BIOSYNTHESIS OF BIOBUTANOL FROM OIL PALM FROND JUICE BY *Clostridium acetobutylicum*

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ABSTRAK

Tenaga dari sumber biomas menjadi semakin penting, kerana ia boleh digunakan untuk menggantikan sumber tenaga konvensional. Industri kelapa sawit Malaysia menjana banyak biomas lignoselulosa yang menyebabkan masalah pelupusan. Oleh itu, jus kelapa sawit (OPF) diperkenalkan sebagai medium dalam proses penapaian Aseton-Butanol-Ethanol (ABE) untuk menghasilkan biobutanol oleh *Clostridium acetobutylicum* ATCC 824. Kajian ini bertujuan untuk mengkaji potensi jus OPF sebagai medium dengan membandingkan pengeluaran butanol oleh gula tiruan sebagai percubaan pada awal kajian. Selanjutnya, kajian ini meneruskan objektif kedua dan ketiga untuk menilai dan mengoptimumkan faktor yang mempengaruhi penapaian ABE oleh *C. acetobutylicum* ATCC 824. Kandungan gula dalam jus OPF ditentukan untuk mengenal pasti kepekatan gula pada awal proses penapaian. Kawalan gerak balas permukaan (RSM) digunakan untuk menyaring dan mengoptimumkan penghasilan butanol. Jumlah gula dalam jus OPF adalah 68.58 g/L, dengan nilai glukosa, sukrosa dan fruktosa 48.19 g/L, 8.48 g/L dan 11.91 g/L. Hasil butanol dihasilkan oleh gula sintetik dalam eksperimen kawalan seolah-olah sama berbanding dengan penapaian dalam jus OPF, dengan hanya 11.25% lebih tinggi daripada jus OPF. Dalam analisis faktorial, kepekatan ekstrak yis adalah faktor tertinggi yang mempengaruhi proses penapaian dengan sumbangan 8.20%. Faktor-faktor sumbangan tertinggi kedua dan ketiga adalah saiz inokulum dan suhu inkubasi. Tiga faktor ini dioptimumkan menggunakan RSM. Keadaan optimum penapaian itu didapati pada 10% saiz inokulum, suhu pengeraman 37°C dan kepekatan ekstrak yis 5.5 g / L. Secara keseluruhan, penapaian ABE untuk menghasilkan biobutanol menggunakan jus OPF oleh *C. acetobutylicum* ATCC 824 mempunyai potensi tinggi yang kemudiannya boleh digunakan sebagai medium komersial dalam industri biominyak.

ABSTRACT

Energy from biomass resources is becoming increasingly important, since it can be used to partly displace conventional sources of energy. The Malaysian oil palm industry generates huge quantities of lignocellulosic biomass which created a major disposal problem. Therefore, oil palm frond (OPF) juice was introduced as a substrate in Acetone-Butanol-Ethanol (ABE) fermentation to produce biobutanol by *Clostridium acetobutylicum* ATCC 824. This study aims to investigate the potential of OPF juice as a substrate for the butanol production. During preliminary study, the production in OPF juice was compared with synthetic sugar as control experiment. Next, this study proceeds with the second and third objective to screen and optimize the factors affecting ABE fermentation by *C. acetobutylicum* ATCC 824. Sugar content in OPF juice was determined to identify the initial sugar concentration for the fermentation. Response Surface Methodology (RSM) was employed to screen and optimize the butanol production. The total sugar analyzed using High Performance Liquid Chromatography (HPLC) in OPF juice was 68.58 g/L, with glucose, sucrose and fructose value 48.19 g/L, 8.48 g/L and 11.91 g/L, respectively. The culture produced 9.24 g/L of biobutanol using OPF juice with 0.24 g/g biobutanol yield. Meanwhile, 10.91 g/L biobutanol produced using synthetic sugars as control experiment with 0.27 g/g biobutanol yield. The biobutanol yield produce by synthetic sugar in control experiment seem comparable to the fermentation in OPF juice, with only 11.25% higher than OPF juice. In factorial analysis, yeast extract concentration was the highest factor affecting the fermentation process with 8.20% contribution. The second and third highest contribution factors was inoculum size and incubation temperature. These three factors were then optimized using RSM. The optimum condition of the fermentation was found out at 10% inoculum size, 37°C incubation temperature and 5.5 g/L yeast extract concentration which 0.2992 g/g biobutanol yield was obtained in validation process. These experimental findings were in close agreement with the model prediction, with a difference only 9.76%. Overall, ABE fermentation to produce biobutanol using OPF juice by *C. acetobutylicum* ATCC 824 has high potential which later can be used as commercial substrate in biofuel industry.

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LIST OF SYMBOLS

g	Gram
g/g	Gram per gram
g/L	Gram per liter
g/L/h	Gram per liter per hour
hr	hour
R^2	R-squared
°C	Degree celcius
%	Percentage
v/v	Volume per volume

LIST OF ABBREVIATIONS

ABE	Acetone-Butanol-Ethanol
ANOVA	Analysis of Variance
CCD	Central Composite Design
DNS	Dinitrosalicyclic acid
DOE	Design of experiments
GC	Gas chromatography
HPLC	High performance liquid chromatography
OPF	Oil palm frond
RSM	Response surface methodology
OPT	Oil palm trunk
EFB	Empty fruit bunches
POME	Palm oil mill effluent
FFB	Fresh fruit bunches
CDW	Cell dry weight
FFD	Full factorial design
MPOB	Malaysian palm oil board
NaOH	Sodium hydroxide
HCL	Hydrochloric acid
$(NH_4)2SO_4$	Ammonium sulfate
KH_2PO_4	Monopotassium phosphate
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
$Fe(NO_3)_3.9H_2O$	Iron(III) nitrate nonahydrate
$(MnSO_4.4H_2O)$	Manganese(II) sulfate tetrahydrate
ATCC	American type of culture collection
OD	Optical density
RPM	Rotation per minute
YE	Yeast extract

CHAPTER 1

INTRODUCTION

1.1 Background of study

Recently, energy crisis is one of the most serious threats throughout the world. The demand for petroleum-derived fuels is increasing. Due to extensive oil consumption and its increasing price trend over the past decades, the use of biofuels as a partial replacement for fossil fuels has gained great attention. Biobutanol is a good candidate as a biofuel for its interesting advantages: higher energy content, lower vapor pressure making it safer to use, and lower hygroscopy; thus make it easy to preserve and distribute as it does not absorb water (Qureshi & Ezeji, 2008). Moreover, biobutanol has properties which can be applied in pure form or blended in any concentration with gasoline or diesel, can be used in any automobile engine without modifications and can be converted to valuable chemical compounds such as acrylate, methacrylate esters, glycol ethers and butyl acetate (Tashiro & Sonomoto, 2010). As stated in Table 1.1, Niemisto et al. (2013) compared and clarified that biobutanol is advances compared to bioethanol.

Table 1.1 Fuel properties of biobutanol and advances compared to bioethanol

Property	**Advances compared to ethanol**
Blending ability	Can be blended with gasoline in any percentage, all the way up to 100% or diesel fuel in higher concentrations without the need of vehicle retrofitting. With higher concentrations also the share of renewable components is increased in the final fuel mixture.
Energy content, octane values and air-to-fuel ratio	Values are closer to gasoline than ethanol: better fuel economy (kilometers per liter) than with ethanol.
Less evaporative	Safer to use and handle than ethanol and generates lower amounts of volatile organic compound (VOC) emissions.

Table 1.1 Continued

Property	Advances compared to ethanol
Lower water solubility	Compared to ethanol, decreased tendency of corrosion in pipelines and fuel tanks, and if spills or leaks happen, the tendency for spreading in the groundwater is reduced. Ethanol cannot be shipped through pipelines, because it could be contaminated with water.
Lower vapor pressure	Compared to ethanol, decreased tendency of corrosion in pipelines and fuel tanks.
Biodegradability	Butanol is more biodegradable than ethanol and will biodegrade in the environment under aerobic conditions.

Biobutanol is produced by acetone-butanol-ethanol (ABE) fermentation from renewable biomass products using several genera of bacteria, particularly *Clostridium* species in anaerobic conditions. ABE fermentation ranked second only to ethanol fermentation in the first part of the 20th century, but disappeared in the second part due to the increasing costs of the substrate molasses and rise of the petrochemical industry. With the depletion of fossil fuels ABE fermentation becomes interesting again. The fermentation uses *Clostridium* species to consume the sugars and convert them to variety of solvents such as acetone, butanol, ethanol, acetic acid and butyric acid. *C. saccharoperbutylacetonicum, C. acetobutylicum, C. beijerinckii,* and *C. aurantibutyricum* are most capable to produce significant amounts of solvents among the *Clostridium* species (Raganati et al., 2012).

Renewable energy has been identified globally as a key driver to achieve economic growth while ensuring minimal environmental harm. Therefore, the exploration of crop wastes and agricultural residues as a fermentation substrate is expected to increase rapidly. Since Malaysia is located in the equatorial region, has a tropical rainforest climate and it is being hot and humid throughout the year; oil palm grows significantly. It is reported that the total palm oil plantation area in Malaysia has increased from 5.74 million hectares in 2016 to 5.81 million hectares in 2017 (MPOB, 2018). OPF is one of lignocellulosic biomass which mainly originates from waste stream, thus, there is no harvesting cost required. This lignocellulosic biomass produced value-added bio-products like phytochemicals in a bioethanol refinery which can save the environment and also add economic value to the oil palm industry (Boateng et al., 2014).

Preliminary study has been done by Zahari et al. (2014) proposed, sugars derived from OPF juice can be potential fermentation substrate for biobutanol production. OPF juice has been identified as a good carbon source for the production of value-added products such as the production of poly (3-hydroxybutyrate), P(3HB) (Zahari et al., 2012).

1.2 Problem Statement

The Malaysian oil palm industry generates an estimated 77.24 million tonnes biomass in a year (Ng et al., 2011). The biomass includes oil palm trunks (OPT), oil palm fronds (OPF), empty fruit bunches (EFB) and palm pressed fibres (PPF), palm shells and palm oil mill effluent palm (POME). The presence of these oil palm wastes has created a major disposal problem. It is disheartening to note that a large portion of oil palm biomass is either air burnt or left at the plantations, thereby constituting environmental hazards to lives (Onoja et al., 2018). Among those biomass, OPF occupied the largest portion comprising 44.84 million tonnes of oil palm fronds, 13.97 million tonnes of palm trunks, 6.93 million tonnes of EFB, 4.21 million tonnes of oil palm shell and 7.29 million tonnes of mesocarp (Ng et al., 2011). The Malaysian government further forecasts that the supply of biomass from oil palm will increase to 85-111 million tonnes of solid biomass and 70-110 million tonnes of POME by 2020 (MPOB, 2018).

For per hectare of oil palm plantation, about 10 tonnes of dry palm fronds are produced. OPF is available daily when the palms are pruned during the harvesting of fresh fruit bunches for oil production. The OPF are pruned regularly and left on the ground for natural decomposition, soil conservation, erosion control and ultimately the long-term benefit of nutrient recycling which is a slow and uneconomical process. Open burning or simply abandon the waste away is a great loss of energy source since this biomass have significant energy content. With the large quantity of fronds produced even by the small farmers, this material has a very promising market as a source of renewable energy. Recently, major studies from previous work tend towards exploitation of agro-wastes as a substrate in fermentation for biobutanol production by *Clostridium species*. Selection of fermentation substrate is concerned with composition of sugars in the biomass where high amount of sugars eventually will give in high titre of biobutanol production.

Research has been carried out on sugars production from OPF juice by Zahari et al. (2012). Characterization of the OPF juice revealed that large amount of sugars (76.09 g/L) can be obtained simply by pressing the OPF. Furthermore, the OPF juice is rich in minerals and nutrients which are essential for bacterial growth during fermentation. They found that high amount of available sugars in OPF juice can be used as renewable feedstock for the production of valuable products such as lactic acid, bioethanol and biobutanol.

Moreover, biobutanol has significant potential as a "next generation" biofuel due to its cleaner and superior fuel properties compared to bioethanol and biodiesel. Biobutanol production from waste fermentation would enable a clean and environmental friendly technology for energy generation and waste treatment at the same time. In this study, the used of OPF juice as a fermentation substrate for biobutanol was investigated. The hypothesis was OPF juice can be a great carbon source for the fermentation of biobutanol production, same as those biomasses mentioned before. Since OPF is an abundant solid waste at oil palm plantation, it has great potential to be used as sustainable, renewable and cheap fermentation feedstock for the production of biobutanol. Hence this study was done in order to screen and optimize the factors affecting of biobutanol from OPF juice.

1.3 Research Objective

The objectives of this research are: -

i. To evaluate the potential production of OPF juice as a renewable carbon substrate for biobutanol production by *Clostridium acetobutylicum* ATCC 824.

ii. To analyse the factors which affect the production of biobutanol from OPF juice.

iii. To optimize the significant factors that affects the production of biobutanol from OPF juice.

1.4 Scope of Study

As a follow-up to the objectives, the scopes of this study are:

i. To determine the total sugars concentration in OPF juice by using high performance liquid chromatography (HPLC) to be used as a substrate for biobutanol production by *C. acetobutylicum* ATCC 824.

ii. To observe the *C. acetobutylicum* ATCC 824 growth in the fermentation process.

iii. To screen five parameters affecting fermentation process which are 5 – 7 of pH value, 40 – 60 g/L of total sugars concentration in OPF juice, 1 – 20% of inoculum size, temperature at 32 – 42 ℃, and 1 – 10 g/L of yeast extract concentration by applying fractional factorial design (FFD) using Design Expert 7.1.6.

iv. To optimize the parameters which highly contribute to the biobutanol production that obtain from screening study. These parameters were examined using central composite design (CCD) using Design Expert 7.1.6.

v. To validate the optimum condition obtained in optimization study.

1.5 Significance of Study

This study create waste to wealth solution by utilization of renewable biomass such as OPF for the production of value-added product of biobutanol. This work can reduce the huge volume of biomass generated from the oil palm plantation, which is one of the problematic issues related to the palm oil industry. Other than that, producing biobutanol from OPF juice is an alternative to substitute the usage of edible food sources. Further investigation on the factors affecting the fermentation process is to get the maximum value of biobutanol produced.

1.6 Structure of this Thesis

The thesis structured into five main chapters. The first chapter represents the background of the research which includes the problem statement, objectives, scopes and significance. Chapter 2 is composed of the literature review about biobutanol production from OPF juice by *C. acetobutylicum*, where it describes the biobutanol, OPF juice, factors affecting the fermentation and experimental design. Chapter 3 explains the materials and methods employed throughout the experiment. Chapter 4 discuss on the results of the research and finally, conclusion and recommendations for future works have been drawn in Chapter 5.

CHAPTER 2

LITERATURE REVIEW

2.1 Biobutanol

Butanol is a four carbon alcohol consisting of a saturated, linear and a hydroxyl group on the last carbon on the chain as in Figure 2.1. It is also known as butane-1-ol (IUPAC), 1-Butanol, n-Butanol, butyl alcohol, butyl hydroxide, methylolpropane, and propylcarbinol. The molecular formula, molecular weight and boiling point as $CH_3(CH_2)2CH_2OH$, 74.1216 and 118.8°C, respectively. Butanol is a clear, flammable and colourless liquid with a distinct odour. It is currently used as an important industrial chemical or solvent with a variety of applications, including latex surface coating, enamels, lacquers, as well as production of antibiotics, vitamins and hormones (Lee et al., 2008). There are four structural isomers of which 1-butanol (n-butanol) is the most important commercial isomer. This isomer occurs in nature and is primarily used industrially as a solvent or component in surface coatings.

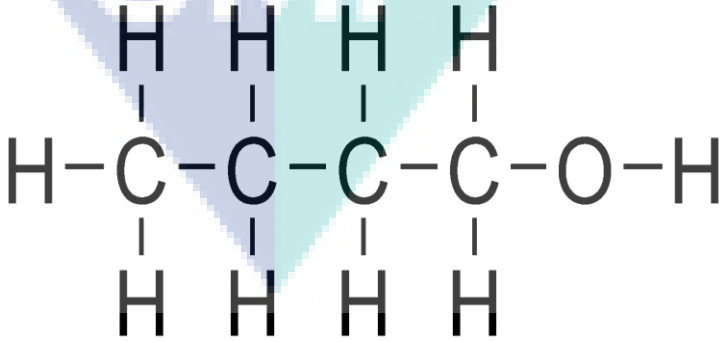

Figure 2.1 Chemical structure of butanol

Butanol can be produced from biomass (as "biobutanol") as well as fossil fuels (as "petrobutanol"); biobutanol and petrobutanol have the same chemical properties. Biobutanol are produced by alcoholic fermentation of simple sugars from the agricultural feedstocks such as corn, wheat, sugar beet, cassava and sugarcane. First generation biofuels are made from crops that could be used as food, such as from sugars and conversion of food grade oils to biodiesel. Second generation biofuels refer to conversion of lignocellulosic biomass to fuels. Land that could be used to produce food is used for growing lignocellulosic crops for second generation biofuels. Third generation biofuels are fuels produced in areas not available for growing food, such as high-lipid algae to biodiesel (USDA, 2009).

2.1.1 Main applications of butanol

A relatively new, but very important application is butanol as a biofuel. Recently, butanol has been considered as an alternative transportation fuel to replace gasoline because its properties are very similar to gasoline. Jin et al. (2011) discovered eight main advantages of butanol that indicates butanol has the potential in biofuel industry. First, having higher heating value which reduce the consumption of fuel and a better mileage can be obtained with butanol. Butanol has low volatility that means it will have less tendency towards cavitation and vapour lock problem especially during the summer or winter. Besides, it also less in ignition problem and intersolubility. Less ignition problem is easy to start the engine in cold weather, meanwhile, intersolubility makes the butanol blend with diesel or gasoline without any co-solvents and very well. Furthermore, butanol has high viscosity and safer since it has very low vapour pressure point and a high flash point. It makes butanol as a safe fuel to use in high temperature. Jin et al. (2011) also mentioned that butanol is easy distribution. It can tolerate water contamination and is less corrosive thus, it is more suitable for distribution through pipelines and facilitates storage. Last but not least, fermentation of butanol generated hydrogen and easily recovered. This will increase the energy yield. Based on these advantages, butanol can be used safely with a specific blend ratio with gasoline or diesel fuel in engines.

Besides, butanol also used primarily as an industrial solvent and in the production of butyl esters. In food industry, it is used to add artificial flavour to a variety of food products. Apart from that, butanol is used as a chemical precursor for plastic solvents, waxes, resins, perfumes, rayon, detergents, polymers, paints, hydraulic fluids, and as an

extractant in pharmaceutical processes of antibiotics, hormones, drugs and vitamins (Durre, 2007). In cosmetic industry also used butanol as ingredients in manufacturing of lipsticks, eye makeup and nail coats.

2.2 Fermentation process of biobutanol

Acetone-Butanol-Ethanol or ABE fermentation is an anaerobic process which uses microorganism which produced the major end-products of acetic acid, butyric acid, acetone, butanol, ethanol, carbon dioxide and hydrogen (Figure 2.2). Biobutanol production via ABE fermentation is a typical biphasic process, including acidogenic phase in which acetate, butyrate, carbon dioxide, and hydrogen are produced during the exponential phase through the activation of acids synthesis pathways, and solventogenic phase in which acetone, butanol, and ethanol are produced during stationary phase via the reassimilation and reutilization of acetic and butyric acids (Lee et al., 2008). Generally, *C. acetobutylicum* and *C. beijerinckii* has been used for this fermentation. The maximum concentration of products in the process does not exceed 20 g/L in the batch reactor and the weight ratio of the three products i.e. acetone, butanol and ethanol is in ratio of 3:6:1. Out of the 20 g/L of pure solvent, butanol is about 8-13 g/L in concentration.

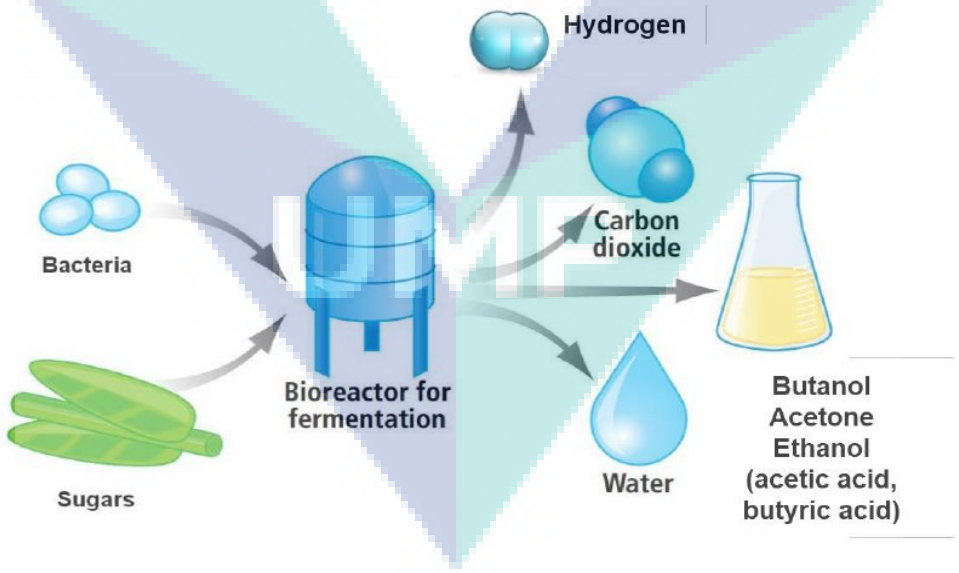

Figure 2.2 Biobutanol production via ABE fermentation

Source: Rohani (2013)

2.2.1 History of ABE fermentation

Pioneered by Chaim Weizmann in UK at the time of World War I, the industrial acetone-butanol-ethanol (ABE) production using solventogenic clostridia was a very successful industrial fermentation. Historically, the fermentation continued in USA until about 1950. Its decline has been caused by increasing substrate costs and the availability of much cheaper feedstocks for chemical solvent synthesis by the petrochemical industry. In 1950-1960s, the fermentation process completely ceased in Europe and North America. Meanwhile, in China, it was first established in 1950s and peaked in the 1980s (Ni & Sun, 2009). The so-called oil crisis in 1973 led to renewed interest in novel fermentation and product recovery technologies as well as in the metabolism and genetics of the bacterial species involved. In order to reintroduce this fermentation process, there were three major problems have been overcome throughout 1990s; the high costs of the substrate, the low product concentration and the high product recovery cost.

Cheap agriculture-waste-based feedstocks have been exploited for their potential as novel substrates such as apple pomace, jurusalem artichokes, lignocellulose, whey, low-grade potatoes, rye, peat and palm oil mill effluent. The other possibility to improve the traditional process in 1990s is to reduce costs of product recovery. Adsorption, gas stripping, liquid-liquid extraction, membrane evaporation, perstraction, pervaporation and reverse osmosis were listed out as novel product recovery techniques for clostridial solvent fermentation (Durre, 1998). All the methods have their own advantages and disadvantages.

As reported in 1982s, agricultural residues such as bagasse and rice straw have been used for production of acetone-butanol by C. *saccharoperbutylacetonicum*. The butanol produced to the extent of 16 g/L. In the process, the bagasse and rice straw have been hydrolyzed by mixed culture, *Trichoderma reesei* and *A. wentii* to obtain the fermentable sugar. After removed the impurities, the substrate had undergone acetone-butanol fermentation (Soni et al., 1982). George et al. (1983) screened thirty-four strains representing 15 species of anaerobic bacteria for acetone, isopropanol and n-butanol (solvent) production. Several strains of *C. beijerinckii* and *C. aurantibutyricum* produced at least 49mM n-butanol. While, *C. acetobutylicum* strains produced up to 41mM n-butanol. From the screening process, the most promising strain was *C. beijerinckii* VPI

13436 or NRRL B-592. This strain has been chosen for further studies on the cellular control.

Conversion of alkali-pretreated wheat straw into butanol and acetone by *C. acetobutylicu*m has been achieved in a one-step hydrolysis and fermentation process involving the use of cellulase from *T. reesei*. In this experiment, it was discovered that acidogenesis took place during the first six hours with a rapid pH drop followed by solventogenesis which ended 38 hours with a final solvent production of 17.3 g/L. The highest production was 7.4 g/L acetone, 10.3 g/L butanol, less than 0.1 g/L ethanol, 1.8 g/l acetic acid and 1.3 g/L butyric acid. This shows that on 1984s, researchers have discovered that the use of a single step process for hydrolysis and fermentation of pretreated straw can present several advantages which gains in equipment and time (Marchal et al., 1984).

On 1985s, the research on this field has been studied systematically for industrial optimization of both strain selection, and fermentation operation. Marchal et al. (1985) have investigated the utilization of Jerusalem artichokes possibilities of the optimization to produce acetone and butanol. In the project, they used two selected strains of *C. acetobutylicum* NCIB 8052 (ATCC 824) which were IFP 902 and IFP 904. Three conditions of fermentation were conducted; fermentation of chemically hydrolysed Jerusalem artichoke juice, fermentation of enzymatically hydrolysed Jerusalem artichoke juice, and simultaneous hydrolysis and fermentation with controlled pH. The most important result reported that the pH was to hold during part of the growth stage at a favorable pH in the range of 6 to 6.5 then let it drop by self-acidification to obtain the conditions for optimal solvent production (Marchal et al., 1985).

The research on solvent production has drawn intention to the improvement of the fermentation either by altering the medium conditions or removing the inhibitory factors involved. In 1987, Soni et al. (1987) revealed that intracellular and extracellular inhibitors, including metabolic end-products, caused the inhibition of cell growth and solvent production. The writers claimed that butanol at the level of 13 g/L was completely inhibitory to the growth of cells, wheareas butyric acid totally inhibited the cell growth at a concentration of 8.7 g/L. Investigations was carried out on the effect of addition of culture filtrate concentrate and cell-free extract concentrate. Therefore, the effect indicated that non-volatile inhibitors produced by cells were also inhibitory for

bioconversion. The butanol production was found to be reduced by 15%-20% on addition of cell-free extract concentrate. Last but not least, Soni et al. (1987) performed the fermentation by adding heat-sterilized concentrates on growth which resulted in a reduction of inhibition.

The other inhibitor effect reported during this era, was by Ballongue et al. (1987) which found that acetic acid, butyric acid, butanol and gases produced by bacteria act as inhibitors of specific growth rate. While ethanol and acetone were not reported to be toxic at physiological concentrations. The inhibition of growth is reflected not only in a slowing down the growth rate but also in a decrease of the final biomass of the fermentation. Therefore, the arising of biosynthesis of ABE fermentation in 1980s, the researchers start to discover and develop in both scientific and engineering aspects in this clostridial solvent fermentation. Cheap agricultural-waste-based feedstocks have been exploited for their potential as novel substrates. Product recovery technologies as well as the metabolism and genetics of the bacterial species also involved.

2.2.2 Microorganisms producing biobutanol

Clostridia are Gram-positive bacteria and typically strict anaerobes. Clostridia form robust endospores which are resistant to oxygen, heat and alcohol. Most clostridia species are motile and have flagella projecting in all directions used for propulsion (Andreesen et al., 1989). The Clostridium genus of bacteria is found in soil, sewage, vegetation, plant and animal products and the digestive tracts of many animals. Most are obligate anaerobes that grow best in a temperature between 30ºC and 37ºC and pH between 6.5 and 7.0. Most Clostridia produce non-vegetative spores that are able to withstand high temperatures, oxygen contamination, acidic or basic conditions (Sneath et al., 1986). Many of the early strains of solventogenic Clostridia were isolated from river mud, sewage, soil, manure, roots, rotted wood and corn stalks and still used in butanol fermentation research until today (Beesch, 1952). By 1927, the practice of harvesting and isolating solventogenic Clostridium species from these matrices was well established.

Solvent-producing Clostridia are separated into five distinct groups: *C. acetobutylicum, C. butyricum C. saccharoperbutylacetonicum, C. beijerinckii,* and *C. pasteurianum.* All of these solventogenic Clostridium bacteria can produce acetone, butanol and ethanol but in different concentrations. This is because of some strains have

an additional enzyme, a secondary alcohol dehydrogenase, that converts acetone to isopropanol. All five species of solventogenic Clostridia are known to ferment glucose, fructose, sucrose, arabinose, xylose, mannose, cellobiose, lactose, maltose, raffinose, salicin, amygdalin, starch and dextrin (Keis et al. 2001). There are three types well-known solventogenic strains for ABE fermentation which are *C. acetobutylicum, C. beijerinckii* and *C. saccharoperbutylacetonicum.* Clostridium species are classified into pathogenic and non-pathogenic. The three types mentioned earlier are non-pathogenic which capable of producing acetic acids, butyric acid and gasses (H_2 and CO_2) and capable of converting them to solvents, acetone, butanol and ethanol (Morris, 1994).

Recently, Shanmugam et al. (2018) discovered a newly isolated *Clostridium sp.* WST from the mangrove sediment to be able produced biobutanol as high as 0.54 and 0.55 g/g from glucose and galactose in low concentration of substrates. This production determined to be highest within the reported batch fermentation by Clostridial strains. The fermentation process was done without the requirement of pH control and with the negligible level of generated ethanol and acids. Therefore, the strain is the great potential to offer an economically feasible option for the large-scale sustainable biobutanol production in future.

Other than Clostridia, there are several genetically modified microorganisms that have been developed for biobutanol production. Sakuragi et al. (2015) and Kuroda & Ueda (2016) have found that *Saccharomyces cerevisiae* inserted with genes related was successfully developed for biobutanol production. They discovered this yeast is easy to handle and has a higher tolerance to many stresses as compared to Clostridia, but the biobutanol production is still relatively low. Meanwhile, Krivoruchko et al. (2013) reported that the manipulation of acetyl-CoA in *S. cerevisiae* increased biobutanol titre 6.5 times higher than the previous value reported for biobutanol produced by yeast. However, this value is still low as compared to the biobutanol produced by Clostridia. Besides yeast, the other genetically modified microorganisms reported were *Lactobacillus sp.* (Berezina et al., 2010), *Lactococcus sp.* (Liu et al., 2010), *Pseudomonas sp.* (Ruhl et al., 2009), and *Escherichia coli* (Saini et al., 2016) produced biobutanol in the range (0.066–0.303) g/L only.

2.2.2.1 *Clostridium acetobutylicum*

Historically, in 1862, Louis Pasteur used a microbe called "Vibrion butyrique", which was most likely a mixed culture containing a solventogenic *Clostridium* species to produce a C4 alcohol (Durre, 2007). In 1893, Martinus Beijerinck isolated and gave a detailed description of a similar strain of solventogenic bacterium which he called *Granulobacter saccharobutyricum*. In 1926, these solventogenic bacteria were classified as *Clostridium acetobutylicum* (Durre, 2007). *C. acetobutylicum* is an anaerobic, gram-positive, and spore-forming microorganism. It has an ability to produce acetone, butanol, ethanol, as a final product under anaerobic condition, using different carbohydrate sources including monosaccharides and polysaccharide. To date, the ability of *C. acetobutylicum* to produce biobutanol was investigated by Alla et al. (2017), Aliyu et al. (2017), Lu et al. (2012), Wang et al. (2014), Wang & Chen (2011), Razak et al. (2013), Japar et al. (2013), Raganati et al. (2013), Foda et al. (2010) and others.

C. acetobutylicum is primary solventogenic clostridial species that have been evaluated for ABE production. Metabolic engineering of *C. acetobutylicum* has been studied extensively to better understand the gene expression and regulation for enhanced butanol production (Lutke- Eversloh and Bahl, 2011). *C. acetobutylicum* will be used for fermentation. The choice of strain for use in ABE fermentation is based on the nature of the raw material used, the ratio of end products required, the need for additional nutrients, and phage resistance. The most common ratio for solvent production is ABE in the ratio of 6:3:1. (Jones & Woods, 1986).

In recent years, ABE fermentation has been initiated in Malaysia but still not widely and commercially viable because of high capital cost and operating cost. *C. acetobutylicum* NCIMB 13357 was performed fermentation on palm oil mill effluent (POME) without addition of nutrients and started to produce solvents after 48 hr incubation (Kalil et al., 2003). The studied were carried out on effect of concentration of sedimented POME, the effect of initial culture pH, and the use of immobilized cells for ABE production. They discovered that the optimum conditions of fermentation for sedimented POME were 90% concentration and initial pH 5.8 in order to get high yield of solvent. Either free growing cells or immobilized cells of Clostridia can ferment POME to produce ABE. Another similar work done on POME also proved that *C.*

acetobutylicum NCIMB 619 can utilize POME as the main medium to produce solvents (Japar et al., 2013).

Futhermore, past few years, Khamaiseh et al. (2011) studied on biobutanol production by *C. acetobutylicum* NCIMB 13357 in modified medium using date fruit as carbon source. In early stage of screening process, the results showed that 40 g/L of date fruit under initial pH 7 at 35ºC were the optimum conditions for fermentation process. However, later, they studied on the effect of some parameters for the fermentation and found out the medium containing 30 g/L of date fruit at 35ºC incubation temperature with initial medium pH 7.0 gave the highest concentration of solvents (Khamaiseh et al., 2013). In order to enhance the ability of *C. acetobutylicum* NCIMB 13357 production of biobutanol on date fruit, the use of P2 medium was investigated Khamaiseh et al., (2014). The results obtained from this study showed that the yield of biobutanol and ABE increased compared to products yield measured in previous studies using modified date fruit medium. Consequently, P2 medium had favorable supplements for the growth of *C. acetobutylicum* to produce more butanol and solvents and to cause higher productivity.

C. acetobutylicum P262 from NCIMB Ltd. was used by Guvenilir & Deveci (1996) to produce ABE from corn mash and molasses. It was reported that the culture was produced in 24 hr and the solvents production increased up to 30 hr. However, butanol production reached a maximum level of 48 hr. Meanwhile, Foda et al. (2010) used *C. acetobutylicum* DSM 792 and *C. acetobutylicum* AS 1.224 to investigate the suitability of cheese whey for biobutanol production. This strain is corresponding to ATCC strain 824. In early stage of experiment, the authors compared the ability of the two strains in lactose medium. It was found that production of acetic and butyric acid by *C. acetobutylicum* DSM 792 was higher amounts and slightly increased by prolonging the incubation time from 50 to 75 hrs. Ethanol and acetone production were decreased, while butanol amount was not changed. By *C. acetobutylicum* AS 1.224, acetic acid and ethanol production was increased after 50 hrs, while other compounds did not detect. Therefore, the fermentation was proceed by *C. acetobutylicum* DSM 792 only and butanol was observed after one day increased up until five days with cheese whey medium. It can be interpreted that both strain *C. acetobutylicum* P262 and DSM 792 have similar production profile which increase up after 24 hours and stop depends on the substrates.

C. acetobutylicum ATCC 824 was succesfully produced solvents by the fermentation using kudzu roots and isoflavane extraction from kudzu fermentation residue (KFR) after 96 hr fermentation (Wang & Chen, 2011). It was discovered that the culture was inhibited when the sugar concentration more than 60 g/L. This might be due to butanol inhibition. Therefore, inhibition in the fermentation almost occured when the sugar concentration in the substrate were too high. Razak et al. (2013) also used *C. acetobutylicum* ATCC 824 in their works. Razak et al. (2013) studied the optimization conditions of biobutanol production from oil palm decanter cake hydrolysate and found out the maximum biobutanol yield 0.13 g/g with 8.17 g/L concentration.

Besides as discussed above, Table 2.1 shows different species of *C. acetobutylicum* used for biobutanol production from 2013 until 2018. Therefore, based on these convincing results reviewed earlier, in this study, *C. acetobutylicum* ATCC 824 was chosen to produce biobutanol using OPF juice.

Table 2.1 *C. acetobutylicum* for biobutanol production

Microorganism	**Substrate**	**Biobutanol (g/L)**	**Biobutanol yield (g/g)**	**Reference**
Clostridium sp. WST	Glucose	16.20	0.54	Shanmugam et al. (2018)
C. acetobutylicum ATCC 824	Corn starch	4.76	NS	Alla et al. (2017)
C. acetobutylicum ATCC4259	OPF juice	8.61	0.29	Aliyu et al. (2017)
C. acetobutylicum ATCC 824	Microalgae-based carbohydrates	13.03	0.45	Wang et al. (2014)
C. acetobutylicum ATCC 824	Oil palm decanter cake hydrolysate	8.17	0.13	Razak et al. (2013)
C. acetobutylicum NCIMB 619	Palm oil mill effluent	0.43	0.03	Japar et al. (2013)
C. acetobutylicum ATCC 824	Sago pith residue	2.23	0.11	Linggang et al. (2013)

*NS is not stated

2.2.3 Metabolic pathway of Clostridia

During the ABE fermentation, two different growth phases of *C. acetobutylicum* will occur as shown in Figure 2.3. Phase one is called the exponential acidogenic phase and the other phase is solventogenic phase. During acidogenic phase, the growth of cell is at maximum and it produces organic acids (butyric acid and acetic acid) and hydrogen

gas (Jones and Woods, 1986). Ethanol and acetone are formed in small volumes. The production of the acids results in a low pH which can threat of cell death. Imminent death is evaded by a major metabolic shift that takes place at the end of the exponential growth phase. This also marks the end of the acidogenic phase and the start of the solventogenic phase. The acids are converted into butanol and acetone in solventogenic phase. Conversion of butyrate and acetate into solvents increases the pH again, which means the cells can stay metabolically active for a longer time. The pH typically rises and falls slightly towards the end of the fermentation as the microorganism attempts to establish equilibrium between acidogenesis and solventogenesis. However, the solvents are also killing the cells, with butanol being the most toxic. Solvents inactivate the membrane proteins and destroy the membranes of the cells. The carbon sources used for fermentation are initially converted to pyruvate prior to the generation of the solvent end products.

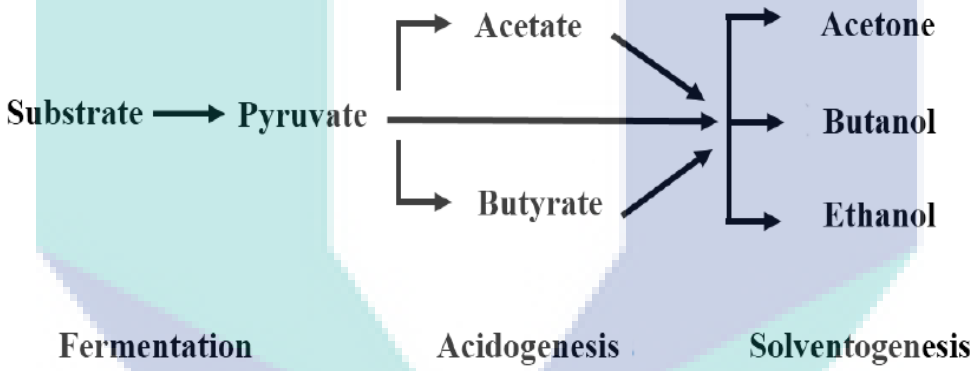

Figure 2.3 Phases of ABE fermentation process
Source: Qureshi and Ezeji (2008)

The metabolic pathways for acidogenesis and solventogenesis of *C. acetobutylicum* are shown Figure 2.4. The fermentation begins with glucose is catabolized to undergo glycolysis to produced pyruvate by the Embden-Meyerhof-Parnas pathway. Meanwhile, pentoses (xylose and arabinose) are catabolized by the pentose phosphate pathway to produce pyruvate. The resulting pyruvate is converted into acetyl-coenzyme A (acetyl-CoA) by pyruvateferredoxin oxidoreductase (PFOR).

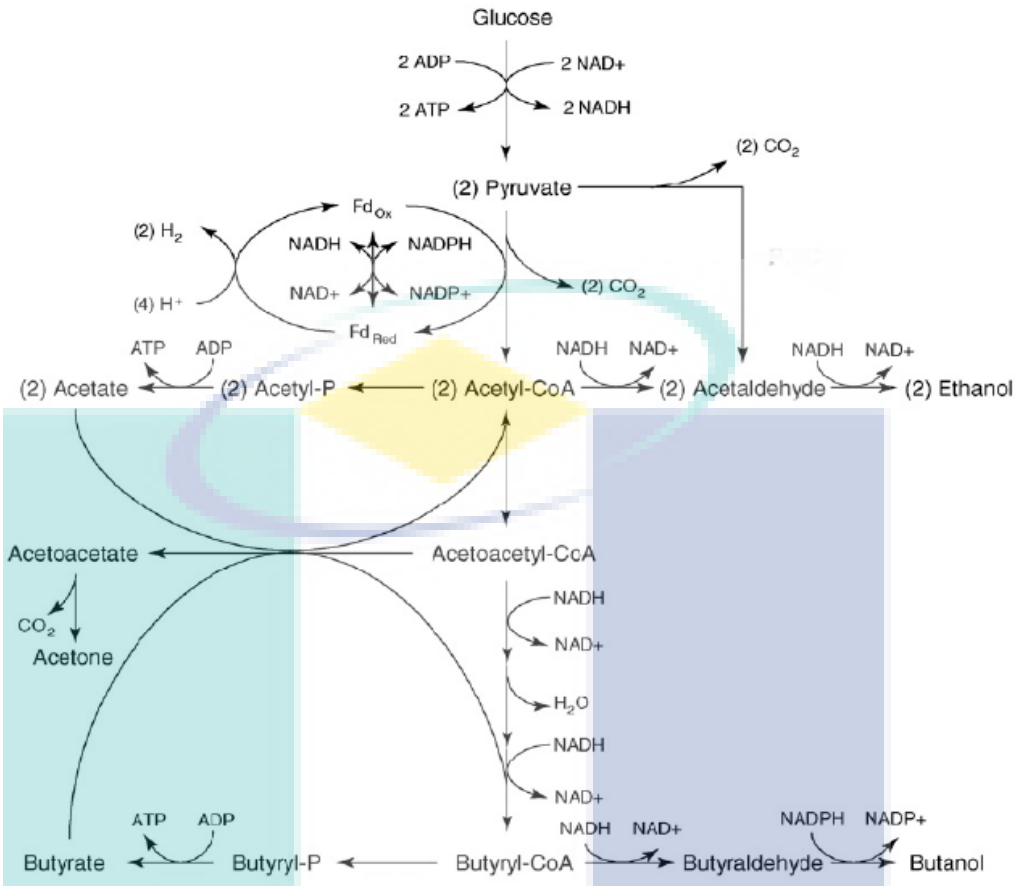

Figure 2.4 Metabolic pathway of ABE fermentation for biobutanol production
Source: Durre (1998)

Acetyl-CoA is further converted into oxidized products (acetone, acetate or CO_2) or butyryl-CoA following the pathway analogous to fatty acid biosynthesis in order to form butanol or butyrate. Activities of enzymes involved in the pathway fromacetyl- CoA to butyryl-CoA are important for both butyrate and butanol production. Butyrate will only be produced subsequently if there are sufficiently high levels of the enzymes involved in the pathway from butyryl-CoA to butyrate present. In addition, some factors affecting the fermentation environment are required for the shift from acid to butanol and acetone production. The factors including pH, growth phase of culture and incubation temperature. These facts have to be considered for the future researchers to maximize butanol concentration on solvent.

2.3 Biomass as substrate for biobutanol production

Clostridia have the ability to utilize a wide range of substrates, including hexose and pentose sugar, starch-based crops, lignocelluloses and hydrolysate. The use of cheap

substrates such as agriculture waste would be beneficial. Some of the agriculture wastes have been used were corn steep liquor, soy molasses, palm oil mill waste, wheat straw, barley straw hydrolysate and sugar cane juice. Previous studies reported that *Clostridium* species are able to ferment sugars and starches from a variety of real world sources as tabulated in Table 2.2.

Table 2.2 Different biomass as substrate used for biobutanol production

Substrate	**Microorganism**	**Biobutanol (g/L)**	**Biobutanol yield (g/g)**	**Reference**
Switchgrass	*C. saccharoperbutyl-acetonicum* N1-4	8.6	0.16	Wang et al., 2019
Sugarcorn juice	*C. beijerinckii*	8.3	0.31	Flores et al., 2018
Coffee silverskin hydrolysate	*C. beijerinckii* CECT 508	7.02	0.27	Valsero et al. (2018)
Bambusa bambos	*C. beijerinckii* ATCC 55025	8.34	0.21	Kumar & Banerjee. (2018)
Food waste	*Clostridium sp.* HN4	5.23	0.09	Qin et al. (2018)
Pea pod waste	*C. acetobutylicum* NRRL B-527	3.82	0.13	Nimbalkar et al. (2018)
Potato peel	*C. saccharoperbutyl-acetonicum* DSM 2152	8.11	0.20	Valsero et al. (2018)
Sugarcane industry waste utilization (Press mud)	*C. acetobutylicum* NRRL B-527	4.43	0.13	Nimbalkar et al. (2017)
Corn starch	*C. acetobutylicum* ATCC 824	4.76	NS	Alla et al. (2017)
Sugarcane field residue	*C. beijerinckii* YVU1	16.5	0.27	Reddy et al. (2017)
OPF juice	*C. acetobutylicum* ATCC 4259	8.61	0.29	Aliyu et al. (2017)
Apple pomace	*C. beijerinckii* CECT 508	9.11	0.28	Valsero et al. (2017)
Microalgae-based carbohydrates	*C. acetobutylicum* ATCC 824	13.03	0.45	Wang et al. (2014)
Oil palm decanter cake hydrolysate	*C. acetobutylicum* ATCC 824	8.17	0.13	Razak et al. (2013)
Palm oil mill effluent	*C. acetobutylicum* NCIMB 619	0.43	0.03	Japar et al. (2013)

Table 2.2 Continued

Substrate	Microorganism	Biobutanol (g/L)	Biobutanol yield (g/g)	Reference
Sago pith residue	*C. acetobutylicum* ATCC 824	2.23	0.11	Linggang et al. (2013)
Cane molasses	*C. saccharobutylicum* DSM 13864	13.4	NS	Ni et al. (2012)
De-oiled rice bran	*C. saccharoperbutyl-acetonicum* N1-4	7.72	0.27	Al-Shorgani et al. (2012)
Kudzu roots	*C. acetobutylicum* ATCC 824	11.20	0.21	Wang & Chen (2011)

*NS is not stated

Most of the substrates listed are agricultural residue and waste with high content of cellulose and hemicellulose with low lignin content. This criterion is the ideal selection of substrate for biobutanol production. It can be obtained from wood and fibrous materials from organic sources, agricultural wastes, organic municipal wastes and organic industrial wastes. However, major studies from previous work tend towards investigation of agro-wastes as a substrate for biobutanol production

2.4 Palm oil and oil palm biomass

Oil palm belongs to the species *Elaeis guianensis* is widely cultivated oil bearing tropical palm tree originates from West Africa and was introduced to South East Asia in the last century (Ofori-Boateng & Lee 2013). The top five global palm oil producers are Indonesia (53%), Malaysia (36%), Thailand (3%), Nigeria (2%) and Colombia (2%) (Index Mundi, 2013). Malaysia as the world's second largest palm oil producer and occupied huge plantations area up to 5.038 million hectares (MPOB, 2011). Towards 2014, according to statistics, the oil palm plantation area increasing to 5.5 million hectares in 2013. Hence, resulted being the largest plantation and one of the biggest exporters of palm oil to the world (MPOC, 2010).

This large plantation subsequently gives rise to Malaysia generates approximately 80 million tonnes of dry solid biomass from the oil palm industry in 2010. This figure is expected to reach up to 110 million tonnes in the year 2020 (AIM, 2011). The main type of the biomass waste includes empty fruit bunches (EFB), palm-pressed fibre (PPF), palm oil trunks (OPT), palm oil fronds (OPF) and palm oil mill effluent (POME). which can actually utilize efficiently into value-added product. However, the most abundant biomass

generated from oil palm plantation is oil palm frond (OPF) which currently contributes to nearly 60% of the volume. Wan Zahari et al. (2004) reported that oil palm industries in Malaysia generated 54.17 million tons and 54.24 million tons of OPF in 2010 and 2011, respectively. The increment of OPF waste produced was 19 million tons from 2004 to 2011 (Wan Zahari et al., 2004). Normally, after harvesting, the OPFs are left to rot in the plantation in order to fertilize the soil. However, the huge amount of OPF generated globally may be considered underutilized and become unmanageable waste. Therefore, this study evaluates on production of biobutanol from OPF juice as the fermentation substrate.

2.4.1 Potential used of OPF juice for biobutanol production

As shown in Figure 2.5, during the harvesting of fresh fruit bunches, this OPF is felled in between the inter rows of the oil palm plant. The OPF is approximately 2 – 3 meters long and weighs about 10 kg (wet weight). It consists of the petiole (the stem) and many long leaflets on either side of the stem.

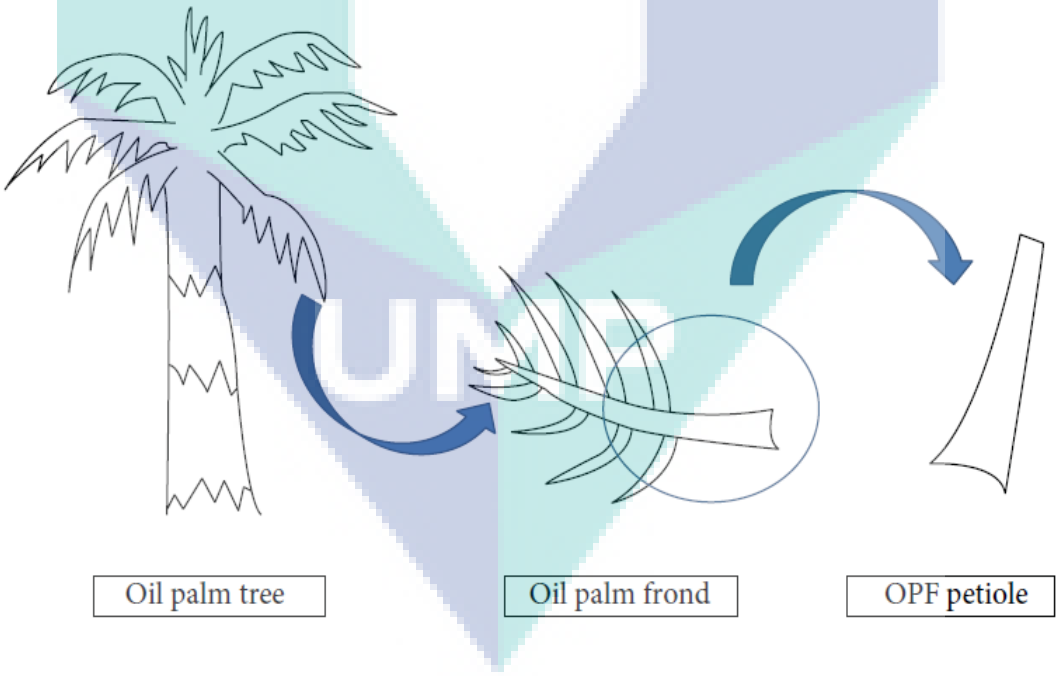

Figure 2.5 OPF petiole collection from the oil palm plantation.
Source: Maail et al. (2014)

The basal part (lower third) of the OPF petiole contain 66% of the total sugars in OPF, while the nutrients mostly centered at the top two thirds of the fronds (AIM, 2011). Therefore, it is expected that the desirable content for production of biobutanol is only the basal portion. Meanwhile, the other part of the OPF would remain and will not interfere with the nutrients supply to the soil in the plantations as fertilizer. Nordin et al. (2016) also discovered the bottom part of the OPF has the best properties compared to the middle and top part. Furthermore, abundant supply of OPF petioles is guaranteed from plantation because fronds are constantly available during fronds pruning for fruit harvesting. The petiole of the OPF contains the fibre (mainly lignocellulosic materials) covered with a hard epicarp. OPF is found to contain high amount of carbohydrates which are convertible to simple sugars, biofuels, etc. OPF is reported to contain higher holocellulose (about 84% dry matter content comprising cellulose and hemicelluloses) compared to the fibres of pineapple leaves, coconut leaves and banana stem (Lee & Boateng, 2013).

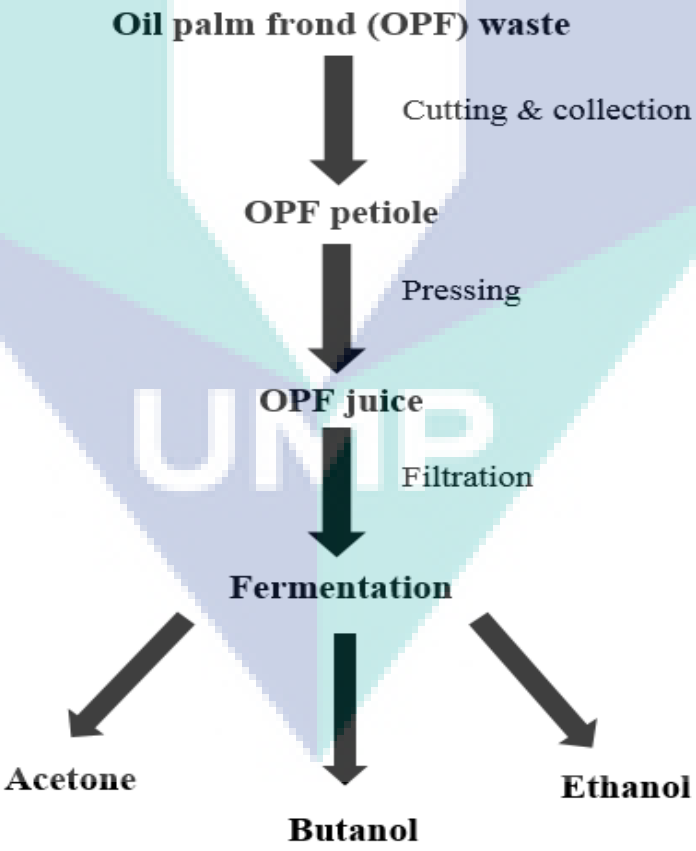

Figure 2.6 A general process for biobutanol production from OPF juice

Figure 2.6 shows a general process for biobutanol production from OPF juice. Zahari et al. (2012) discovered a substantial amount of sugars can be obtained simply by pressing the OPF. The total sugars obtained was 76.09 g/L comprising 1.68 g/L fructose, 53.95 g/L glucose and 20.46 g/L sucrose. Moreover; OPF juice is rich in minerals and nutrients which are essential for bacterial growth during fermentation. Maail et al. (2014) also suggested that OPF juice could be a potential renewable substrate for fermentation process considering that the free sugars are obtainable with a simple mechanical treatment, are consistently available since OPF is pruned daily, do not inhibit microbial growth, and contain no impurities.

2.5 Factors affecting biobutanol production

Environmental factors within ABE fermentation is very important and greatly influence the process because of the typical difficult biphasic fermentation characteristics. Moreover, the initiation of sporulation, both of which can be profoundly affected by various environment control parameters including pH, temperature, inoculum size, medium components, as well as substrate and product inhibition (Papoutsakis, 2008). For example, acid crash with no solvent generation due to poor process control such as pH instability can be frequently observed during ABE fermentation by solventogenic clostridia (Sillers et al., 2008). Therefore, a specific culture conditions for a specific comparative study might be close to optimum for some species and strains.

2.5.1 Initial pH

Appropriate control strategy for pH in ABE fermentation is crucial towards the shift of a process from acid production phase to a phase for solvents formation, leading to a higher production of biobutanol (Jones and Woods, 1986). Substrate is converted into acids during the acidogenesis (acetic acid and butyric acid are produced by exponentially growing cells). Under certain conditions, the bacterial culture stops growing and solventogenic phase is stimulated. In fermentation broth, when the acidogenesis phase is completed and it enters solventogenic phase to produce butanol, acetone and ethanol. Unfortunately, overproduction of acids leads to inactivation of the microbial culture and absence of solvent formation. Thus, the initial pH of fermentation broth must be appropriate for the cell culture.

As reported by Linggang et al. (2013), the ABE production by *C. acetobutylicum* ATCC 824 is the highest at initial pH 5.0 (7.35 g/L) as compared to the other pH values 5.5, 6.0 and 6.5. Similar to Geng and Park (1993) mentioned that high yield of solvent (0.39 g/g) were obtained at initial pH 5.0. Meanwhile, the total solvent was very low at pH 6.5 (1.48 g/L) but the acids accumulation up to 13.31 g/L. From this previous studied, it was found that the increasing in initial pH, will increase the production of acids, not the solvents. According to Ibrahim et al. (2012), accumulation high of organic acids between 5 and 13 g/L in fermentation system can disrupt the membrane protein function in the cell by acidifying clostridia cells cytoplasm. This condition may result in inhibition of metabolic functions in the fermentation process.

However, the higher production of butanol by *C. acetobutylicum* NCIMB 13557 using modified date fruit medium was observed at initial pH 7 (Khamaiseh et al., 2011). Khamaiseh et al. (2011) compared the production at initial pH 6 and 7. They found out at initial pH 7, the butanol produced was 5.31 g/L compare to 4.36 g/L at pH 6. The other study by Al-Shorgani et al. (2015) obtained the butanol and ABE increased when the initial pH raised from 4 to 5.8. However, increasing the pH of the culture more than 5.8, the production was decreased. This study indicated that *C. saccharoperbutylacetonicum* N1-4 was capable of growing and producing butanol at a wide range of initial pH value (4.0–8.0). This can be concluded that the initial optimum pH for solvent production varies with strain, culture and substrate conditions. Therefore, in this study, initial pH of 5.0 to 7.0 was chosen to screen the effect for biobutanol production.

2.5.2 Temperature

Suitable temperature of a medium during fermentation influenced the total yield, the ratios of solvents and the rate at which solvents are produced. The temperature can effect on the enzymatic pathway of the *C. acetobutylicum* and this lead to loss of its ability to produce acids in acidogenesis and also to convert the acids to solvent in. solventogenesis. Al-Shorgani et al. (2015) and Chen et al. (2013) discovered similar pattern of incubation temperature of *C. saccharoperbutylacetonicum* N1-4. They observed that the butanol production was increased when the temperature ranging from 25 to 30°C. Whereas, at higher temperature at 35 to 45°C, the butanol production was reduced.

Khamaiseh et al. (2011) also discovered the most favourable temperature for their works is at 35°C. They used *C. acetobutylicum* NCIMB 13357. However, the butanol production by *C. acetobutylicum* NRRLB527 was highest at temperature 30°C (Mane and Deshmukh, 2013). The other study by Ni et al. (2012) used *C. saccharobutylicum* DSM 13864 discovered 37°C as the optimum temperature. Therefore, 32°C - 42°C was chosen as incubation temperature for biobutanol production in this study.

2.5.3 Inoculum size

Inoculum size is one of important factor affecting ABE fermentation by Clostridia. Al-Shorgani (2015) observed, arise in inoculum size from 5% to 15% resulted in an enhanced butanol and ABE concentration and a further increase in the inoculum size from 15% to 20% led to a constant value of ABE concentration. Ranjan et al. (2013) mentioned this phenomenon can be attributed to the fact that an increase in the inoculum size more than the critical level (optimum value) had no considerable effects on lag phase of microbial growth and cell activity, which consequently resulted in the production of butanol in concentrations close to optimum inoculum size.

Futhermore, Razak et al. (2013), discovered an optimum inoculum size of 16.2% for butanol production by *C. acetobutylicum* from oil palm decanter cake hydrolysate. However, the production of butanol by *C. acetobutylicum* MTCC481 from rice straw hydrolysate was investigated by Ranjan et al. (2013) and they revealed that an inoculum size of 5% was the optimum inoculum size for the production of butanol in ABE fermentation. Apart from that, studies performed by Mane and Deshmukh (2013) found that the maximum butanol production by *C. acetobutylicum* NRRLB527 from glucose was achieved at an optimum inoculum size of 10% (v/v). Therefore, the optimum critical value of inoculum size varies on each fermentation process. From this review, 1% - 20% of inoculum size was chosen to investigate the effect for biobutanol production in this work.

2.5.4 Initial sugar concentration in substrate

Both low and high present of initial sugar concentration in the fermentation substrate could lead to lessen microbial growth, thereby making the process for the solvents formation unfavorable. As studied by Linggang et al. (2013), they investigated the performance of ABE production by *C. acetobutylicum* ATCC 824 using different

concentration of hydrolysate (30, 50 and 70 g/L) containing 23, 40 and 57 g/L glucose, respectively. They found that the highest production was at 50 g/L of hydrolysate with 40 g/L glucose content. Moreover, Linggang et al. (2013), observed similar trends with the study done by Al-Shorgani et al. (2012), who reported that increasing glucose concentration up to 50 g/L tend to increase the solvent production. But with the higher value more than 50 g/L, resulted in decreasing of production.

Komonkiat & Cheirsilp (2013) also observed the highest butanol production was at 50 g/L of initial sugar concentration in the substrate. They use oil palm sap as the substrate for ABE fermentation by *C. acetobutylicum* DSM 1731 and found out the production of solvents drastically decreased when increased the initial sugar more than 50 g/L. This may due to the inhibitory effect of a high concentration of sugar and also a high concentration of acids produced during acidogenesis at high sugar concentration. Therefore, 40 g/L – 60 g/L was chosen as initial sugars concentration for biobutanol production in this study.

2.5.5 Yeast extract concentration

Microbial growth rate will increase when supplemented with organic nitrogen sources which contains compound like protein, yeast extract, amino acid, and glutamic acid in a culture medium (Abou-Zied and Yassein, 1976). Welsh et al. (1987) mentioned that combining certain organic and inorganic nitrogen sources could facilitate the microbial rate of growth, utilization of substrates and solventogenesis phase. Alla et al. (2012) reported the combination of organic nitrogen source, yeast extract (5 /L) and inorganic nitrogen source, ammonium nitrate (1.6 g/L) to spoilage date fruits homogenate significantly enhanced ABE production. The effect of yeast extract in ABE fermentation was due to the degradation of the amino acids in yeast extract, which is the major source for growth (Zhang and Wiegel, 1990). Li et al. (2012) was investigated the cause for the delay of phase shift in ABE fermentation in cassava substrate and finally found out that low nitrogen contents was the reason. They observed the starch consumption was accelerated, almost completely used and the phase shift occurred smoothly after adding yeast extract into the substrate.

Chua et al. (2013) discovered the effect of butanol production in medium supplemented with 0.1%, 0.4% and 1.0% of yeast extract and the production significantly

improved with 0.4% and 1.0%. Besides, the optimum yeast extract concentration by butanol-producing microflora identified as *Clostridium sps.* reported by Cheng et al. (2012) was at 5.13 g/L. The other study by Al-Shorgani et al. (2016) discovered increasing the yeast extract up to 4 g/L enhanced biobutanol production but more than 4 g/L of yeast extract decreased biobutanol production by *C. acetobutylicum* YM1. Meanwhile, Linggang et al. (2013) evaluated the effect of yeast extract concentration ranging from 0 to 5 g/L on ABE production in sago pith residues hydrolysate. In contrast, they discovered the highest performance of ABE fermentation by *C. acetobutylicum* ATCC 824 was achieved when 0.5 g/L of yeast extract was supplied into the fermentation medium. However, Choi et al. (2012) observed high cell density of *C. acetobutylicum* when 10 g/L yeast extract was supplemented. It was suggested that the best condition for solvent production occurs when the nutrient supply is just sufficient for growth, depends on the type of strain and substrate.

Previous study by Li et al. (2014), they used corn steep liquor as nitrogen sources in ABE fermentation in cassava substrate. The results from this study indicated that the phase shift occurred smoothly when corn steep liquor was added into the fermentation substrate, which enhanced butanol and total solvent production. In general, organic nitrogen sources such as corn steep liquor also can provide various amino acids, minerals, growth factors and vitamins that promote good growth of microorganisms. However, yeast extract, is the most common used nitrogen sources for cell culture and fermentation processes. Thus, 1 g/L – 10 g/L was chosen as initial yeast concentration for biobutanol production in this study.

2.6 Experimental design

In this study, to get the optimum production of biobutanol from OPF juice, optimization was done to select the best fermentation condition from a set of available parameters. The optimization was used the response surface methodology (RSM). For an optimization to be successful, the selected parameters were screened to identify the factors with high contribution for the fermentation. Factorial analysis was applied in the screening process of this study using fractional factorial design (FFD). Factorial analysis helps to screen out the factors affecting. RSM is then applied for the optimized biobutanol production.

The software Design Expert version 7.1.6 is used in the statistical discussions for both factorial design and the RSM. Design Expert is a statistical software tool which is developed by Stat-Ease, Inc. This software provides assistance in statistical research be it at two-level factorial designs or mixture design technique.

2.6.1 Fractional factorial design (FFD)

Factorial analysis was used to determine the influence of several factors on the response (Golshani et al., 2013). A lot of information was provided with minimum run of experiment (Rozet et al., 2013). Besides, non-significant variables were eliminated in the process for further optimization process. Factorial analysis studies all of the main factors, interactions between the factors and its effect to the responses (Montgomery, 2004). The experimental design is constructed by setting all factors at two levels that were used as the limits of the values range (Bingol et al., 2010). The two levels of the factors are low level (-1) and high level (+1).

When having a lot of factors, there is a large amount of test runs needed even without using replication. As for an example, a design with 10 factors at two levels will result in a 210 or 1024 combination of runs. At certain point, this is not practical with the high amount of cost for the operation and raw material. In order to overcome this problems, fractional factorial design (FFD) is applicable as it offers efficient and economical results and also have less runs compared to a full factorial design (Bezerra et. al., 2008). FFD experiments are alternatives to complete factorial analysis when budgetary, time, or experimental constraints without the execution of complete factorial experiments without neglecting the interaction effects between factors (Mason et al., 2003).

2.6.2 Response surface methodology (RSM)

Response surface methodology (RSM) is one of the most efficient method for experimental design to find the optimum condition for the factors could either be a maximum or a minimum. Optimization is a mean to select the best factor or effect from a set of available parameters and to explain the relationships between the factors and their response. RSM is a collection of mathematical and statistical techniques that is extensively used to predict the optimum response with good precision for the combination of several factors that influence the process (Aslan, 2008). RSM were used in this

experiment after FFD for developing, improving and optimizing the process. For RSM, one of the commonly used optimization procedures is based on full factorial central composite design (CCD).

CCD is the fraction of three-level factorial experiments that can rotatable. Three best factors were used in CCD to measure interaction and produces the optimum condition. In CCD, there is response surface plot which is a three dimensional graph that illustrate the relationship between the factors and the response. The graph is plotted based on two factors while the other factors are constant at the optimum points (Tabaraki and Nateghi, 2011).

CHAPTER 3

METHODOLOGY

3.1 Overall Methodology

The research methodology for biobutanol production from OPF juice by *C. acetobutylicum* ATCC 824 was separated into three main parts; preliminary study, the factor screening process and the optimization process. The brief overall process method for this research is illustrated in the flowchart on Figure 3.1. The process started with the collection and preparation the OPF juice. The fresh OPF (without leaves) were collected from the oil palm plantation in Felda Lepar Hilir, Kuantan, Pahang and directly extracted by pressing the frond using a conventional sugarcane press machine. The determination of the sugar content in OPF juice was the next process. As for this process, the initial sugar content of OPF juice can be prepared to the required sugar concentration for each experiment needed.

The next process was the fermentation of *C. acetobutylicum* ATCC 824 from OPF juice to produce biobutanol as the first objective for this research. The analysis of factors affecting the biobutanol production was investigated as the second objective. The five factors that were analysed; initial pH, incubation temperature, initial sugar concentration, inoculum size and yeast extract concentration. The consecutive steps were categorized under the third objective of this research; the optimization of biobutanol production process. The third objective started with the selection of factors that have the highest contribution to production during factor analysis process. These selected factors were optimized in order to achieve the third objective. The validation of the experimental data was performed at suggested optimum condition.

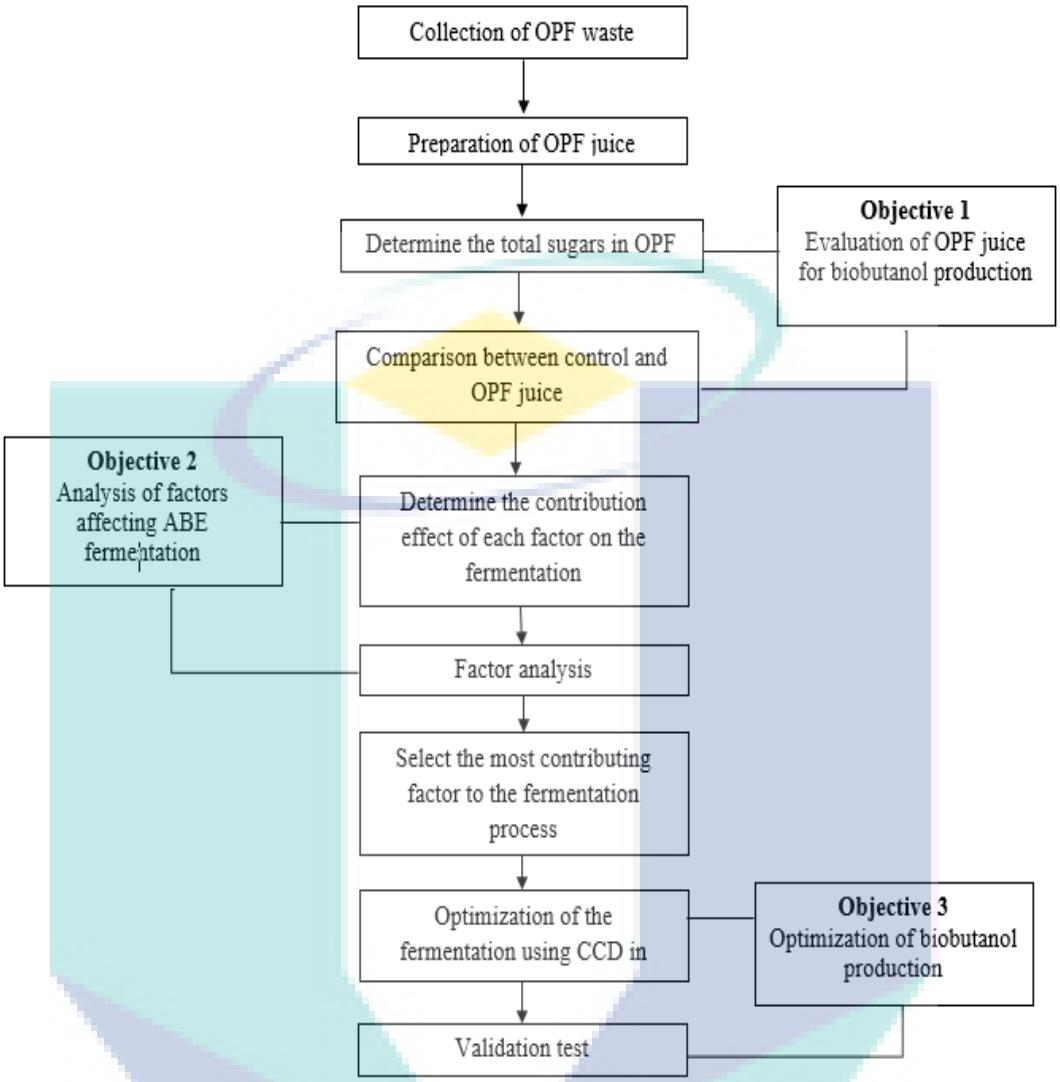

Figure 3.1 A brief process of the ABE fermentation from OPF juice

3.2 Chemicals and materials

3.2.1 Chemicals and reagents

The chemicals in this study were purchased from various suppliers. The list of the chemical was in Appendix A.

3.2.2 Raw material (oil palm frond juice)

Oil palm frond (OPF) juice was prepared by pressing the fresh OPF (without leaves), and centrifuge to remove the precipitate. 50 kg of fresh OPF (without leaves) was collected from oil palm plantation at Felda Lepar Hilir, Kuantan, Pahang. OPF was cut into small size (40 cm – 60 cm) as in Figure 3.2 and pressed using sugarcane pressing machine. Then, OPF juice was centrifuged at 10,000 rpm for 10 min and filtered to remove the solid particles. The precipitate (pellet) was decanted and the supernatant (OPF juice) as in Figure 3.3 was used in the fermentation. From 50 kg of OPF, 25 L of OPF juice obtained. OPF juice was stored at -4^{o}C until it wants to be used.

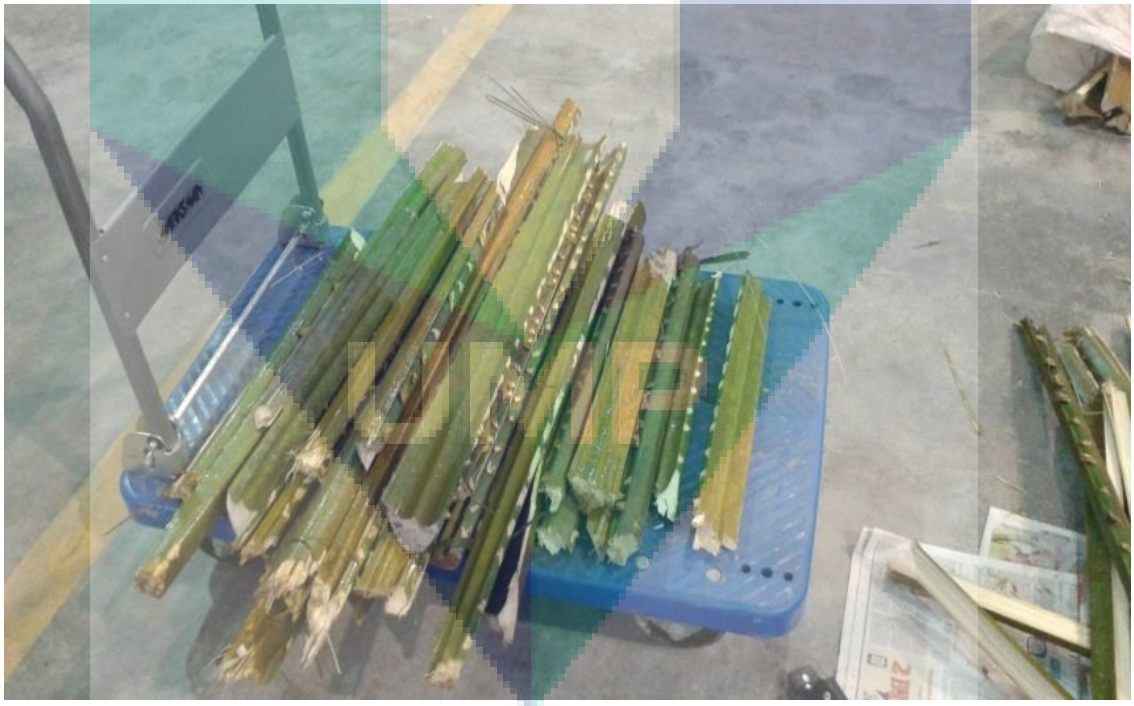

Figure 3.2 Fresh OPF (without leaves)

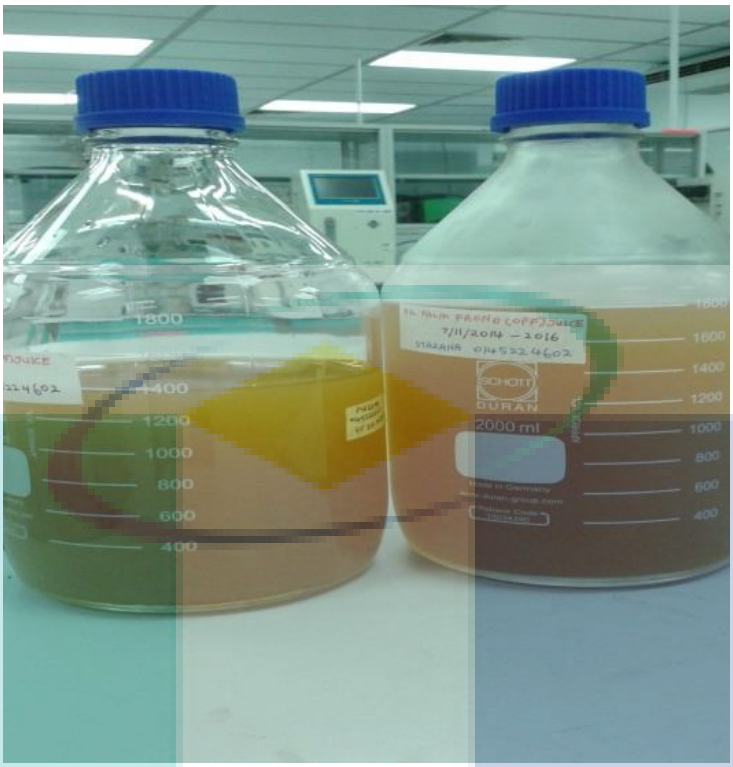

Figure 3.3 OPF juice after centrifugation

3.3 Microorganism

On reviving the bacteria, the freeze-dried *C. acetobutylicum* was mixed to 100 mL sterile deoxygenated, reinforced clostridial medium (RCM) in a serum bottle. RCM was used for reviving the bacteria and for inoculum development. The cultures were incubated at 37ºC for 20-24 hr to its exponential phase at static condition. The grown cultures will be ready when the pH is between 5.2-5.5 and are observed to be healthy and motile under a microscope at 400x magnification. The bacteria were then maintained and preserved in glycerol-lactose solution at -80ºC.

3.3.1 Glycerol stock preparation

Glycerol-lactose solution was prepared by mixing 20 mL of glycerol with 10 g of lactose. 80 mL of distilled water was added into the mixture and the mixture was then sterilized at 121ºC for 15 min. On preparing the glycerol stocks, 100 mL of grown cultures were mixed with 100 mL glycerol-lactose solution, under sterile condition. 1 mL of the mixture was then aliquot to each of sterile cryovial tubes and stored at -80ºC.

3.3.2 Inoculum preparation

Each cryovial tube which consists of 1 mL of glycerol stock was mixed with 100 mL of sterile deoxygenated, RCM in a serum bottle under sterile conditions. It was then incubated at a static condition for 72 hours at 37°C. A spore suspension was produced and can be stored at 4°C. Prior to usage, the spore suspension was heat-shocked by immersing in 90°C water bath for 90 s. 10 mL of spore suspension was inoculated into 90 mL sterile deoxygenated RCM aseptically. The mixture was incubated in static conditions for between 18-20 hr at 37°C until it was ready to be used.

The inoculum was seemed to be ready for fermentation when there were numerous bubbles of carbon dioxide (CO_2) had been produced, which indicates active respiration by the growing cells as shown in Figure 3.4. Other than that, the rod-shaped motile bacteria were present under a microscope at 400x magnification, while other bacterial morphologies were absent. The pH and optical density at 600 nm of the inoculum should be in the range 5.2-5.5 and 1.5-2.0; respectively.

Figure 3.4 Grown inoculum of *C. acetobutylicum* ATCC 824

3.4 Medium preparation

3.4.1 Growth medium (RCM)

RCM was used for reviving the bacteria and for inoculum preparation. RCM was prepared by dissolving 38 g of RCM powder in a litre of distilled water. Solutions were boiled for 1 min while being stirred with a magnetic stirrer to ensure homogeneity. The liquid media were then distributed each into 125 mL serum bottles, sparged with oxygen-free nitrogen gas, sealed and sterilized at 121^{o}C for 15 min.

3.4.2 Biobutanol production medium

OPF juice was distributed up to required working volume into 125 mL serum bottles, adjust the pH, sparged with oxygen-free nitrogen gas, sealed and sterilized at 121°C for 15 min as shown in Figure 3.5. For enhancement of biobutanol production, P2 synthetic media was added to OPF juice. The components of the P2 medium are listed in Table 3.1 below (Qureshi & Blaschek, 1999).

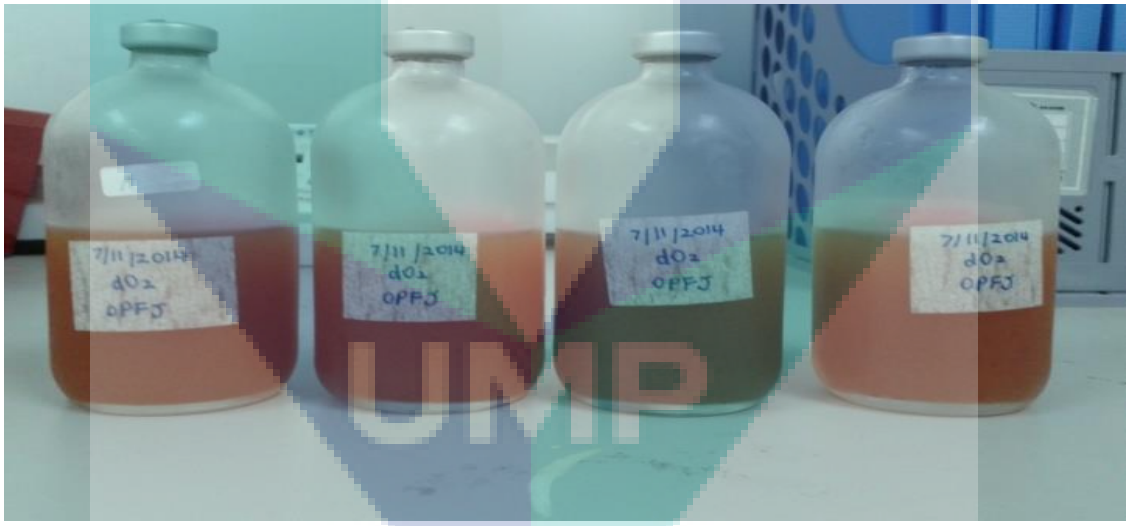

Figure 3.5 Sterile OPF juice

P2 medium developed by Monot et al. (1982) has been widely used until now. The P2 medium is actually designed for saccharolytic clostridia containing buffer, minerals, vitamins and yeast extract (Annous and Blaschek, 1990). Enhancement in biobutanol production has been found when supplementing P2 medium with different substrates such as cassava starch (Thang et al., 2010), maltodextrins (Formanek et al., 1997) and starch packing peanuts (Ezeji et al., 2003). Al-Shorgani et al. (2012) reported

that tryptone yeast extract acetate medium (TYA) and P2 medium are the most favorable medium composition for biobutanol production as compared to reinforced clostridial medium (RCM) and anaerobic sugar medium (AnS).

Table 3.1 Composition of nutrient content of P2 medium

Source: Qureshi & Blaschek (1999)

Nutrient content		Composition (g/L)
	Yeast extract	1
Buffer	KH_2PO_4	0.5
	K_2HPO_4	0.5
	Ammonium acetate	2.2
Mineral	$MgSO_4.7H_2O$	0.2
	$MnSO_4.7H_2O$	0.01
	$FeSO_4.7H_2O$	0.01
	NaCl	0.01
Vitamin	Para-amino-benzoic acid	0.001
	Thiamine	0.001
	Biotin	0.00001

The yeast extract solution was autoclaved at 121°C and then cooled to room temperature. A stock solution of other nutrients (buffer, minerals, and vitamins) was filter sterilized (0.2µm) and added to the yeast extract and OPF juice as carbon source. The pH of each solution was adjusted to the required pH using 0.5M Sulphuric Acid (H_2SO_4) and 0.5M Sodium Hydroxide (NaOH).

3.5 Sugar analysis of OPF juice

Glucose, sucrose, and fructose were analyzed by high performance liquid chromatography (HPLC) (Agilent 1200 series, U.S.A) using Rezex ROA – organic acid H+ (8%) column (Phenomenex) (300 x 7.80 mm) with a flow rate of 0.5 mL/min and refractive index detector (RID) detector at 30°C. The mobile phase running consists of 0.005M H_2SO_4, recommended for the column used. The column was set at 60°C for a 30 min run time and an injection of 10 µL (Langenberg et al., 2012). Standards of glucose, fructose and sucrose were used to make the standard curve for each sugar using the HPLC. The standard calibration graphs were in Appendix B.

3.6 Preliminary study of biobutanol production

This research was started with determination of growth profile of the bacteria, sugar analysis of OPF juice, and comparison study of biobutanol production by *C.*

acetobutylicum ATCC 824. This set of experiment was done at static condition, pH 6, 37°C and 50 g/L initial sugar concentration. The results were compared with control experiment which is using analytical grade of sugar.

The determination of growth profile of the bacteria was conducted in 120 mL serum bottle, with 100 mL working volume. Growth media was prepared according to Table 3.1 (P2 medium) and 50 g/L initial sugar concentration with 10% (volume of inoculum per volume of media) of actively growing inoculum developed earlier in section 3.3.2. Fermentations were preceded at 37°C without agitation. Over the course of the fermentation, 2 mL samples were taken intermittently every 24 hr, until 144 hr for analytical procedures; off-line pH reading, optical density (OD) measurement and cell dry weight (CDW).

The sugar analysis of OPF juice was determined using OPF juice obtained in section 3.2.2. The method of HPLC used as in subtopic 3.5. The last part for this preliminary study was to compare the biobutanol production by *C. acetobutylicum* ATCC 824 in control fermentation (P2 medium) with OPF juice. The fermentation was conducted in 120 mL serum bottle, with 100 mL working volume. Growth media was prepared according to Table 3.1 (P2 medium) and 50 g/L initial sugar concentration with 10% (volume of inoculum per volume of media) of actively growing inoculum developed earlier in section 3.3.2. Fermentations were done at 37°C without agitation. Over the course of the fermentation, 2 mL samples were taken intermittently every 24 hr, until 144 hr for analytical procedures; off-line pH reading, optical density (OD) measurement, sugar and products (solvents and acids) determination.

3.7 Factorial analysis method

The experimental design for factorial analysis was performed using Design Expert software (Version 7.1.6, Stat-Ease, Inc., Minneapolis, MN) program. The experimental table was constructed in two-level 2^{5-1} factorial design. In factorial analysis, five factors were studied with 21 runs of experiment. The main factors and the interactions between the factors were determined in factorial analysis. Information about the most contribution factors influenced the production of biobutanol from OPF juice also provided in this analysis.

3.7.1 Experimental setup for factorial analysis

Five variables were evaluated including the pH, total sugars in OPF juice, inoculum size, temperature and yeast extract concentration using 2-level half factorial design. The initial pH value for the fermentation process was varied from five to seven. The total initial sugars in OPF juice was set between 40 to 60 g/L. The initial sugars concentration for fermentation was diluted to the concentration required from the original sugar concentration of OPF juice determined in section 3.5, result showed in Table 4.1. The inoculum size was used between 1 to 20% prior to total working volume, 100 mL. The incubator temperature for the fermentation process was set between 32 to 42°C. The concentration of yeast extract was varied from 1 to 10 g/L. Table 3.2 shows the level for each factor for in factorial analysis.

Table 3.2 Factors applied in 2^{5-1} design, the coded levels and actual values.

Factors	Symbols	Units	Level		
			Low (-1)	Middle (0)	High (+1)
pH	A	-	5	6	7
Total sugars in OPF juice	B	g/L	40	50	60
Inoculum size	C	%	1	10.5	20
Temperature	D	°C	32	37	42
Yeast extract concentration	E	g/L	1	5.5	10

3.8 Optimization method

The experimental design for optimization was done by using Design Expert software (Version 7.1.6, Stat-Ease, Inc., Minneapolis, MN). The experimental table was constructed by using central composite design (CCD) in response surface methodology (RSM). CCD was applied to identify the relationship between the factors and their response. The selection and range of three factors for the optimization were chosen from the factorial analysis. Based on these three factors, 20 runs of experiments were generated including 6 centre points.

3.8.1 Experimental setup for optimization

Temperature, yeast extract concentration and inoculum size were selected for the optimization to determine the optimum conditions for biobutanol production. The incubation temperature was set between 34 to 40°C, yeast extract concentration 3.5 to 7.5

g/L and inoculum size from 8 to 12%. The experimental table for optimization was constructed with five levels of numeric factors. The five levels consisted of plus and minus alpha (axial point), plus and minus 1 (factorial points), and the center point. Table 3.3 show the level for each factor in optimization.

Table 3.3 Experimental range and levels of the independent variables.

Independent variables	**Range and levels**				
	-α	**-1**	**0**	**+1**	**+α**
Temperature (°C)	31	34	37	40	43
Yeast extract (g/L)	1.5	3.5	5.5	7.5	9.5
Inoculum size (%)	6	8	10	12	14

3.8.2 Validation experiment for optimization

The validation experiment was conducted from the highest biobutanol yield produced in the optimization experiments generated from CCD. Table 3.4 shows the condition for each of the factors that were used in this experiment. The predicted and experimental values were compared to determine the validity of the model. Eq. (3.1) was used to calculate the percentage error between the values.

Table 3.4 Model validation of the biobutanol production.

Parameters	**Value**
Inoculum size (%)	10
Temperature (°C)	37
Yeast extract (g/L)	5.5

$$\% \, Error = \frac{(Actual \; value - Predicted \; value)}{Actual \; value} \times 100\% \qquad 3.1$$

3.9 Analysis procedures

2 mL of the samples were used for measures the optical density (OD) at 600nm to evaluate the bacterial growth. The other 3 mL of sample was used for determination of solvents and sugars by gas chromatography (GC) and high performance liquid chromatography (HPLC).

3.9.1 Determination of acetone, biobutanol, ethanol and acids

Samples withdrawn from the fermentation serum bottle at 0 hr and 144 hr were dispensed into 1.5 mL eppendorf tubes and were spun down for 5 min at 15,000 x g. The supernatant was withdrawn using 1 mL syringe and passed through a nylon membrane filter 0.20 µm (Milipore, USA).

Solvent concentrations (acetone, butanol and ethanol) and acids (acetic and butyric) were determined by gas chromatography (Agilent Technologies, 6890N network GC system). An external standard method was used which 0.7 mL of methanol was mixed with 0.7 ml of sample's supernatant liquid. A microliter of the mixture was injected into a 30 m x 0.32 mm x 0.5 µm HP-INNOWax capillary column with 0.25µm film thickness. The column carrier was helium, 40 cm/sec, 11.7 psi (60°C) with 2.5 ml/min constant flow. The injection was split 50:1 with a flow of 50 mL/min through the column. Initial temperature of 45°C was held for 2.5 min, then raised by 10°C/min up to 150°C, held for 14 minutes, and raised by 10°C/min up to 250°C, held for a minutes for a total run time of 17.222 minutes. The analytes were determined with a flame ionization (FID) detector held at 275°C (Lin et al., 2013). Standards for acetone, butanol, ethanol, acetic acid and butyric acid were made for each analyte tested using the gas chromatograph. The standard calibration graphs were in Appendix D.

3.9.2 Residual sugar determination using HPLC

Samples withdrawn from the fermentation serum bottle at 0 hr and 144 hr to determine the residual sugars. Samples were dispensed into 1.5 mL eppendorf tubes and were spun down for 5 min at 15,000 x g. The supernatant was withdrawn using 1 mL syringe and passed through a nylon membrane filter 0.20 µm (Milipore, USA). The filtrate will be analyzed using HPLC (Agilent Series 1200, USA) as explained in Section 3.5, page 34. The standard calibration graphs were in Appendix C.

3.9.3 Cell concentration determination

The concentration of *C. acetobutylicum* was determined by the cell dry weight method. A correlation between the cell dry weight (CDW) and optical density at 600 nm (OD_{600}) needed to be established. First, the optical density of sample (fermentation broth) was determined by measuring at 600 nm using UV/visible spectrophotometer. Next, 2.0 mL from the same sample was centrifuged at 13,300 for 5 min and the pellet was washed once with distilled water and leave to dry at 80°C for 48 hr.

The weight of the empty centrifuge tube was measured before the sample was filled (W1). Then, the dried cells were kept in the desiccators and weigh until consistent reading was recorded (W2). The CDW can be calculated from equation (3.2). CDW of corresponding OD_{600} was obtained as in Appendix E. A correlation between the CDW and the OD_{600} was developed, that is CDW (g/L) = (1.2017 × OD_{600}) + 0.3753. Cell concentration at CDW basis can be determined from this correlation.

$$\text{CDW (g/L)} = \frac{(\text{W2} - \text{W1})\ \text{g}}{2\ \text{mL}} \times 1000\ \text{mL} \qquad 3.2$$

3.9.4 Evaluation of Solvents and acid fermentation in Batch Culture

Data obtained from GC and HPLC from the experiment were evaluated to calculate the biobutanol production yield. The yield can be calculated based on equation 3.3.

$$\text{Biobutanol yield (g/g)} = \frac{\text{Biobutanol concentration (g/L)}}{\text{Sugar consumption (g/L)}} \qquad 3.3$$

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Sugars content in OPF juice

The OPF is waste generated from oil palm industry which consists of petiole (the stem) and many long leaflets on both side of the stem, rich in cellulosic materials and sugars. The renewable sugars from OPF juice can simply be obtained by pressing the OPF as stated in subtopic 3.2. The example HPLC chromatograms of sugar as shown in Appendix B. It was observed that the composition of the sugars contained glucose as major component in OPF juice at 48.19 g/L. The other sugars present were sucrose (8.48 g/L) and fructose (11.91 g/L) as stated in Table 4.1. These sugars are among some of the common sugars favorable for bacteria consumption.

As reported by Jiang et al. (2014), glucose-based fermentation produced the highest ABE production, far ahead of other sugars that they had tested, arabinose, mannose, xylose and cellobiose. This agrees with Grimmler et al. (2010) and Servinsky et al. (2010), stated that glucose is the most efficient as carbon source for the transcriptional regulation and metabolism genes which can be seen through consistency of ABE production comparing with other sugars in their works. The result obtained is similar to sugars compositions in OPT sap, which reported by Kosugi et al. (2010), Yamada et al. (2010), and Komonkiat & Cheirsilp (2013) where glucose has been found to be the major sugar component in the sap. However, Obahiagbon and Osagie (2007) who reported different sugars compositions that oil palm sap collected in Nigeria containing sucrose as the dominant sugar. The discrepancies may be due to the differences in varieties, species and cultivating conditions.

Table 4.1 Compositions of oil palm frond (OPF) juice from other researchers

Research	Sugars (g/L)			Total sugar (g/L)
	Glucose	Sucrose	Fructose	
This research	48.19	8.48	11.91	68.58
Zahari et al. (2012)	53.95	20.46	1.68	76.09
Che Maail et al. (2014)	32.70	7.17	4.93	44.8
Aliyu et al. (2015)	40.50	NM	NM	40.50

*NM is Not Mentioned

Table 4.1 shows the differences of sugar content from Zahari et al. (2012), Che Maail et al. (2014) and Aliyu et al. (2015). The sugar composition was also similar with the findings reported by Zahari et al. (2012). However, Zahari et al. (2012) showed slightly higher total sugars concentration (76.09 g/L) with glucose 53.95 g/L and sucrose content was higher than fructose with the value 20.46 g/L and 1.68 g/L, respectively. Meanwhile, as showed in Table 4.1, in this study, the sugars composition was reported as; 68.58 g/L with glucose 48.19 g/L and fructose was higher than sucrose, 11.91 g/L and 8.48 g/L, respectively. The differences between this study and the study conducted by Zahari et al. (2012) was 10.95%. Aliyu et al. (2015) also used OPF juice and found out the highest glucose concentration was 40.5 g/L. There was a slight difference in total sugars concentration by Zahari et al. (2012), Aliyu et al. (2015) and the findings of this thesis. Each OPF is individually different from others at different plantations may differ in sugar concentration. The OPF in this work was obtained from the oil palm plantation in Gambang, Pahang, meanwhile Zahari et al. (2012) obtained from Serdang, Selangor and Aliyu et al. (2015) from Batu Pahat, Johor.

Futhermore, the oil palm tree age difference also gives different amount of sugar, which older tree contains lesser sugar (Murai & Kondo, 2010). Aliyu et al. (2015) reported that the OPF petiole with the age limit of 15-25 years gives higher sugars concentration of 40.5 g/L as compared with 17.85 g/L of 5-10 years. The high sugar content indicates its suitability as a renewable carbon source for the production of biobutanol through ABE fermentation. The total sugar concentration was then diluted into the required concentration based on each experiment. In general, glucose and fructose were reported as the preferred substrates over sucrose consumed simultaneously by *C. acetobutylicum* (Servinsky et al., 2010).

4.2 Comparison study

In this study, OPF juice was chosen as potential substrate to produce biobutanol by *C. acetobutylicum* ATCC 824. The fermentation experiments for both using synthetic sugars as control and OPF juice were done to compare the capability of solvents production. The control batch fermentation was conducted using 50 g/L synthetic sugars as the carbon source, same as sugar concentration used in OPF juice, 50 g/L. The sugars consumption and solvents production were monitored every 24 hr for 144 hr to study the metabolism of the fermentation process. The chromatograms of standard solvents produced which determined by GC as in Appendix B.

The control and OPF juice batch fermentation were conducted with same parameters; initial pH 6, inoculum size 10%, initial sugars concentration 50 g/L and at incubation temperature 37°C. For this part, the samples were taken at 72 hr for analysis. As shown in Table 4.2, the culture produced 4.87 g/L acetone, 9.24 g/L butanol, and 1.59 g/L ethanol with total solvents 15.70 g/L using OPF juice. The fermentation using synthetic sugars resulted in 5.43 g/L acetone, 10.91 g/L butanol and 1.35 g/L ethanol with total solvents 17.69 g/L. The ABE production using synthetic sugars is slightly higher than fermentation using OPF juice by 11.25%. The results showed that the biobutanol and ABE production from OPF juice were almost comparable with that produced from the synthetic sugars at similar total initial sugars concentration.

Table 4.2 Comparison of biobutanol production using OPF juice and synthetic sugars.

Parameters	**Medium**	
	Control	**OPF juice**
Acetone (g/L)	5.43	4.87
Butanol (g/L)	10.91	9.24
Ethanol (g/L)	1.35	1.59
Total ABE (g/L)	17.69	15.70
Acetic acid (g/L)	2.06	3.86
Butyric acid (g/L)	1.04	0.60
Total acid (g/L)	3.10	4.46
Sugar consumption (g/L)	40.95	38.29
Butanol productivity (mg/L/h)	75.76	64.14
ABE productivity (mg/L/h)	122.85	108.96
Butanol yield (g butanol/g sugar)	0.27	0.24
ABE yield (g ABE/g sugar)	0.43	0.41

Based on 72 hr fermentation time, 76.58% of sugars in OPF juice was consume by the strain, leaving 11.71 g/L of residual sugars in the medium. Meanwhile, 81.9% of synthetic sugars was consumed in control experiment. The 5.32% difference sugars consumption is negligible since the OPF juice used is a mixture of other components. The ABE yield and productivity obtained using OPF juice were 0.41 g/g and 108.96 mg/L/h, respectively. This results almost comparable to the result obtained using synthetic sugars which the ABE yield and productivity was 0.43 g/g and 122.85 mg/L/h, respectively. The comparison showed that there was no significant difference among the fermentations suggesting that OPF juice did not inhibit *C. acetobutylicum* ATCC 824 growth.

In the glucose fermentation by *C. beijerinckii* BA 101 reported by Qureshi et al. (2008), they used 55 g/L glucose as carbon source. Total ABE was produced 18.1 ± 1.5 g/L which 13.2 g/L was butanol, leaving behind 7.8 g/L residual glucose. The fermentation resulted in ABE yield of 0.38 ± 0.006 g/g. The results obtained in this research showed 13.16% higher ABE yield with 0.43 g/g values. *C.acetobutylicum* ATCC 824 seems to be more capable in the ABE fermentation process. The other ABE fermentation using synthethic glucose by *C. acetobutylicum* ATCC 824 by Razak et al. (2013) reported ABE concentration and yield 13.68 g/L and 0.22 g/g, respectively. The lower ABE yield by Razak et al. (2013) was due to the fact that the growth of *C. acetobutylicum* ATCC 824 is inhibited at butanol concentration 7-13 g/L, thus the culture cannot undergo the solventogenic phase at this level (Haggstorm, 1985).

Harde et al. (2016) found the ABE fermentation of enzymatic hydrolysates of *C. forskohlii* roots by *C. acetobutylicum* NCIMB 2877 only produced 0.55 g/L solvents, 4.29 g/L of total acids with 31.46 g/L sugar concentration. However, the control ABE fermentation using synthetic glucose by same culture, under same condition, it was observed that 6.95 g/L of total solvents and 1.59 g/L of total acids. This occur may be due because of inhibitors such as phenolic compounds in root hydrolysate used as substrate are reported to be antimicrobial (Baydar et al., 2004). Futhermore, *C. forskohlii* is an herbal plant belonging to Lamiaceae family which known to contain higher phenolic contents and Sunitha et al. (2013) also reported the extracts from these plants to be rich in phenolic compounds. From the control fermentation, Harde et al. (2016) compared and conclude that the substrate is not suitable for ABE fermentation, not the culture. In this

work, the fermentation using OPF juice is comparable to the control fermentation since the difference of production only 11.25%.

Various lignocellulosic feedstocks for biobutanol production via ABE fermentation have been used in previous studies but few of these studies discuss as in Table 4.3. The biobutanol production in this study was comparable to Wang & Blaschek (2011). Wang & Blaschek (2011) reported on ABE fermentation using 42.2 g/L sugar concentration in tropical maize stalk juice by *C. beijerinckii* NCIMB 8052. The butanol production and yield were 11.5 g/L and 0.27 g/g, respectively. There is not much difference with the production of butanol in this work, which the butanol yields 0.24 g/g. According to Wang & Blaschek (2011), the mixed sugars obtained from tropical maize stalk juice were composed of high concentrations of sucrose, glucose and fructose. The content of mixed sugars was similar with OPF juice. These type of sugars are all easily degraded during the microbial processes.

Table 4.3 Comparison of butanol production by *Clostridium* species using other substrate.

Substrate	**Culture**	**Biobutanol produced (g/L)**	**Biobutanol yield (g/g)**	**Reference**
OPF juice	*C. acetobutylicum* ATCC 824	9.24	0.24	This research
Palm oil mill effluent	*C. saccharoperbutyl-acetonicum* N1-4	0.9	0.18	Al-Shorgani et al. (2015)
Date fruit	*C. acetobutylicum* NCIMB 13357	11	0.48	Khamaiseh et al. (2014)
Oil palm sap	*C. acetobutylicum* DSM 1731	7.29	0.36	Komonkiat & Cheirsilp (2013)
Oil palm decanter cake	*C. acetobutylicum* ATCC 824	6.04	0.11	Razak et al. (2013)
Sago starch	*C. saccharoperbutyl-acetonicum* N1-4	6.20	0.12	Al-Shorgani et al. (2012)
Tropical maize stalk juice	*C. beijerinck*ii NCIMB 8052	11.5	0.27	Wang & Blaschek (2011)

The other biobutanol production from various solventogenic *Clostridia* and different types of biomass hydrolysate was comparable to the studies by Al-Shorgani et al. (2012), Razak et al. (2013), Komonkiat & Cheirsilp (2013), Khamaiseh et al. (2014) and Al-Shorgani et al. (2015). Those studies used *C. saccharoperbutylicum* N1-4, *C. acetobutylicum* ATCC 824, *C. acetobutylicum* DSM 1731 and *C. acetobutylicum* NCIMB 13357. *C. acetobutylicum* NCIMB 13357 produced the highest biobutanol yield with 0.48

g/g and butanol concentration of 11 g/L. They used date fruit as the substrate (Khamaiseh et al., 2014). As compared to the same strain used as in this study, Razak et al. (2013) reported lower value of butanol yield and concentration, 0.11 g/g and 6.04 g/L, respectively. Therefore, this can be described that OPF juice are more capable to produce biobutanol than oil palm decanter cake as substrate. The differences in ABE production may be due to the differing nature of the agricultural substrates, the sugar content and the different *Clostridium* strains. These may have contributed to the inefficient utilization of the sugars for solvent production. Different substrate sources showed different fermentation behavior, although all fermentations were done under the same conditions (Al-Shorgani et al., 2012). The results obtained from batch fermentation of OPF juice and synthetic sugars showed that OPF juice without pretreatment is a suitable carbon source for biobutanol production by *C. acetobutylicum* ATCC 824.

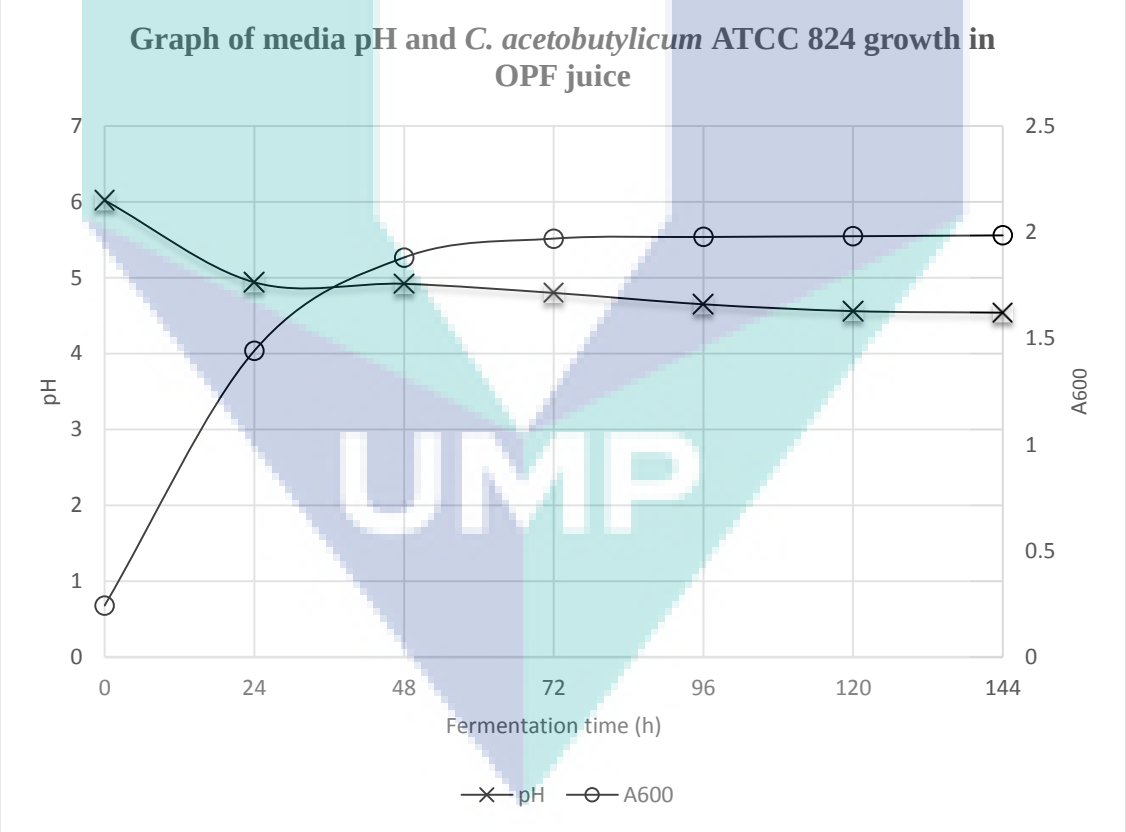

Figure 4.1 Graph of media pH and *C. acetobutylicum* ATCC 824 growth in OPF juice

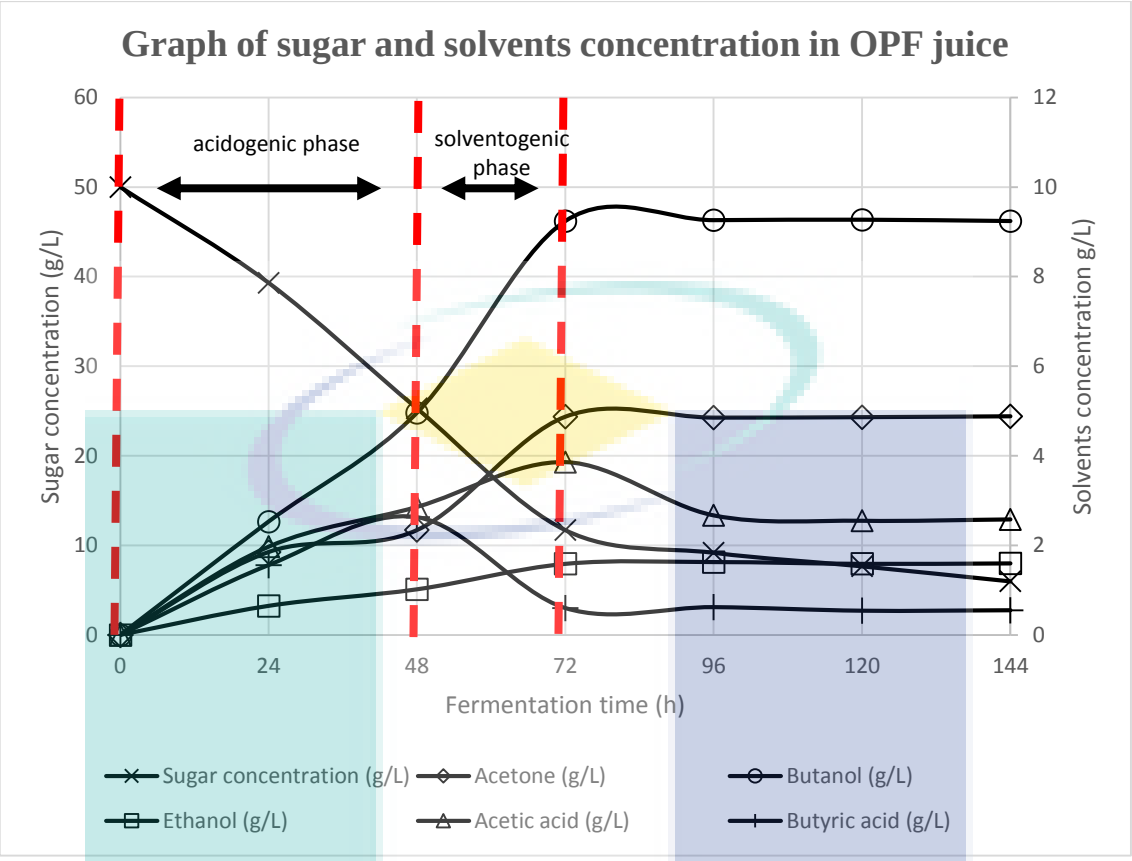

Figure 4.2 Graph of sugar and solvents concentration in OPF juice

In order to assess the capability of OPF juice as carbon source on ABE fermentation by *C. acetobutylicum* ATCC 824, the fermentation experiment was regularly monitored every 24 hr within 144 hr. The growth and solvents production was reported as in Figure 4.1 and 4.2. Figure 4.1 illustrates the pH of media and *C. acetobutylicum* ATCC 824 growth in OPF juice. As can be seen, the growth had a lag phase in early hours of fermentation. Once rapid growth began, stationary phase was achieved in just over 48 hours. The pattern that was seen in the fermentation was a fall in pH followed by pH increase between 24 hr to 72 hr as the acids were converted to solvents. During the exponential phase, the cell density reached OD_{600} 1.97 at 72 hr, before entered stationary phase. The exponential growth was a result of sugar consumption by the bacterial strain.

Figure 4.2 illustrates the sugar and solvents concentration throughout the fermentation of *C. acetobutylicum* ATCC 824 in OPF juice. The initial sugars concentration drops dramatically from the beginning of the fermentation towards 72 hr. Comparing the graph in Figure 4.1 and Figure 4.2, C. *acetobutylicum* ATCC 824 starts to produce solvents at the end of the exponential growth phase, which coincides with a

switch from acidogenesis to solventogenesis. Butyric acid decreased, meanwhile the butanol increased from 48 hr to 72 hr.

In this experiment, the initial pH was observed decreased from 6 to 4.8 within 72 hr. This can be discussed because of the formation of acetic and butyric acids in acidogenic phase. This phase was observed at 48 hr of fermentation where acetic acids and butyric acids were at highest value of 3.86 g/L and 2.63 g/L, respectively. A high accumulation of the acids during the transition phase of 72 hr. The formation of acids preceded the production of solvents and that part of the acids formerly produced was subsequently consumed. This behavior could be explained with the production of the solvents mainly on acetone and butanol. Linggang et al. (2013) also reported high production of acetic and butyric acid was obtained within 72 hr of ABE fermentation by *C. acetobutylicum* ATCC 824 using sago pith residue hydrolysate. The ABE production and yield was reported as high as 4.22 g/L and 0.2 g/g (Linggang et al., 2013). Therefore, the transition phase is same as in this work, as can be observed in Figure 4.2, within 48 to 72 hr, butyric acid decreases from 2.63 g/L to 0.6 g/L. On the other hand, butanol production increase from 4.96 g/L to 9.24 g/L.

However, Sun and Liu (2010) reported less than 1 g/L of total solvents were produced after 72 hr of ABE fermentation by *C. acetobutylicum* ATCC 824 using sugar maple hydrolysate that was treated with phosphoric acid and ammonium and the organic acids found as high as 8.5 g/L. The phase transition from acidogenic to solventogenic phase did not occur completely. Wang and Chen (2011) and Ibrahim et al. (2012) also found a high concentration of organic acids production in their study but with low ABE concentration produced. The ABE fermentation by *C. acetobutylicum* ATCC 824 using steam-exploded corn stover hydrolysate produced 3.71 g/L of solvents and as high as 7.25 g/L organic acids at 72 hr (Wang and Chen, 2011). In Ibrahim et al. (2012) studied, the acids and ABE production by *C. butyricum* EB6 produced up to 6.60 g/L of total organic acid using glucose as carbon source. The acids were mainly produced and the pH decreasing to 4.26 within 24 hr of fermentation. Then, it was observed the acetic acid formed was decreased to 1.55 g/L after 144 hr while the butyric acid was not consumed.

This due to the fact that high concentration of organic acids accumulated in fermentation environment decreased the capability of the culture for uptake and recycling of both acetic and butyric acid (Grimmler et al., 2010). Therefore, this phenomenon

resulting in low solvents production. From the prilimanary study, OPF juice is proven capable to produce biobutanol by *C. acetobutylicum* ATCC 824. As observed in Figure 4.2, although the fermentation was run for 144 hr, the culture stopped producing butanol within 72 hr. The fermentation likely stopped due to butanol inhibition. During 72 hr until 144 hr, the butanol production fluctuated between 9.24 to 9.27 g/L, which at 144 hr the butanol was 9.24 g/L same as 72 hr. Therefore, in this study, 72 hr fermentation time was taken as the maximum hour of solvent production for next experiment. In order to proceed the experiment to know the most influence factor in the fermentation process, five factors were selected based on literature review in Chapter 2. These five factors chosen were initial pH value, total sugars in OPF juice, inoculum size, temperature and yeast extract concentration. The factors and ranges that were chosen for factorial analysis was in Table 3.2.

4.3 Factorial analysis of biobutanol production

The experimental design for factor analysis process was carried out with the aid of the software Design Expert (Stat-Ease Inc., Statistic made easy, Minneapolis, MN, USA, Version 7.1.6). The two-level fractional factorial design study was used in this analysis. This method analysed five factors that were found to be affecting the production of biobutanol. These factors were pH value (A), total sugars in OPF juice (B), inoculum size (C), temperature (D), and yeast extract concentration (E). The purpose of this objective was to determine the influence of these factors towards the production of biobutanol in ABE fermentation. The interaction between factors was also discovered in this objective. This analysis was carried out at certain ranges of value as listed earlier in Chapter 3 in Table 3.2. The experimental lists were generated by the software. Response was analysed by examining the model, interpreting the results and graph generated by the software and finally validating the experiment. This process must be followed by an optimization process as a subsequent experiment to prove the investigation process (Finney, 1946).

4.3.1 Design of experiment for factorial analysis

The design of experiment was applied according to a 2^{5-1} fractional factorial design (FFD). Table 4.4 shows the experimental design and the results of the response variable studied. Twenty-one fermentation runs were carried out with different levels of

each factor according to the design generated. All the experiments were performed in triplicates and the results were recorded as average values of the biobutanol yield.

Table 4.4 Experimental design of fractional factor analysis process using 2^{5-1} factorial design and results obtained with OPF juice.

No	Variables					Biobutanol yield (g/g)
	A	B	C	D	E	
1	6	50	10.5	37	5.5	0.2564
2	7	60	20	32	1	0.2143
3	5	40	20	32	1	0.2206
4	7	40	1	42	10	0.1862
5	7	40	20	42	1	0.0478
6	5	60	1	32	1	0.0065
7	7	40	20	32	10	0.2049
8	5	40	20	42	10	0.0969
9	7	40	1	32	1	0.0154
10	6	50	10.5	37	5.5	0.2414
11	5	40	1	42	1	0.0176
12	7	60	1	42	1	0.0057
13	6	50	10.5	37	5.5	0.2930
14	5	60	20	42	1	0.0397
15	5	60	1	42	10	0.1368
16	6	50	10.5	37	5.5	0.2506
17	7	60	20	42	10	0.0474
18	7	60	1	32	10	0.0287
19	6	50	10.5	37	5.5	0.2829
20	5	40	1	32	10	0.1765
21	5	60	20	32	10	0.2194

The results showed that the production of biobutanol by *C. acetobutylicum* ATCC 824 from OPF juice was ranged between 0.0057 g/g to 0.2930 g/g as shown in Table 4.4. As can be observed, runs 13, 19 and 1 showed the highest biobutanol production with the value as high as 0.2930 g/g, 0.2829 g/g and 0.2564 g/g, respectively. All these three runs were at center points condition where the pH value of 6, total sugars 50 g/L, temperature 37°C, inoculum size 10.5% and yeast extract concentration 5.5 g/L. The lowest biobutanol yield for run 12 with biobutanol yield 0.0057 g/g can be interpreted by the low percentage of inoculum size of 1% and too high incubation temperature at 42°C. The Clostridial cells cannot tolerate and grow at high temperature. The regression model for variables in terms of coded factors as shown in equation given below:

$$\text{Biobutanol yield} = 0.10 - 0.010A - 0.017B + 0.032C - 0.032D + 0.033E + (9.737x10^{-3})\,AD - 0.010AE + 0.011BC - 0.012BE - 0.047CD - 0.027CE + 0.011DE \quad 4.1$$

Where A is the pH value, B, C, D and E are the total sugars in OPF juice, inoculum size, temperature and yeast extract concentration, respectively. A, B, C, D and E are referred as the main effect while AD, AE, BC, BE, CD, CE and DE are the interaction effect.

4.3.2 Screening Factor Analysis

The contribution of the main factor was visually demonstrated by Pareto Chart in Figure 4.3. The bar length is proportional to the value of estimated effect. As observed in Figure 4.3, there was orange and blue bars, t-value limit line and Bonferroni's limit line. The orange bar indicates positive effects, whereas, the blue bar for negative effects. For main effects, an effect is said to be positive when the response increasing with the increase of the factor. Meanwhile, the negative effect is when an increase in its high level will result in a decrease in the response. For interactions, the positive effect is when both factors were a chance to the same level (low or high), the response will increase. The negative effect is when both factors were change to the opposite level (one at its low and the other at its high), the response will increase.

Effects of t-value limit (black line) are considered statistically significant at 95% confidence level while effects below t-value limit are not likely to be significant. Bonferroni's corrected t-test on the individual terms to justify individual terms in models selected by forward selection for any model with a small global p-value (Mee, 2009). Effect above Bonferroni's corrected t-value limit (red line) is almost certainly significant (Anderson, 2008). A quick analysis was performed on the selected effects using Pareto chart to statistically check for significance of the selected effects at 95% confidence level. Factors C, D, E, CD and CE shown to be significant at both t-value limit and Bonferroni's corrected t-value limit. Factors B, BE, DE and BC was shown to be significant at t-value limit only. The others factor A, AE and AD was not significant at both line and it shows that the factors do not contribute to the response.

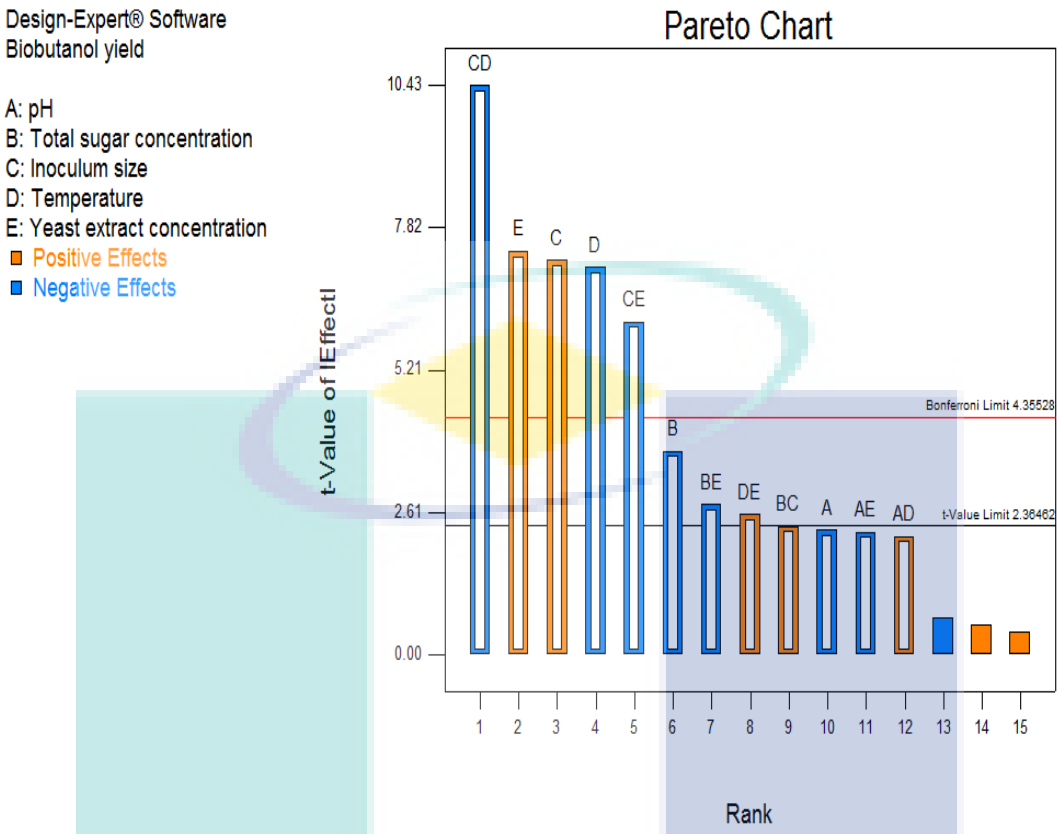

Figure 4.3 Pareto chart of screening factors on biobutanol production

From the Pareto chart, CD, D, CE, B and BE were observed to be factors that give negative effects to the response. For factor total sugar concentration, B and temperature, D, the lower value used in the experiment, the higher response was resulted. However, factors E, C, DE and BC was shown as positive effects. Increasing in inoculum size, C and yeast extract concentration, E used, was increased the value of response. Figure 4.3 demonstrated factor E (yeast extract concentration), C (inoculum size) and D (temperature) are most significantly affect the biobutanol production.

4.3.3 ANOVA for Factor Analysis Process

The statistical significance was evaluated using the statistical test for analysis of variance (ANOVA) to determine the quality of the model. As in Table 4.5, F-values was used to check the significance of a regression equations. Meanwhile, the p-values was used to check the significance of each coefficient (Wang et al., 2012). The p-value tests the null hypothesis that data from the experiment with the identical means. If the p-value was less than 0.05, it shows that only 5% chance of the model could occur because of the

noise and the model is significant. From ANOVA in Table 4.5, calculated model's F value of 29.32 with a probability value (Prob>F) of <0.0001 suggest that the model was significant and fitted well to the experimental data (P<0.05). Meanwhile, the p-value for each of the model terms A, B, C, D, E, BD, CE, CD and DE showed the value less than 0.05. It indicated the contribution of the model was significant (Wang et al., 2012). As for AD, AE and BC, the p-value were observed greater than 0.05. Thus, these interaction factors can be concluded as insignificant and excluded from the model which can improve the model.

The lack of fit is a measure of the failure of a model to represent data in the experimental domain at which data points were not included in the regression model or variations in the models cannot be accounted by random error. If there is a significant lack of fit, the response is not fitted (Shukor et al, 2014). As shown in Table 4.5, the F value for lack of fit is 0.21 and the probability value of 0.08871 implied that lack of fit was insignificant and hence the model was valid for further studies.

Table 4.5 Analysis of variance table (ANOVA) analysis for 2^{5-1} fractional factorial design (FFD)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.11	12	9.396×10^{-3}	29.32	<0.0001	significant
A	1.673×10^{-3}	1	1.673×10^{-3}	5.22	0.0563	
B	4.469×10^{-3}	1	4.469×10^{-3}	13.94	0.0073	
C	0.017	1	0.017	52.25	0.0002	
D	0.016	1	0.016	50.37	0.0002	
E	0.018	1	0.018	54.61	0.0002	
AD	1.517×10^{-3}	1	1.517×10^{-3}	4.73	0.0661	
AE	1.624×10^{-3}	1	1.624×10^{-3}	5.07	0.0591	
BC	1.777×10^{-3}	1	1.777×10^{-3}	5.54	0.0508	
BE	2.426×10^{-3}	1	2.426×10^{-3}	7.57	0.0285	
CD	0.035	1	0.035	108.7	<0.0001	
CE	0.012	1	0.012	37.21	0.0005	
DE	2.111×10^{-3}	1	2.111×10^{-3}	6.59	0.0372	
Curvature	0.099	1	0.099	307.48	<0.0001	Significant
Residual	2.243×10^{-3}	7	3.205×10^{-4}			
Lack of fit	3.009×10^{-4}	3	1.003×10^{-4}	0.21	0.8871	Not significant
Pure error	1.943×10^{-3}	4	4.856×10^{-4}			
Correlated Total	0.21	20				

4.3.4 Main effect for factorial analysis on biobutanol production

One of the aspects that were studied in the factor analysis process is the main effect analysis. The usage of the two-level factorial design allowed this aspect to be studied thoroughly. This analysis was a study to determine which factor contributed the most in ABE fermentation process.

Table 4.6 Percentage contribution of each main factor to the production of biobutanol

Factor	Percentage Contribution (%)
A	0.78
B	2.09
C	7.84
D	7.56
E	8.20

From Table 4.6, it is shown that factor E (yeast extract concentration) contributes the most to the production of biobutanol as much as 8.20% and based on Pareto Chart in Figure 4.3, factor E having positive effect. Komonkiat & Cheirsilp (2013) reported that the production of biobutanol by *C. acetobutylicum* DSM 1731 increasing with the addition of yeast extract as high as 26%. In general, yeast extract is nitrogen source for the fermentation medium to provide various amino acids, minerals, growth factors and vitamins that promote good growth of microorganism. However, Razak et al. (2013) mentioned *C. acetobutylicum* ATCC 824 was able to consume the wide range of yeast extract concentration for higher butanol production. At certain limit, too high yeast extract concentration, can decrease the biobutanol yield. It is because the yeast extract only stimulated the growth of cells, but reduced the tendency to produce the solvents.

The other previous study by Mechmech et al. (2015), they found out no biobutanol production with absence of yeast extract in fermentation using xylose as carbon source by *C. acetobutylicum* ATCC 824. This indicates that the ammonium acetate only as inorganic nitrogen source in P2 medium was unable to induce solvent production. Monot et al. (1982) investigated the effect of the component concentrations of a synthetic medium on acetone and butanol fermentation by *C. acetobutylicum* ATCC 824 was discovered that ammonium acetate greatly affected fermentation. However, they

observed the addition of ammonium acetate at concentrations of over 2.2 g/L had deleterious effects on the production of solvents and no growth occurred in the absence of ammonium acetate. Therefore, both ammonium acetate and yeast extract were needed in P2 medium for the fermentation. Mechmech et al. (2015) also observed the biobutanol production increasing with the increase of yeast extract concentration. The used of 1 g/L of yeast extract resulted the lowest ABE production of 1.25 g/L, compare to the used of 5 g/L and 10 g/L of yeast extract which enhanced the fermentation performance with butanol production of 3.43 g/L and 3.47 g/L, respectively. Kasap (2002) also reported the same phenomenon, which *C. beijerinckii* NRRL B593 grew well in the fermentation medium, but did not produce solvents, unless with the added of yeast extract.

However, Linggang et al. (2013) evaluated the highest ABE production was used only 0.5 g/L of yeast extract supplied into the fermentation medium. The study was carried out the experiment to observe the effect of yeast extract concentration ranging from 0 to 5 g/L using 50 g/L of sago pith residues hydrolysate as substrate by *C. acetobutylicum* ATCC 824. The highest ABE production was 8.84 g/L with 0.5 g/L of yeast extract. Meanwhile, the lowest production was found when no yeast extract was supplied. The addition of the highest value of yeast extract (5 g/L) in the medium, resulted in high organic production, but low solvents production. The results were almost comparable to the other studies by Liu et al. (2010), Qureshi et al. (2008) and Wang and Chen (2011) where the addition of 1, 3 and 5 g/L of yeast extract resulted in ABE concentration of 8.39, 7.41 and 7.35, respectively. These findings occurred because of the fermentation became acidogenic rather than solventogenic when excessive of nitrogen source (yeast extract) Maddox et al. (2000). The production of high amount of acids in early stage of fermentation may cause the inhibition of the cell metabolism which finally cause the solvents to be not produced. High ABE production requires nitrogen limitation and excess of carbon source, so that the nutrient supply is just sufficient for growth (Madihah et al., 2001).

Meanwhile, Al-Shorgani et al. (2016) reported that *C. acetobutylicum* YM1 produced high biobutanol production up to 4 g/L yeast extract concentration. Increasing beyond 4 g/L of yeast extract decreased the production. Production of ABE from spoilage date palm fruits by mixed culture *C. acetobutylicum* and *Bacillus subtilis* required the addition of yeast extract 5 g/L to enhance the solvent production (Alla et al., 2012). This

was comparable to the experiment by Cheng et al. (2012) who found the biobutanol production by mixture cultures optimum with the addition of 5.13 g/L yeast extract. The quantity of yeast extract need to supply may differ based on the type of substrate. Difference substrate contain their own mineral content which can use as nitrogen source. On the other hand, there were studies reported the addition of organic nitrogen source supplemented in the fermentation medium also can enhance the ABE production such as alfalfa juice and corn steep liquor by Mechmech et al. (2015) and Li et al. (2014). Therefore, the factor E, yeast extract concentration will be further studied for optimization to know the optimum value needed by *C. acetobutylicum* ATCC 824 on biobutanol production in OPF juice.

Inoculum size shows as the second factor that contributes to the biobutanol production in this study with the percentage of 7.84%. The fermentation was performed at different inoculum sizes at 1% (v/v) and 20% (v/v) with the center point of 10.5% (v/v) of inoculum. As reported by Shukor et al (2014), the biobutanol production was increasing with the increased of inoculum sizes. This is due to the fact that Clostridial cells decreases the lag phase of growth which improves the microbial cell growth to enhance the solventogenesis phase where the butanol produced (Shukor et al, 2014). Same goes to the study of the effect of inoculation size on the growth and solvent production by Kheyrandish et al. (2015). In the study, they used two different amounts of inoculation size, 3% and 5%. The culture inoculated with 5% inoculum observed to reach the stationary phase faster and produced higher butanol concentration compare to 3%. A similar pattern was reported by Al-Shorgani et al. (2015) who studied the production of butanol by *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) using palm oil mill effluent. They observed that the increased in inoculum size from 5% to 15% resulted in an enhanced butanol concentration. A further increase from 15% to 20% led to a minimal increase in butanol concentration with a constant ABE production.

However, the optimum inoculum size is varied depending on the microorganism and substrate used. As shown in Table 4.4, page 51, the highest butanol yield was achieved at inoculum size 10.5% compare to 20% with 0.2930 g/g. Meanwhile, studies performed by Ranjan et al. (2013) revealed that the maximum butanol production by *C. acetobutylicum* MTCC 481 from rice straw hydrolysate was achieved at an optimum inoculum size 5%. On the other hand, Razak et al (2013) found the optimum inoculum

size of 16.2% for the production of butanol by *C. acetobutylicum* from oil palm decanter cake hydrolysate. This shows that increasing the inoculum size higher than critical level had no important effect on the microbial growth and cell activity which will contribute to the production of butanol (Ranjan et al., 2013).

Based on Table 4.6, temperature contributes as much as 7.56% on the effect of biobutanol production from OPF juice. The optimum incubation temperature in ABE process varies depending on the type of microorganism and substrate utilized as carbon source. Al- Shorgani (2015) and Chen et al. (2013) investigated the effect of culture temperature on butanol production in ABE fermentatation. Both of these previous studies observed similar and comparable pattern of result. Al-Shorgani (2015) investigated in a range 25 to 40°C, whereas Chen et al. (2013) tested 25 to 45°C. Both of them found the butanol production increasing when the temperature increased from 25 to 30°C. Meanwhile, a higher temperature at 35 to 45°C, they found lower butanol produced. Same goes to the study by Mane and Deshmukh (2013) the highest butanol production by *C. acetobutylicum* NRRLB527 was found at a culture temperature of 30°C. One possible explaination was might be due to the fact that *C. acetobutylicum* will lose the ability to produce solvents at high or low temperature because it affects the enzymatic pathway in both acidegonesis and solventogenesis pathways. The cells could not convert the substrate into acids and from acids to solvents (Khamaiseh et al, 2012). Ranjan et al. (2013) also mentioned that a low butanol production at temperature higher than 30°C could be attributed to the fact that high temperature inhibits enzyme catalyzed reactions that are essential for cellular metabolism of acid and solvent production in ABE fermentation. However, the difference has been found on butanol production by *Clostridium saccharobutylicum* DSM 13864 using cane molasses was investigated by Ni et al. (2012). They noted that the optimum temperature for maximum butanol synthesis was 37°C. Thus, the optimum temperature for this study will be determined in the next optimization experiment.

The least effective factors were the total sugars concentration in OPF juice and initial pH medium with the values of 2.09% and 0.78%, respectively. Production of biobutanol in fermentation process is by conversion of sugar in OPF juice such as glucose, fructose and sucrose into organic acids and the acids were converted into butanol. At low concentration of sugar, the production relatively low because of the law of mass action.

Meanwhile, at high concentration of sugars, there was a substrate inhibition through pH depletion because the forming of acids in acidogenesis stage increase (Jones & Woods, 1986). As reported by Madihah et al. (2001), total solvent production increased drastically when used from 10 to 50 g/L carbon source as substrate and decreasing when higher than 60 g/L. In this work, as in Table 4.5, factor B, total sugars concentration used in the experiment was in a range 40 to 60 g/L. This is because of based on most of the previous studies have reported that the solvent production will increase with the increasing of total sugars in the medium. Up to the optimum value, the production will not have significant improvement. Therefore, in this experiment, 50 g/L sugars concentration will be used further in the next experiment since there was not high in contribution percentage as stated in Table 4.6. Based on the response in Table 4.4, the highest biobutanol yield is at 50 g/L of sugar concentration.

Based on ANOVA in Table 4.5, factor A, pH is not significant since the p-value was higher than 0.05. The effect of initial pH of the fermentation process has been discovered as one of the key factors that influence the transition from acidogenesis to solventogenesis phase by *C. acetobutylicum.* As for example, Linggang et al. (2013) discovered that *C. acetobutylicum* ATCC 824 produced high ABE production at initial pH 5 as compared to 5.5, 6.0 and 6.5. This was comparable to the result observed by Geng and Park (1993) who reported the culture tends to produce mostly organic acids with a small amount of ABE at initial pH 6 and above. The high organic acids have an inhibitory effect on cell growth and metabolic functions in the cell (Ezeji et al., 2007). In this work, the pH used in a range 5 to 7. This small range can be concluded not significantly influenced the fermentation process.

4.3.5 Interaction between factors for factorial design

Beside the main factors, there are also interaction effects that play important role in the production of biobutanol by *C. acetobutylicum* ATCC 824. There are ten interactions discovered in this study as shown in Table 4.7. Based on ANOVA in Table 4.5, three interaction factors were not significant since the p-value higher than 0.05. The factors are AD, AE and BC. The interaction effect plot was observed to represent the results of the regression analysis. It was determined by the deviations of the average between the high and low levels for each factor.

As in Table 4.7, the highest percentage interaction is between factor C, inoculum size and D, temperature with 16.31%. These two factors contributed the highest and second highest to the fermentation process. The other two high values of interaction are between C, inoculum size with factor E, yeast extract concentration and factor B, total sugars in OPF juice with factor E, yeast extract concentration with value 5.58% and 1.14%, respectively. The other interactions with low percentage of contribution can be ignored since they give very low effect towards the fermentation process.

Table 4.7 Percentage contribution of each interaction factor to the production of biobutanol

Factor	Percentage Contribution (%)
AB	0.07
AC	0.045
AD	0.71
AE	0.76
BC	0.83
BD	0.026
BE	1.14
CD	16.31
CE	5.58
DE	0.99

Figure 4.4 shows there is an interaction between inoculum size (C) and temperature (D). As can be seen, biobutanol yield was increased with increasing inoculum size at the incubation temperature, 32°C. At 42°C, the biobutanol yield was not significantly decreased with the increasing of inoculum size. The line at 42°C seem to be almost straight line and this concluded that the increasing of inoculum size did not give significant enhancement in the response at this high temperature. Ranjan et al. (2013) reported that high temperature inhibits enzymatic pathway of *C. acetobutylicum* which importance to the metabolism of acid and solvent production. This lead to the loss of ability of the culture to grow and convert the substrate to acids or solvents. This interaction shows that incubation temperature at 32°C is more favorable temperature for the fermentation compared to 42°C.

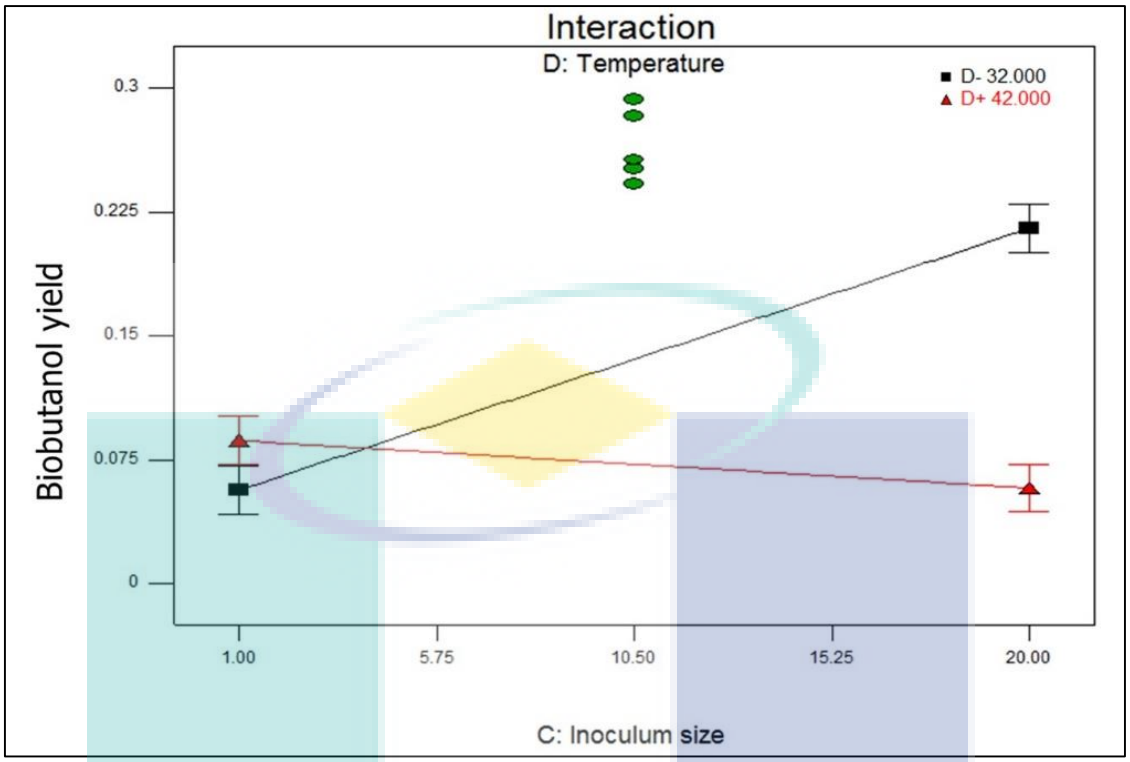

Figure 4.4 The interaction graph between inoculum size (C) and temperature (D)

The interaction between inoculum size and yeast extract concentration can be found in Figure 4.5. The biobutanol yield seems not affected by inoculum size when the yeast extract concentration at 10 g/L. Otherwise, at 1 g/L of yeast extract, the biobutanol yield is increasing gradually when inoculum size increased. This is due to the high concentration of yeast extract into the fermentation medium was stimulated the growth of the cells, and resulted in high organic acids but reduced the ability to produce solvents (Razak et al, 2013). The production of high amount of acids in the early stage of fermentation may inhibit the culture metabolism thus resulting in lower ABE production. Meanwhile, at 1 g/L addition of yeast extract is just sufficient for the growth, not excess of nitrogen source. This finding is in agreement with the results obtained by Linggang et al. (2013) who reported that the highest amount of ABE production was detected when 1 g/L of yeast extract was supplied to the medium compared to 3 and 5 g/L.

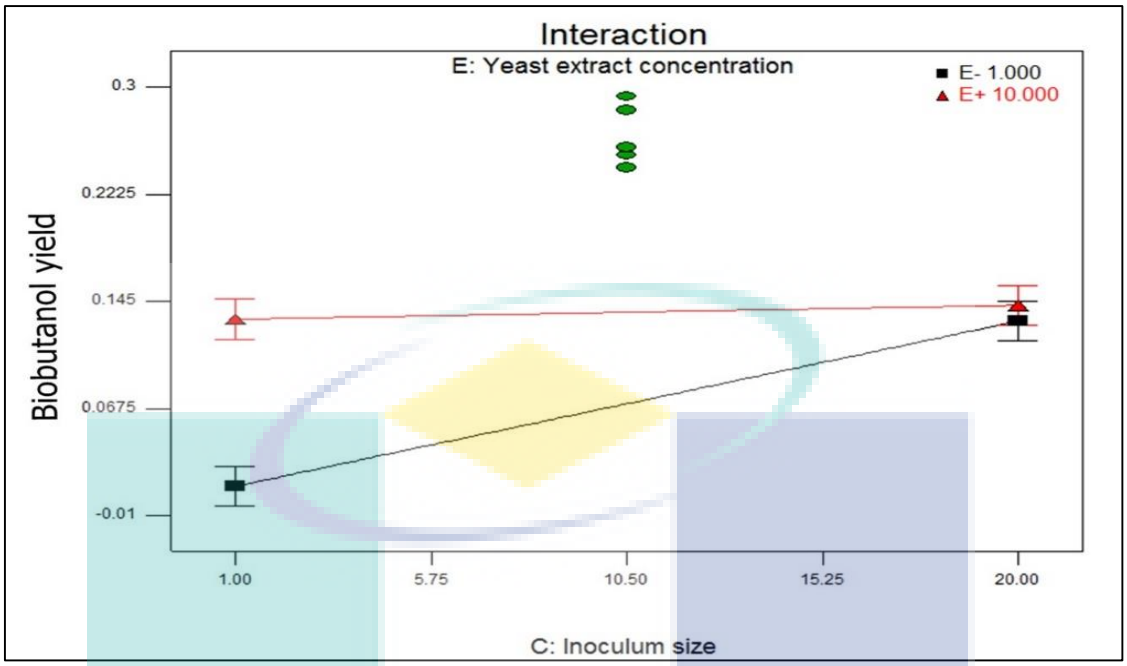

Figure 4.5 The interaction graph between inoculum size (C) and yeast extract concentration (E)

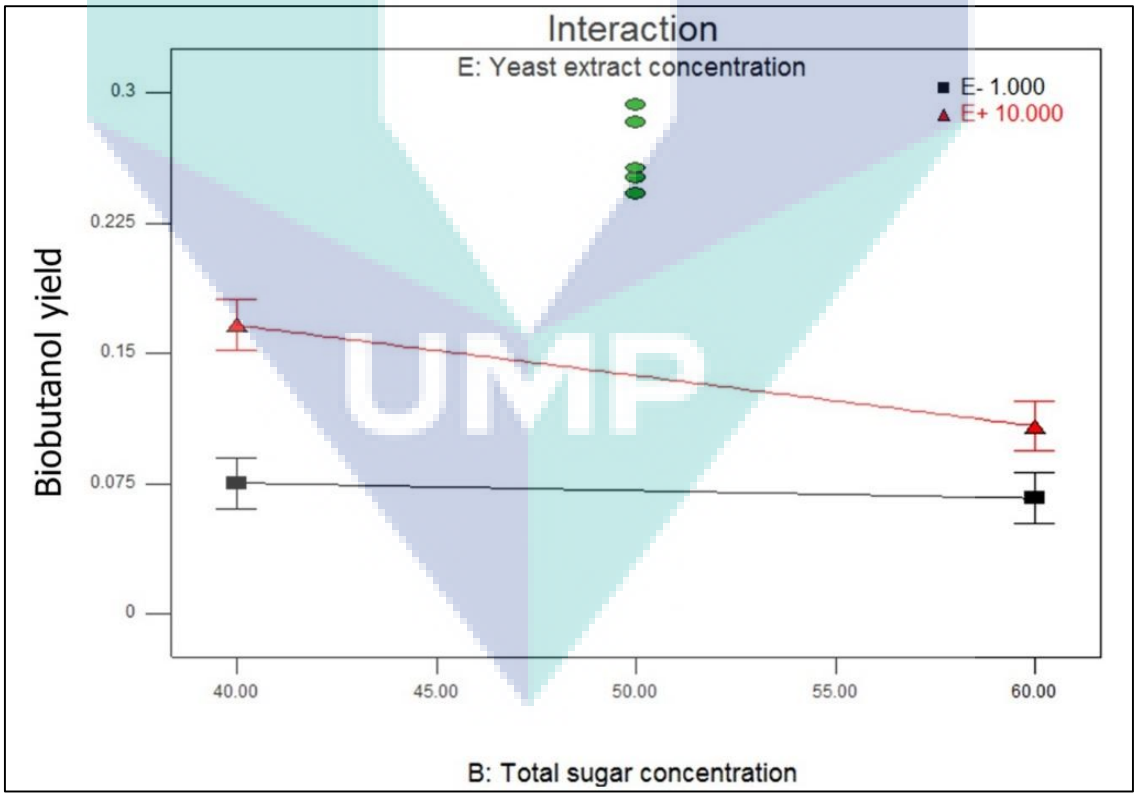

Figure 4.6 The interaction graph between total sugar concentration (B) and yeast extract concentration (E)

Interaction between factor B, total sugar concentration and factor E, yeast extract concentration graphically illustrated as in Figure 4.6. Compare to Figure 4.5, yeast extract at 1 g/L affected and interacted to the factor inoculum size in the fermentation. Meanwhile, for Figure 4.6, interaction between B and E, was observed that yeast extract at 10 g/L responded to the factor total sugar concentration. Biobutanol yield decreasing with the increased of total sugar concentration at 10 g/L of yeast extract. Whereas, at 1 g/L of yeast extract, the biobutanol yield found at the same value although with the increasing of the sugar concentration. From this interaction, it can be evaluated that at *C. acetobutylicum* ATCC 824 can produce ABE production at yeast extract as high as 10 g/L. Further study on optimization will determine the exact value of optimum condition of the factors.

4.4 Optimization of biobutanol production

The experimental design for optimization for biobutanol production by *C. acetobutylicum* ATCC 824 from OPF juice was covered using the response surface methodology (RSM) based on the central composite design (CCD) with the biobutanol yield as the response. Optimizing was done to improve the performance of the system to get the maximum response for the best ABE fermentation performance. Three of the screened factors from the factor analysis process were used as the independent factors which are inoculum size, temperature and yeast extract concentration. With a fit of a second order (quadratic) model for the full factorial CCD, the design consisted of 20 sets of experiments. Five levels of variation of numeric factor were used in the experiments respectively. The five levels consisted of plus and minus alpha (axial point), plus and minus 1 (factorial points), and the centre point as showed in Table 4.8.

Table 4.8 Experimental range and levels of the independent variables

Independent variables	**Range and levels**				
	-α	**-1**	**0**	**+1**	**+α**
Inoculum size (%)	6	8	10	12	14
Temperature (°C)	31	34	37	40	43
Yeast extract (g/L)	1.5	3.5	5.5	7.5	9.5

4.4.1 Design of experiment for optimization of biobutanol production

The selected variable from the factor analysis process were temperature (A), yeast extract concentration (B) and inoculum size (C). These variables were studied and the response of was designated as biobutanol yield (Y). With the aid of Design Expert software, twenty sets of experiments were formed and a quadratic model was proposed. Similar to the factor analysis process, Design Expert software 7.1.6 was used to develop the experimental plan and optimize the regression equation.

Table 4.9 presented the full design of the central composite design for optimization process along with three of its variables, actual response and predicted response.

Table 4.9 Experimental design and results obtained from optimization of biobutanol production

Standard Order	Variables			Biobutanol Yield (g/g)	
	A	B	C	Actual	Predicted
1	8	34	3.5	0.1107	0.1000
2	12	34	3.5	0.1694	0.1600
3	8	40	3.5	0.1091	0.0940
4	12	40	3.5	0.0789	0.0930
5	8	34	7.5	0.1781	0.1700
6	12	34	7.5	0.2199	0.2400
7	8	40	7.5	0.0932	0.1100
8	12	40	7.5	0.1150	0.1300
9	6	37	5.5	0.0419	0.0510
10	14	37	5.5	0.1339	0.1200
11	10	31	5.5	0.1282	0.1400
12	10	43	5.5	0.0257	0.0130
13	10	37	1.5	0.0551	0.0690
14	10	37	9.5	0.1887	0.1700
15	10	37	5.5	0.2289	0.2700
16	10	37	5.5	0.3054	0.2700
17	10	37	5.5	0.2786	0.2700
18	10	37	5.5	0.2437	0.2700
19	10	37	5.5	0.2986	0.2700
20	10	37	5.5	0.2611	0.2700

Actual response is the experimental response which was obtained from the experiment while predicted response was the response obtained from the theoretical calculation. From Table 4.9, the response, biobutanol yield was ranged between 0.0257 g/g and 0.3054 g/g. The maximum biobutanol production of 0.3054 g/g was observed at the center point condition of inoculum size 10%, temperature 37°C and yeast extract concentration 5.5 g/L.

The mathematical relationship for biobutanol production from OPF juice by *C. acetobutylicum* ATCC 824 was developed by considering three independent variables: inoculum size, temperature, and yeast extract concentration; and one dependent variable: biobutanol yield obtained from per gram biobutanol concentration per sugars concentration consumption. The respective models are shown below:

$$Y = +0.27 + 0.017*A - 0.030*B + 0.025*C - 0.014*A*B + 4.388E\text{-}003*A*C - 0.012*B*C - 0.046*A^2 - 0.049*B^2 - 0.037*C^2 \quad 4.2$$

Where Y is biobutanol yield, A is inoculum size, B is temperature, and C is yeast extract concentration. The quadratic model was selected to provide the best fit with the experimental results. Equation 4.2 was formulated using the complete experimental design and response matrix through Table 4.9. Analysis of variance (ANOVA) for response surface quadratic model and parametric conditions for validating the model of biobutanol production process were represented in Table 4.10 and Table 4.11, respectively.

4.4.2 Statistical Modeling and ANOVA for optimization

In order to obtain a good fit for the response function and experimental data, Design Expert was used for the regression analysis. With the aid from ANOVA as in Table 4.10, the quadratic polynomial model for responses was determined.

From the ANOVA, with the model F-value of 20.97 and a Prob > F value was less than 0.0500, the model terms can be identified as significant which led to the case of A2, B2 and C2 being significant model terms. There was only a 0.02% chance that the "Model F-Value" occurred due to noise. The coefficient of determination (R^2) obtained was 0.9497 (94.97%), as well as a high value of the adjusted determination coefficient

(adjusted R^2 = 0.9044). The minimum acceptable amount of R^2 for bio-related work is 85%. It indicated a good agreement between experimental and predicted values.

Table 4.10 Analysis of variance (ANOVA) table (Partial sum of square) for response surface quadratic model

Source	Sum of square	Degree of freedom	Mean square	F value	Prob>F	
Model	0.14	9	0.015	20.97	<0.0001	Significant
A	4.76E-003	1	4.76E-003	6.59	0.0281	
B	0.015	1	0.015	20.48	0.0011	
C	0.010	1	0.010	14.19	0.0037	
AB	1.482E-003	1	1.482E-003	2.05	0.1828	
AC	1.540E-004	1	1.540E-004	0.21	0.6544	
BC	1.193E-003	1	1.193E-003	1.65	0.2280	
A2	0.053	1	0.053	72.92	<0.0001	
B2	0.059	1	0.059	81.89	<0.0001	
C2	0.035	1	0.035	48.36	<0.0001	
Residual	7.23E-003	10	7.23E-004			
Lack of fit	2.631E-003	5	5.26E-004	0.57	0.7229	Not significant
Pure error	4.603E-003	5	9.206E-004			
Cor total	0.14	19				
Standard deviation = 0.027 Mean = 0.16 C. V.% = 16.48			PRESS = 0.028 R^2 = 0.9497 Adjusted R^2 = 0.9044 Pred R^2 = 0.8077 Adequate precision = 13.409			

From Table 4.10, it was clearly shows that the model was statistically significant regarding highest F-value 20.97, very low probability ($p<0.0001$) and sum of square of 0.14. It was also observed that all linear (A, B and C) and quadratic (A^2, B^2 and C^2) coefficients were significant on biobutanol production as the p-values calculated for this factor was less than 0.05. Therefore, changes in this parameter could significantly impact the biobutanol production from OPF juice fermentation.

Meanwhile, all the interaction coefficients were insignificant; indicating that these terms had little impact on butanol production. The most significant effect of the linear coefficients is temperature (B), followed by yeast extract concentration (C) and inoculum size (A). Generally, the lack of fit p-value of 0.7229 implied that the lack of fit is not significant relative to the pure error. The non-significant lack of fit is positive because it demonstrates a good fit of the model to the data. A good fit means that the generated models adequately explained the variation of data.

The actual vs. predicted and residuals vs predicted responses are shown respectively in Figure 4.7 and 4.8.

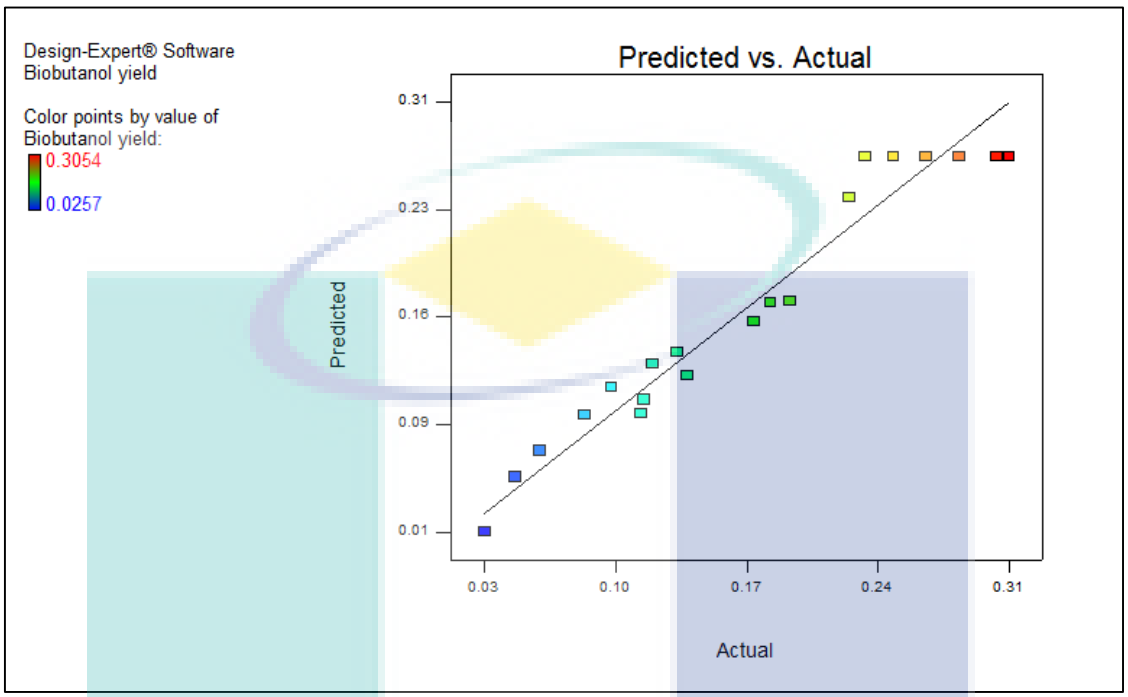

Figure 4.7 Predicted vs. Actual response of biobutanol production

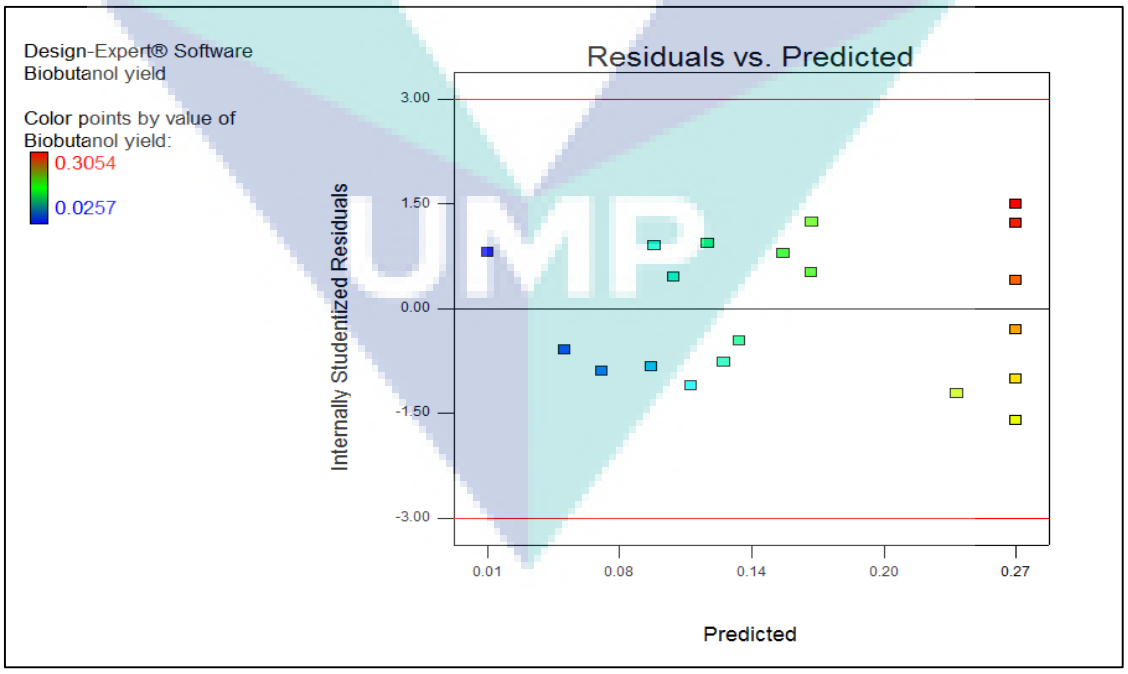

Figure 4.8 Residual plot of biobutanol production

The actual vs. predicted responses exhibit almost a linear relationship which predicts the reasonable precision of fitted empirical model. Residuals vs. predicted responses in Figure 4.8 represent unusual structure and equally scattered points above and below x-axis but all these points are between ±3.0 which imply adequacy and reliability of proposed models. Hence, it can be resolved that developed models are adequate in predicting biobutanol production from OPF juice by *C. acetobutylicum* ATCC 824.

4.4.3 Response surface plot for optimization

Evaluation of the interactions between various factors using RSM quantifies terms of three dimensional response surfaces and contour lines. Figure 4.9, 4.10 and 4.11 were plotted to demonstrate the interactions among the three factors and to estimate biobutanol yield over the independent variables. These plots demonstrate the effects of two factors on the response at a time and assist in arbitration of degree of parametric interaction on the desired responses. Three responses were generated depending on three variables involved in process.

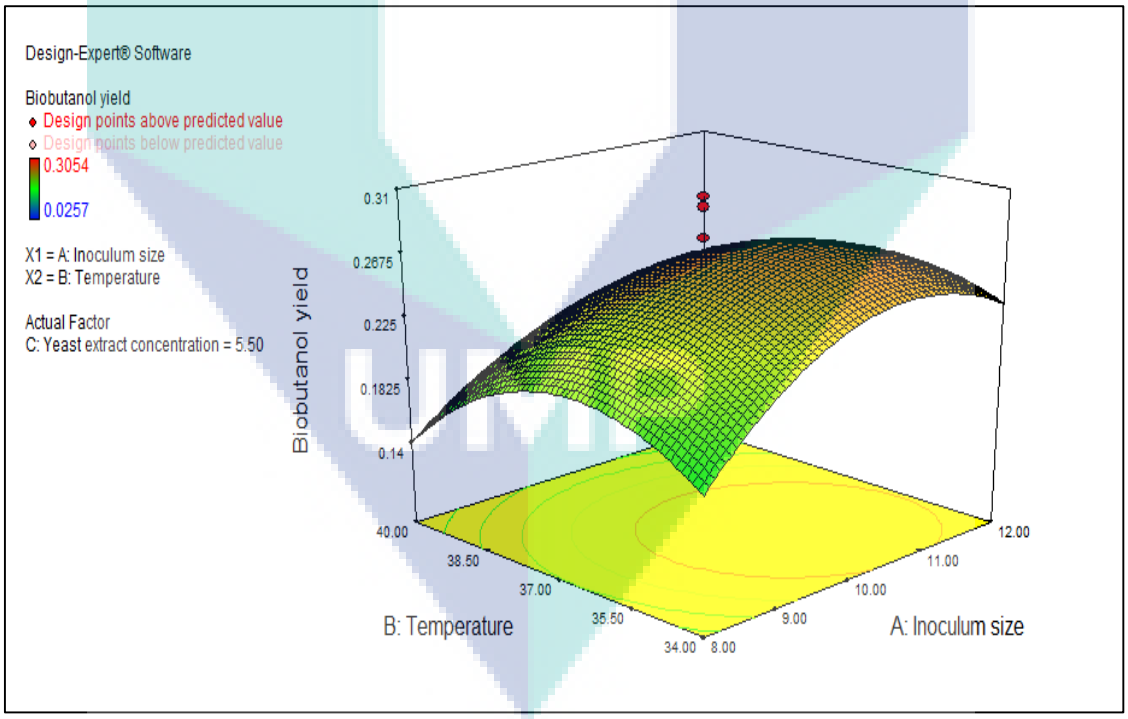

Figure 4.9 Response surface and contour plot showing the effect of inoculum size and temperature on biobutanol yield

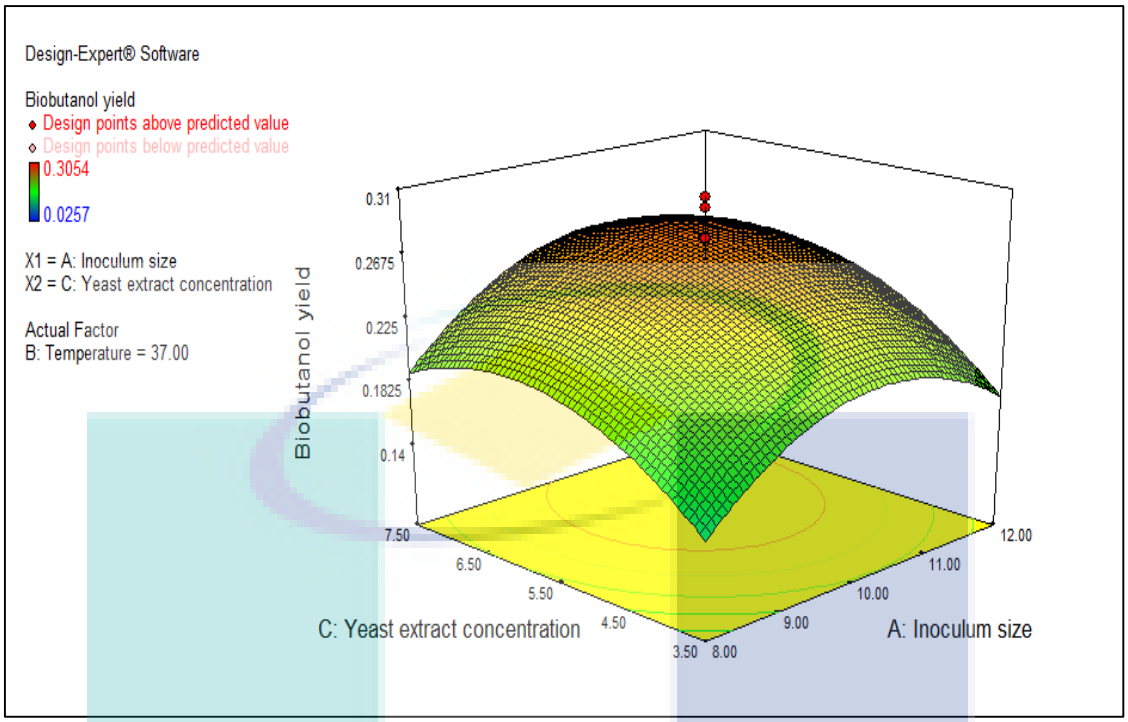

Figure 4.10 Response surface and contour plot showing the effect of inoculum size and yeast extract concentration on biobutanol yield

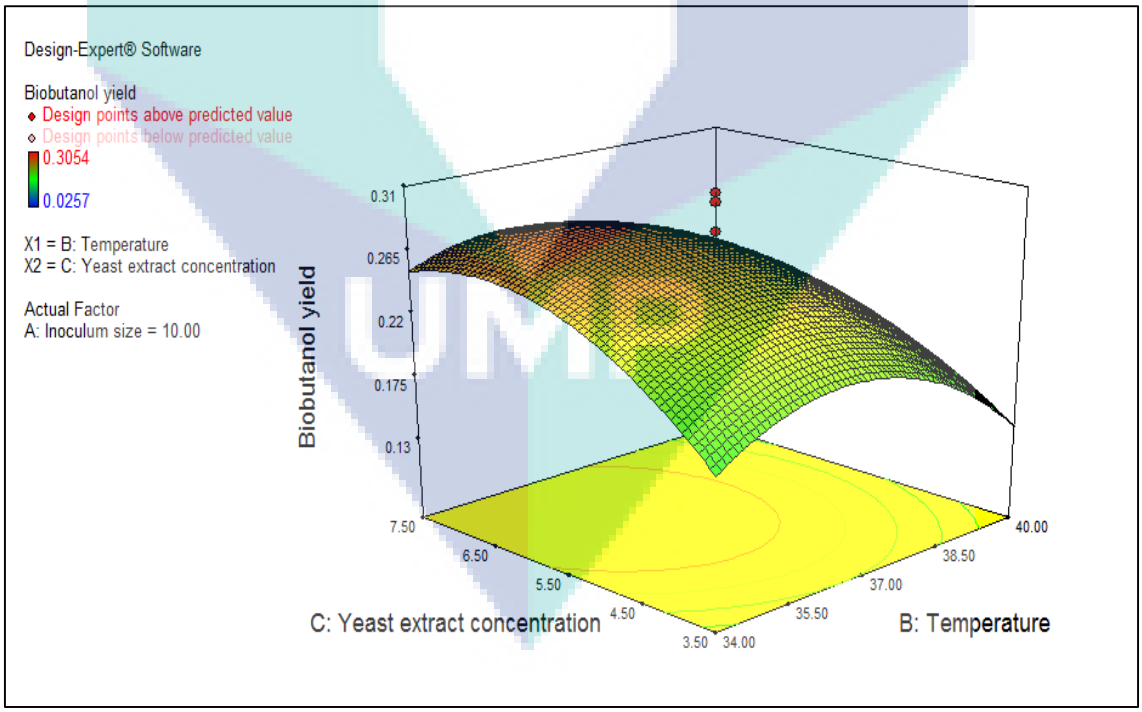

Figure 4.11 Response surface and contour plot showing the effect of temperature and yeast extract concentration on biobutanol yield

Figure 4.9 shows three dimensional response surface relationship between inoculum size and temperature on the biobutanol production at the center level of the fermentation temperature. It can be observed that biobutanol yield increases with increasing of inoculum size and the temperature has no steeper effect on the fermentation process.The three dimensional response surface relationships between inoculum size and yeast extract concentration at the center level of reaction time is illustrated in Figure 4.10. It can be seen that biobutanol yield increases with increasing of inoculum size and yeast concentration. Biobutanol yield then reaches to a maximum point and again starts decrease with increasing the yeast extract concentration.

Figure 4.11 displays three dimensional response surface interactions between temperature and yeast extract concentration at the center level of biobutanol yield. It is evident that biobutanol production increasing with increasing yeast extract concentration but reduces with rising the temperature. The effects of temperature and yeast extract concentration on biobutanol yield are more significant than inoculum size. From Figure 4.9 and 4.11, it can be observed that there were very low biobutanol productions at high temperature. This phenomenan happened could be attributed to the fact that high temperature inhibits enzyme catalyzed reactions that are essential for cellular metabolism of acid and solvent production in the fermentation (Ranjan et al, 2013).

4.4.4 Optimization of biobutanol production

The optimum inoculum size is varied depending upon the microorganism and substrate used. A study was found by Al-Shorgani et al (2015), who observed that the maximum butanol production by *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) from palm oil mill effluent in ABE fermentation is at an optimum inoculum size of 15%. On the other hand, the production of butanol by *C. acetobutylicum* MTCC 481 from rice straw hydrolysate was investigated by Ranjan et al (2013) was found that an inoculum size of 5% as the optimum inoculum size. Besides that, studies performed by Razak et al (2013) revealed that the maximum butanol production by *C. acetobutylicum* from oil palm decanter cake hydrolysate in ABE fermentation was achieved at an optimum inoculum size of 16.2% (v/v).

The improving butanol production with the increasing inoculum size due to the fact that is because of an increase in the inoculation of Clostridial cells decreases the lag

phase of microbial cell growth. This condition improves Clostridial growth resulting in the enhancement of solventogenesis phase and also the butanol production (Shukor et al, 2014). Kumar & Banerjee (2018) observed the biobutanol production increased linearly with increase in the inoculum concentration from 2 to 12% (v/v) only. As can be seen in Figure 4.9 and 4.10, increasing the inoculum size higher than the critical level of 10% did not show any improvement in butanol production. This phenomenan can be related to the fact that an increase in the inoculum size more than the critical level (optimum level) had no considerable effects on lag phase of microbial growth and cell activity, which resulted in the production of biobutanol (Ranjan et al, 2013).

The optimum incubation temperature in this study is observed at 37°C. A similar pattern was observed by several previous works. Ni et al (2012) who cultivated *C. saccharobutylicum* DSM 13864 using cane molasses found the optimum temperature for maximum butanol synthesis was 37°C. Besides, Ranjan et al. (2013) found that the maximum butanol production by *C. acetobutylicum* MTCC 481 from rice straw hydrolysate-based medium was attained at an optimum couture temperature also at 37°C. The maximum butanol production was observed at 37°C with bamboo through simultaneous pretreatment and saccharification hydrolysate as carbon source using *C. beijerinckii* ATCC 55025 (Kumar & Banerjee, 2018). They also observed, the butanol production was decreased significantly, with further increase in temperature. Pretreated bagasse of sweet sorghum was used to produce butanol in simultaneous saccharification and fermentation at 37°C and it was reported that 6.34 g/L of butanol by *C. acetobutylicum* (Jafari et al., 2017).

In contrast, studies performed by Sheng et al. (2011) and Khamaiseh et al. (2013) showed that the highest butanol production by *C. acetobutylicum* from corn straw hydrolysate and date fruit medium was detected at incubation temperature of 35°C. The optimum temperature for simultaneous saccharification and fermentation for biobutanol production using pretreated oil palm empty fruit bunch was also observed at 35°C, in order to compromise with the growth of *C. acetobutylicum* ATCC 824 for maximum biobutanol yield (Razali et al., 2018). In an attempt the effect of temperature on butanol production was studied by Yao et al. (2017) and Mane and Deshmukh (2013) who reported that the optimum temperature for the highest butanol production by *C. saccharoperbutylacetonicum* and *C. acetobutylicum* NRRL B527 was 30°C. The

variations in optimum incubation temperature could be depending to the type of microorganism and substrate used.

Yeast extract, enriched with nitrogen sources as proteins and amino acids, is one of the common used nitrogen sources for cell culture and fermentation processes which can promote phase shift from acidogenesis to solventogenesis and indirectly enhance biobutanol production. Li et al. (2012) was reported that the addition of yeast extract could promote phase shift occurrence and improve fermentation by *C. acetobutylicum* ATCC 824 performance comprehensively when using cassava substrate as high as 80% of productivity. In this study, the maximum biobutanol yield by *C. acetobutylicum* ATCC 824 from OPF juice was produced at 5.5 g/L yeast extract concentration as showed in Table 4.9. This result similar to the study reported by Madihah et al (2001) who observed 0.34 g/g biobutanol yield by *C. acetobutylicum* from gelatinized sago starch with additional 5 g/L of yeast extract in the substrate. Ibrahim et al. (2012) also reported that the ABE production by *C. butyricum* EB6 using oil palm empty fruit bunch as alternative substrate with supplemented basal medium which consisted 6 g/L of yeast extract produce as high as 0.24 g/g of ABE yield. Therefore, yeast extract can stimulate the growth of the cells, resulting in increasing the cells to produce the solvents. However, as in Figure 4.10 and 4.11, at too high yeast extract concentration, more than 5.5 g/L, the production seems to be decreasing. This is because of the extensive sugar consumption by the cells in the growth phase that make the reduction in cells to produce the solvents.

In contrast, Valsero et al. (2018) discovered that there were no significant differences were observed between 1 or 5 g/L yeast extract for biobutanol performance using coffee silverskin hydrolysates as substrate in their study. Therefore, in order to reduce costs, they prefer to add only 1 g/L yeast extract. However, for this thesis, biobutanol production is optimum at 5 g/L of yeast extract.

4.4.5 Validation of optimization

To further validate the accuracy of RSM prediction, an experiment was performed under the optimal conditions obtained in Table 4.9. This validation was also used to verify the accuracy of the model. Validation was carried out with conditions as follows: temperature 37°C, yeast extract concentration 5.5 g/L, and inoculum size 10%. Under the above optimized condition, the maximum production of biobutanol was estimated as 0.27

g/g. The results were verified by triplicate experiments and the maximum biobutanol yield was 0.2992 g/g was obtained from the validation test as in Table 4.11.

Table 4.11 Model validation of the biobutanol production

Parameters		**Value**
Inoculum size (%)		10
Temperature (°C)		37
Yeast extract (g/L)		5.5
Biobutanol yield (g/g)	Experimental	0.2775
	Predicted	0.2700
	Actual	0.2992

These validation findings were in close agreement with the model prediction and experimental value (average from Table 4.9), with a difference only 9.76%. This validated that the RSM approach was effective for optimizing the operational conditions for the ABE fermentation process.

CHAPTER 5

CONCLUSION

5.1 Conclusion

OPF juice can be utilized in the production of biobutanol via ABE fermentation by *C. acetobutylicum* ATCC 824. This study has shown that OPF juice consisted of high sugar content; glucose (48.19 g/L), sucrose (8.48 g/L) and fructose (1.68 g/L). In preliminary study, the ABE production using synthetic sugars as control and OPF juice were comparable. The ABE yield and productivity obtained using OPF juice were 0.41 g/g and 108.96 mg/L/h, respectively. Meanwhile, using synthetic sugars, ABE yield and productivity was found as 0.43 g/g and 122.85 mg/L/h, respectively. The comparison showed that OPF juice is promising to produce biobutanol in this work.

Screening of five independent variables in biobutanol production was studied prior to the optimization. From the result, yeast extract concentration gave the highest contribution at 8.20%, followed by inoculum size at 7.84% and incubation temperature at 7.56%. The total sugar concentration and pH gave low contributions with 2.09% and 0.78% respectively. The model obtained from RSM was significant with p-value <0.0001.

Optimization of three independent variables which are yeast extract, inoculum size and incubation temperature was employed using RSM. 20 experimental runs including 5 replicates of center points were designed by CCD. The optimal conditions obtained was at 5.5 g/L yeast extract concentration, 10% inoculum size and 37°C incubation temperature. The model obtained from CCD was significant with low p-value (<0.0001) and non-significant lack of fit. The model has R^2 of 0.9497, implying a high correlation between the observed and predicted values. In validation experiment, the biobutanol yield that obtained during the experiment was compared with the predicted

values. The FA yield that obtained from the experiment was 0.2992 g/g while the predicted value was 0.2700 g/g. There was 9.76% of error between the predicted and experimental values.

The findings of this study suggest that OPF just is a potential carbon souce for the substrate in fermentation medium for ABE production by *C. acetobutylicum* ATCC 824. Moreover, oil palm waste is very abundant in nature since Malaysia is one of the major palm oil producers. Therefore, fully utilization of OPF is not only beneficial in terms of value-added products, but it also can reduce the environmental pollution problems due to its large accumulation in nature.

5.2 Recommendation

Several recommendations were proposed in this chapter in order to improve the biobutanol production by *C. acetobutylicum* ATCC 824 from OPF juice. The recommendations are listed below.

1. Immobilization cell to increase cell concentration in the reactor. Low cell concentration in ABE fermentation can cause low productivity of the production of solvents. The productivity of the ABE fermentation increased up to 10-50 times greater than that obtained during normal batch fermentation, with the increase of cell concentration in the reactor (Ezeji et al., 2004). Thus, resulting in a major economic advantage.

2. Using a coextractant to remove the butanol while fermentation takes place to boost butanol yield. This is because of butanol production is limited to concentrations of about 13 g/L due to the toxicity of the butanol produced in during fermentation. A nonpolar solvent can be used to extract the butanol from the aqueous solution. A coextractant which continuously remove butanol during fermentation was able to boost yields of production.

3. Apply gas stripping technique for in situ butanol recovery during the ABE fermentation (Ezeji et al., 2006; Ezeji et al., 2005). It is a process whereby a gas (or gases) is passed through the fermentation broth to capture the solvents. The solvents are recovered from the gas by cooling it off in a condenser, thereby condensing the solvents, where after it is collected in a receiver vessel. The gas is

recycled back to the fermenter to capture more solvents. This process continues until all the fermentable sugars are utilized by the culture, or until there is a rapid decrease in productivity. This recovery technique also because of the toxicity of butanol itself to the culture at 13 g/L.

4. Use fed-batch fermentation technique. Fed-batch fermentation is started in a batch mode with a low medium volume (usually less than 50% of fermenter volume) and a low substrate concentration (non-inhibitory to the culture). As the substrate is used by the culture, it is replaced by adding a concentrated substrate solution at a slow rate, thereby keeping the substrate concentration in the fermenter below the toxic level for the culture and increasing the culture volume in the reactor over time. This fermentation technique must be simultaneously applied with one of product recovery techniques as for example gas stripping mentioned in section 5.2.3. Therefore, the butanol concentration in the fermentation substrate will always not exceed more than the toxicity level.

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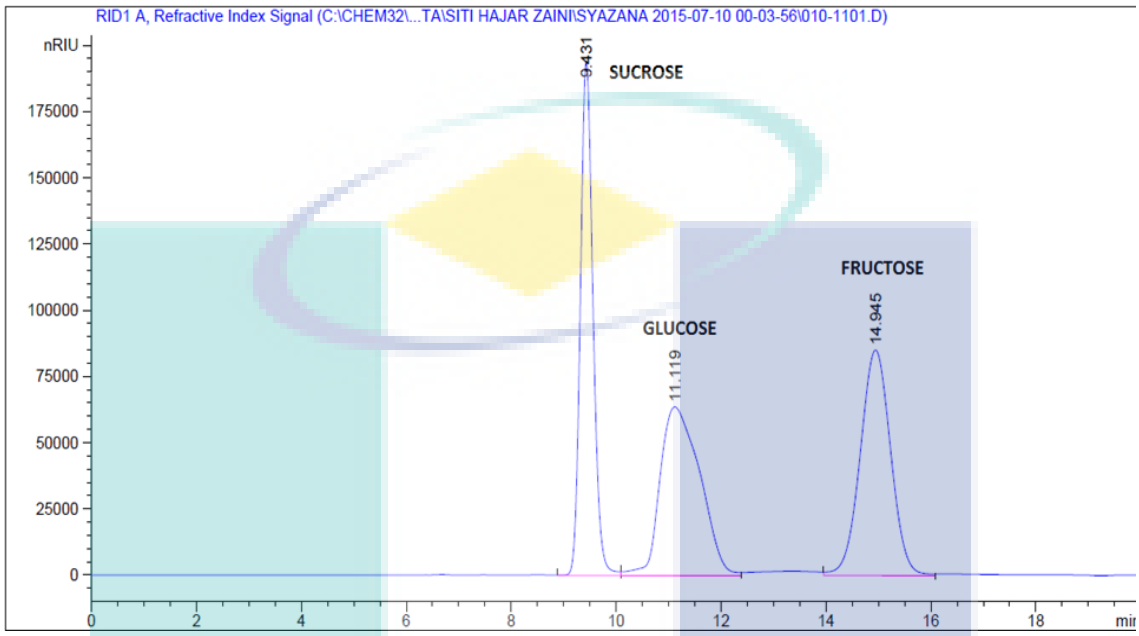
APPENDIX A

A1 List of chemicals

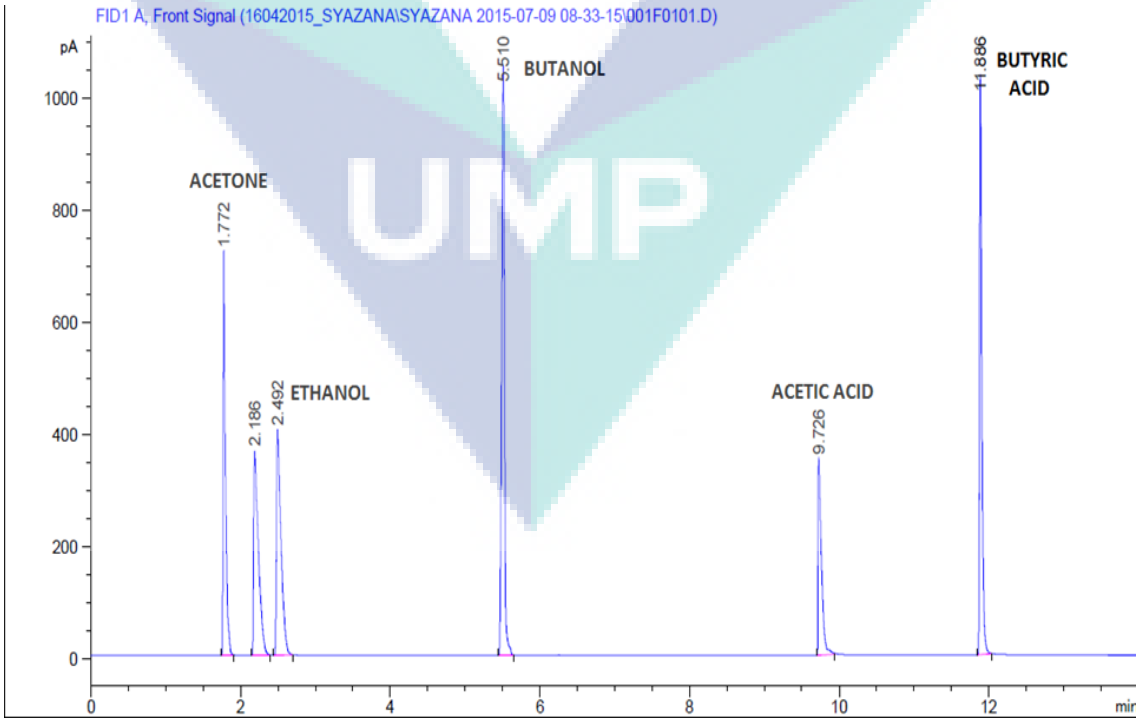
No	Chemical name	Manufacturer	Country origin
1	Ammonium acetate	Merck	United States
2	Sodium chloride	Merck	United States
3	Potassium dihydrogen phosphate	Merck	United States
4	Di-potassium hydrogen phosphate	Merck	United States
5	Sodium hydroxide	Merck	United States
6	Calcium carbonate	Merck	United States
7	Acetic acid	Fisher Scientific	United Kingdom
8	Butyric acid	Sigma Aldrich	United States
9	Acetone	Fisher Scientific	United Kingdom
10	1-Butanol	Fisher Scientific	United Kingdom
11	Ethanol	Merck	United States
12	Methanol	Merck	United States
13	Lactose monohydrate	Merck	United States
14	Glycerol	Thermo Scientific	United Kingdom
15	Yeast extract	Oxoid	United Kingdom
16	Reinforced clostridial medium (RCM)	Oxoid	United Kingdom
17	Reinforced clostridial agar (RCA)	Oxoid	United Kingdom
18	Thamine hydrochloride	Sigma aldrich	United States
19	4-aminobenzoic acid	Merck	United States
20	Biotin	Sigma aldrich	United States
21	Magnesium sulfate heptahydrate	Fisher Scientific	United Kingdom
22	Manganese sulfate heptahydrate	Merck	United States
23	Iron (II) sulfate heptahydrate	Sigma aldrich	United States
24	D-Glucose anhydrose	Fisher Scientific	United Kingdom
25	D (-) Fructose	Fisher Scientific	United Kingdom
26	Sucrose	Fisher Scientific	United Kingdom

APPENDIX B

B1 Chromatograms of mixed standard solution of sugar (HPLC)

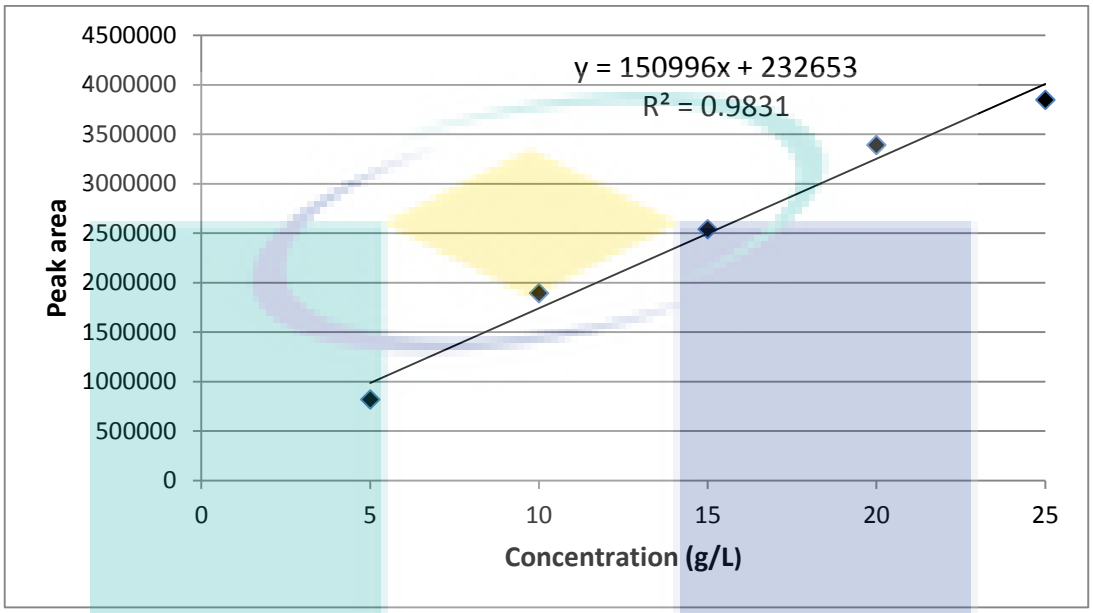


B2 Chromatograms of mixed standard solution of solvents (GC-FID)

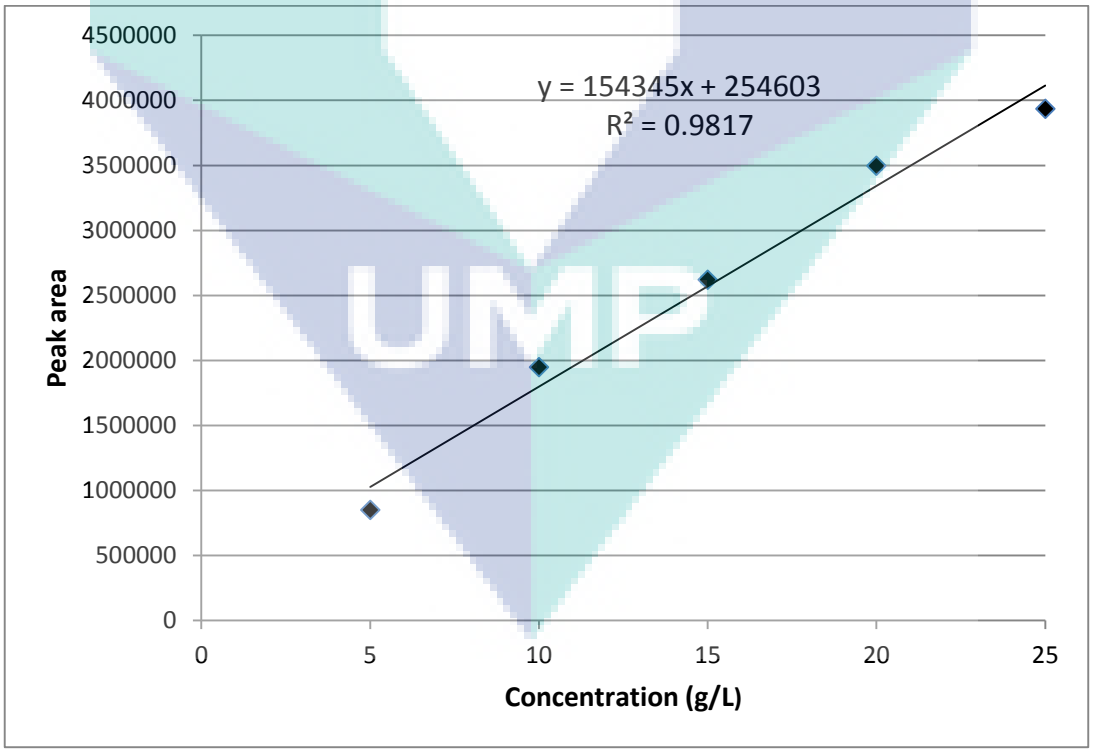


APPENDIX C

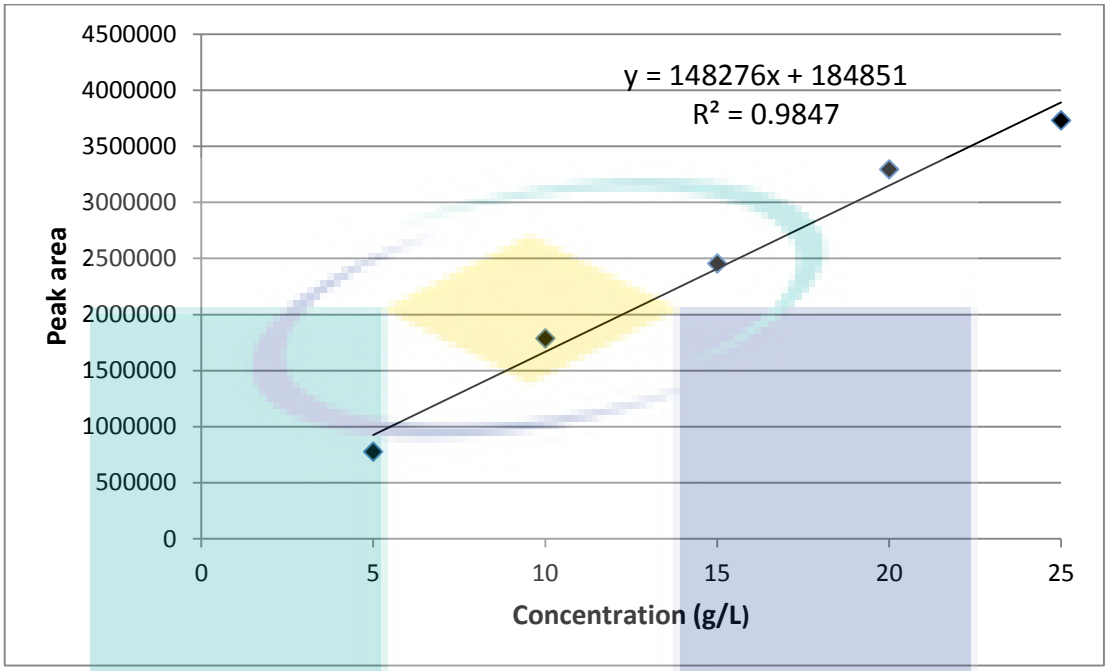
C1 Calibration curve for glucose



C2 Calibration curve for sucrose

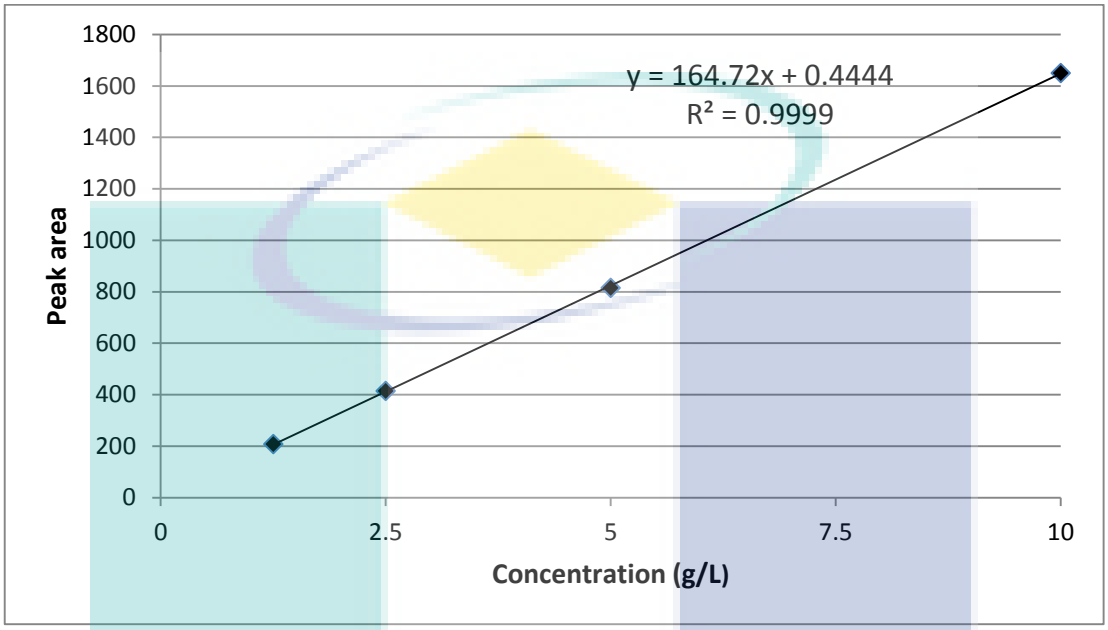


C3 Calibration curve for fructose

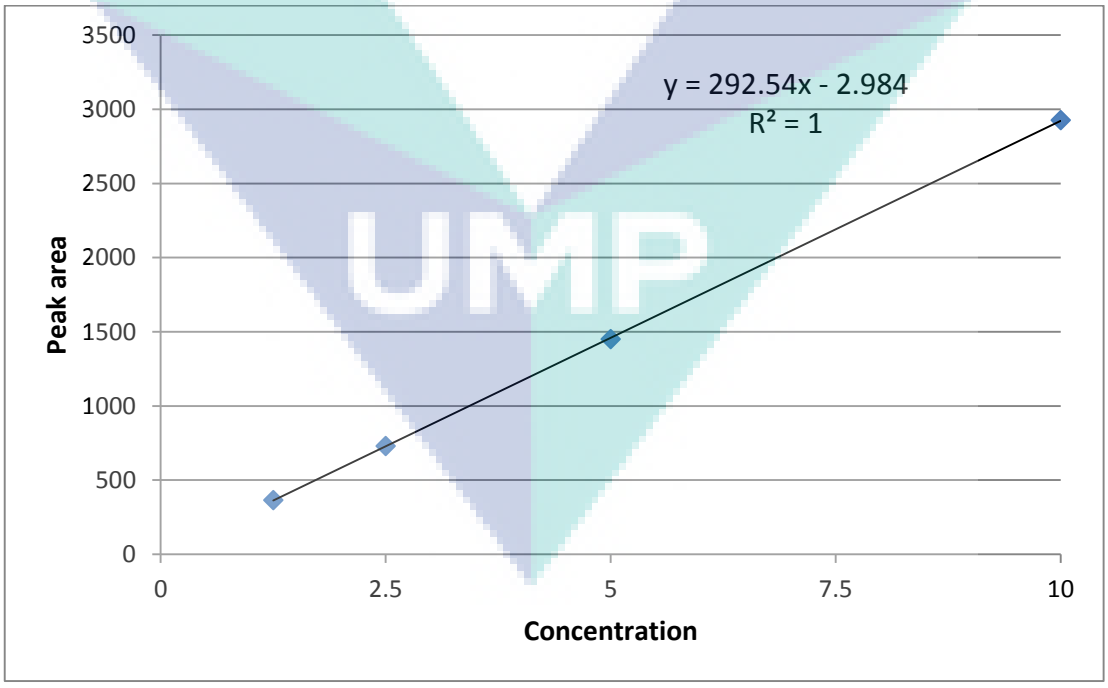


APPENDIX D

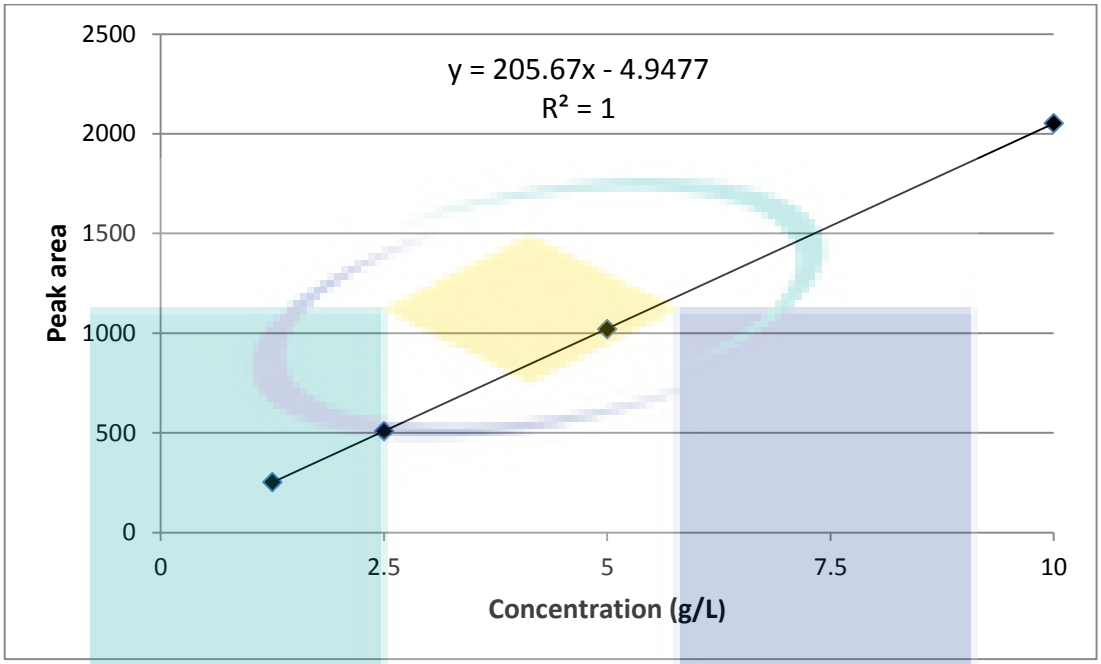
D1 Calibration curve acetone



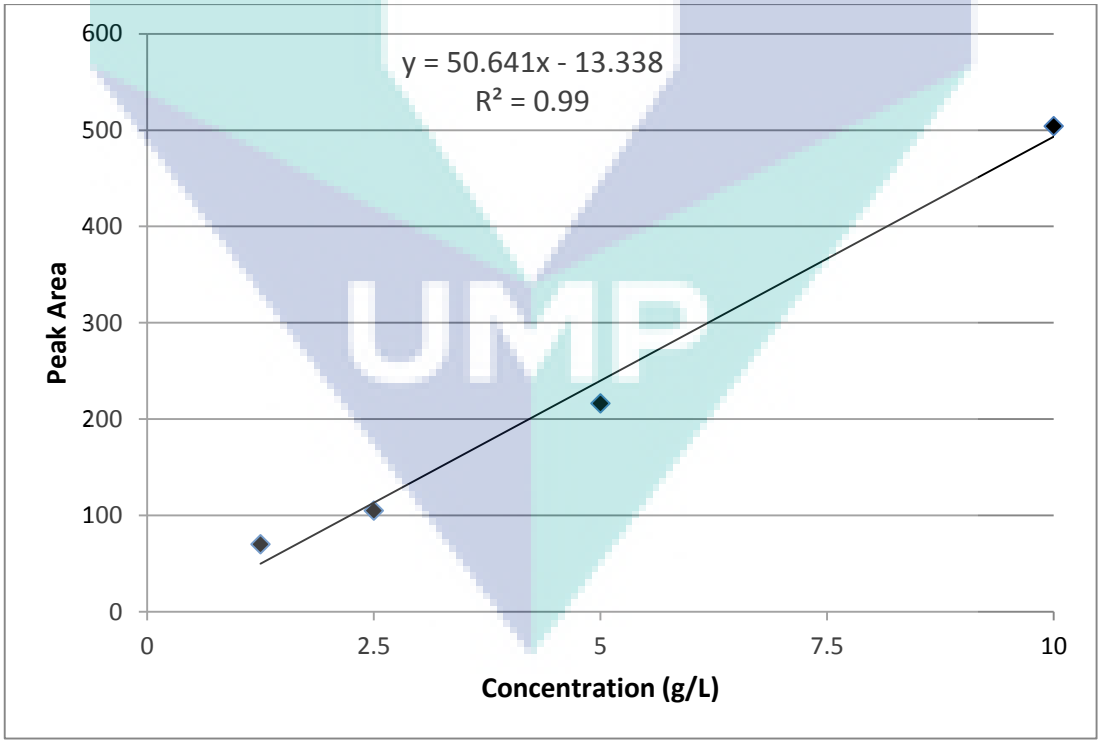
D2 Calibration curve butanol



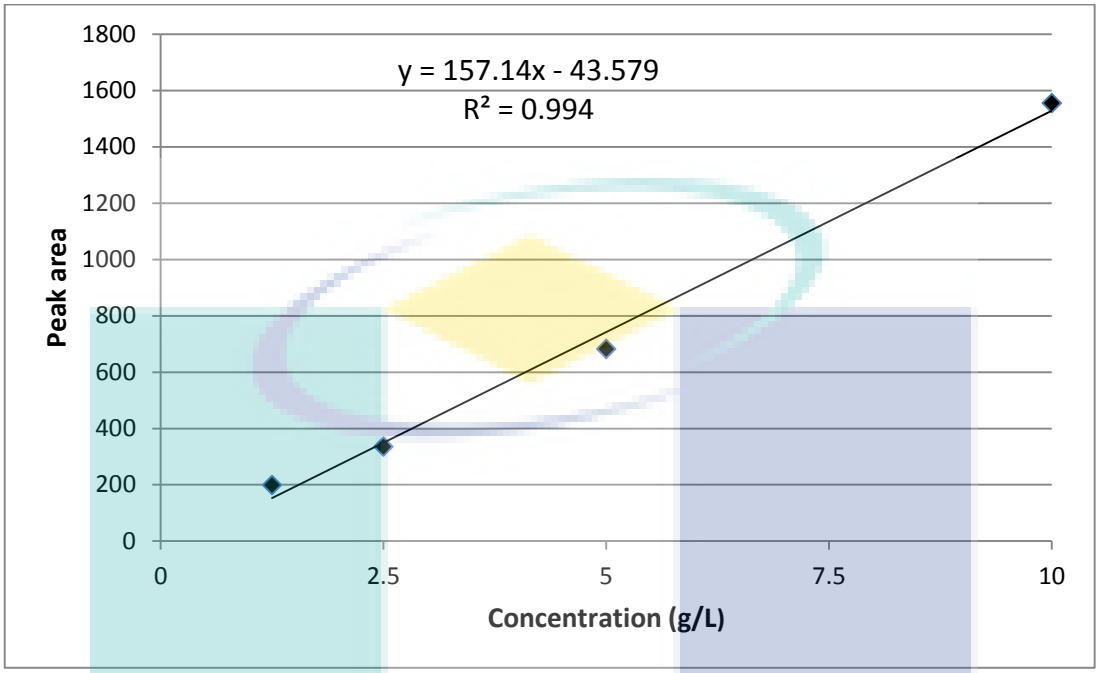
D3 Calibration curve ethanol



D4 Calibration curve acetic acid

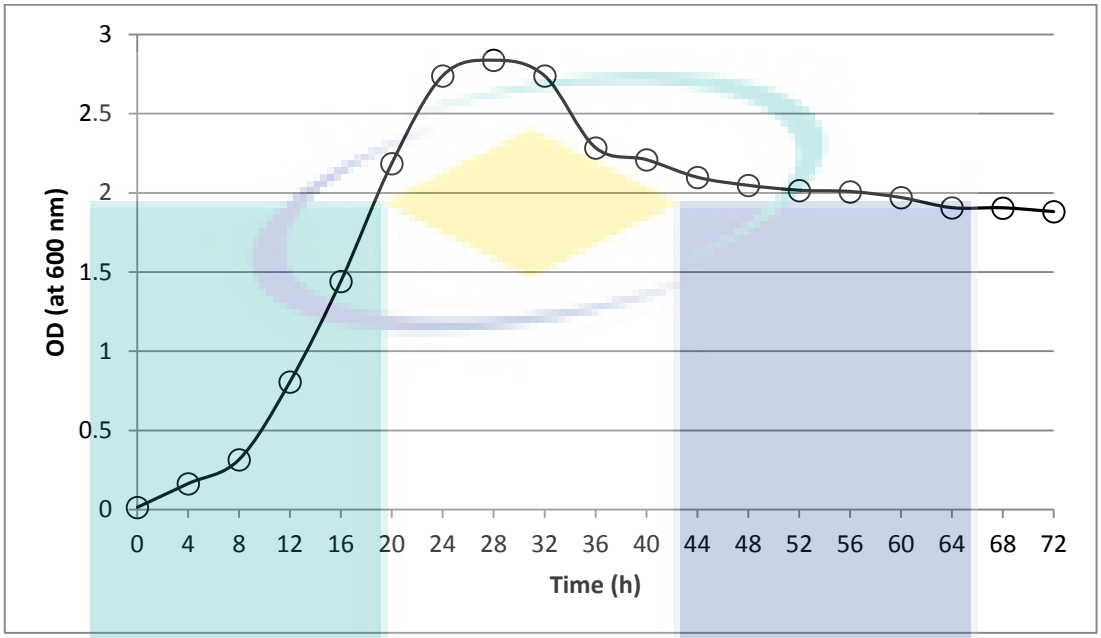


D5 Calibration curve butyric acid

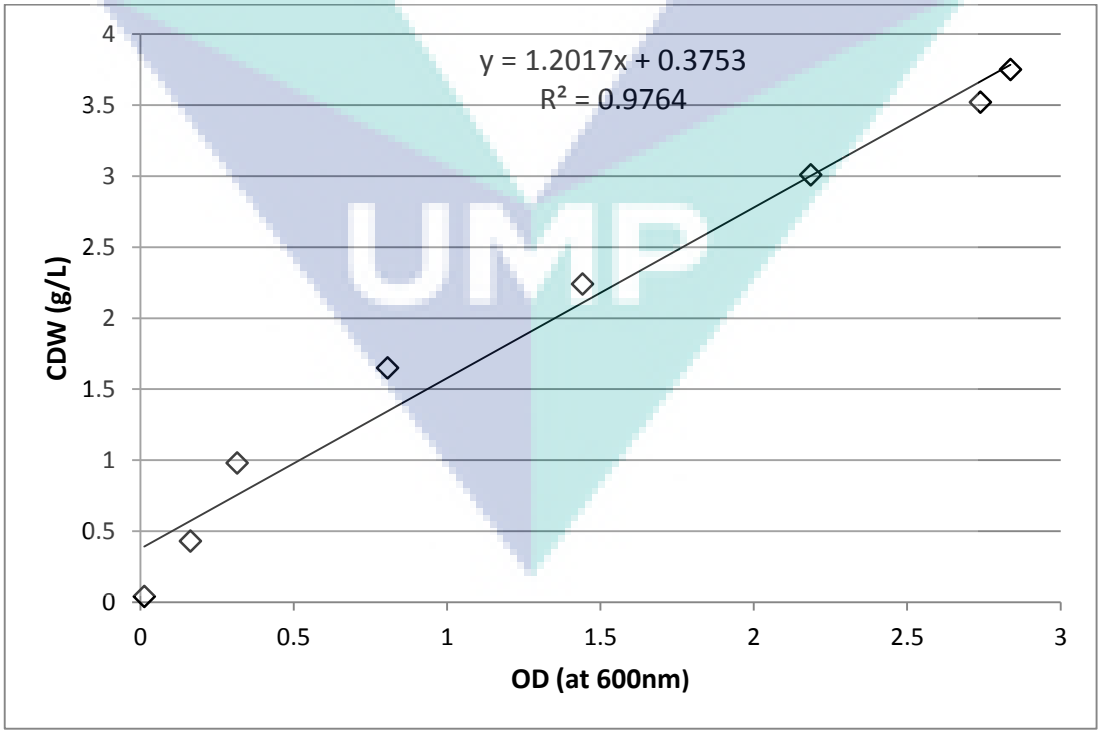


APPENDIX E

E1 Growth profile of *Clostridium acetobutylicum* ATCC 824



E2 Biomass standard curve of *Clostridium acetobutylicum* ATCC 824



APPENDIX F

F1 Factorial Analysis

Main effect list for factorial analysis from Design Expert software

Use your mouse to right click on individual cells for definitions.

Response 1 Biobutanol yield

Hierarchical Terms Added after Manual Regression

D

ANOVA for selected factorial model

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

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.11	12	9.396E-003	29.32	< 0.0001	significant
A-pH	*1.673E-003*	*1*	*1.673E-003*	*5.22*	*0.0563*	
B-Total sugar	*4.469E-003*	*1*	*4.469E-003*	*13.94*	*0.0073*	
C-Inoculum s	*0.017*	*1*	*0.017*	*52.25*	*0.0002*	
D-Temperatu	*0.016*	*1*	*0.016*	*50.37*	*0.0002*	
E-Yeast extra	*0.018*	*1*	*0.018*	*54.61*	*0.0002*	
AD	*1.517E-003*	*1*	*1.517E-003*	*4.73*	*0.0661*	
AE	*1.624E-003*	*1*	*1.624E-003*	*5.07*	*0.0591*	
BC	*1.777E-003*	*1*	*1.777E-003*	*5.54*	*0.0508*	
BE	*2.426E-003*	*1*	*2.426E-003*	*7.57*	*0.0285*	
CD	*0.035*	*1*	*0.035*	*108.70*	*< 0.0001*	
CE	*0.012*	*1*	*0.012*	*37.21*	*0.0005*	
DE	*2.111E-003*	*1*	*2.111E-003*	*6.59*	*0.0372*	
Curvature	0.099	1	0.099	307.48	< 0.0001	significant
Residual	2.243E-003	7	3.205E-004			
Lack of Fit	*3.009E-004*	*3*	*1.003E-004*	*0.21*	*0.8871*	*not significant*
Pure Error	*1.943E-003*	*4*	*4.856E-004*			
Cor Total	0.21	20				

ANOVA for factorial analysis

The Model F-value of 29.32 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

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

The "Curvature F-value" of 307.48 implies there is significant curvature (as measured by difference between the average of the center points and the average of the factorial points) in the design space. There is only a 0.01% chance that a "Curvature F-value" this large could occur due to noise.

The "Lack of Fit F-value" of 0.21 implies the Lack of Fit is not significant relative to the pure error. There is a 88.71% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.018	R-Squared	0.9805
Mean	0.14	Adj R-Squared	0.9470
C.V. %	12.58	Pred R-Squared	0.8992
PRESS	0.012	Adeq Precision	17.926

The "Pred R-Squared" of 0.8992 is in reasonable agreement with the "Adj R-Squared" of 0.9470.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 17.926 indicates an adequate signal. This model can be used to navigate the design space.

Final Equation in Terms of Coded Factors:

Biobutanol yield =

+0.10

-0.010 * A

-0.017 * B

+0.032 * C

-0.032 * D

+0.033 * E

+9.737E-003 * A * D

-0.010 * A * E

+0.011 * B * C

-0.012 * B * E

-0.047 * C * D

-0.027 * C * E

+0.011 * D * E

Final Equation in Terms of Actual Factors:

Biobutanol yield =

+0.43462

-0.069969 * pH

-1.33106E-003 * Total sugar concentration

+0.037719 * Inoculum size

-0.010531 * Temperature

+0.022279 * Yeast extract concentration

+1.94750E-003 * pH * Temperature

-2.23889E-003 * pH * Yeast extract concentration

+1.10921E-004 * Total sugar concentration * Inoculum size

-2.73611E-004 * Total sugar concentration * Yeast extract concentration

-9.82368E-004 * Inoculum size * Temperature

-6.38596E-004 * Inoculum size * Yeast extract concentration

+5.10556E-004 * Temperature * Yeast extract concentration

F2 Optimization

Fit summary for Optimization from Design Expert software

Response 1 Biobutanol yie Transform: None

*** WARNING: The Cubic Model is Aliased! ***

Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	0.53	1	0.53			
Linear vs Mean	0.030	3	9.949E-003	1.40	0.2799	
2FI vs Linear	2.830E-003	3	9.432E-004	0.11	0.9525	
Quadratic vs 2FI	0.10	3	0.035	47.86	< 0.0001	Suggested
Cubic vs Quadra	2.533E-003	4	6.331E-004	0.81	0.5628	Aliased
Residual	4.701E-003	6	7.835E-004			
Total	0.68	20	0.034			

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

Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Linear	0.11	11	9.939E-003	10.80	0.0083	
2FI	0.11	8	0.013	14.46	0.0046	
Quadratic	2.631E-003	5	5.262E-004	0.57	0.7229	Suggested
Cubic	9.828E-005	1	9.828E-005	0.11	0.7571	Aliased
Pure Error	4.603E-003	5	9.206E-004			

*"Lack of Fit Tests": Want the selected model to have insignificant lack-of-fit.

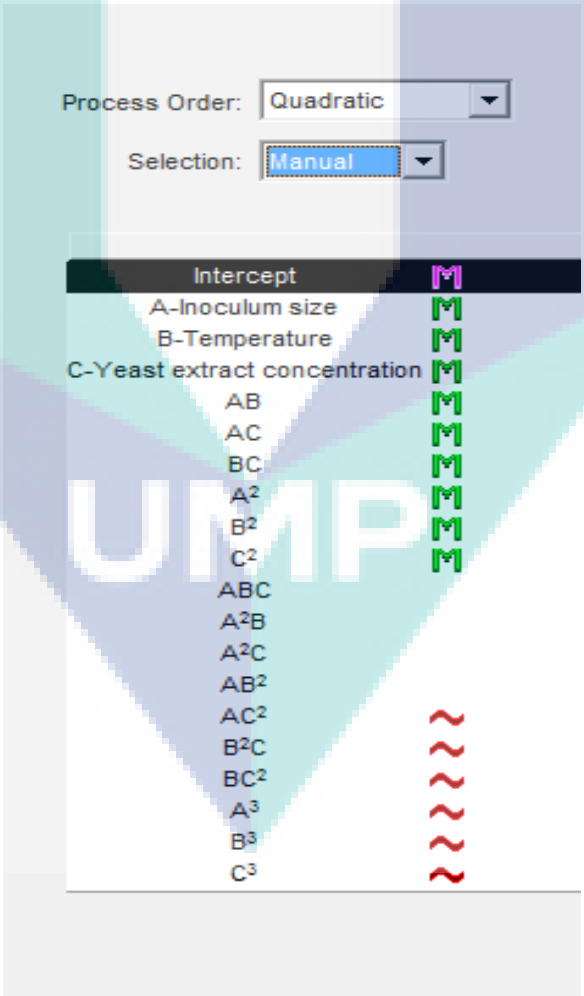
Fit summary for Optimization from Design Expert software continued

Model Summary Statistics

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	0.084	0.2076	0.0590	-0.1237	0.16	
2FI	0.092	0.2273	-0.1294	-0.3734	0.20	
Quadratic	0.027	0.9497	0.9044	0.8077	0.028	Suggested
Cubic	0.028	0.9673	0.8965	0.8143	0.027	Aliased

"Model Summary Statistics": Focus on the model maximizing the "Adjusted R-Squared" and the "Predicted R-Squared".

Central composite design model



ANOVA for Optimization

Use your mouse to right click on individual cells for definitions.

Response 1 Biobutanol yield

ANOVA for Response Surface Quadratic Model

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

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.14	9	0.015	20.97	< 0.0001	significant
A-Inoculum siz	*4.764E-003*	*1*	*4.764E-003*	*6.59*	*0.0281*	
B-Temperature	*0.015*	*1*	*0.015*	*20.48*	*0.0011*	
C-Yeast extrac	*0.010*	*1*	*0.010*	*14.19*	*0.0037*	
AB	*1.482E-003*	*1*	*1.482E-003*	*2.05*	*0.1828*	
AC	*1.540E-004*	*1*	*1.540E-004*	*0.21*	*0.6544*	
BC	*1.193E-003*	*1*	*1.193E-003*	*1.65*	*0.2280*	
A^2	*0.053*	*1*	*0.053*	*72.92*	*< 0.0001*	
B^2	*0.059*	*1*	*0.059*	*81.89*	*< 0.0001*	
C^2	*0.035*	*1*	*0.035*	*48.36*	*< 0.0001*	
Residual	7.234E-003	10	7.234E-004			
Lack of Fit	*2.631E-003*	*5*	*5.262E-004*	*0.57*	*0.7229*	*not significant*
Pure Error	*4.603E-003*	*5*	*9.206E-004*			
Cor Total	0.14	19				

The Model F-value of 20.97 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

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

The "Lack of Fit F-value" of 0.57 implies the Lack of Fit is not significant relative to the pure error. There is a 72.29% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.027	R-Squared	0.9497
Mean	0.16	Adj R-Squared	0.9044
C.V. %	16.48	Pred R-Squared	0.8077
PRESS	0.028	Adeq Precision	13.409

The "Pred R-Squared" of 0.8077 is in reasonable agreement with the "Adj R-Squared" of 0.9044.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 13.409 indicates an adequate signal. This model can be used to navigate the design space.

ANOVA for Optimization continued

Final Equation in Terms of Coded Factors:

Biobutanol yield	=
+0.27	
+0.017	* A
-0.030	* B
+0.025	* C
-0.014	* A * B
+4.388E-003	* A * C
-0.012	* B * C
-0.046	* A^2
-0.049	* B^2
-0.037	* C^2

Final Equation in Terms of Actual Factors:

Biobutanol yield	=
-9.51601	
+0.31555	* Inoculum size
+0.42284	* Temperature
+0.17959	* Yeast extract concentration
-2.26875E-003	* Inoculum size * Temperature
+1.09687E-003	* Inoculum size * Yeast extract concentration
-2.03542E-003	* Temperature * Yeast extract concentration
-0.011451	* Inoculum $size^2$
-5.39331E-003	* $Temperature^2$
-9.32557E-003	* Yeast extract $concentration^2$

APPENDIX G

Product Sheet of *C. acetobutylicum* ATCC 824

ATCC

Product Sheet

Clostridium acetobutylicum
(ATCC® 824™)

Please read this FIRST

Storage Temp.
Frozen: -80°C or colder
Freeze-Dried: 2°C to 8°C
Live Culture: See Propagation Section

Biosafety Level
1

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: *Clostridium acetobutylicum* (ATCC® 824™)

American Type Culture Collection
PO Box 1549
Manassas, VA 20108 USA
www.atcc.org

800.638.6597 or 703.365.2700
Fax: 703.365.2750
Email: Tech@atcc.org

Or contact your local distributor

Description

Designation: [CCRC 10639, CCUG 42182, DSM 792, IAM 19013, IFO 13948, JCM 1419, KCTC 1790, L.S. McClung 2291, LMG 5710, McCoy and McClung strain W, NCCB 29024, NCCB 84048, NCIMB 8052, VKM B-1787]
Deposited Name: *Granulobacter pectinovorum* (Stormer) Beijerinck
Product Description: Type strain. Degrades xylan, produces acetone, acidolysin, butyl alcohol [butanol], autobacteriocin, and restriction endonuclease Cac8241. Also used in the assay of p-aminobenzoic acid. Organism used in genome sequencing project.

Propagation

Medium
ATCC® Medium 2107: Modified Reinforced Clostridial
ATCC® Medium 260: Trypticase soy agar/broth with defibrinated sheep blood

Growth Conditions
Temperature: 37°C
Atmosphere: Anaerobic

Propagation Procedure

1. Open vial according to enclosed instructions or visit www.atcc.org for instructions.
2. Under anaerobic conditions aseptically rehydrate the entire pellet with approximately 0.5 mL of #2107 broth. Aseptically transfer the entire contents to a 5-6 mL tube of #2107 broth. Additional test tubes can be inoculated by transferring 0.5 mL of the primary broth tube to these secondary broth tubes. Best practice dictates the use of pre-reduced media.
3. Use several drops of the primary broth tube to inoculate a #260 plate and/or #260 agar slant.
4. Incubate in an anaerobic atmosphere at 37°C for 24-48 hours. Incubate one agar plate aerobically at 37°C to check for contamination.

ANAEROBIC CONDITIONS:

- Anaerobic conditions for transfer may be obtained by the use of an anaerobic gas chamber or placement of test tubes under a gassing cannula system connected to anaerobic gas.

Anaerobic conditions for incubation may be obtained by any of the following:

- Loose screw caps on test tubes in an anaerobic chamber
- Loose screw caps on test tubes in an activated anaerobic gas pack jar
- Use of sterile butyl rubber stoppers on test tubes so that an anaerobic gas headspace is retained

Notes

Purified genomic DNA of this strain is available as ATCC® 824D-5™.
Additional information on this culture is available on the ATCC® web site at www.atcc.org.

References

References and other information relating to this product are available online at www.atcc.org.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the current publication of the *Biosafety in Microbiological and Biomedical Laboratories* from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes for Health.

ATCC Warranty

ATCC® products are warranted for 30 days from the date of shipment, and this warranty is valid only if the product is stored and handled according to the information included on this product information sheet. If the ATCC® product is a living cell or microorganism, ATCC lists the media formulation that has been found to be effective for this product. While other, unspecified media may also produce satisfactory results, a change in media or the absence of an additive from the ATCC recommended media may affect recovery, growth and/or function of this product. If an alternative medium formulation is used, the ATCC warranty for viability is no

ATCC

Product Sheet

Clostridium acetobutylicum (ATCC® 824™)

Please read this FIRST

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longer valid.

Disclaimers

This product is intended for laboratory research purposes only. It is not intended for use in humans.
While ATCC uses reasonable efforts to include accurate and up-to-date information on this product sheet, ATCC makes no warranties or representations as to its accuracy. Citations from scientific literature and patents are provided for informational purposes only. ATCC does not warrant that such information has been confirmed to be accurate.
This product is sent with the condition that you are responsible for its safe storage, handling, and use. ATCC is not liable for any damages or injuries arising from receipt and/or use of this product. While reasonable effort is made to insure authenticity and reliability of materials on deposit, ATCC is not liable for damages arising from the misidentification or misrepresentation of such materials.

Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org

Additional information on this culture is available on the ATCC web site at www.atcc.org.

APPENDIX H

H1 Published paper

1. **Nur Syazana Muhd Nasrah,** Mior Ahmad Khushairi Mohd Zahari, Nasratun Masngut and Hidayah Ariffin. (2017). Factorial experimental design for biobutanol production from oil palm frond (OPF) juice by *Clostridium acetobutylicum* ATCC 824. Chemical Engineering Research Bulletin. 19(2017), 36 – 42.

2. **Nur Syazana Muhamad Nasrah,** Mior Ahmad Khushairi Mohd Zahari, Nasratun Masngut and Hidayah Ariffin. (2017). Statistical Optimization for Biobutanol Production by *Clostridium acetobutylicum* ATCC 824 from Oil Palm Frond (OPF) Juice Using Response Surface Methodology. MATEC Web of Conference. 111(03001), 1 – 8.

3. **Nur Syazana Muhd Nasrah,** Mior Ahmad Khushairi Mohd Zahari and Nasratun Masngut. (2016). Biobutanol Production by *Clostridium acetobutylicum* ATCC 824 Using Oil Palm Frond (OPF) Juice. In: Proceedings of The National Conference for Postgraduate Research (NCON-PGR 2016), 34 -38.

H2 Attended conference

1. International Conference on Fluids & Chemical Engineering (FLUIDSCHE2017), 4-6 April 2017, TH Hotel Kota Kinabalu, Sabah, Malaysia. (Oral presentation)

2. International Conference of Chemical Engineering & Industrial Biotechnology (ICCEIB 2016), 28-30 November 2016, Bayou Lagoon, Melaka, Malaysia. (Oral presentation)

3. The National Conference for Postgraduate Research (NCON-PGR 2016), 24-25 September 2016, Universiti Malaysia Pahang (UMP), Pekan, Pahang. (Oral presentation)

4. Creation, Innovation, Technology & Research Exposition (CITREX 2016), 7-8 March 2016, Universiti Malaysia Pahang (UMP), Gambang, Pahang. (Poster presentation)