# Optimization of Ethanol Production from Orange Peels using Response Surface Methodology

by

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(12217)

Dissertation submitted in partial fulfillment of
the requirements for the
BACHELOR OF ENGINEERING (Hons)
(CHEMICAL ENGINEERING)

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# CERTIFICATION OF APPROVAL

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Approval by:

_____

(Dr Oh Pei Ching)

UNIVERSITI TEKNOLOGI PETRONAS
TRONOH, PERAK
September 2012

## CERTIFICATION OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this project, that the original work is my own except as specified in the references and acknowledgements and that the original work contained herein have not been undertaken or done by unspecified sources or persons.

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TUAN NORAZIHA BT TUAN ZAKARIA

## ABSTRACT

The factors of high oil price, the need for increased energy security and concern over greenhouse gas emissions from fossil fuels make bioethanol the focal point of the public and researchers. Bioethanol is a form of renewable energy that can be produced from agriculture feedstock. The recent study has come out with a new feedstock for the production of ethanol, which is using orange peels. The production of ethanol using orange peels is preferable to be studied due to the existing production method produce a heavy carbon footprint and also high in cost. Besides, it is also related to the disposal problem and environment concern as the wastes from processed orange are just left over and commonly are burnt.

Thus, in this project, research was done to produce ethanol from orange peel using two stage hydrolysis and fermentation studies, to study the effects of yeast concentration and temperature on ethanol production from orange peel and to optimize the concentration of yeast and temperature using Response Surface Methodology (RSM) method. In order to achieve these objectives, experiment was conducted which comprises the two stages hydrolysis process, preparation of yeast cells, fermentation and optimization using RSM.

For the first part of experiment, the primary and secondary hydrolysis of orange peel was carried out at acid concentration of 0 to 1.0% (w/v). At acid concentration of 0.5 and 0.75% (w/v) was the highest glucose yield for primary and secondary hydrolysis, respectively. For the fermentation, the range of temperature and yeast concentration of 30℃ to 40℃ and 0.1% to 0.5% (w/v) respectively, was selected to be studied. The pH and fermentation time was fixed at optimum condition which is pH5 and 15h accordingly. Response Surface Methodology (RSM) using two factors and two level central composite design was employed to optimize the effect of temperature and yeast concentration on ethanol production from orange peel. Based on the results obtained, the highest ethanol yield is around 6-6.2 g/L at temperature of 39-40℃ and yeast concentration of 0.25-0.3% (w/v). So, with this finding, it shows promise for scale up studies for larger industry.

## ACKNOWLEDGEMENT

First and foremost, praise to Allah the Almighty for his guidance and giving me strength to finish my Final Year Project in two semesters. I do not think I would make it this far without Him. I believe everything has been planned carefully by Him, as the best of planners is Allah (Quran, 8-30).

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## ABBREVIATION

| | |
|---|---|
| RSM | Response Surface Methodology |
| OPP | Orange Peel Powder |
| CCD | Central Composite Design |
| GYE | Glucose Yeast Extract |
| HPLC | High Performance Liquid Chromatography |
| RI | Refractive Index |
| HMF | Hydroxymethylfurfurals |
| ANOVA | Analysis of Variance |

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# CHAPTER 1
# INTRODUCTION

## 1.1 Background of Project

Biofuel is a type of fuel whose energy is derived from carbon fixation and it is also known as renewable energy sources. Biofuel commonly is produced from living organisms or from metabolic byproducts which are obtained from organic or food waste products. The fuel that is derived from biomass conversion, as well as solid biomass, liquid fuels, and various biogases is considered biofuels. The factors of high oil price, the need for increased energy security, concern over greenhouse gas emissions from fossil fuels, and support from government subsidies make biofuel the focal point of the public and researchers. According to NREL (May 18, 2012), the two most common types of biofuel in use today are ethanol and biodiesel.

For this project, the ethanol or ethanol fuel is highlighted to be studied. Ethanol fuel is often used as motor fuel, mainly as a biofuel additive to gasoline or petrol. It is a form of renewable energy that can be produced from agriculture feedstock such as sugar cane, potato, manioc and corn. Recent study has come out with a new method for the production of ethanol, which is using orange peels. The method used for producing ethanol from the orange peels is much greener and less expensive as compared to the current method available to run vehicles on the fuel. The method uses plant-derived enzyme to break down orange peels into sugar, which is then fermented into ethanol.

Therefore, we decided to conduct an experiment in order to study about the optimization of ethanol production from orange peels, focusing on the parameters such as temperature and enzyme concentration. The Response Surface Methodology is used in order to optimize the selected parameters.

## 1.2 Problem Statement

In recent years, few studies were conducted to produce ethanol fuel from orange peel. As a low cost renewable agriculture residue, conversion of orange waste to ethanol seems to be a good solution for domestic energy supply which can meet the local demand, while avoiding disposal related problems.

In addition, the existing production method, for example the conventional corn based ethanol production produce a heavy carbon footprint, which means it releases high concentration of greenhouse gases. Besides, Scientific American (2012) reported that the existing production method requires high cost.

It is also reported that the existing production feedstock uses food-based feedstock instead of conversion from waste. For example, the production of ethanol from corn, cassava, sugar cane and potato utilizes the whole part of the feedstock, which has a high demand in other relevant industries.

Besides, orange wastes posed disposal-related problem and environment concern as the wastes from processed orange including peels, segment membranes, and seed are just left over after juice extraction and commonly are burnt. In this regard, high energy is required in order to burn the orange wastes.

Therefore, a study should be carried out in order to produce ethanol from orange peels as this method is much greener and less expensive. The optimum temperature, pH, enzyme concentration and fermentation time need to be studied through fermentation studies with the use of Response Surface Methodology for optimization.

## 1.3 Objectives

i) To produce ethanol from orange peel using two stage hydrolysis and fermentation studies.
ii) To study the effects of yeast concentration and temperature towards ethanol production from orange peel.
iii) To optimize the yeast concentration and temperature using Response Surface Methodology method.

## 1.4 Scope of Study

For the first objective, experiment is conducted to produce ethanol from orange peel using two stage hydrolysis and fermentation studies. The primary stage and secondary stage of hydrolysis are carried out in order to analyze the sugar content in the orange peel sample. For the second objective, the effect of yeast concentration and temperature is analyzed from a series of experiment. For the last objective, the design expert software is used to optimize the selected parameters which are yeast concentration and temperature. The method used for the optimization study is the Response Surface Methodology Method (RSM).

## 1.5 Feasibility of the Project within Scope and Time Frame

The optimization of yeast concentration and temperature will be conducted using RSM method by using Design Expert Software by StatEase. The scope of study will be focused on the effect of yeast concentration, temperature and fermentation study of ethanol production from orange peel. The study will be conducted in a few stages. The first stage is doing research regarding the bioethanol itself, orange production, and fermentation experiment. The second stage will be the experimental design and then is followed by conducting the experiment. This project will be carried out in the given time frame and will cover the scope of study.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Introduction

Malaysia is one of the countries in Asia that practiced agriculture as one of its major industries of economic importance. With the significant amount of agricultural activities, agricultural wastes have become a very promising alternative source for bioethanol production. Tye et al. (2011) reported that, the estimated availability of the biomass and its potential energy generated in Malaysia are 50,919 dry kton/year and 13,343 kton/year, respectively. The estimated energy generated from biomass can contribute to approximately 21.5% of the national energy requirement. Furthermore, the potential bioethanol market in Malaysia is much larger than the market for biodiesel. This is because, a much larger portion of vehicles in Malaysia run on gasoline.

## 2.2 Bioethanol as a Renewable Energy

According to Vogelbusch Biocommodities (2012) bioethanol is a readily available fuel, made from plant-based feedstocks and is a clean fuel for combustion engines. It produces considerably lower emissions on combustion and it only releases the same amount of carbon dioxide as plants bound while growing. Due to this, Goh et al. (2010) stated that, bioethanol is 'carbon neutral' which means free from sulfur and aromatics that are harmful to living organisms. Besides producing less harmful emission during combustion, bioethanol also emits less green house gas (Dhabekar and Chandak, 2010). Bioethanol is also frequently used as petrol substitute for road transport vehicle. Bioethanol is mainly produced by sugar fermentation process. So, the main source of sugar required to produce ethanol comes from fuel or energy crops like maize, corn, saw dust, red canary grass, cord grasses, jurusalem artichoke, sorghum plants and orange peel. Talebnia (2008) reported that in the future, fuel will come from fruits, weeds and

sawdust which comprise of biomass in the form of cellulose and lignocelluloses since they are suitable for bio ethanol production (Sanchez and Cardona, 2008).

On the other hand, ethanol or ethyl alcohol ($C_2H_5OH$) which is a clear colourless liquid, is biodegradable and causes little environment pollution. In petrol, it is used to replace lead as an octane enhancer since ethanol is a high octane fuel. Besides, The Green Car Website (2012) stated that bioethanol is considered an alternative to petrol and diesel, so its popularity is emerging as a fuel for cars and is well establish in Brazil. According to Himmel et al. (2007), the negative impacts of fossil fuel on the environment and the unstable oil market are the factors that lead to the constant search for alternative fuels.

Thus, second-generation bioethanol is a great and potential alternative to replace fuels without causing feud to food-fuel supply as they are derived from non edible sources (Sun and Cheng, 2002).

## 2.3 Bioethanol Production

Ethanol can basically be produced from biomass by the hydrolysis and fermentation processes. Biomass wastes contain a complex mixture of carbohydrate polymers from the plant cell walls known as cellulose and lignin. In order to produce sugars from the biomass, the biomass is pre-treated with acids or enzymes in order to reduce the size of the feedstock and to open up the plant structure. According to Grohman et al (1994), pretreatment of either chemical, biological or mechanical, is required to break down cellulose, hemicellulose, and pectin polymers present in the cell walls of orange peels and convert them to their sugars monomers. So, the cellulose and the hemi cellulose portions are broken down (hydrolysed) by enzymes or dilute acids into sucrose sugar which is then fermented into ethanol. Besides, the lignin which is also present in the biomass is normally used as a fuel for the ethanol production plants boilers. There are three principle methods of extracting sugars from biomass. They are concentrated acid hydrolysis, dilute acid hydrolysis and enzymatic hydrolysis.

### 2.3.1 Concentrated Acid Hydrolysis

The concentrated acid hydrolysis is the arkanol process where the concentrated sulphuric acid is added into biomass that has been dried up to 10% moisture content. This technique is considered as an old technique since it was available at the end of the $19^{th}$ century (Sheehan and Himmel,1999). A concentrated acid is applied at a moderate temperature to break the hydrogen bonding between cellulose chains. The advantages of this method are it can be perform at low temperature and results in high yields. However, concentrated acid hydrolysis also has the disadvantages such as the large amount of acid which need to be recovered or reused to make it economically viable, take longer reaction time and it requires high cost for neutralization. Galbe and Zacchi (2012) also said that this method result in equipment corrosion problem. Therefore, this method will not be used in this project due to the hazardous concern and cost factor.

### 2.3.2 Enzymatic Hydrolysis

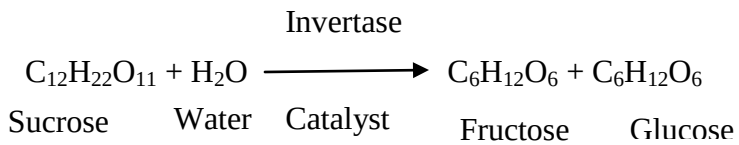
For this type of hydrolysis, the enzyme is used to breakdown the biomass, instead of using acid to hydrolyze it into sucrose. This process works in a similar way with the hydrolysis process which uses acid. However, this process is considered to be very expensive since it is still in the early development stage. Besides, according to Grohmann et al. (1992), this method is efficient to release almost all carbohydrates present in orange peel but it is hampered by the high cost of enzyme and the slow rate of depolymerization reaction that makes this method less attractive for this project.

### 2.3.3 Dilute Acid Hydrolysis

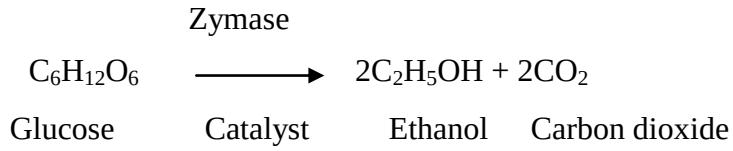
The dilute acid hydrolysis process is one of the oldest, simplest and most efficient methods of producing ethanol from biomass. Dilute acid is used to hydrolyze the biomass to sucrose. Alriksson (2006) reported that the advantages of dilute acid hydrolysis are fast reaction rate and low acid consumption. Besides, as it is a dilute acid, it is less hazardous as compared to concentrated acid hydrolysis method. Nevertheless, the conversion of cellulose into glucose is low, thus, we need to perform two steps of hydrolysis which is primary hydrolysis and secondary hydrolysis. These two steps of hydrolysis will be carried out in this project as dilute acid hydrolysis is cheaper, less hazardous and the most efficient method to hydrolyze biomass.

### 2.3.4 Sugar Fermentation Process

The hydrolysis process breaks down the cellulostic part of the biomass into sugar solutions that can then be fermented into ethanol. Yeast is added to the solution, which is then heated. The yeast contains an enzyme called invertase, which acts as a catalyst and helps to convert the sucrose sugars into glucose and fructose. The chemical reaction is shown below:




The fructose and glucose sugars then react with another enzyme called zymase, which is also contained in the yeast to produce ethanol and carbon dioxide. According to El Facto (2012), the chemical reaction of alcoholic fermentation to produce ethanol is shown as follows:




The most commercially used yeast for ethanol production is *Saccharomyces cerevisiae* (Jefferies, 2006). It has been genetically engineered to ferment xylose, one of the major fermentable sugars present in cellulosic biomass. For a successful economic production of ethanol, the optimization of important parameters such as yeast concentration, temperature and knowledge of the interaction between these variables are very important.

## 2.4 Orange Peel as a Source of Biomass

Orange is considered as the most important fruit that is consumed all over the world. It is commonly produced in tropical and subtropical regions across the globe. From the research made, it is said that orange is the major citrus fruit and its production has increased since 1980s. According to Plessas et al. (2007), orange production is predicted to approach 66.4 million tonne by 2010, representing a 14% increase within 12 years. Index Mundi (2011) reported that the production of orange fruits in Malaysia maintain a steady growth rate from 2006 until 2011 which is 12000 metric tonne per year. However, up to now, we could not find any commercial importance for the orange residues, which are the orange peels. The orange peels commonly are disposed and largely underutilized for the cattle feed. Grohman, Cameron and Buslig (1995) studied that orange peels are rich in fermentable sugars which is glucose, fructose and sucrose, along with insoluble polysaccharides cellulose and pectin. Because of this, the orange peel is considered as a new finding of biomass source in order to produce ethanol.

## 2.5 Response Surface Methodology (RSM)

Response Surface Methodology or RSM is a collection of mathematical and statistical techniques which are very beneficial for modeling and analysis of problems. Commonly, a response of interest is influenced by several variables and the objective is to optimize the response (D.C Montgomery,1997). Optimization and the study of the effect of important parameters for fermentation such as yeast concentration and temperature are important for successful economic yield of ethanol. Liu et al. (2007) stated that, in biological systems, RSM has been successfully employed for the optimization of parameters for the production of enzymes and ethanol. Besides, according to Kabbashi et. al (2007), the central composite design (CCD) is used in the experimental design for the optimization of process conditions.

In designing an experiment, the optimal design allows variables to be estimated without bias. Besides, optimal design which is provided by RSM also promotes a lower cost for experimentation since it allows the statistical models to be estimated by fewer experimental runs. Noordin et al. (2004) reported that, in order to determine the relationship between factors and the response variables investigated, the analysis of the data collected must be done in a statistical manner using regression. A regression is performed based on a functional relationship between the estimated variable, Y and one or more regressor input variable $x_1$,$x_2$….$x_i$. So, to fit a model equation, the least square technique is used by minimizing the residual error measured by the sum of square deviations between the actual and estimated responses.

For this project, two factors is considered to be studied which are yeast concentration and temperature. 13 runs of experiments are conducted according to the variables designed using Design Expert software. When the experiment is performed, the result which is ethanol concentration is analyzed by RSM for the optimization.

# CHAPTER 3

# METHODOLOGY

## 3.1 Introduction

This project is initiated with a literature review regarding the general view of bioethanol, the production of bioethanol and source of recent biomass. Details regarding the methodology for each part are discussed in the next section. Figure 3.1 shows the work sequence for this project:

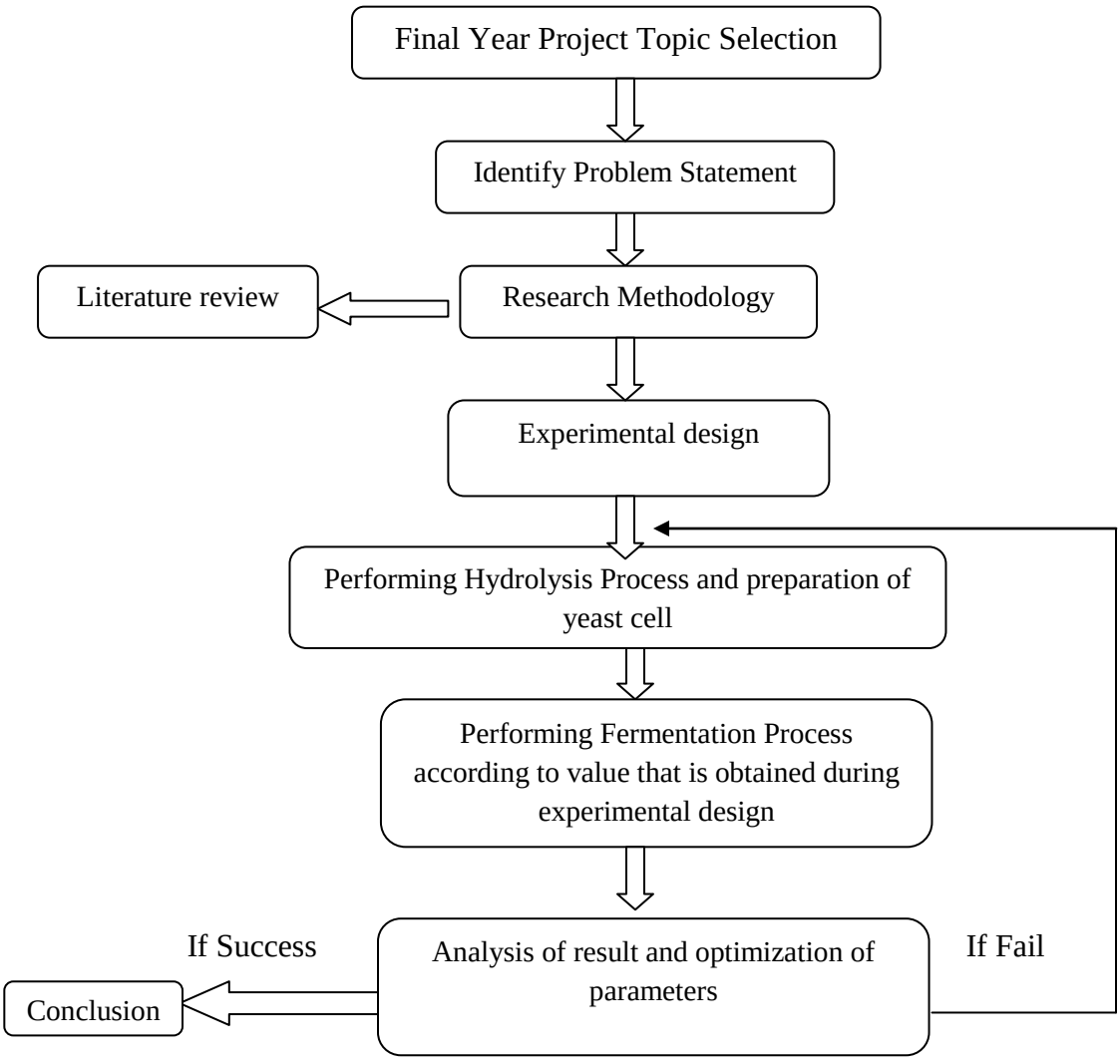



**Figure 3.1: Sequence of Work for Final Year Project**

### 3.2 Experimental Design

As this project is aimed to study the effects of yeast concentration and temperature on ethanol production from orange peel, so the value for each parameter needs to be obtained. This can be done by using Central Composite Design (CCD) by RSM. A two factor and two level CCD consisting of 13 experimental runs for ethanol production is employed. The experimental design is generated by Design Expert Software, based on the range decided for each parameter. The range for yeast concentration and temperature is set at 0.1-0.5% (w/v) and 30-40℃ respectively. Table 3.2 shows the tabulated value for variables generated by Design Expert software in terms of coded and uncoded.

**Table 3.2: Experimental Design**

| Run | T(°C), $x_1$ | Yeast Conc (% w/v), $x_2$ |
|---|---|---|
| 1 | 27.93(-1.414) | 0.3(0) |
| 2 | 30(-1) | 0.1(-1) |
| 3 | 35(0) | 0.3(0) |
| 4 | 30(-1) | 0.5(1) |
| 5 | 42.07(1.414) | 0.3(0) |
| 6 | 35(0) | 0.3(0) |
| 7 | 40(1) | 0.5(1) |
| 8 | 40(1) | 0.1(-1) |
| 9 | 35(0) | 0.3(0) |
| 10 | 35(0) | 0.3(0) |
| 11 | 35(0) | 0.02(-1.414) |
| 12 | 35(0) | 0.58(1.414) |
| 13 | 35(0) | 0.3(0) |

### 3.3 Performing Hydrolysis Process

**Hydrolysis of Orange Peel**

1) 13g of orange peel powder (OPP) as in Figure 3.3(a) is weighed and transferred into each of the 20 polycarbonated baffle flasks.
2) 87g of deionized water is added into polycarbonated flask containing OPP to produce 12% w/v OPP as in Figure 3.3(b).
3) Sulphuric acid is added at 0, 0.25, 0.5, 0.75 and 1.0% w/v to the 12% w/v OPP solution.
4) Figure 3.3(c) is when the solution is put in autoclave at 121°C for 15 min for treatment and sterilization.
5) The solution is filtered using vacuum filtration using coarse filter paper as in Figure 3.3(d).
6) The hydrolysate is collected in receiver flask like in Figure 3.3(e).
7) Sugars are being analyzed using refractometer. The value of refractive index of the solutions is used in order to calculate the concentration of glucose in the samples, according to equation given by Marker T.L et al (n.d). The equation is shown in Appendix.
8) The treatment that resulted in the highest amount of sugar is selected for fermentation.

**(a) (b) (c) (d) (e)**

**Figure 3.3: Primary and Secondary Hydrolysis Procedure**

### 3.4 Preparation and Propagation of Yeast Cells

1) Dried yeast powder is added into sterilized 150 ml Erlenmeyer flask containing 50 ml glucose yeast extract (GYE) as in Figure 3.4 (b). GYE is shown in Figure 3.4(a).
2) The flask is put in the incubator and is incubated at 30°C for 48hr at 100 rpm as in Figure 3.4(c).
3) The inoculums are transferred into 250 ml Erlenmeyer flask which contains 100 ml GYE broth.
4) 50 ml of prepared culture is transferred into 1L flask containing 500 ml of sterilized GYE broth.
5) The flask is put into an incubator and is incubated at 30°C for 24 hr and 100 rpm.
6) The cells are transferred to sterilized 50 ml centrifuge tube.
7) Then, it is centrifuged at 10000g at 4°C for 10 min in centrifuge as in Figure 3.4(d). Figure 3.4(e) shows the cells after centrifuged.

The hydrolysate and yeast is subsequently used for fermentation process. The procedure is shown in Figure 3.4 below:

**(a)** **(b)** **(c)** **(d)** **(e)**

**Figure 3.4: Procedure for yeast propagation**

### 3.5 Performing Fermentation Process

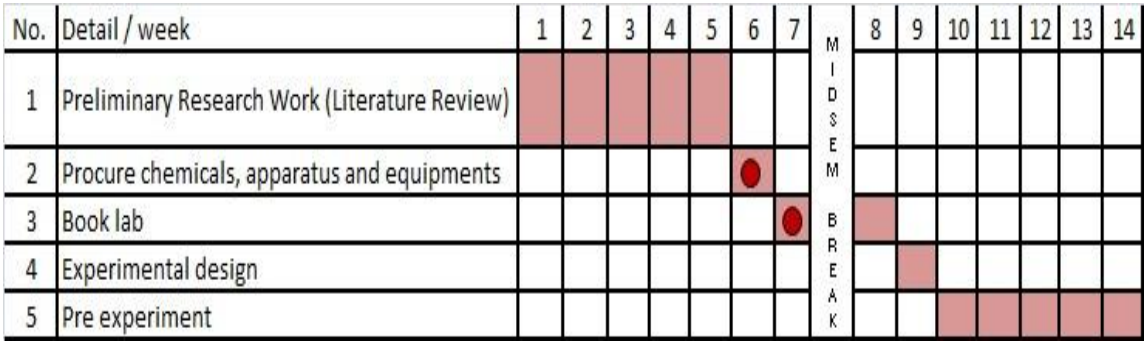
When the hydrolysate and yeast cells are prepared, the fermentation process will be carried out according to the procedures below:

**Batch Fermenter Experiments**

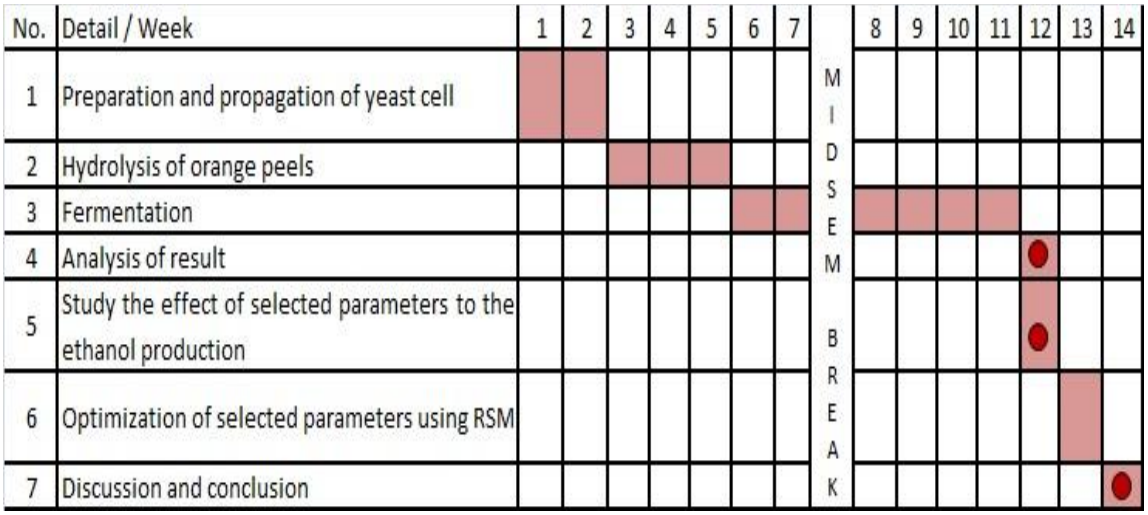
1) 1.2L of hydrolysate is collected from selected primary pretreatment.
2) Then, the 1.2L is put into 2L of batch fermenter.
3) The hydrolysate is neutralized and supplemented with a concentrated nutrient solution, to have final concentration of 0.3% w/v of yeast extract and 0.2% w/v peptone.
4) The residual pretreated biomass is collected in a sterile bag and is stored frozen for secondary hydrolysis.
5) The fermenter containing hydrolysate is heated to a temperature of 80°C for 30 min and is agitated at 250 rpm.
6) The fermentation is performed at temperature, pH and time according to the runs obtained from Design Expert at the beginning of project.
7) The fermenter is inoculated with 120 mL of yeast inoculums at concentration of $1x10^9$ cells/mL.
8) The agitation speed is maintained at 200 rpm.
9) The pH is maintained using sterilized 5N HCl and 10N NaOH.
10) The sample is drawn at 3h intervals and analyze for sugar and ethanol concentration.

The concentration of ethanol yield is determined by using refractometer. Three concentrations of standards are prepared and the refractive index (RI) for each sample is analyzed. Then the RI for samples are checked and compared with standard to find the concentration of samples. The concentration yield is then tabulated into the RSM design for further optimization.

### 3.5 Gantt Chart and Key Milestone (FYP 1 and FYP 2)




**Final Year Project I (May 2012)**




**Final Year Project II (Sept 2012)**

Process

Key Milestone

### 3.6 Chemicals, Materials and Tools Required

The chemicals, materials and tools that are required for this project are listed in Table 3.7(i) and Table 3.7(ii) below:

**Table 3.7(i): Materials and tools required**

| Apparatus | Equipments | Software |
|---|---|---|
| • Beaker<br>• Flask<br>• Measuring cylinder<br>• Buchner funnel<br>• Centrifuge tube<br>• Spatula<br>• Knife | • Oven<br>• Blender<br>• Incubator<br>• Centrifuge<br>• Autoclave<br>• Weighing scale<br>• HPLC column<br>• Refractometer | • Design Expert by StatEase |

**Table 3.7(ii): Chemicals required**

| Chemical Reagents | Assay | CAS Number | Supplier |
|---|---|---|---|
| Sodium hydroxide | ≥ 50% | 1310-73-2 | Sigma-Aldrich |
| Sulphuric acid | ≥ 97% | 7664-93-9 | Sigma-Aldrich |

# CHAPTER 4
# RESULT AND DISCUSSION

## 4.1 Chemical Analysis of Orange Peel

For the chemical analysis of orange peel, the sugar content in the pretreatment process is analyzed. The pretreatment process includes primary and secondary hydrolysis of orange peel. The resulted amount of sugar content for five samples in primary and secondary hydrolysis are tabulated in Table 4.1(a) and 4.1(b) respectively and is visualized in Graph 4.1(a) and 4.1(b):

**Table 4.1(a): Sugar content in primary hydrolysis**

| Sample (% $H_2SO_4$) | Refractive index | Conc (% glucose in water) |
|---|---|---|
| 0 | 1.33586 | 0.4729 |
| 0.25 | 1.42394 | 1.1191 |
| 0.5 | 1.57077 | 2.1964 |
| 0.75 | 1.4387 | 1.2274 |
| 1 | 1.33759 | 0.4856 |

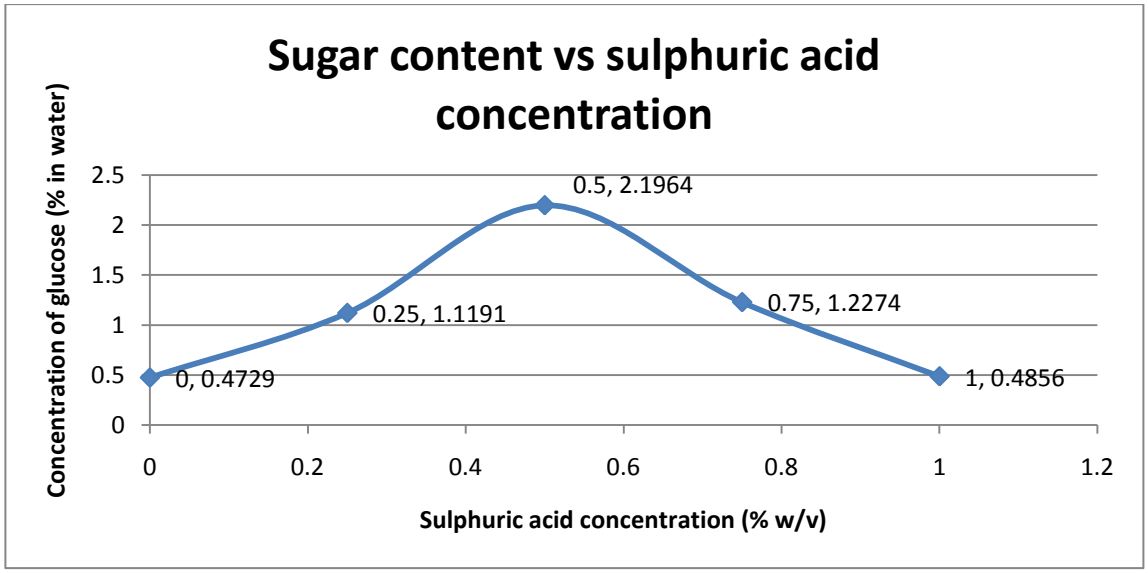



**Figure 4.1(a): Sugar content in primary hydrolysis**

**Table 4.1(b): Sugar content in secondary hydrolysis**

| Sample (% $H_2SO_4$) | Refractive index | Conc (% glucose in water) |
|---|---|---|
| 0 | 1.3375 | 0.4850 |
| 0.25 | 1.3391 | 0.4970 |
| 0.5 | 1.3397 | 0.5011 |
| 0.75 | 1.3415 | 0.5143 |
| 1 | 1.3408 | 0.5092 |

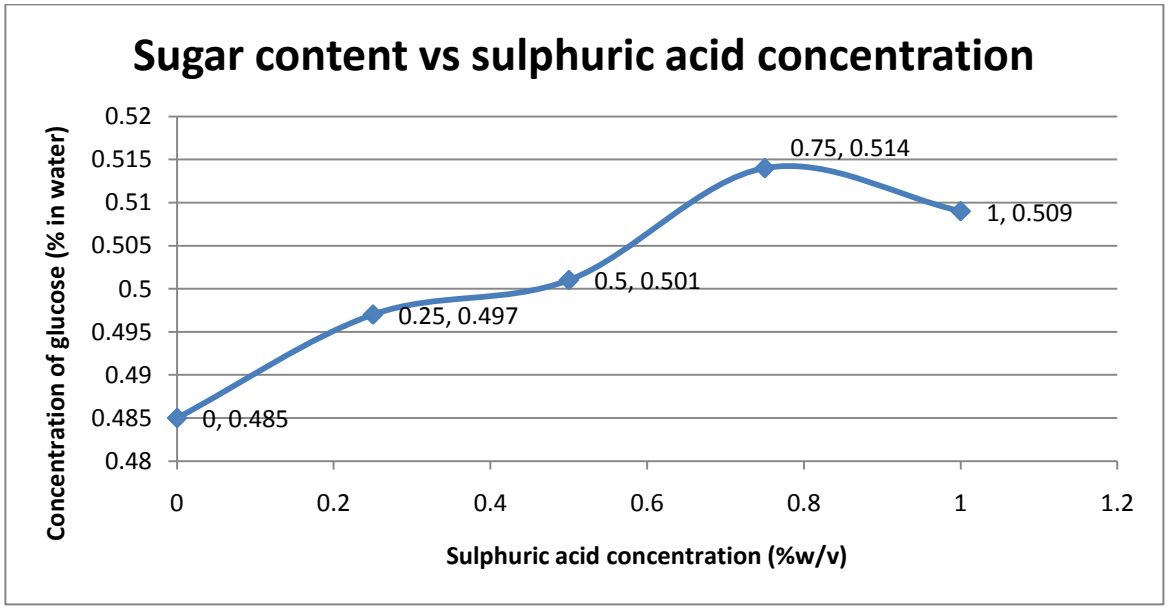



**Figure 4.1(b): Sugar content in secondary hydrolysis**

Chemical analysis of orange peel needs to be carried out in order to analyze the amount of sugar release during the pretreatment process. A pretreatment process is required for the hydrolysis of cellulosic and glycosidic bonds in pectin to release sugars for fermentation.

### 4.1.1 Primary Hydrolysis

As mentioned in methodology section, the pretreatment process is carried out in two stages, primary and secondary. The treatment resulting in the highest amount of sugar is selected for fermentation. As for primary hydrolysis, the orange peel powder is diluted with distilled water and is pretreated using 0, 0.25, 0.5, 0.75 and 1.0% (w/v) of sulphuric acid ($H_2SO_4$). Then, the samples are subjected to sterilization pretreatment at 121°C for 15 min.

Figure 4.1(a) shows the yield of glucose increases rapidly over the first three acid concentrations but then reached a peak at 0.5% (w/v) of sulphuric acid. Since then, the glucose yield has quickly dropped in which the sugar concentration decline to the lower value as the acid level increases from 0.5 to 0.75 and 1.0% (w/v). According to Oberoi H.S et al (2010), as the acid level increases, the glucose degrades to Hydroxymethylfurfurals (HMFs). So, this is why the glucose concentration declines to the lower value as the concentration of sulphuric acid increases.

Thus, the hydrolysis using 0.5% (w/v) of sulphuric acid is selected for the primary treatment. The hydrolysate from this selected pretreatment is collected for the fermentation.

### 4.1.2 Secondary Hydrolysis

As for secondary pretreatment, the same method and same concentration of acid from the primary pretreatment is used. The only difference between these two steps is; the secondary hydrolysis is carried out using the residual pretreated biomass from primary hydrolysis and the time for sterilization is increased to 30 min.

From Figure 4.1(b), it shows that the yield of glucose increases slightly from concentration of 0 to 0.75% (w/v), but decrease when the concentration is 1% (w/v). So, the secondary hydrolysis also resulted in an increase in the sugar concentration at increased acid level, until 0.75% (w/v). However, like the previous pretreatment, a further increase in acid level resulted in decline in the sugar concentration due to the glucose degrades to HMFs. So, for secondary hydrolysis, the pretreatment using 0.75% (w/v) is selected for fermentation.

Therefore, from the result obtained from primary and secondary hydrolysis, we can conclude that at the acid level of 0.25% (w/v) and below, the effectiveness to yield sugar from orange peel biomass is low. The hydrolysate of pretreatment is shown in Figure 4.1.2:

**Figure 4.1.2: Hydrolysate of primary and secondary pretreatment**

Therefore, the presence of glucose (fermentable sugar) as shown in Table 4.1(a) and 4.1(b) in a significant amount promotes a good potential for use of orange peel as a

substrate for fermentation-based products, which is ethanol. So, this finding proof that the orange peel is a good source of biomass for ethanol production.

### 4.2 Propagation of Yeast Cells

The yeast is successfully propagated and cultured in the laboratory. The glucose yeast extract (GYE) is prepared first as it is needed to be used for the propagation of yeast cells. The GYE produced is shown in Figure 4.2(a) below:

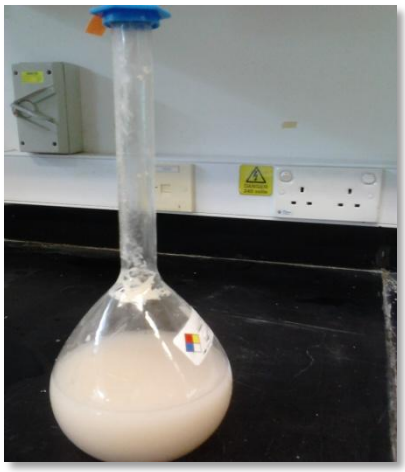


**Figure 4.2(a): Glucose Yeast Extract**

When the yeast is cultured for about 3 days, the solution is centrifuged at 10000g at 4°C and for 10 min. The resulted inoculums after centrifuge are as in Figure 4.2(b) below:

**Figure 4.2(b): Yeast solution after centrifuge**

The supernatant (liquid) is separated from the solid, and is collected for fermentation. The yeast solution produced gases and pungent smell that indicates the existence of yeast.

### 4.3 Response Surface Methodology (RSM) results

#### 4.3.1 Model Fitting and ANOVA

The analysis of ethanol concentration is the most important part for this project as the optimization of the yeast concentration and temperature is based on this result. Table 4.3.1(i) shows the ethanol concentration yield according to experimental design.

**Table 4.3.1(i): Ethanol concentration yield according to experimental design**

| Run | T(°C), $x_1$ | Yeast Conc (% w/v), $x_2$ | Ethanol Conc (g/L) |
|---|---|---|---|
| 1 | 27.93 | 0.3 | 5.40 |
| 2 | 30.00 | 0.1 | 5.00 |
| 3 | 35.00 | 0.3 | 5.80 |
| 4 | 30.00 | 0.5 | 6.00 |
| 5 | 42.07 | 0.3 | 5.80 |
| 6 | 35.00 | 0.3 | 6.20 |
| 7 | 40.00 | 0.5 | 5.90 |
| 8 | 40.00 | 0.1 | 5.90 |
| 9 | 35.00 | 0.3 | 6.00 |
| 10 | 35.00 | 0.3 | 5.90 |
| 11 | 35.00 | 0.02 | 5.30 |
| 12 | 35.00 | 0.58 | 5.10 |
| 13 | 35.00 | 0.3 | 5.90 |

The RSM software analyzed the data and fit the data to various models such as linear, two-factorial and quadratic. The resulted analysis of variance (ANOVA) suggested that the quadratic model is the most suitably described for this kind of interaction. The second-order effect of yeast concentration was the significant terms obtained from statistical analysis of RSM using Design Expert Software, as shown in Table 4.3.1(ii).

**Table 4.3.1(ii): ANOVA for synthesis variables pertaining to response percent yield**

| Source | Sum of Squares | Mean Square | *F*-value | Prob > *F* |
|---|---|---|---|---|
| Model | 1.15 | 0.29 | 4.09 | <0.0429[a] |
| $x_1$ | 0.23 | 0.23 | 3.32 | 0.1058[b] |
| $x_2$ | 0.064 | 0.064 | 0.92 | 0.3665[b] |
| $x_1x_2$ | 0.25 | 0.25 | 3.56 | 0.0958[b] |
| $x_2^2$ | 0.6 | 0.6 | 8.56 | <0.0191[a] |
| Lack of fit | 0.47 | 0.12 | 5.1 | 0.0718[b] |
| pure error | 0.092 | 0.023 | | |

[a]Significant at "Prob > *F*" less than 0.05
[b]Insignificant at "Prob > *F*" more than 0.05

The Model *F*-value of 4.09 implies that this model is significant. There is only 4.29% chance that a "Model *F*-value". This large value could occur due to noise. Values of Prob > *F* less than 0.0500 indicated that the model terms were significant. In this case, $x_2^2$ was significant model term. The lack of fit *F*-value of 5.10 implied that there was a 7.18% chance that "lack of fit *F*-value", due to noise.

So, the significant term contributed to a quadratic model, as given in Equation 4.3(i) and 4.3(ii) in terms of coded and uncoded (actual) respectively.

$$\mathbf{y\ (g/L) = 5.89 - 0.29x_2^2} \qquad \mathbf{(4.3i)}$$

$$\mathbf{y\ (g/L) = 1.27708 - 7.28261x_2^2} \qquad \mathbf{(4.3ii)}$$

The *P*-value obtained from the analysis of ANOVA was very low (0.0718) indicating a good reproducibility of experimental data. Besides, the reliability of the regression model to sufficiently represent the actual relationship between response and the significant variable is confirmed by the high values of coefficient of determination, $R^2$ (0.6716), Adj- $R^2$ (0.5074) and Pred- $R^2$ (-0.3409) as shown in Table 4.3.1(iii).

**Table 4.3.1(iii): Summary of ANOVA and regression analysis for ethanol yield**

| Model | Significant Model Term | Standard Deviation | $R^2$ | Adj-$R^2$ | Pred-$R^2$ | Adequate Precision |
|---|---|---|---|---|---|---|
| Quadratic | $x_2^2$ | 0.26 | 0.6716 | 0.5074 | -0.3409 | 6.350 |

### 4.3.2 Mutual Effect of Parameters

Based on mathematical analysis of the experiment data, the interaction between independent process factors and their respective response was plot graphically. The three-dimensional surface counter plot and counter plot for ethanol yield are shown in Figure 4.3.2(i) and 4.3.2(ii) accordingly.

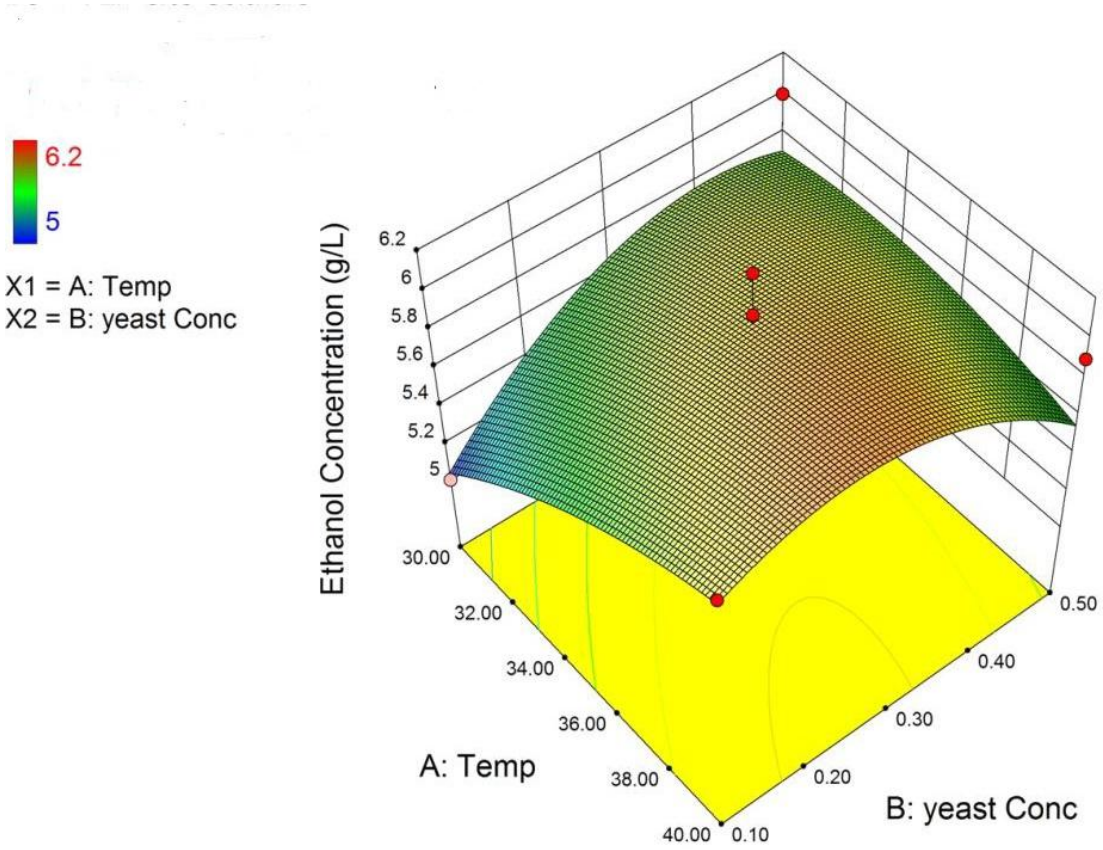



**Figure 4.3.2(i): Response surface plot of temperature, yeast concentration and ethanol yield**

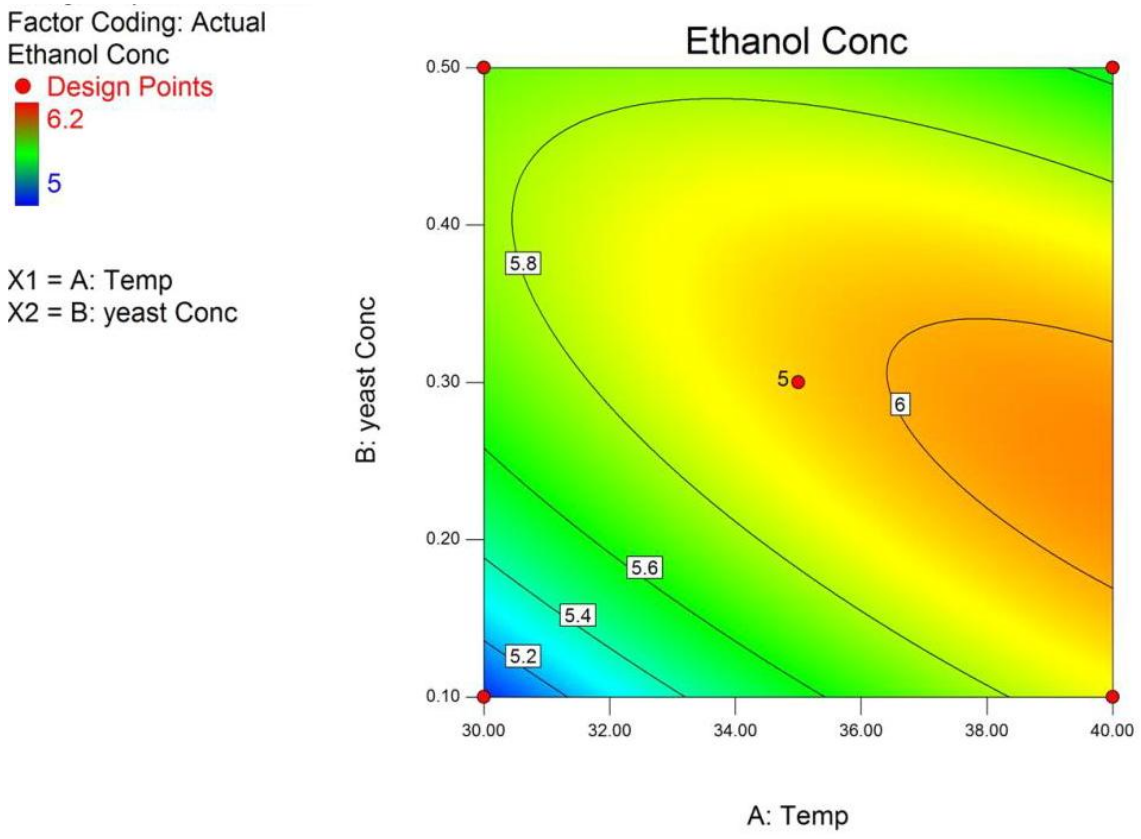



**Figure 4.3.2(ii): Response surface contour plot of temperature, yeast concentration and ethanol yield**

The response surfaces shown in Figure 4.3.2(i) and 4.3.2(ii) based on the model in which the two variables which are temperature and yeast concentration are varied in the range of 30-40℃ and 0.1-0.5%(w/v) respectively, while the pH and fermentation time are fixed to 5 and 15h respectively. From the figure, it is clearly shows that the ethanol concentration goes higher as the colour of contour goes from blue to red, which is from 5 g/L to 6.2 g/L. Besides, obviously we can see that the highest ethanol yield is at the red contour, which is at temperature of 39℃ to 40℃ while the yeast concentration is 0.24 to 0.3%.

For the fermentation, Russell (2003) stated that at a range of 5-5.2 of pH value is the ideal pH for fermentation as it is the best environment for yeast to grow. So, this is why the pH value is fixed to pH 5 for this experiment. Besides, for the fermentation

time, the earlier studies made by Oberoi et al (2010), they found that the optimum fermentation time is 15h.

According to Peggy (2012), the ideal temperature for yeast growth is around 37°C to 46°C. The yeast begins to die at temperature of 49°C. From the Figure 4.3.2(i), we can see that the ethanol production start to vigorously produced around temperature of 37.5-40°C with the ethanol production of 6 g/L. However, at the temperature around 34°C to 36°C, the ethanol production nearly reaches the red contour, but with a higher yeast concentration. So, this indicates that to produce higher concentration of ethanol at lower temperature, we need to use higher concentration of yeast. At the meantime, for the temperature around 30°C to 32°C, it is just a condition where the yeast starts to grow. Thus, the concentration of ethanol around this temperature is obviously low.

### 4.3.3 Validation of Statistical Model and Diagnostic

RSM can be used to observe the interaction effects among independent variables. Figure 4.3.3(i) shows the interaction between temperature and yeast concentration. From this figure, the spread of the points on the right side of the figure where the temperature is high is lower than the spread of points at the left side of the figure where temperature is low. It means that, the effect of yeast concentration ($x_2$) was less significant at high level of temperature ($x_1$). This is due to the dying of yeast at a very high temperature.

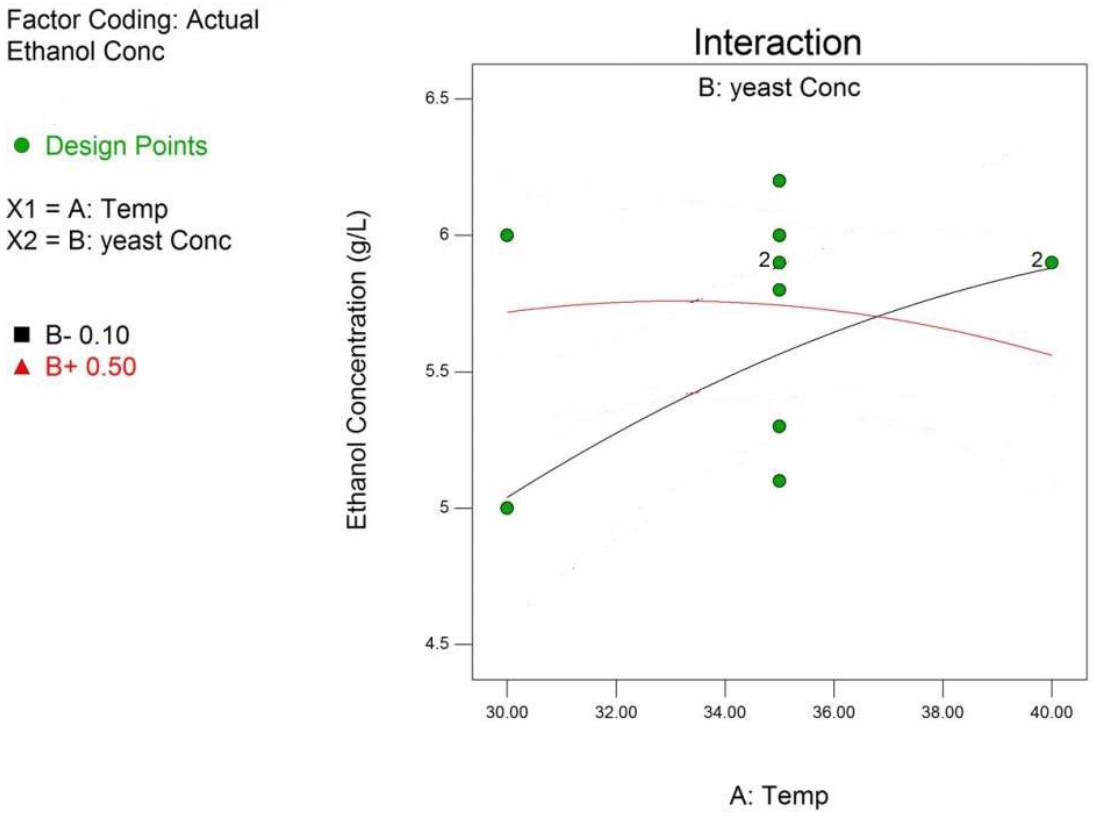



**Figure 4.3.3(i): Interaction plot of temperature and yeast concentration**

To ensure that the statistical assumptions fit to the analysis data for ANOVA, the diagnostic plots were employed by creating a scatter plot with the theoretical percentiles for residual analysis of the response surface design. Figure 4.3.3(ii) shows the normal probability in percentage which can be used to clarify whether the standard deviations between actual and predicted response values follow a normal distribution or not. So, from Figure 4.3.3(ii), the points are scatter in a straight line which means that there are no abnormalities of the experimental results.

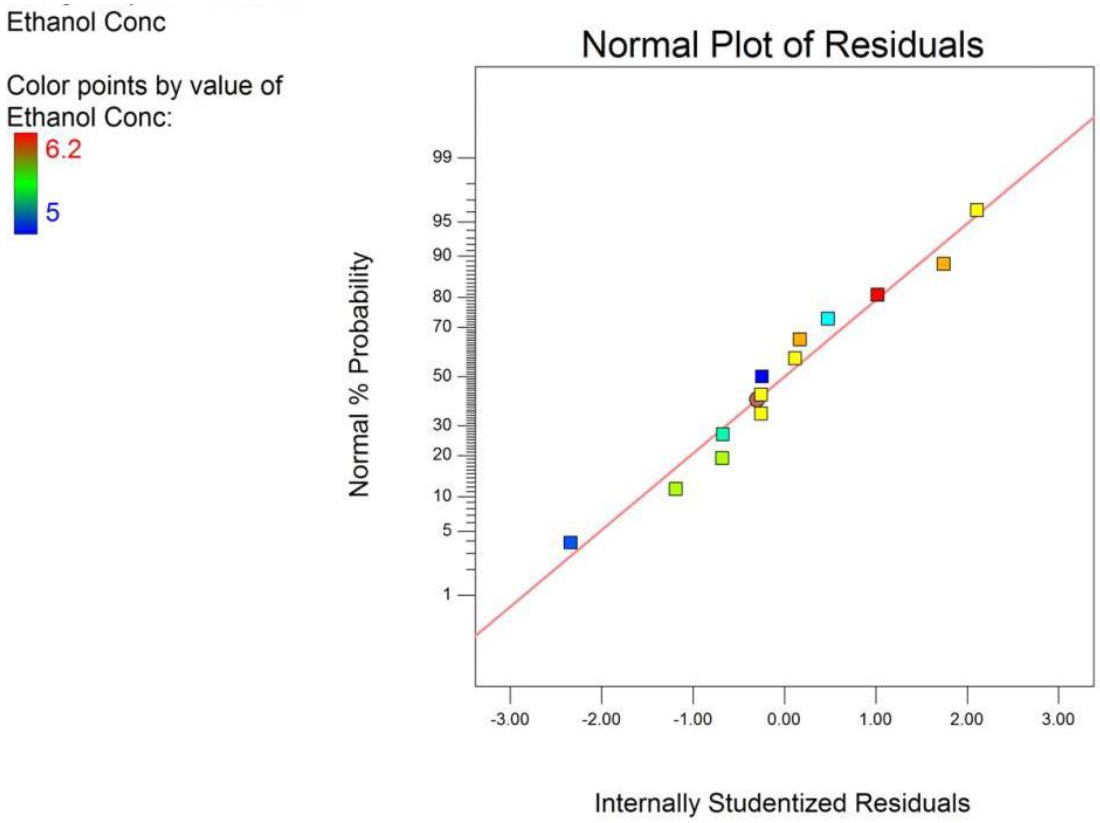



**Figure 4.3.3(ii): Normal probability plot of studentized residuals for ethanol yield**

Figure 4.3.3(iii) shows that all points of the experimental run were scattered randomly within a constant range of residual across the graph, which was within the area of ±3.00. So, we can say that the suggested model was adequate and the assumption of constant variance was confirmed.

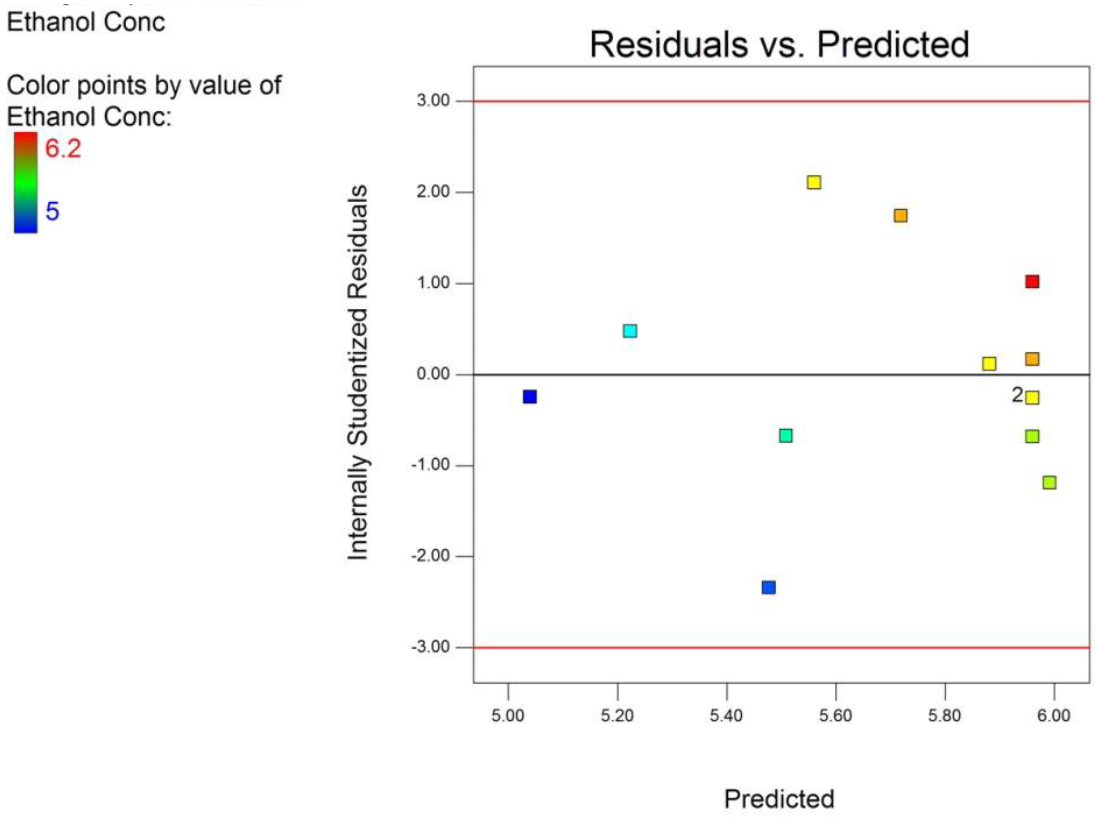



**Figure 4.3.3(iii): Plot of residuals versus predicted response for ethanol yield**

Figure 4.3.3(iv) below shows a positive interrelation between the response predicted by the model equation and the actual results that is obtained from the experiment. The points that are above the diagonal line represent those over-estimated. From the figure, we can see that quite several points scatter far from the diagonal line. This might be due to the failure of equipment. The refractometer was used to analyze the ethanol concentration instead of HPLC, due to HPLC failure to function. So, using refractometer might be less accurate as compared to HPLC.

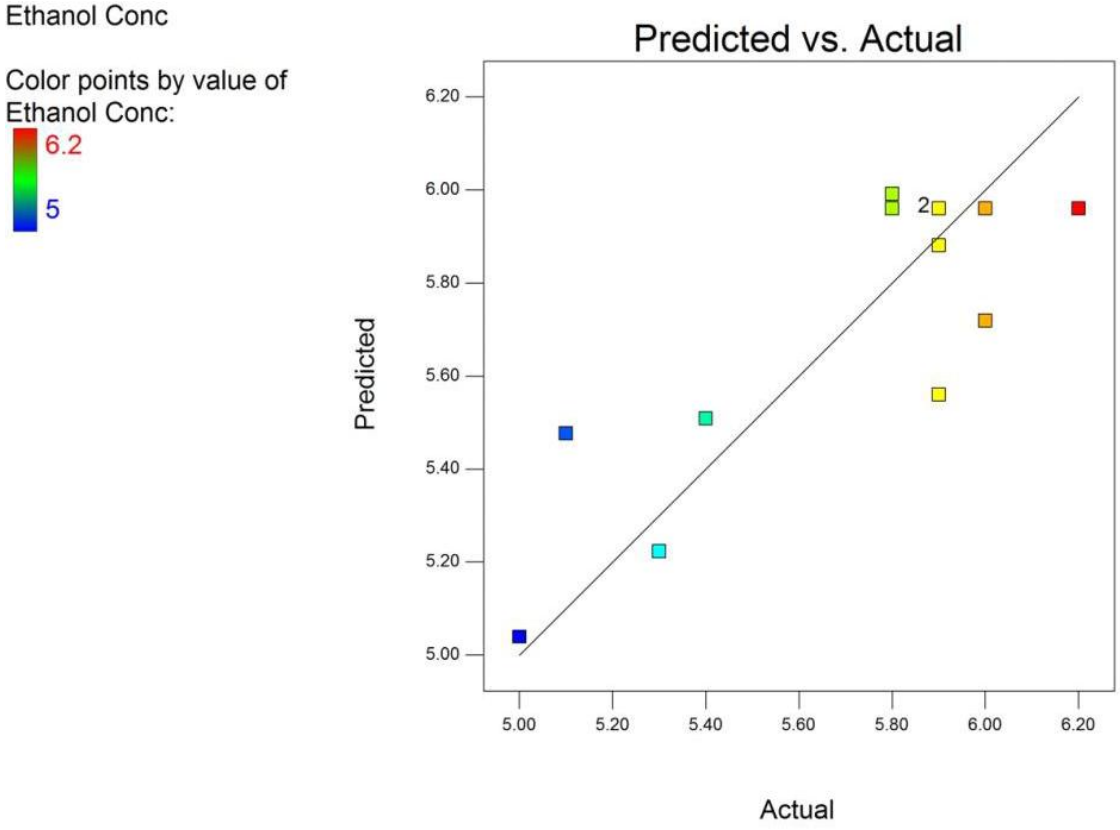



**Figure 4.3.3(iv): Plot of predicted versus actual values for ethanol yield**

### 4.3.4 Response surface optimization and verification

In order to synthesize the maximum ethanol production at the optimum temperature and yeast concentration, we used the numerical optimization to achieve this. As the main objective of this project is to optimize the ethanol yield, so the analysis of the selected parameters which are yeast concentration and also temperature are important. This numerical optimization gives the highest desirability which indicates the highest ethanol yield at the optimum condition of temperature and yeast concentration. Table 4.3.4(i) shows the most desirable operating condition was at temperature of 40.00°C and yeast concentration of 0.24% (w/v).

**Table 4.3.4(i): Numerical optimization for RSM**

| Reaction Condition | Temperature (°C) | Yeast concentration % (w/v) | Predicted yield (g/L) | Desirability |
|---|---|---|---|---|
| 1 | 40.00 | 0.24 | 6.07973 | 0.712 Selected |
| 2 | 40.00 | 0.25 | 6.07972 | 0.712 |
| 3 | 40.00 | 0.24 | 6.07953 | 0.712 |
| 4 | 40.00 | 0.27 | 6.07385 | 0.711 |
| 5 | 40.00 | 0.41 | 5.86965 | 0.699 |
| 6 | 40.00 | 0.42 | 5.84712 | 0.698 |

Therefore, to verify the optimal points given by the numerical optimization, three additional experimental runs were carried out at temperature 40°C and 0.24% (w/v) yeast concentration. Table 4.3.4(ii) shows the result for ethanol concentration yield which is in good agreement with the values predicted by RSM.

**Table 4.3.4(ii): Verification of response obtained from experimental study**

| Run | Temperature (°C) | Yeast concentration % (w/v) | Actual yield (g/L) | Average yield (g/L) | Predicted yield (g/L) | Standard deviation (%) |
|---|---|---|---|---|---|---|
| 1 | | | 6.07856 | | | |
| 2 | 40.00 | 0.24 | 6.07542 | 6.07430 | 6.07973 | 0.089 |
| 3 | | | 6.06892 | | | |

So, from the tabulated data in Table 4.3.4(ii), the error estimations between the predicted and values is 0.089% which fell below 1%. This denotes that the numerical optimization is reliable in order to produce ethanol with high concentration yield.

# CHAPTER 5
# CONCLUSION AND RECOMMENDATION FOR FUTURE WORK

## 5.1 Conclusion

The experimental works for this project is successfully done in laboratory. The results obtained for pretreatment process is in agreement with findings made by Oberoi et al (2010) which shows the highest yield of glucose in primary and secondary hydrolysis is at 0.5% (v/v) and 0.75% (w/v) concentration of sulphuric acid, respectively.

Besides, from the pretreatment experiment, it is confirmed that the orange peel biomass yield glucose from the break down process, which will be used to produce ethanol in the fermentation experiment. So, the first objective is successfully achieved, which is to produce ethanol from orange peel using two stage hydrolysis and fermentation studies.

This project also proved that the fermentation also depends on yeast concentration and temperature instead of pH and fermentation time. So, the second objective is achieved. RSM successfully generate the optimum condition for yeast concentration and temperature in order to yield the maximum concentration of ethanol production which is at 0.24-0.3% w/v of yeast concentration with temperature around 38- 40°C.

## 5.2 Recommendation for Future Work

For this project, the measurement should be done correctly, as the main parameter that needs to be studied is related to the measurement technique.

To get a more accurate concentration, the ethanol produced should be analyzed using HPLC. So, for future work, we should avoid this equipment failure problem in order to get a more accurate result.

A problem with two parameters is actually is not fit enough to be optimized using RSM. So, in future works we can add up the number of parameters such as pH and fermentation time instead of fixed them to the optimum value, to get a more reliable result.

The optimum conditions that have been studied for ethanol production can be applied in large industry by increasing the scale of consumption in order to produce the desired amount of ethanol.

# APPENDIX

Equation given by Marker T.L et al (n.d) which is:

**n = 0.1363x + 1.2714**

Where:
n = Refractive index (RI)
x = conc of glucose (% in water)

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