of oil palm trunk sap are also reviewed. Furthermore, a literature review on microorganisms used in fermentation is presented too. Besides that, a review on the addition of inorganic nutrients on bioethanol yield from sugar fermentation is presented. Finally, the method of analysing sugar and bioethanol is also reviewed in this chapter.

Chapter 3 specifically presents on the materials and methods engaged in this study which includes the source of raw materials and chemicals. Details on all type of equipment, experimental methods and procedures are also presented. Chapter 4 discusses on the results and discussion for the composition of sugar in OPT sap, the screening of bioethanol production in OPT sap by six different microorganisms and the screening and optimisation of nutrient addition in OPT sap using the selected strain.

Finally, Chapter 5 presents the conclusion of the study and recommendations for future research.

CHAPTER 2

LITERATURE REVIEW

2.1 BIOETHANOL

Germany and France has been using bioethanol in internal combustion engines as early as 1894 and started to be used as a fuel in Brazil since 1925 (Balat and Balat, 2009). Since 1980s, bioethanol become the promising alternative fuel in many countries (Balat, 2005; Demirbas 2004) due to the oil crises of the 1970s (Balat and Balat, 2009) and the urge to reduce greenhouse gas emission. Table 2.1 presents the properties of bioethanol as fuels.

Table 2.1: Some properties of bioethanol as fuels

Fuel property	Ethanol
Cetane number	8
Octane number	108
Auto-ignition temperature (K)	606
Latent heat of vaporization (MJ/Kg)	0.91
Lower heating value (MJ/Kg)	26.7

Source: Balat and Balat (2009)

Bioethanol is applicable as a vehicle fuel because of its high octane number, broader flammability limits, higher flame speeds and higher heats of vaporization than gasoline. This will apply for a higher compression ratio, shorter burn time, and leaner burn engine in an internal combustion engine (Balat and Balat, 2009). The most imperative thing is that 5% bioethanol can be blended with the petroleum products (Balat and Balat, 2009) and burned in traditional combustion engines with virtually no

modifications needed (Loppacher and Kerr, 2005). At higher bioethanol level, E85 which consists of 85% bioethanol and 15 % gasoline is the major blended fuel (Balat, 2007) but requisite for engine modification (Demirbas, 2007).

Energy demand in Malaysia is having a rapid increase as shown in Figure 2.1. It is estimated in 2030, energy demand will reach nearly 100 Mtoe (million tonne of oil equivalent). From Table 2.2, Malaysia energy consumption in 2012 depended heavily on fossil fuels (crude oil, natural gas, coal and coke) and only 0.3 % of energy came from renewable sources such as biomass, biodiesel and biogas (excluding hydropower). If this trend keeps on going, energy security in Malaysia will be jeopardized as Malaysia fossil fuel reserves is forecast to last only for another 30-40 years (Hassan *et al*., 2005). The price of crude oil is getting more expensive each year and a lot of money has been spent on subsidy to keep the cost of energy low. Due to this matter, Malaysian government are beginning to look for consistent source of renewable energy instantly to support the energy demand particularly in transportation fuel.

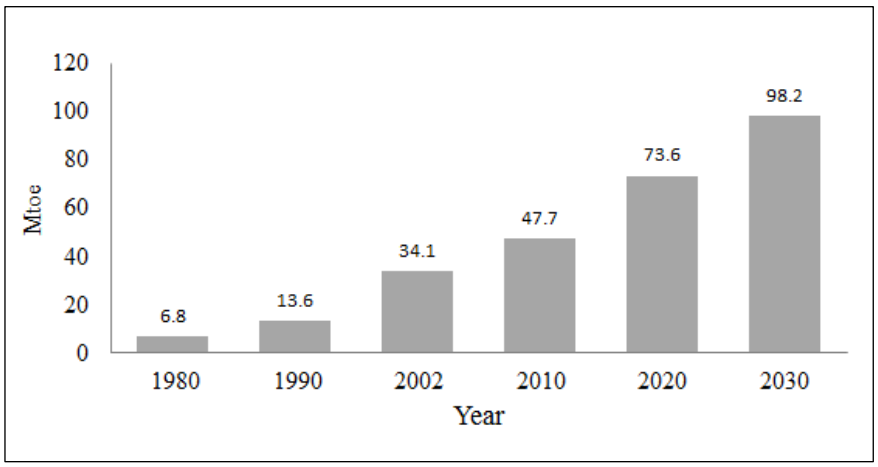

Figure 2.1: Energy demand in Malaysia

Source: APEC (2006)

Table 2.2: Malaysia energy consumption in 2012

Energy source	Percentage (%)
Crude oil	33.4
Natural gas	46.0
Coal and coke	18.9
Hydropower	2.6
Biodiesel	0.1
Biomass	0.2
Biogas	0.0

Source: MEIH (2014)

Malaysia is the world largest producer and exporter of palm oil which consequently produce vast amount of biomass from its plantation and milling activity. Oil palm biomass includes empty fruit bunches, fronds and trunks can thus have a good potential to be converted into renewable energy sources. Remarkably, the production of biofuel in Malaysia is still low as motor vehicles is still depending on fossil fuel. However, due to increase in crude oil price and global temperature, Malaysian government is creating a lot of interest in biofuel from renewable sources to decrease dependency towards fossil fuels. The National Biofuel Policy (2005) introduced a biodiesel fuel blend (B5) in late 2009 by establishing biodiesel B5 standards and deploying biodiesel at selected petrol stations (Mekhilef *et al*., 2011). However, this plan was not successful in Malaysia generally due to the competition from the food industry and the high price of palm oil (Daud, 2009).

Production of bioethanol using oil palm biomass (the second-generation of bioethanol) has a greater potential in substituting fossil fuels as it is not derived from the edible sources (the first-generation of bioethanol). Hence, there is no issue on food versus fuel as second-generation of bioethanol does not compete with the human food supply. If biomass were fully exploited to produce second-generation bioethanol, liquid bioethanol can replace fossil fuels in vehicle and fulfil 35.5 % of the Malaysia's energy demand with a cleaner and sustainable renewable energy (Goh *et al*., 2010). Since many vehicles in Malaysia operate using gasoline, the bioethanol market in Malaysia may

potentially be greater compared to biodiesel. A forecast of complete substitution of fossil fuels with bioethanol has been computed in Table 2.3.

Table 2.3: Malaysia's population and potential bioethanol demand

State	Population	Energy demand for transportation (Gj/day)	%	Area of oil palm plantation (Ha)	%	Potential Bioethanol demand (ton/day)
Johor	3,101,200	21,480	11.9	595,524	16.0	796
Kedah	1,848,100	12,800	7.1	71.934	1.9	474
Kelantan	1,505,600	10,428	5.8	79,146	2.0	386
Labuan	83,500	578	0.3	-	0.0	21
Melaka	713,000	4,938	2.7	45,816	1.0	183
N. Sembilan	846,300	5,862	3.3	149,879	4.0	217
Pahang	1,427,000	9,884	5.5	563,809	15.0	366
Perak	2,256,400	15,628	8.7	323,535	9.0	58
Perlis	224,500	1,555	0.9	258	0.0	377
Pulau Pinang	1,468,800	10,173	5.6	13,010	0.0	579
Sabah	2,931,700	20,306	11.3	1,151,698	31.0	752
Sarawak	2,312,600	16,018	8.9	513,306	14.0	593
Selangor & Putrajaya	4,736,100	32,804	18.2	120,563	3.0	1,215
Terengganu	1,016,500	7,041	3.9	135,911	4.0	261
Kuala Lumpur	1,556,200	10,779	6.0	-	0.0	399
Total	26,027,500	180,274	100	3,764,389	100	6,677

Source: Goh *et al*. (2010)

2.2 OIL PALM

In Malaysia, the oil palm (*Elaeis guineensis*) tree was originated from West Africa where it was found growing wild and afterwards developed into an agricultural crop (Wahid *et al*., 2004; Sumathi *et al*., 2008). It is a tropical palm tree which can be cultivated easily in Malaysia to produce palm oil. A single-stemmed, mature tree can grow up to 20 m tall (Figure 2.2). Its leaves are pinnate with size between 3 to 5 m long. The flowers are produced in dense clusters with each individual small flower consists of

three sepals and three petals. From pollination to maturity, the fruit takes 5 to 6 months to mature (Figure 2.3). The outer layer of the fruit (the pericarp) is fleshy and oily with a single seed (kernel) which is also rich in oil. Due to these properties, oil palm tree was cultivated commercially for the production of palm oil.

Figure 2.2: Old oil palm tree

Figure 2.3: Oil palm fruit bunches

For efficient oil productivity, oil palm tree must be replanted at average of 25 years intervals (Lim *et al*., 1997; Yusoff, 2006; Yamada *et al*., 2010). This is mainly because old oil palm becomes too tall to harvest economically (Wahid *et al*., 2004). When replanting, old palms tree are cut down and most of them are discarded or burnt at the plantation site. As a result, the felled palm trunks become one of the most important biomass resources in Malaysia (Sumathi *et al*., 2008; Shuit *et al*., 2009).

About 10 % of the total 5.23 million hectares of oil palm plantation in Malaysia must be replanted yearly (MPOB, 2013) and this will produce about 70 million old palm trees that will be felled in Malaysia (Lim *et al*., 1997). From this replanting activity, over 15 million tonnes of oil palm trunks will be generated annually (Mumtaz *et al*., 2010). Besides the felled OPT, extraction of palm oil also produces many leftover biomasses such as empty fruit bunches, fibres and fronds. According to Sulaiman *et al*. (2011), each kg of palm oil roughly produced another 4 kg of dry biomass, where approximately a third of that is found in empty fruit bunch derived wastes and the other two thirds is represented by trunks and frond materials.

The OPT structure is not strong enough for use as lumber, and thus only the outer part of the trunk, which is relatively strong, is partially utilized for plywood manufacturing (Sulaiman *et al*., 2008). In the plywood production process, the inner part is discarded in large amounts due to its extremely weak physical properties. Kosugi *et al*. (2010) has attempted to produce bioethanol and lactic acid from the felled trunk in order to utilize the OPT felled for replanting, especially the inner part. The sap in the trunk contains high glucose content; thus, enables it to be used as feedstock for bioethanol production. Apart from that, there are also other components in the squeezed sap that may enhance fermentation, which are amino acids, organic acids, minerals and vitamins. The findings from Kosugi *et al*. (2010) shows that the oil palm sap squeezed from felled trunks is a very promising feedstock for bioethanol and lactic acid production.

Based on the results obtained from Kosugi *et al*. (2010), glucose was found to be dominant sugar in all part of the trunks, approximately 86.9 %, 86.3 % and 65.2 % of the total free sugars contained in the inner, middle and outer parts, respectively. In addition to glucose, significant amounts of sucrose, fructose and other sugars were contained in the sap as shown in Table 2.4.

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CHAPTER 3

METHODOLOGY

3.1 RAW MATERIAL

In this study, OPT sap was obtained from OPT (*tenera* species), approximately 30 years old from the Federal Land Development Authority (FELDA) Jengka 14, Pahang in October 2012. A 10 cm thick disc was taken from the middle part of the trunk, which ranged in length from 9-13 m. The discs (33-42 cm in diameter) were cut into 4-6 pieces and squeezed through sugarcane press machine to collect the sap. All the squeezing process was done within 12 hour after the tree was cut down. The OPT sap 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. Prior to use, the sap was centrifuged at 6,000 rpm for 15 min at 4 °C (Eppendorf, Centrifuge 5810 R, Germany) and the supernatant was used in the fermentation. Figure 3.1 shows the overall process in the preparation of OPT sap.

Figure 3.1: OPT sap preparation. (a) Oil palm tree was cut down (b) The trunk was cut into pieces (c) The trunk bark was removed (d) The middle part of the trunk was cut into smaller pieces (e) The trunk pieces was squeezed through the sugarcane machine (f) The sap obtained was filtered and stored.

3.2 CHARACTERISATION OF OPT SAP

The initial OPT sap sugar composition was checked for sugar composition analysis using HPLC as described in Chapter 3.10.1. The differences on composition of fresh OPT sap collected within 12 hour oil palm tree was felled and after 12 months of storage in -20 °C freezer were analyzed.

3.3 CHEMICAL REAGENTS

The analytical grade chemicals used in this study were ammonium sulphate, magnesium sulphate and calcium chloride from Fisher Scientific (U.K), whereas di-ammonium hydrogen phosphate, β-alanine and potassium dihydrogen phosphate were obtained from Merck (Germany). Medium component such as yeast extract were purchased from Fisher Scientific (France), peptone and tryptone from Sigma-Aldrich (USA) and agar powder by Sigma-Aldrich (Spain). Other chemicals like sulphuric acid and sodium hydroxide were bought from Fisher Scientific (U.K). HPLC grade sucrose were bought from Sigma-Aldrich (Switzerland), glucose and fructose were sourced from Merck (Germany), while ethanol and acetonitrile from Fisher Scientific (U.K).

3.4 MEDIUM PREPARATION

Two types of medium were used throughout this research namely, YPD agar and YPD broth. 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. Both flask were then covered with aluminium foil and was sterilized in an autoclave (Hirayama HVE-50, Japan) for 20 min at 121 °C. After autoclave, 100 mL of filter-sterilized 20% w/v glucose was added in the agar to avoid Maillard reaction. Then, agar was poured into sterilized Petri dish after the temperature has dropped to 60 °C. The agar was left solidified and all plates were sealed before being kept in the refrigerator at 4 °C until further use. To make YPD broth, the procedure was the same except no agar powder was added.