ETHANOL FERMENTATION FROM *Leucaena leucocephala* SEEDS

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ETHANOL FERMENTATION FROM *Leucaena leucocephala* SEEDS

ABSTRACT

Nowadays, utilization of renewable fuel from biomass such as bioethanol is gaining a worldwide attention. Due to the global demand for bioethanol production, edible biomass such as corn and sugarcane could not be the feedstock to solve this issue as their primary use is for food production. Hence, *Leucaena leucocephala* (non-edible biomass) would be a possible alternative to replace edible biomass. This research deals with seeds of *Leucaena leucocephala* because there is a limited study on conversion of cellulose obtained from *Leucaena leucocephala* seeds into bioethanol and the seeds are normally left unused. In this study, therefore, evaluation of fermentation parameters such as days of fermentation, amount of sugar, amount of yeast and pH range was studied in order to find optimum parameters. Optimum days of fermentation, amount of sugar, amount of yeast and pH range were 3 days, 20 g, 3 g as well as between 4.0 – 4.9. Optimum parameters are important to produce high yield bioethanol from cellulose obtained from *Leucaena leucocephala*.

PENAPAIAN ETANOL DARIPADA BIJI BENIH *Leucaena leucocephala*

ABSTRAK

Pada masa kini, penggunaan bahan api boleh diperbaharui daripada biojisim seperti bioetanol semakin mendapat perhatian seluruh dunia. Oleh kerana permintaan global untuk pengeluaran bioetanol, biojisim yang boleh dijadikan makanan seperti jagung dan tebu tidak boleh menjadi bahan mentah untuk menyelesaikan isu ini kerana penggunaan utama biojisim tersebut adalah untuk pengeluaran makanan. Oleh itu, *Leucaena leucocephala* (biojisim tidak boleh dimakan) akan menjadi alternatif yang mungkin menggantikan biojisim yang boleh dimakan. Penyelidikan ini berkaitan dengan biji benih *Leucaena leucocephala* kerana terdapat kajian yang terhad pada pemprosesan selulosa yang diperoleh daripada biji benih *Leucaena leucocephala* kepada bioetanol dan biji benih ini biasanya dibiar tanpa sebarang kegunaan. Dalam kajian ini, oleh itu, penilaian parameter bagi penapaian seperti hari penapaian, jumlah gula, jumlah yis dan julat pH telah dikaji untuk mencari parameter yang optimum. Hari penapaian yang optimum, jumlah gula, jumla yis dan julat pH masing-masing adalah 3 hari, 20 g, 3 g serta antara 4.0 dan 4.9. Semua parameter yang optimum adalah penting untuk membuat penghasilan bioetanol yang tinggi daripada selulosa yang diperoleh daripada *Leucaena leucocephala*.

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LIST OF SYMBOLS AND ABBREVIATIONS

β	:	Beta
CO_2	:	Carbon dioxide
°C	:	Degree Celcius
g	:	Gram
He	:	Helium
HP	:	Hewlett-Packard
μm	:	Micrometer
mL	:	Milliliters
min	:	Minute
%	:	Percentage
pH	:	Potential of hydrogen
rpm	:	Revolutions per minute
ρ	:	Rho
Σ	:	Summation
$\sqrt{}$	:	Square Root
vs.	:	Versus

CHAPTER 1: INTRODUCTION

1.1 Background of Study

Ethanol is an organic compound which is also known as ethyl alcohol (Sanchez and Cardona, 2008). Sarkar et al. (2012) stated that renewable fuel such as bioethanol could be produced from sugar-containing plants such as sugar cane and could be utilized in blends with the petroleum-based fuel. The cellulose from plants would then become one of the resources for the production of bioethanol. Meanwhile, Hahn-Hägerdal et al. (2006) reported that ethanol could also be totally utilized as fuel without mixing in engines. Lignocellulose consists of hemicellulose, cellulose, and lignin (Lee, 1997). Cellulose is then could be converted into sugar which is applicable for the production of ethanol (Kasavi et al., 2012). An enzyme which is produced from yeast such as *Saccharomyces cerevisiae* is needed in conversion of cellulose into sugar (Sun and Cheng, 2002). On the other hand, xylose which was originated from hemicellulose would not be able to be converted into ethanol by *Saccharomyces cerevisiae*.

However, the genetically engineered *Saccharomyces cerevisiae* could be able to convert xylose into ethanol (Ho et al., 1998). Meanwhile, process of conversion from sugar into ethanol is called as fermentation. During fermentation process, different parameters are needed to be controlled (Lin et al., 2012). The fermentation process could also convert toxic compound contained in the hydrolysis product that cause low enzyme inhibition (Tengborg et al., 2001) which is then increased the yield of ethanol (Söderström et al., 2005). Yield of ethanol is also being compared by using various conditions (Lin et al., 2012). The presence of ethanol could be determined by several analyses including the use of chromatograph headspace.

1.2 Problem Statement

Currently, bioethanol is produced from crop residue (Hattori and Morita, 2010) and sugar cane (Dias et al., 2012). However, utilization of sugar cane for bioethanol production could be problematic (Pimentel, 2003) as these raw materials are consumed by human as well as animal feed. Hence, the high demand for ethanol fuel could not be supported (Hahn-Hägerdal et al., 2006). Another alternative way which can replace this edible plant is by using the non-edible plants such as *Leucaena leucocephala*. There is very limited study on conversion of sugars from seeds of this plant into bioethanol.

1.3 Significance of Study

Leucaena leucocephala could potentially be used for the production of bioethanol (Feria et al., 2011). This bioethanol could then be used directly as fuel in engines. *Leucaena leucocephala* is a non-edible plant which could replace the use of edible plant as bioethanol feedstock. The edible plant, such as corn and sugarcane, could then totally be consumed for food production. Thus, the full utilization of *Leucaena leucocephala* seeds for bioethanol production could solve the issue of energy demand as well as preventing food crisis for human.

1.4 Objectives

The objectives of this study are:

1. To evaluate the parameters for the fermentation of anhydrous glucose with yeast.
2. To compute the yield of ethanol from *Leucaena leucocephala* seeds using pre-determined conditions.
3. To analyze the yield of ethanol from *Leucaena leucocephala* seeds by using GC/MSD headspace.

1.5 Hypothesis

The highest amount of ethanol may be produced from short period days of fermentation, high amount of glucose, high amount of yeast, low pH range, high amount of *Leucaena leucocephala* seeds and high amount of cellulose obtained from *Leucaena leucocephala* seeds.

CHAPTER 2: LITERATURE REVIEW

2.1 *Leucaena leucocephala*

Leucaena leucocephala is known as lead tree and belongs to the family of legume (Benjakul et al., 2014). *Leucaena leucocephala* is a plant which grows rapidly (Aderibigbe et al., 2011). Due to its high mineral contents, *Leucaena leucocephala* become the source of food to the farm animals (Nirmal and Benjakul, 2011). According to Shelton and Brewbaker (1998), it is a non-edible plant as it contains mimosine, a toxic substance. Hence, there is a limit for feeding to the farm animals because it could be resulted to death. So, it is not used for food production and thus, known as non-edible plant. Mimosine is an amino acid but not a protein which is consisted of β-(3-hydroxy-4-pyridon-1-yl)-*L*-alanine (Lalitha and Kulothungan, 2006). It could also hinder the activity of tetramer that contains four copper atoms for each molecule and binds with two aromatic compounds and oxygen (Nirmal and Benjakul, 2011).

Despite its toxicity, the plant grows well in arid and marginal lands region (Ngongolo et al., 2017). *Leucaena leucocephala* has been used in different ways including for medication purpose (Syamsudin et al., 2010). Besides that, *Leucaena leucocephala* seeds could also be able to reduce the excessive production of melanin in shrimp when shrimp is being stored in cold temperature (Nirmal and Benjakul, 2011). Furthermore, its brown seeds could also be the new resource of natural antioxidant (Benjakul et al., 2014). Moreover, the viscous secretion in the seed could be used as adhesive for the formulation of pill (Verma and Balkishen, 2007). In addition, a carbohydrate found in the seed could be able to delay the spread of cells to other parts of body and deter the progress of abnormal cell growth (Gamal-Eldeen et al., 2007).

On the other hand, seeds extracts could be beneficial in the dismissal of internal parasites from body without host injury and acts as sleep-influencing effect that could be able to slow the activity of brain (Syamsudin et al., 2010). Meanwhile, according to Aderibigbe et al. (2011), its seed oil is usable for skin medication. It is could also be able to inhibit the growth of the microorganisms. This is because both gram-negative and gram-positive bacteria are responsive to antibiotics found in the oil. Seed oil could also be useful to dissolve synthetic chemicals in the fats of membrane (Idowu et al., 2009). The oil from seeds of *Leucaena leucocephala* has been also manipulated for biodiesel production (Hakimi et al., 2017).

Leucaena leucocephala have flowers, pods and leaves (Figure 2.1). The seeds of Leucaena are located in the pods. Aderibigbe et al. (2011) stated that Leucaena seeds is egg-shaped. The main part of Leucaena seed is brown in colour while the soft part of Leucaena seed is yellow in colour. Leucaena seed is hard because it is covered by wax and needs to be damaged before seedlings start to grow (Shelton and Brewbaker, 1998). About 24.5% to 46% of protein contained in the seeds and it consist of amino acids such as methionine, leucine and lysine (Ahmed and Abdelati, 2009). Meanwhile, *Leucaena leucocephala* seeds contain 35 to 45% carbohydrate (Sotelo, 1980). Carbohydrate is a group that includes cellulose, starch and sugar. It is also proved by Syamsudin et al. (2010) that, there are compounds which are consisted of galactose group bounded with other sugars in the seeds. On the other hand, there are 2.6% of lignin, 15.0% of cellulose and 7.9% of hemicellulose found in seeds of *Leucaena leucocephala* which is reported by Gupta and Raheja (1986).

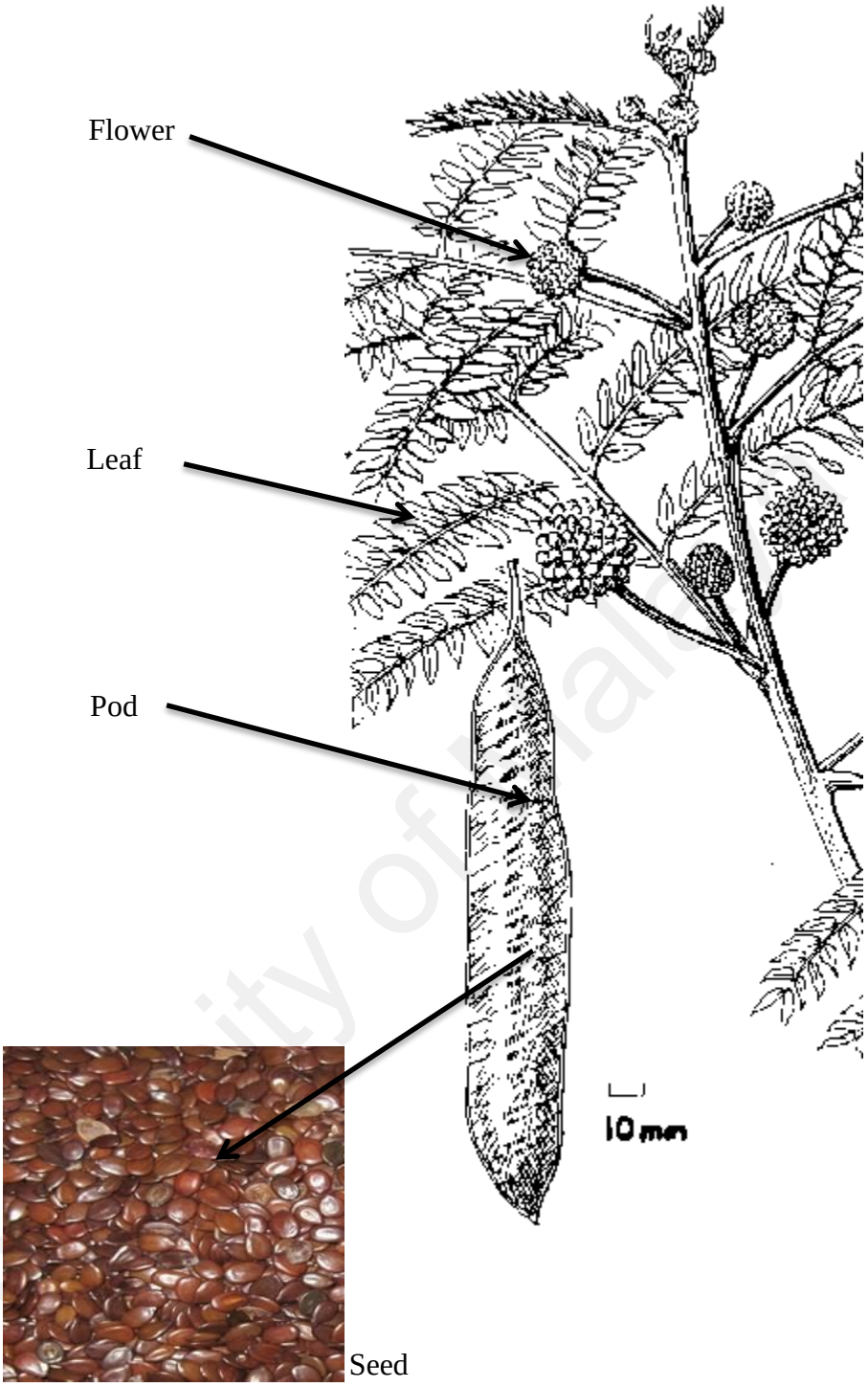

Figure 2.1: *Leucaena leucocephala* with flowers, leaves, pod and seeds (Shelton and Brewbaker, 1998)

2.2 Lignocellulosic

The focus of this study is on the potential utilization of cellulose obtained from *Leucaena leucocephala* seeds because these seeds are normally left unused. However, there are a limited number of studies on conversion of cellulose obtained from *Leucaena leucocephala* seeds into ethanol. Nevertheless, since there is cellulose in Leucaena seeds as reported by Gupta and Raheja (1986), it is then could be able to hydrolyze into glucose (Sreenath and Jeffries, 2000) before fermentation is taken place to produce ethanol (Sun and Cheng, 2002; Mussatto et al., 2008). This study is carried out based on production of ethanol from corn (MaDonald et al., 2002) as there is cellulose contained in the corn (Gray et al., 2006). This conversion process is highly encouraged for scientific and research activity in order to improve the industrial process (Lanzafame et al., 2012).

Cellulose could also be obtained in large quantities from plants (Gan et al., 2003) and it is the polymeric substances found in large quantity in the world (Wada et al., 2010), which is renewable (Sun et al., 2009). The cellulose could also be obtained from the wood of herbs and trees for conversion into sugar (Kasavi et al., 2012) and further conversion of sugar into ethanol. It is very large molecule with structure of crystal which is consists of repeated (1-4)-linked β-D-glucose units (Sun et al., 2009) and connected by glycosidic linkages (Lanzafame et al., 2012). Wada et al. (2010) stated that its diameter is only 1×10^{-9} m. So, it is called as small fibril (Laureano-Perez et al., 2005) in the wall of cell and it is surrounded by lignin along with hemicellulose. Cellulose could not be easily harmed by acidic compounds (Sun et al., 2009).

Meanwhile, according to Mussatto et al. (2008), cellulose is correlated with lignin chemically and physically as well as correlated with hemicellulose physically in lignocellulose materials where about 55% to 75% of carbohydrates are contained in lignocellulose (Mosier et al., 2005). Hence, lignin, cellulose and hemicellulose are the materials for build up a structural of lignocellulose (Gan et al., 2003). *Leucaena leucocephala* seeds could be a lignocellulosic source in the production of ethanol due to the presence of cellulose in seeds of *Leucaena leucocephala* which is stated by Sotelo (1980). Lignocellulose could become the renewable and low cost raw materials (Alvira et al., 2010) for fuel ethanol production (Mosier et al., 2005) without competition with production of food (Wada et al., 2010).

The study is compared with corn where lignocellulose of corn consisted of 25% to 35% of hemicellulose, 40% to 50% of cellulose and 15% to 20% of lignin (Gray et al., 2006). Although most of ethanol is produced from corn at United States of America in the year of 2000 (MaDonald et al., 2002), the utilization of lignocellulosic material could reduce the cost for ethanol production (Sun and Cheng, 2002). Lignocellulosic could be able to be used in biomass conversion process to produce value-added chemicals and fuels efficiently (Centi et al., 2011; Lanzafame et al., 2011). Hemicellulose would make more firms in terms of bonding between itself, lignin and cellulose (Laureano-Perez et al., 2005). The presence of hydrogen bonds in hemicellulose also caused the structure of plant cell wall to become stronger (Mosier et al., 2005).

Hendriks and Zeeman (2009) stated that hemicellulose contained glucose, xylose and sugar acids. Mannose is the most dissolved compound which is found in hemicellulose followed by xylose, glucose, arabinose and galactose while xylan in hemicellulose would be the easiest to being extracted. The existence of lignin in plant cell wall would prevent any fluids to pass through in and out of plant cell, support the structure of plant, deter the consequences from the activity of reactive oxygen species and impede the invasion of microbes. Coniferyl, paracoumaryl alcohol and sinapyl alcohol are phenylpropane units which would then build up the lignin. Lignin would also make higher degree of connectivity in plant because it is an amorphous polymer. These compounds would also affect the solubility of lignin (Grabber, 2005).

The prompt increase in converting large quantity of cellulose into bioethanol could progressively substituted petroleum-based fuels energy (Sun and Cheng, 2002; Rostrup-Nielsen, 2005). However, there would be some limitations in the yield of ethanol when the cost of production need to be combined with cost of the most favorable components and development of competent pretreatments (Sun and Cheng, 2002) and retaining the activity of genetically engineered microorganisms, (GEMs) during fermentation (Dipardo, 2000). There are four main steps in the production of ethanol from lignocellulosic which are pretreatment, hydrolysis, fermentation and separation (Mosier et al., 2005; Hendriks and Zeeman, 2009). The last step in production of ethanol from lignocellolusic after separation is post-treatment (Hendriks and Zeeman, 2009).

2.3 Pretreatment

According to Mussatto et al. (2006), the competency of hydrolysis would be decreased due to the existence of lignin and hemicellulose. The hardship of breaking down cellulose into sugars for further fermentation is also caused by the formation of hydrogen bonds found in cellulose (Jarvis, 2003). The covering of cellulose by hemicellulose and lignin, accessibility of surface region, diversity in characteristics of biomass particles as well as degree of structural order in cellulose would cause uncooperative in hydrolysis of lignocellulosic materials (Chang and Holtzapple, 2000). Nevertheless, there would be some ways to deal with cellulose structure for further processing of sugars hydrolysis and later continued with the production of ethanol (Dadi et al., 2006). Meanwhile, different solutions in breaking down of lignocellulosic had to be considered due to the ability in changes of lignocellulosic composition and structure (Mosier et al., 2005).

Thus, elimination of both lignin and hemicellulose are needed by using alkaline treatment such as sodium hydroxide, NaOH (Sharma et al., 2002; Mosier et al., 2005) where low concentration of alkali solutions is required (Sun and Cheng, 2002; Wada et al., 2010). The alkali solution used could be able to break down ester bond which is connected between lignin and a group of hemicellulose such as xylan (Sun and Cheng, 2002). Besides that, alkali could also be able to eliminate sugar acids and acetyl group from hemicellulose where cellulose surface would be accessible to the enzyme (Chang and Holtzapple, 2000). The elimination of hemicellulose would cause the size of pores on substrates becomes larger and hence, cellulose could be able to being hydrolyzed (Palonen et al., 2004).

According to Mosier et al. (2005), although alkali pretreatment would produce salt at the end of pretreatment and period of pretreatment time taken is long, but it is applicable under immediate condition, less pressure and low temperature. According to Hendriks and Zeeman (2009), the first step in alkaline pretreatment is solvation and saphonication where the size of lignocellulosic become larger and round in shape. This condition would make it easily to being accessed by bacteria and enzymes. If the concentration of alkali given is potent, disappearance of carbon in polysaccharides in lignocellulosic could be occurred. The high recovery of hemicellulose in lignocellulosic is due to the low number of hemicellulose molecules (Laser et al., 2002). Hendriks and Zeeman (2009) also stated that xylan could be able to being eliminated by using potassium hydroxide, (KOH). However, the utilization of alkali by lignocellulosic would occur in alkaline pretreatment.

Alkaline pretreatment would change the degree of structural order in cellulose of lignocellulosic and solubilized lignin in lignocellulosic. The changes and solubilization of lignin would then increase the exterior region of lignocellulosic. Alkaline pretreatment could be able to prevent the production of hindrance compounds (Ramos, 2003) but disagreed by Hendriks and Zeeman (2009) where they stated that it has hindrance effect. They also stated that, although alkaline pretreatment has hindrance effect which would be less favor able in the production of ethanol, but it has good effect in terms of cellulose degradation. Cost-saving is another positive effect in using alkaline pretreatment (Hendriks and Zeeman, 2009).

Generally, pretreatment is the changes of cellulose structure where it is being done in order to make it accessible to the enzyme before further process is taken place as stated by Mussatto et al. (2008) and it is less costly (Bothast and Schlicher, 2005). Mosier et al. (2005) stated that pretreatment would then ensure the high production of glucose in a short period of time. Meanwhile, different ways of pretreatment (Sun and Cheng, 2002) would be implemented if there is the production of lignin co-product. The necessity for pretreatment to occur is the prevention in formation of secondary products, capable in producing sugars by hydrolysis and prevention in disappearance of carbohydrates (Sun and Cheng, 2002). Pretreatment is also needed to produce good results without costing a lot of money (Sun and Cheng, 2002) where it had to be balanced with the cost of operation, cost of biomass, cost of capital and cost of downstream process (Palmqvist and Hahn-Hägerdal, 2000).

Cellulose would be easier to expose with cellulase if lignin and hemicellulose are being eliminated. This is because, according to Pan et al. (2005), there is the shaping of physical impediment by lignin. Thus, lignin could not be able to restore the adhesion of cellulase on to the cellulose (Mansfield et al., 1999). If the exterior area is much lower than area of interior, cellulase could be able to being trapped in the pores (Zhang and Lynd, 2004). Besides that, according to Mussatto et al. (2008), fibers of cellulose would firstly break up from the beginning of structural attachment when both lignin and hemicellulose are being eliminated. Then, the elevation in open space (Sun and Cheng 2002) and surface region of fibers cause exposure to the action of cellulase on fibers easily. The toxic materials such as phenolic, acetic acid and so on are not appeared in the product of hydrolysis or known as hydrolysate (Mussatto et al., 2008).

Hence, the high competency of fermentation process would achieve (Mussatto and Roberto, 2004). The high production of glucose is also enviable for activity of fermentation (Yeh et al., 2010). Mussatto et al. (2008) stated that the soft cellulose could be able to produce glucose at the superior value which is at 85.6%. It is 310% more elevated than non-treated material where there is presence of both lignin and hemicellulose while 17.4% slightly elevated than elimination of hemicellulose. According to Mussatto et al. (2008), the hydrolysate would contain low concentration of glucose as the primary product compared to the high concentration of xylose and arabinose as the secondary products of non-treated material. This is because hemicellulose may contain polymers either pentoses such as xylose and arabinose or hexoses such as glucose (Mosier et al., 2005; Hendriks and Zeeman, 2009).

Hence, the higher content of lignin and hemicellulose, the less competency of cellulose hydrolysis. Nevertheless, the way to obtained high production of glucose is the need to eliminate both lignin and hemicellulose completely. Nonetheless, according to Mansfield et al. (1999), the existence of lignin (Mosier et al., 2005; Hendriks and Zeeman, 2009) and hemicellulose in plant would be unsusceptible to the invasion of microbial In addition, Sun and Cheng (2002) stated that there are a few of other enzymes which could also invade hemicellulose instead of cellulase which only invade cellulose. Those enzymes are β-xylosidase and xylanase. These enzymes would discharge arabinose and xylose. However, xylose could also be converted into ethanol by using genetically engineered microorganisms (GEMs). GEMs could also be able to downsized cost for yield of ethanol and enhanced the competency of yield.

GEMs are where the gene interest of cellulase is being located and copied out of deoxyribonucleic acid, DNA extracted from the organism into another microorganism such as fungi in order to produce new production system of cellulase. According to Mosier et al. (2005), the final objectives of lignin and hemicellulose separation from cellulose will cause cheaper and easier. Besides that, there would be necessary in improving two pretreatment methods which are physical and chemical in order to produce more intended results. The improvement would allow the corresponding occurs between biomass and process of pretreatment.

2.4 Hydrolysis

After cellulose is being separated from lignin and hemicellulose, according to Zhang et al. (2010), cellulose is then being hydrolyzed by fixing of catalytic domain of cellulase on the substrate during attachment between cellulose and cellulose binding domain. Thus, the enzyme which is adhered on the cellulose would be the competent way in the production of sugars. Hydrolysis could be simply explained as the process of breaking down of cellulose into glucose units by cellulase (Mosier et al., 2005) as shown in figure 2.3 where cellulase is an enzyme which would be produced by fungi such as *Saccharomyces cerevisiae* as shown in figure 2.2 (Sun and Cheng, 2002). Even so, cellulose could be able converted directly into ethanol which is stated by Szczodrak and Fiedurek (1996). However, due to the structure of cellulose, the production of sugars which is aided by enzyme would be less than 20% (Mosier et al., 2005).

Yeasts are single cell or known as unicellular microorganisms and categorized as eukaryotic in the fungus kingdom. They would be able to develop false hyphae or known as pseudohyphae (Kurtzman and Fell, 2005) by budding reproduction (Balasubramanian et al., 2004). The hyphae are the feature of multicellular for yeasts (Kurtzman and Fell, 2005). Yeast is also used in the production of ethanol from feedstocks such as sugar cane (Sedlak and Ho, 2004) instead of *Leucaena leucocephala*. Yeast is suitable for yield of ethanol as it could be able to being modified genetically easily, generally regarded as safe or abbreviated as GRAS and has an ability to tolerate with the ethanol (Hahn-Hägerdal et al., 2001; Byrne et al., 2005).

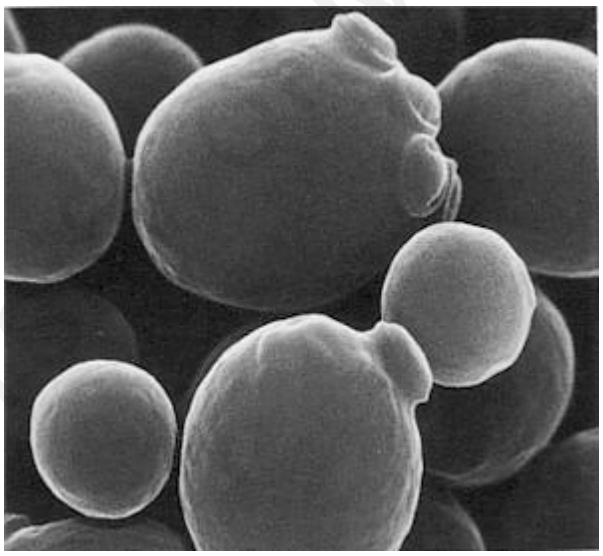

Figure 2.2: *Saccharomyces cerevisiae* (Robin, 2010)

Hydrolysis could also be done by using chemicals instead of cellulase which is also stated by Hendriks and Zeeman (2009). Hydrolysis would be able to remove excess of reactant easily and hence, it is known as solid-phase reaction (Sun et al., 2009).

Nevertheless, it is a time-consuming based process (Gan et al., 2003; Zhang and Lynd, 2003). On the other hand, the rate of breaking down process for glucose is faster than the rate of breaking down for cellulose (Sun et al., 2009). Sun and Cheng (2002) also stated that the breaking down of cellulose could be occurred with the adhesion of cellulase on to cellulose surface before the disintegration of cellulose into sugars is done by fungi, and lastly, the release of cellulase from cellulose is occurred. The sugars produced would be available for fermentation process. According to Hendriks and Zeeman (2009), the six carbon sugars are easily to being converted into ethanol while five carbon sugars are only converted into ethanol if the conversion is aided by several microorganisms. However, production of sugars would decrease because microorganisms which play the role in conversion is hindered by ethanol produced.

The process of hydrolysis could also be explained by Chang and Holtzapple (2002) and Cao and Tan (2002), where enzyme would first, spread out from the bulk of solution. After that, it would diffuse through the lignin which is acted as a barrier. Then, it would bind on to the substrate. Next, the acceleration of hydrolysis took place. Sun and Cheng (2002), Cao and Tan (2002), Prasad et al. (2007) and Kumar et al. (2008) stated that there are three groups of cellulases which are involved in the breaking down of cellulose. There are β-glucosidase, β-1-4-exoglucanases and β-1-4-endoglucanases. Firstly, endoglucanases would break down the inner chains of cellulose. Then, exoglucanases would break down the final part of bond and cause the discharge of cellobiose. Lastly, β-glucosidase would break down cellobiose and discharge glucose units.

Hence, hydrolysis is known as a heterogeneous reaction (Chang and Holtzapple, 2002; Cao and Tan, 2002) and the hydrolysates would be cellobiose along with glucose (Yeh et al., 2010). Nonetheless, according to Sun and Cheng (2002), the hindrance of cellulase activity is done by cellobiose. Thus, the usage of β-glucosidase, increased in enzyme concentration as well as simultaneous saccharification and fermentation (SSF) are the ways to overcome the hindrance of cellulase. SSF is the process where breaking down of cellulose into glucose with the existence of fermentative microorganisms is carried out (Mosier et al., 2005). According to Sun and Cheng (2002), SSF has some benefits which are the removal of glucose promptly, less necessary of enzyme, speedy of time taken, application of one reactor only, elevation in rate of hydrolysis as well as high production of sugars.

However, there are some limitations in usage of SSF process where growth hindrance of microorganism by ethanol, irreconcilable of fermentation and hydrolysis temperature and enzyme hindrance by ethanol. The statement of hindrance of cellulase stated by Sun and Cheng (2002) is also supported by Yeh et al. (2010) where the rate of hydrolysis for cellobiose would decrease while production of glucose would increase due to the high ratio of enzyme to substrate. On the other hand, the hindrance of β-glucosidase is only due to glucose while exoglucanases and endoglucanases are not reticent by both glucose and cellobioase (Peri et al., 2007). The less activity of β-glucosidase could also reduce the production of glucose which is lower than cellobiose production (Wen et al., 2004). The production of glucose from low quantity of cellobiose by β-glucuronidase would also be done after the uncovering of cellulose towards endoglucancase and cellobiohydrolase (Zhang et al., 2010).

Yeh et al. (2010) stated that the factors that affect the efficiency of hydrolysis are the adhesion of substrate (Sun and Cheng, 2002), level of polymer formation, interaction between enzyme (Sun and Cheng, 2002), rates of reaction that occurred naturally (Sun and Cheng, 2002; Zhang and Lynd, 2004) and degree of structural order in solid (Laureano-Perez et al., 2005). The pH and temperature are the parameters which could be able to affect hydrolysis (Sun and Cheng, 2002). Sun and Cheng (2002) stated that the substrates are the primary factor for activity of cellulose hydrolysis. The structural characteristics of substrates, surface region of cellulose, number of monomeric units in cellulose (Chang and Holtzapple, 2000; Laureano-Perez et al., 2005), degree of solid structural order of lignin and constituents of lignin could also be able to affect the vulnerability of cellulose with cellulase.

However, the increase of hydrolysis rate is because of high cellulase dosage although it would be costly. Cellulase could also be affected by the concentration and type of substrates. The high contents of glucose are also depending on the high content of cellulose (Mussatto et al., 2006). Degree of solid structural order or crystallinity of cellulose could also be able to affect the hydrolysis (Chang and Holtzapple, 2000) where the high crystallinity of hydrolyzed product depends on the slow conversion of crystallinity cellulose (Zhang and Lynd, 2004).

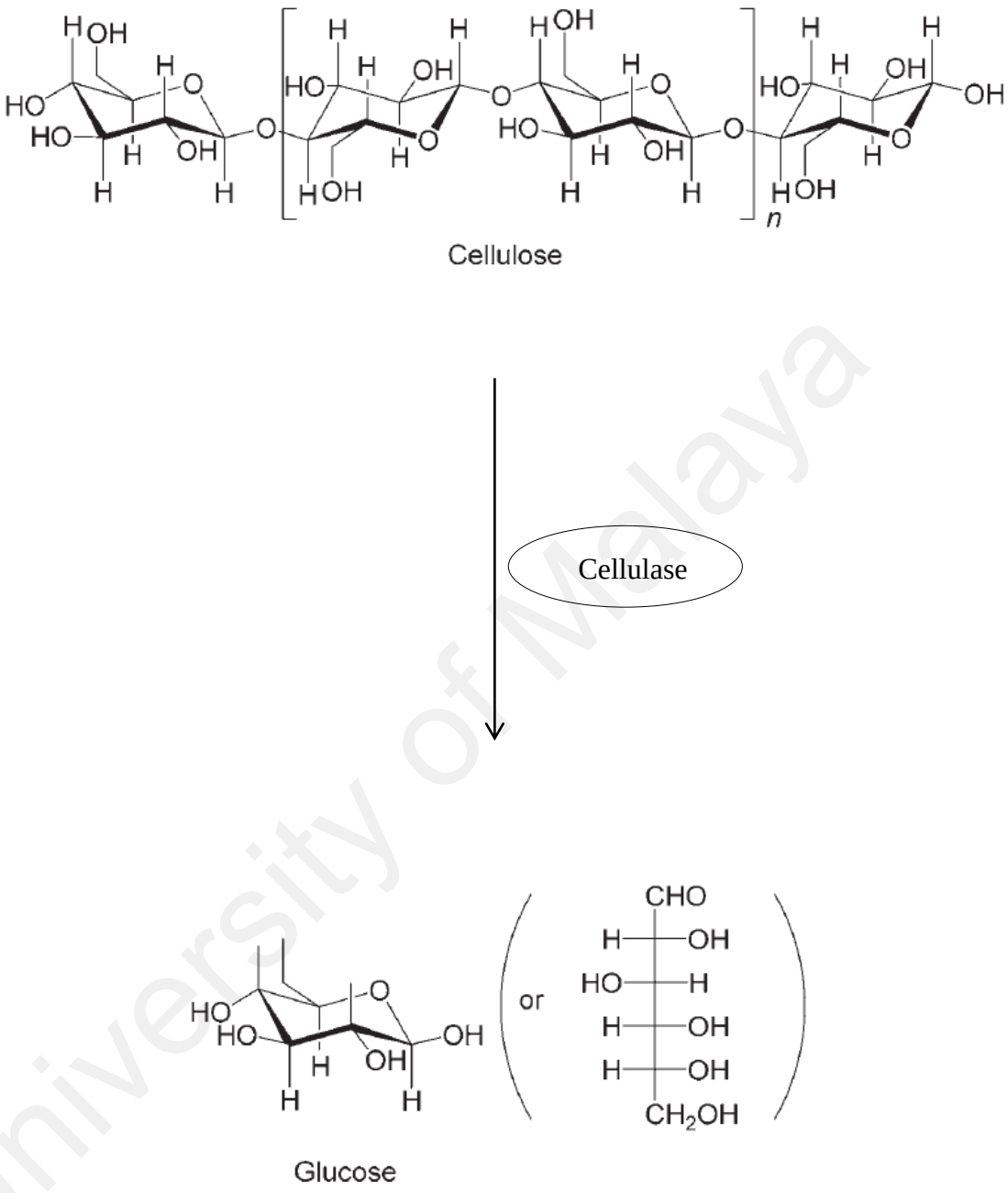

Figure 2.3: Hydrolysis of cellulose into glucose (Fukuoka and Dhepe, 2006)

Glucose would then be converted into two molecules of pyruvate through glycolysis process (Figure 2.4). All cells including yeasts undergo glycolysis process. Schwender et al. (1996) stated that, the first step of glycolysis begins with phosphorylation of glucose into glucose-6-phosphate. After that, glucose-6-phosphate is isomerized into fructose-6-phosphate. Then, fructose-6-phosphate is phosphorylated to become fructose-1,6-bisphosphate. Next, fructose-1,6-bisphosphate will split into two fragments which are dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Dihydroxyacetone phosphate is then converted into glyceraldehyde-3-phosphate. Hence, there are two products of glyceraldehyde-3-phosphate.

After that, glyceraldehyde-3-phosphate is oxidized into 1,3-bisphosphoglycerate (Verhees et al., 2003). During this step, reduction from nicotinamide adenine dinucleotide (NAD^+) into nicotinamide adenine dinucleotide hydride (NADH) would also take place before 1,3-bisphosphoglycerate is converted into 3-phosphoglycerate. Isomerization of 3-phosphoglycerate into 2-phosphoglycerate then occurs. After releasing one molecule of water from 2-phosphoglycerate, it would become phosphenolpyruvate (PEP). The production of pyruvate and adenosine triphosphate or known as ATP would occur if there is transfer of phosphate group from PEP to adenosine diphosphate (ADP).

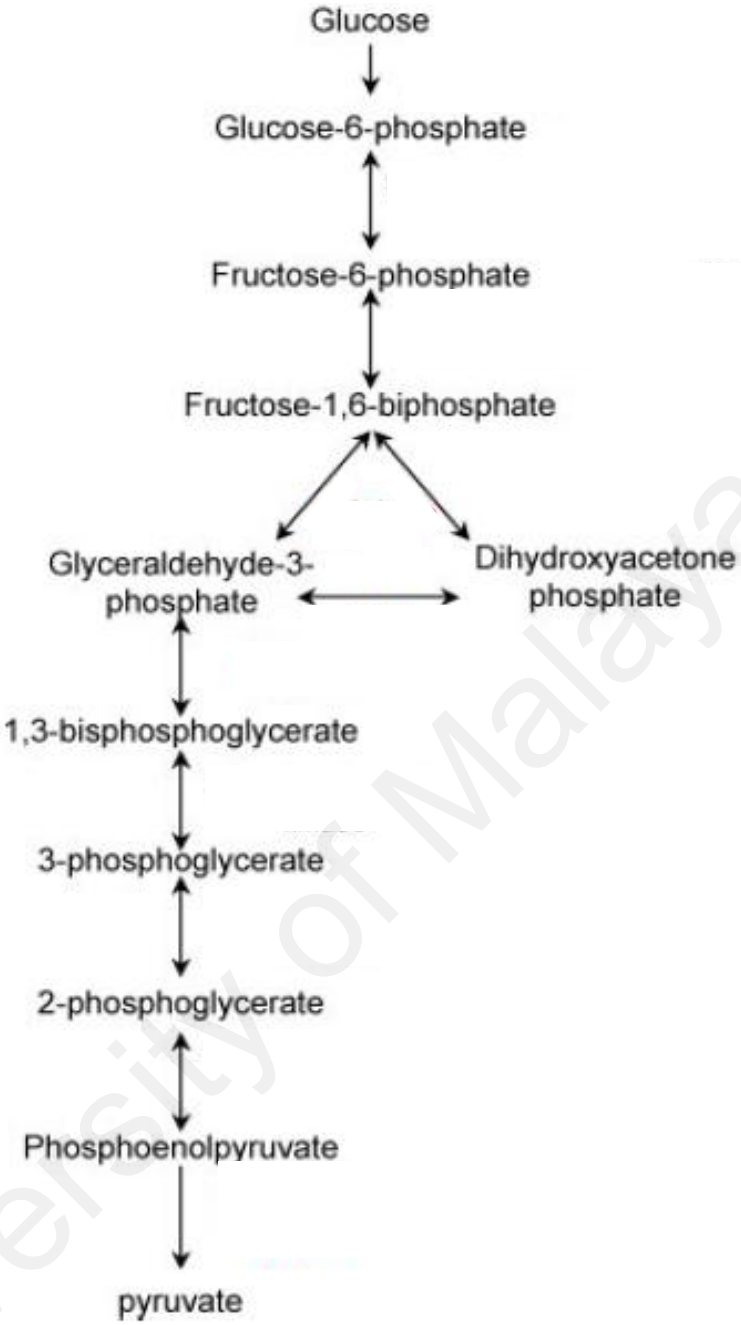

Figure 2.4: Glycolysis pathway (Shao et al., 2007)

2.5 Fermentation

In an alcoholic fermentation, two molecules of pyruvate would undergo two steps to be converted into ethanol using yeast. Pyruvate could also be able to change into ethanol by using genetically engineered microorganisms, (GEMs). The first step in an alcoholic fermentation is the decarboxylation of two molecules of pyruvate into two acetaldehydes. Two molecules of carbon dioxide (CO_2) would be product from the first step. Next, two acetaldehydes would be converted into two ethanol by using hydrogen ion (H^+) which is donated from NADH before it become NAD^+. NAD^+ would then be able to regenerate into NADH by yeasts.

The conversion from glucose into ethanol (fermentation) as stated by Hahn-Hägerdal et al. (2006) could be summarized as shown in Figure 2.5. The microorganisms are needed in the conversion of sugars into bioethanol (Katahira, 2006). The best yeast (*Saccharomyces cerevisiae*) is used in the fermentation process (Talebnia et al., 2010) as this yeast is healthy and strong (Hahn-Hägerdal et al., 2001; Galbe and Zacchi, 2002; Byrne et al., 2005). Hahn-Hägerdal et al. (2006) stated that the capability of yeast in terms of conversion, activity of hindrance compounds and standard in monitoring process would give an impact on the productivity of fermentation. The five-membered aromatic rings or known as furan, a partially dissociation of acids and an aromatic hydrocarbon group bounded directly with hydroxyl group or known as phenols are example of hindrance compound where microorganism could be able convert glucose into ethanol (Palmqvist and Hahn-Hägerdal, 2000).

Besides that, amount of ethanol produced from fermentation would increase (Tengborg et al., 2001) because reduction in toxic compounds which are acted as hindrance compounds for enzymes found in hydrolysate are occurred during fermentation process (Söderström et al., 2005). However, the hindrance of fermentation would occur if there are the existence lignin compounds (Laser et al., 2002). There is another drawback in yield of ethanol where yeast could be less resistance to the hindrance compound such as hydroxymethylfurfural or known as HMF (Hendriks and Zeeman, 2009).

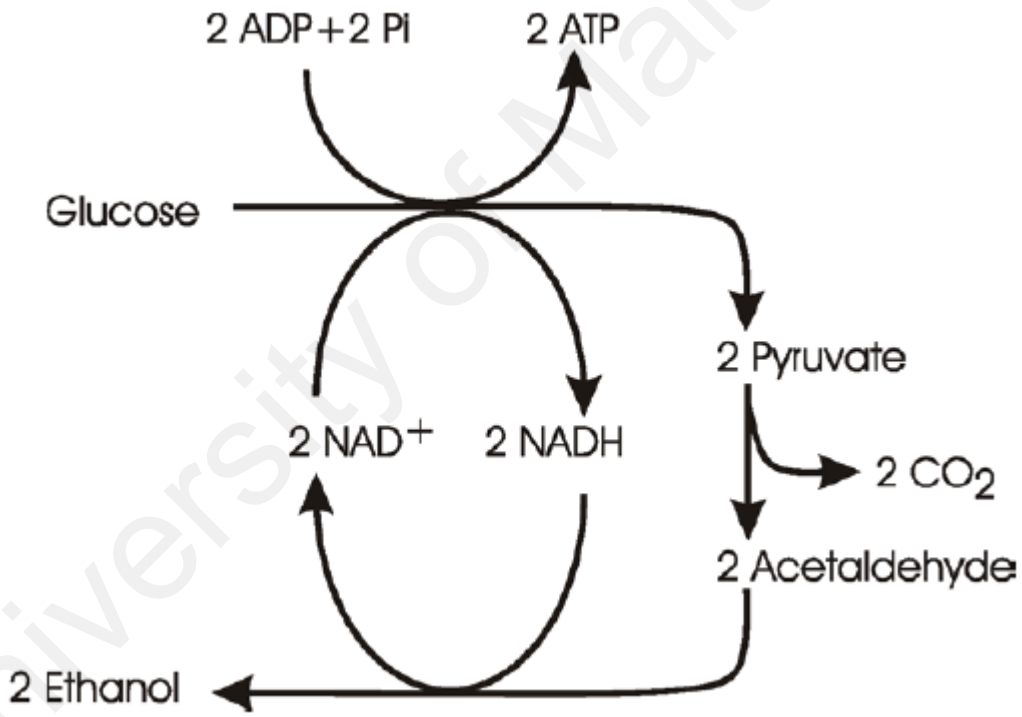

Figure 2.5: Overall pathway of conversion from glucose into ethanol (He, 2015)

Figure 2.5 could also be summarized by using equation of ethanol fermentation as shown in Figure 2.6 where ethanol and carbon dioxide are produced from fermentation of sugar. The equation is used in computing the maximum theoretical yield of ethanol (Lin et al., 2012). Then, it is applicable in finding the yield of ethanol in percentage by comparing it with the actual yield of ethanol.

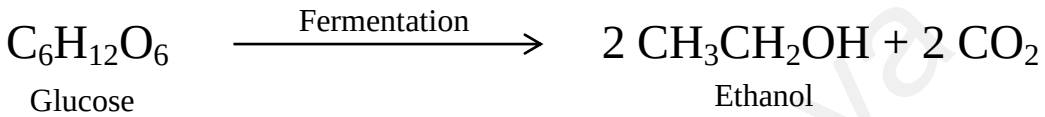

Figure 2.6: Equation for ethanol fermentation process

2.6 Ethanol

Ethanol is also known as bioethanol, grain alcohol, and ethyl alcohol. Ethanol consists of two carbon (C) atoms, five hydrogen (H) atoms and one hydroxide ion (OH^-) and thus, can be written as CH_2CH_5OH as shown in Figure 2.6 while its chemical structure is shown in Figure 2.7. Its liquid is clear, has no colour and could be able to evaporate easily at normal temperature or known as volatile. Its boiling point is at 78.29°C while its melting point is at -114.14°C. Gray et al. (2006) stated that ethanol could be provided from lignocellulosic biomass and hence, it is renewable source which could replace ethanol production from starch (Hahn-Hägerdal et al., 2006) and non-renewable resources such as petroleum. The worldwide production of ethanol would be expected to increase to 100 x 10^9 liters in 2015 (Taherzadeh and Karimi, 2007). The widely research on ethanol production from lignocellulose was done over 20 years (Binod et al., 2010).

However, petroleum which is one of the fossil fuels are less costly in production of fuel than biomass fuel such that ethanol (Sarkar et al., 2012). Although there are disadvantages in ethanol production, but according to Hahn-Hägerdal et al. (2006), ethanol is being widely produced from lignocellulose because starch which is used in yield of ethanol or known as starch-based ethanol could decrease the greenhouse gas or abbreviated as GHG where GHG is played a role in climate change (Farrell et al., 2006). Hence, ethanol production would reduce global warming (Sarkar et al., 2012). In the meantime, the production of ethanol from lignocellulose is higher than worldwide ethanol production (Kim and Dale, 2004). Besides that, starch-based ethanol which has a high request in using it as a fuel in vehicle (Demirbaş, 2005) would be inadequate for both request for human requirements and animal food source.

On the other hand, ethanol could be utilized in replacement to petroleum (Gray et al., 2006) as it could be able to be used directly or mixed with petroleum in engines because ethanol has a capability in increasing the amount of heat absorbed during vaporization of liquid. Furthermore, ethanol could also become a high standard measure of performance of the engine or known as octane rating. Moreover, it is also enriched with oxygen which could minimize gas particles in the engines (Hansen et al., 2005). Meanwhile, ethanol fuel could be able to combine with gasoline to become gasohol in the same tank of flexible-fuel vehicle or abbreviated as FFV. FFV is the vehicle that contained engines which could be able to operate numerous of alternative fuel such that gasohol. The statement of combination between ethanol and gasoline stated by Hahn-Hägerdal et al. (2006) is different with Sarkar et al. (2012) where gasoline could be substituted with ethanol in the market of fuel vehicle.

Hahn-Hägerdal et al. (2006) also stated that the competency of cost in utilizing lignin, competency of ethanol production from hexose and pentose sugars including hindrance compounds, reduction of energy necessity by using developed in process of integration and competency in breaking down of hemicellulose and cellulose to sugars are essential characteristics which is necessary to be done in production of ethanol from lignocellulose compared to starch-based ethanol.

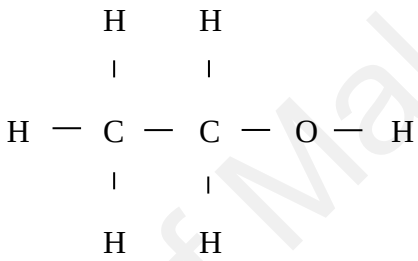

Figure 2.7: Chemical structure of ethanol

CHAPTER 3: METHODOLOGY

3.1 Samples, Chemicals, Instrumentation and Computerization

Two types of samples were used in this study. The first one is cellulose which is extracted from *Leucaena leucocephala* seeds (CLLS) and non-extracted *Leucaena leucocephala* seeds (LLS). The usage of both samples will allow comparison in efficiency of the fermentation. Pretreatment prior to cellulose extraction from Leucaena seeds for CLLS was done at University of Malaya while LLS was blended by using Waring blender, USA. The extraction and blending for both CLLS and LLS were done in order to reduce the size of the samples. The competency in conversion of cellulose into ethanol would then be achieved. The comparison between extracted and non-extracted seeds was then being studied. Meanwhile, Mauri-Pan yeast, Friendemann Schmidt anhydrous glucose, sodium hydroxide (NaOH) pellets, concentrated hydrochloric acid (HCl) was purchased from a local chemical supplier.

On the other hand, instrumentation used are Shimadzu digital weighing scale, Philippines, SK 300 orbital shaker, Hve-50 autoclave, Mettler Toledo LE409 pH meter, Favorit distillation set, PLT Scientific Company and Agilent gas chromatograph mass selective detector or abbreviated as GC/MSD. Meanwhile, purification step would be done by using distillation set. On the other hand, qualitative analysis was done by using Agilent GC/MSD which was carried out by engineers at LabAlliance Company. On the other hand, computerization is about computation of quantitative analysis by using statistical package for the social science or abbreviated as SPSS software in Windows 10.

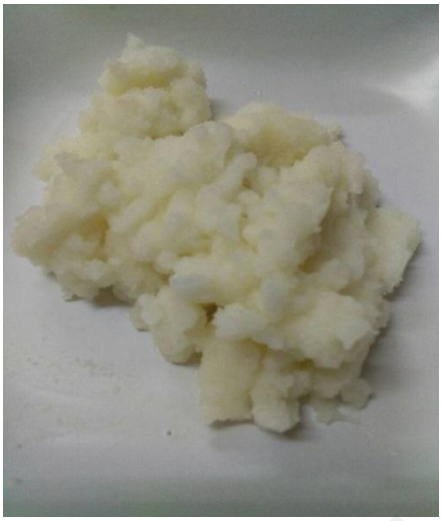

Figure 3.1: Cellulose extracted from *Leucaena leucocephala* seeds

Figure 3.2: Non-extracted *Leucaena leucocephala* seeds

3.2 Parameters

Fermentation would be carried out by controlling different parameters. Parameters are the measurable factors which affect output of experimentation. During fermentation, parameters such as the best pH value for microorganism growth and the yield of ethanol are recorded (Demirbaş, 2004). The production of ethanol and growth of yeast could also be affected by parameters such as concentration of sugar, oxygen, and temperature (Tesfaw and Assefa, 2014) as well as pH (Kasemets et al., 2007). However, this study focuses only on four parameters such as period of time taken for the successful fermentation to occur, amount of yeast, amount of glucose and pH range. The similar study on days of fermentation also carried out by Tahir et al. (2010) while amount of yeast and pH was done by Periyasamy et al. (2009). Optimum parameters are defined as the process to maximize one of the desired process specifications, while keeping factors such as temperature within their constraints.

Before optimum parameters are obtained, the process of quantity consideration which is called as evaluation is carried out. Then, the highest yield of liquid from each purification step of evaluation in this experiment would be the optimum parameter. The computation for the yield of liquid in percentage is known as quantitative analysis where the analysis is defined as the measurement of constituent quantities which are present any substances. Meanwhile, optimum parameters could reduce the costs of experimentation, allow parameters to be estimated without bias, suitable for multiple factors of study and experimental data would be able to identify the unknown parameters. On the other hand, qualitative analysis would also be carried out in order to identify constituents present in a substance of liquid produced from purification process.

3.3 Control Variables

Control variable is the constant or unchanged variable in order to testing the relationship between independent variables in this experiment such as evaluation in amount of glucose, amount of yeast and pH range and dependent variable such as yield of liquid in percentage. Control variables in this experiment were conditions of flask sterilization, condition of fermentation process, condition of recovery steps, computation method (Figure 3.3 and Figure 3.4) in quantitative analysis and operating conditions of GC/MSD. The first step in all experimentation was started with sterilization of empty flasks by using autoclave at 121°C for 2 hours and 30 minutes. The parameters on autoclave were automatically being set. Parameters are used in order to eliminate or remove any bacteria which could exist on surface of the flask. If there is presence of bacteria on flask, it would affect the fermentation process carried by *Saccharomyces cerevisiae*.

Then, flasks were cooled down before proceeding to the next step. Meanwhile, fermentation process was occurred in flask which was placed on an orbital shaker at 80 rpm and 30°C. This is because fermentation was suitable occurred in between 28°C – 35°C (Szczodrak and Fiedurek, 1996). These conditions were fixed for all fermentation process. After the flasks in all experiment were being collected from the orbital shaker, filtration was done on each of mixture from different flasks. Then, the recovery was covered on filtration and purification where filtration was used filter paper with size pore about 4.0 μm to obtain filtered product. The miniature compounds were then contained in filtered product so that the high competency for the next step of purification was achieved.

Meanwhile, the volume of filtered product which was in the liquid form would be range between 55 and 175 mL based on combination of both amount of tap water and warm water in all experimentation steps. The product was then kept in a refrigerator for 10 days maximally prior to the purification process to inhibit fermentation. The purification on the filtered product was then taken place by using distillation with temperature range between 78°C to 80°C which was controlled manually by using thermometer to obtain pure liquid form from the filtered product. The range of temperature was used because boiling point of filtered product, which would be ethanol, is at 78.29°C. The quantitative analysis for all evaluation experiment except for the real study was then carried out by computing manually the yield of liquid in percentage as shown in Figure 3.3.

$$Yield\ of\ Liquid\ (\%) = \frac{Actual\ Yield\ x\ 100}{Theoretical\ Yield}$$

Figure 3.3: Formula for yield of liquid

The exception was because real study had been suggested to analyzed directly by using GC/MSD with operating condition (Column: HP-5ms, Eluent: He (gas), Flow-rate: 1 mL min^{-1}, Temp: 60°C). These conditions had been referred to analysis of ethanol done by Shimadzu Corporation (2011) where temperature of 60°C used in order to heat up the sample. Quantitative analysis from computation manually was also analyzed by using SPSS for plotting the scatter between independent and dependent variables for all evaluation experiment as well as the real study.

After plotting the data, SPSS analysis was still being applied in finding the strength of relationship between both variables for all evaluation experiment including the real study. According to Bluman (2014), the data on strength of relationship was about the computation of correlation coefficient which is symbolized as *r* and it was showed in Figure 3.4 as well as *t* test for *r* value in Figure 3.5. The determinations in value of *r* and *t* test were conducted for evaluation in days of fermentation, amount of glucose, amount of yeast and pH range. When the value of *r* was significant, equation of regression line which was written as $y' = a + bx$ could be determined and the formula in computing value of *a* and *b* were showed in Figure 3.6. The last step in quantitative analysis for all experiment of evaluation except for the real study was the determination in standard error of the estimate by using the equation of regression line, y' where its formula was showed in Figure 3.7. On the other hand, the multiple correlation coefficient, *R* was determined in real study on amount of CLLS and LLS. The formula of value of *R* was showed in Figure 3.8. Next *F* test computed based on Figure 3.9 in order to test the significance of *R*.

$$r = \frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{[n(\Sigma x2) - (\Sigma x)2][n(\Sigma y2) - (\Sigma y2)]}}$$

Figure 3.4: Formula for the linear correlation coefficient, *r*

$$t = r\sqrt{\frac{n-2}{1-r2}}$$

$$d.f. = n-2$$

Figure 3.5: Formula of the *t* test for *r* value

$$y' = a + bx$$

$$a = \frac{n(\Sigma y)(\Sigma x2) - (\Sigma x)(\Sigma xy)}{n(\Sigma x2) - (\Sigma x)2}$$

$$b = \frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{n(\Sigma x2) - (\Sigma x)2}$$

Figure 3.6: Formula for the regression line

$$Sest = \sqrt{\frac{\Sigma(y - y')2}{n-2}}$$

Figure 3.7: Formula for the standard error of the estimate

$$R = \frac{r2yx1 + r2yx2 - (2ryx1 \times ryx2 \times rx1x2)}{1 - r2x1x2}$$

Figure 3.8: Formula for the multiple linear correlation coefficient, *R*

$$F = \frac{R2/k}{(1-R2)/(n-k-1)}$$

Figure 3.9: Formula of the *F* test for *R* value

Where,

n = number of data pairs

x = independent variable

y = dependent variable

$d.f.$ = degrees of freedom

k = number of independent variables

a = *y'* intercept

b = slope of the line

Liquid obtained from purification step was then kept in the vial, sealed with parafilm and kept in refrigerator. The sealing and storage process is a precautionary step so that liquid would not be vaporized easily. After quantitative analysis was done, qualitative analysis on the liquid obtained in all experiment was also carried out by using GC/MSD with the similar operation condition as the real study. However, results for qualitative analysis were only showed the highest yield of liquid obtained from all evaluation experiment.

3.4 Evaluation

3.4.1 Evaluation in Days of Fermentation

The whole experiments were beginning with the evaluation in days of fermentation. The evaluation in days of fermentation was labeled as the first evaluation. The first step in the first evaluation was flask sterilization. Then, 6 g of anhydrous glucose and 30 mL of tap water were mixed with 5 g of yeast and 25 mL of warm water in the sterilized flask. This statement was explained by using similar ratio of 1:5 for both amount of glucose to amount of tap water and amount of yeast to amount of warm water. The warm water is used was because the optimum temperature range for fermentation carried out by yeast is at about 35°C. The temperature range was also caused tap water to be warm and hence, it would inhibit other activity of bacteria existed in tap water.

After 1 day of fermentation, the flask with its contents was collected. The filtration and purification of the products was then carried out before quantitative analysis was done followed up by the qualitative analysis. All the steps were repeated by using a similar amount of glucose and yeast for 3, 6, 8 and 10 days of fermentation. The highest yield of ethanol based on the number days of fermentation would be the optimum days of fermentation. The days of fermentation was selected so that the trend which was then being plotted on graph would easily to being observed and predicted. Meanwhile, amount of glucose and amount of yeast were different from other evaluation steps because these amounts were act as control variables in obtaining the optimum day. The similar optimum day obtained was then applied to the other evaluation steps.

3.4.2 Evaluation in Amount of Glucose

The evaluation in amount of glucose was labeled as the second evaluation. The first step in the second evaluation was sterilization of flask. After that, 5 g of anhydrous glucose and 25 mL of tap water were mixed with 5 g of yeast and 25 mL of warm water was added into the flask. Both conditions also used similar ratio of 1:5. Next, the fermentation process was occurred. After the optimum days of fermentation, the flask was collected. The purification of products was then carried out after filtration of products was done. Both quantitative and qualitative analyses of the liquid obtained were then being implemented. All the steps were repeated using the same amount of yeast but different amounts of glucose. These amounts of glucose employed were 10 g, 15 g, 20 g and 30 g which were then mixed with 50 mL, 75 mL, 100 mL and 150 mL of tap water respectively based ratio of 1:5. The variation in amounts of tap water was needed to ensure both amount of glucose and water are constantly added. The highest yield of liquid acquired from evaluation in amount of glucose would be the optimum amount of glucose.

3.4.3 Evaluation in Amount of Yeast

The evaluation in amount of yeast was labeled as the third evaluation. Before the third evaluation was started, flask was being sterilized under certain conditions. Next, optimum amount of glucose and amount of tap water were mixed with 1 g of yeast and 5 mL of warm water and then added into the sterilized flask. After that, the fermentation process was taken place.

After the optimum number of fermentation days, the flask was collected in order to filtered mixture in flask before it was being purified. Liquid which was gained from purification would then being analyzed thoroughly in terms of quantitative and qualitative. All the steps were repeated using the same optimum amount of glucose but with different amounts of yeast. Those amounts of yeast would be 2, 3, 4 and 5 g of yeast which were then mixed with 10, 15, 20 and 25 mL warm water respectively based on multiples of five. The highest yield obtained from evaluation in amount of yeast would be the optimum amount of yeast.

3.4.4 Evaluation in pH Range

The evaluation in pH range was labeled as the fourth experiment. The sterilization of flask was carried out in the beginning of the fourth experiment. The optimum amount of glucose and tap water with its pH range between 3.0 – 3.9 were mixed with the optimum amount of yeast and warm water before adding into the sterilized flask. After a certain number of fermentation days, the flask was collected. Liquid was then being obtained by purification after filtration step was done before proceeding to quantitative and qualitative analysis of liquid. All the steps were repeated using different pH ranges between 4.0 – 4.9, 5.0 – 5.9, 6.0 – 6.9 and 7.0 – 7.9. The trend which was then plotted on graph would easily to being observed and predicted by using the chosen pH ranges. The highest yield of liquid obtained from fermentation would be the optimum pH range.

3.5 Real Study

The real study began with flask sterilization. After that, the amount of CLLS was evaluated based on the optimum amount of glucose. This is because cellulose consists of a chain of glucose units (Sun et al., 2009). Hence, amount of CLLS could be similar with optimum amount of glucose. An amount of CLLS and an amount of water with its optimum pH range was then mixed with an optimum amount of yeast and added into the sterilized flask. Next, the fermentation process was taken place. After optimum number of fermentation days, the flask was collected. The filtration of products and purification of liquid were then done sequentially followed up by analysis of liquid without computation. All the steps were repeated using an amount of LLS instead of the amount of CLLS which is similar with optimum amount of glucose. The similarity was because LLS contained carbohydrate (Sotelo, 1980) where cellulose in LLS is one of the carbohydrate macromolecules. Cellulose is made up of glucose units (Sun et al., 2009). Thus, both amount of LLS and optimum amount of glucose would be the same due to the similar contents of carbohydrate.

3.6 Recovery

3.6.1 Filtration

After fermentation process from the first, second, third, fourth evaluations and the real study were done, fermented product was obtained. The fermented product which consists of a mixture of glucose with yeast or a mixture of cellulose with yeast was then recovered by filtration before purification was taken place. After the filtration was occurred, the filtered product was achieved.

3.6.2 Purification

Purification is carried out by using distillation. Distillation is an activity where the remaining of any elements, enzyme, lignin, hemicellulose, cellulose, organisms and ash are left at the bottom of distillation set (Mosier et al., 2005). During distillation process, evaporation of the liquid forces the vapor upward past the thermometer and into the condenser. The vapor was then condensed into liquid in a cooling condenser and the liquid was flown downward into a receiving flask or known as small round bottom flask. The liquid was collected in small round bottom flask and subsequently kept in sample vials as showed in Figure 3.10. Although filtration and purification of liquid were done in the real study, both quantitative and qualitative analyses were done simultaneously by using GC/MSD.

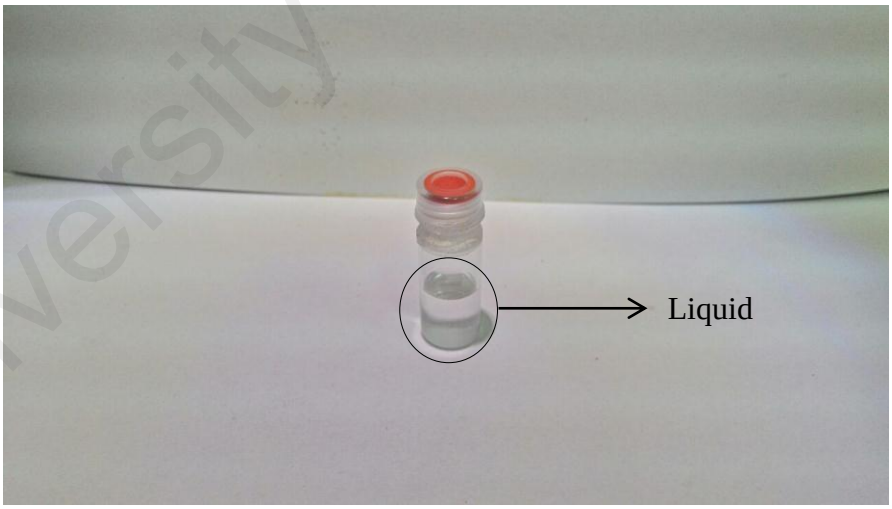

Figure 3.10: Liquid in vial

3.7 Computation

Quantitative analysis is defined as the measurements of constituent quantities which are present any substance. The analysis for all evaluation except for the real study was begun by pouring the liquid collected from small round bottom flask into small measuring cylinder for measurement purpose. As the amount of liquid was being identified, yield of liquid in percentage was then could be able to computed. After keeping liquid in vials inside the refrigerator, liquid was further analyzed by using SPSS before GC/MSD analysis was done detection purpose.

3.8 SPSS Analysis

After computation for yield of liquid in percentage was done, quantitative analysis was continued by plotting the scatter on graph for all experiment of evaluation except for the real study. According to Bluman (2014), scatter plot was a graph of the ordered pairs (x, y) of numbers consisting of independent variable, x such that either days of fermentation, amount of glucose, amount of yeast or pH range and dependent variable, y such that yield of ethanol. This plot is used to determine whether the relationship is positive linear, negative linear, curvilinear or no relationship based on below Figure 3.11. Then, the fit line either in the quadratic or cubic shape was drawn by using SPSS.

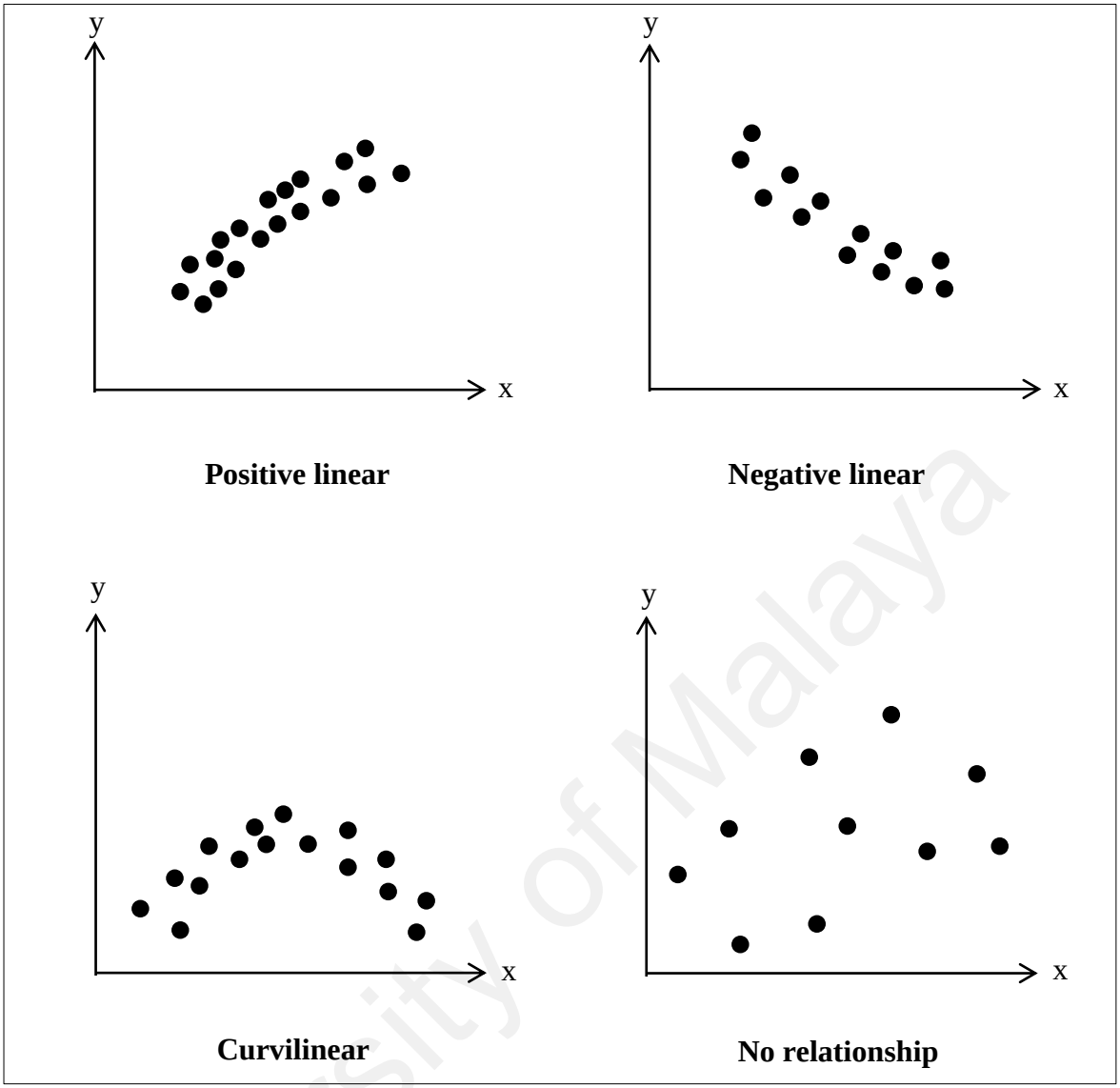

Figure 3.11: Graphs of scatter plot for trend of relationship

The next step was about finding the correlation coefficient, *r* in the first, second, third and fourth evaluations. The *r* value was used in determination of strength of linear relationship between two variables where value of *r* which was near with +1 or –1 would cause the linear relationship was strong. However, there is weak relationship or no linear relationship if the value *r* was equal to 0.

After that, *t* test was conducted in order to test the significance of *r* value. Both variables were normally distributed if there is the significance in value of *r*. Then, *t* test was computed after the graph was plotted. Before *t* test was computed, two hypothesis all experiment of evaluation including the real study were stated such that H_0 and H_1. H_0 is where ρ was equal to 0. This null hypothesis means that there is no correlation between *x* and *y* variables in the population. Meanwhile, H_1 is where ρ was not equal to 0. This alternative hypothesis means that there is a significance correlation between the variables in the population. Then, the similar critical values ±3.182 were obtained from Table 3.1 for the first, second, third and fourth evaluations except for the real study when using two-tailed of α which was equal to 0.05, confidence interval was equal to 95% and there were 5 – 2 = 3 degrees of freedom.

When the value of *r* was significance, the equation of the best fit line of data or known as regression line, *y'* could be determined. The regression line was used in order to predict the data by observing the trend of graph. However, prediction made by using regression line was meaningless if the value of *r* was not significant. The predictions in values of *y'* in regression line equation were then computed in order to find the standard error of the estimate. It was used in order to measure how the data points deviated from the regression line which similar with the standard deviation. Based on formula in Figure 3.7, the unexplained variation was due to the difference between the observed values, *y* and the expected values, *y'* divided by *n* – 2. Hence, the standard error of the estimate would small when the observed values were closer to the predicted values.

Table 3.1: The critical values for *t* test distribution (Beyer, 1986)

Table F The *t* Distribution

d.f.	Confidence intervals	80%	90%	95%	98%	99%
	One tail, α	0.10	0.05	0.025	0.01	0.005
	Two tails, α	0.20	0.10	0.05	0.02	0.01
1		3.078	6.314	12.706	31.821	63.657
2		1.886	2.920	4.303	6.965	9.925
3		1.638	2.353	3.182	4.541	5.841
4		1.533	2.132	2.776	3.747	4.604
5		1.476	2.015	2.571	3.365	4.032
6		1.440	1.943	2.447	3.143	3.707
7		1.415	1.895	2.365	2.998	3.499
8		1.397	1.860	2.306	2.896	3.355
9		1.383	1.833	2.262	2.821	3.250
10		1.372	1.812	2.228	2.764	3.169
11		1.363	1.796	2.201	2.718	3.106
12		1.356	1.782	2.179	2.681	3.055
13		1.350	1.771	2.160	2.650	3.012
14		1.345	1.761	2.145	2.624	2.977
15		1.341	1.753	2.131	2.602	2.947
16		1.337	1.746	2.120	2.583	2.921
17		1.333	1.740	2.110	2.567	2.898
18		1.330	1.734	2.101	2.552	2.878
19		1.328	1.729	2.093	2.539	2.861
20		1.325	1.725	2.086	2.528	2.845
21		1.323	1.721	2.080	2.518	2.831
22		1.321	1.717	2.074	2.508	2.819
23		1.319	1.714	2.069	2.500	2.807
24		1.318	1.711	2.064	2.492	2.797
25		1.316	1.708	2.060	2.485	2.787
26		1.315	1.706	2.056	2.479	2.779
27		1.314	1.703	2.052	2.473	2.771
28		1.313	1.701	2.048	2.467	2.763
29		1.311	1.699	2.045	2.462	2.756
30		1.310	1.697	2.042	2.457	2.750
32		1.309	1.694	2.037	2.449	2.738
34		1.307	1.691	2.032	2.441	2.728
36		1.306	1.688	2.028	2.434	2.719
38		1.304	1.686	2.024	2.429	2.712
40		1.303	1.684	2.021	2.423	2.704
45		1.301	1.679	2.014	2.412	2.690
50		1.299	1.676	2.009	2.403	2.678
55		1.297	1.673	2.004	2.396	2.668
60		1.296	1.671	2.000	2.390	2.660
65		1.295	1.669	1.997	2.385	2.654
70		1.294	1.667	1.994	2.381	2.648
75		1.293	1.665	1.992	2.377	2.643
80		1.292	1.664	1.990	2.374	2.639
90		1.291	1.662	1.987	2.368	2.632
100		1.290	1.660	1.984	2.364	2.626
500		1.283	1.648	1.965	2.334	2.586
1000		1.282	1.646	1.962	2.330	2.581
(*z*) ∞		1.282[a]	1.645[b]	1.960	2.326[c]	2.576[d]

On the other hand, value of multiple correlation coefficient, *R* was obtained from the relationship between two or more independent variables, x_1, x_2, x_3 and x_4 such that days of fermentation, amount of glucose, amount of yeast and pH range with one dependent variable, *y* such that yield of ethanol in the real study on CLLS and LLS. The range of value for *R* was between 0 and +1 where it always positive.

The stronger the relationship between both variables, the closer value *R* with +1 while the closer value *R* with 0 would cause the weaker the relationship between variables. Before *F* test was carried out, two hypotheses were stated same as *t* test value but ρ was represented population correlation coefficient for multiple correlations. The null hypothesis would be rejected if the *F* value was more than critical value obtained from Table 3.2 by using α which was equal to 0.05.

Table 3.2: The critical values for *F* test distribution (Merrington and Thompson, 1943)

Table H *(continued)*

d.f.D.: degrees of freedom, denominator	$\alpha = 0.05$																		
	d.f.N.: degrees of freedom, numerator																		
	1	2	3	4	5	6	7	8	9	10	12	15	20	24	30	40	60	120	∞
1	161.4	199.5	215.7	224.6	230.2	234.0	236.8	238.9	240.5	241.9	243.9	245.9	248.0	249.1	250.1	251.1	252.2	253.3	254.3
2	18.51	19.00	19.16	19.25	19.30	19.33	19.35	19.37	19.38	19.40	19.41	19.43	19.45	19.45	19.46	19.47	19.48	19.49	19.50
3	10.13	9.55	9.28	9.12	9.01	8.94	8.89	8.85	8.81	8.79	8.74	8.70	8.66	8.64	8.62	8.59	8.57	8.55	8.53
4	7.71	6.94	6.59	6.39	6.26	6.16	6.09	6.04	6.00	5.96	5.91	5.86	5.80	5.77	5.75	5.72	5.69	5.66	5.63
5	6.61	5.79	5.41	5.19	5.05	4.95	4.88	4.82	4.77	4.74	4.68	4.62	4.56	4.53	4.50	4.46	4.43	4.40	4.36
6	5.99	5.14	4.76	4.53	4.39	4.28	4.21	4.15	4.10	4.06	4.00	3.94	3.87	3.84	3.81	3.77	3.74	3.70	3.67
7	5.59	4.74	4.35	4.12	3.97	3.87	3.79	3.73	3.68	3.64	3.57	3.51	3.44	3.41	3.38	3.34	3.30	3.27	3.23
8	5.32	4.46	4.07	3.84	3.69	3.58	3.50	3.44	3.39	3.35	3.28	3.22	3.15	3.12	3.08	3.04	3.01	2.97	2.93
9	5.12	4.26	3.86	3.63	3.48	3.37	3.29	3.23	3.18	3.14	3.07	3.01	2.94	2.90	2.86	2.83	2.79	2.75	2.71
10	4.96	4.10	3.71	3.48	3.33	3.22	3.14	3.07	3.02	2.98	2.91	2.85	2.77	2.74	2.70	2.66	2.62	2.58	2.54
11	4.84	3.98	3.59	3.36	3.20	3.09	3.01	2.95	2.90	2.85	2.79	2.72	2.65	2.61	2.57	2.53	2.49	2.45	2.40
12	4.75	3.89	3.49	3.26	3.11	3.00	2.91	2.85	2.80	2.75	2.69	2.62	2.54	2.51	2.47	2.43	2.38	2.34	2.30
13	4.67	3.81	3.41	3.18	3.03	2.92	2.83	2.77	2.71	2.67	2.60	2.53	2.46	2.42	2.38	2.34	2.30	2.25	2.21
14	4.60	3.74	3.34	3.11	2.96	2.85	2.76	2.70	2.65	2.60	2.53	2.46	2.39	2.35	2.31	2.27	2.22	2.18	2.13
15	4.54	3.68	3.29	3.06	2.90	2.79	2.71	2.64	2.59	2.54	2.48	2.40	2.33	2.29	2.25	2.20	2.16	2.11	2.07
16	4.49	3.63	3.24	3.01	2.85	2.74	2.66	2.59	2.54	2.49	2.42	2.35	2.28	2.24	2.19	2.15	2.11	2.06	2.01
17	4.45	3.59	3.20	2.96	2.81	2.70	2.61	2.55	2.49	2.45	2.38	2.31	2.23	2.19	2.15	2.10	2.06	2.01	1.96
18	4.41	3.55	3.16	2.93	2.77	2.66	2.58	2.51	2.46	2.41	2.34	2.27	2.19	2.15	2.11	2.06	2.02	1.97	1.92
19	4.38	3.52	3.13	2.90	2.74	2.63	2.54	2.48	2.42	2.38	2.31	2.23	2.16	2.11	2.07	2.03	1.98	1.93	1.88
20	4.35	3.49	3.10	2.87	2.71	2.60	2.51	2.45	2.39	2.35	2.28	2.20	2.12	2.08	2.04	1.99	1.95	1.90	1.84
21	4.32	3.47	3.07	2.84	2.68	2.57	2.49	2.42	2.37	2.32	2.25	2.18	2.10	2.05	2.01	1.96	1.92	1.87	1.81
22	4.30	3.44	3.05	2.82	2.66	2.55	2.46	2.40	2.34	2.30	2.23	2.15	2.07	2.03	1.98	1.94	1.89	1.84	1.78
23	4.28	3.42	3.03	2.80	2.64	2.53	2.44	2.37	2.32	2.27	2.20	2.13	2.05	2.01	1.96	1.91	1.86	1.81	1.76
24	4.26	3.40	3.01	2.78	2.62	2.51	2.42	2.36	2.30	2.25	2.18	2.11	2.03	1.98	1.94	1.89	1.84	1.79	1.73
25	4.24	3.39	2.99	2.76	2.60	2.49	2.40	2.34	2.28	2.24	2.16	2.09	2.01	1.96	1.92	1.87	1.82	1.77	1.71
26	4.23	3.37	2.98	2.74	2.59	2.47	2.39	2.32	2.27	2.22	2.15	2.07	1.99	1.95	1.90	1.85	1.80	1.75	1.69
27	4.21	3.35	2.96	2.73	2.57	2.46	2.37	2.31	2.25	2.20	2.13	2.06	1.97	1.93	1.88	1.84	1.79	1.73	1.67
28	4.20	3.34	2.95	2.71	2.56	2.45	2.36	2.29	2.24	2.19	2.12	2.04	1.96	1.91	1.87	1.82	1.77	1.71	1.65
29	4.18	3.33	2.93	2.70	2.55	2.43	2.35	2.28	2.22	2.18	2.10	2.03	1.94	1.90	1.85	1.81	1.75	1.70	1.64
30	4.17	3.32	2.92	2.69	2.53	2.42	2.33	2.27	2.21	2.16	2.09	2.01	1.93	1.89	1.84	1.79	1.74	1.68	1.62
40	4.08	3.23	2.84	2.61	2.45	2.34	2.25	2.18	2.12	2.08	2.00	1.92	1.84	1.79	1.74	1.69	1.64	1.58	1.51
60	4.00	3.15	2.76	2.53	2.37	2.25	2.17	2.10	2.04	1.99	1.92	1.84	1.75	1.70	1.65	1.59	1.53	1.47	1.39
120	3.92	3.07	2.68	2.45	2.29	2.17	2.09	2.02	1.96	1.91	1.83	1.75	1.66	1.61	1.55	1.50	1.43	1.35	1.25
∞	3.84	3.00	2.60	2.37	2.21	2.10	2.01	1.94	1.88	1.83	1.75	1.67	1.57	1.52	1.46	1.39	1.32	1.22	1.00

3.9 Gas Chromatograph Mass Selective Detector

The solely observation on sample of liquid produced from purification process in Figure 3.4 would be doubtful. Hence, qualitative analysis is mandatory to be implemented so that results which are gained from quantitative analysis are valid and rigid. The qualitative analysis is about the detection of unknown composition and constituents present in a substance. Gas chromatograph mass selective detector (GC/MSD) was employed to analyze repeating-remove the samples obtained. The GC/MSD began the qualitative analysis by injecting the sample from vials before combination of sample with water was done. Then, gas chromatograph was heated the sample to 60°C for about 5 minutes in the chamber. This step was carried out so that the headspace was obtained. Headspace was the unfilled space above the sample in the chamber. It consisted of gas which was evaporated from its sample form. The heating method was suitable for volatile compound. The evaporated gas was then pulled out of chamber. After that, it entered into two separating columns (Tiscione et al., 2011).

The first column was known as trap where the gas was condensed into sample. The sample was then passed through the volatile interface before it was passed through the second column. The second column would maintain the sample molecules by allowing molecules travelling through it. The molecules were then being removed from column at different time which was known as retention time. The removal activity would allow mass selective detector to dissociate each molecule into ionized fragments. The fragmentation process would cause the formation of pattern where it presented distribution of ions by mass in mass spectrum or known as mass-to-charge ratio. This pattern would be able to determine the information of molecular structure (McLafferty and Turecek, 1993) or detected the composition of the sample.

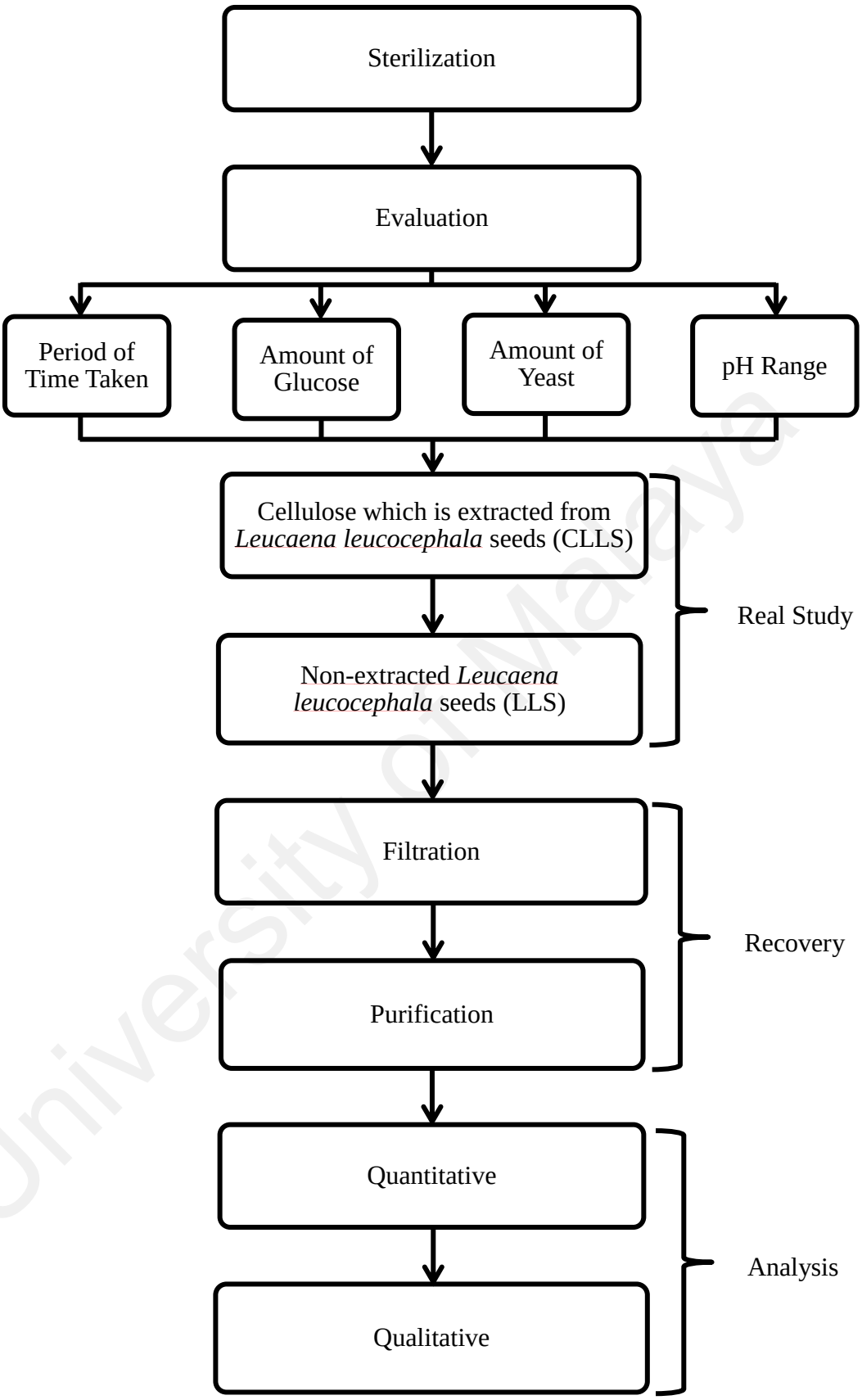

Figure 3.12: Overall activity of experiments

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Introduction

The chapter is consisted of two major sections which are quantitative and qualitative analysis. The section of quantitative analysis is then being divided into two minor sections such that determination of optimum parameters, determination in yield of ethanol obtained from CLLS and LLS as well as relationship between yield of ethanol with optimum days of fermentation, amount of glucose, amount of yeast and pH range in all experiment of evaluation. The determination of optimum parameters section is then being separated into four main sections which are optimum days of fermentation, amount of glucose, amount of yeast and pH range.

4.2 Quantitative Analysis

The analysis is about the calculation on yield of liquid after the purification process. The liquid should be ethanol based on observation made on its physical characteristics where it was colourless and odor like wine.

4.2.1 Determination of Optimum Parameters

The amount of ethanol which was gained from purification step was then being measured where the highest amount of ethanol would be optimum for each parameter.

4.2.1.1 Optimum Days of Fermentation

The first computation on yield of ethanol in percentage obtained from the first evaluation was done. Table 4.1 shows the yield of ethanol when various number of days were employed for fermentation. Based on Table 4.1, percentages of yield of ethanol from day 1 to 3 were increased. However, yield of ethanol decreased from day 8 to 10.

Table 4.1: Yield of ethanol for the first evaluation

Period of Time Taken, days	Yield of Ethanol, %
1	5.07
3	5.43
6	5.07
8	4.35
10	2.90

It was observed that day 3 was the optimum days of fermentation as ethanol is produced at the highest yield which was 5.43%. The graph in Figure 4.1 was plotted and quadratic fit line was drawn by using SPSS based on Table 4.1. Based on Table 4.1 and Figure 4.1, Tahir et al. (2010) stated that ethanol production initially began at a low level. The yield of ethanol is then increased as the number of yeast cells increased. After optimum days of fermentation, the amount of sugar would decrease and the yield of ethanol would be inhibited. Fermentation would continue until almost all of the sugar is utilized by the yeast.

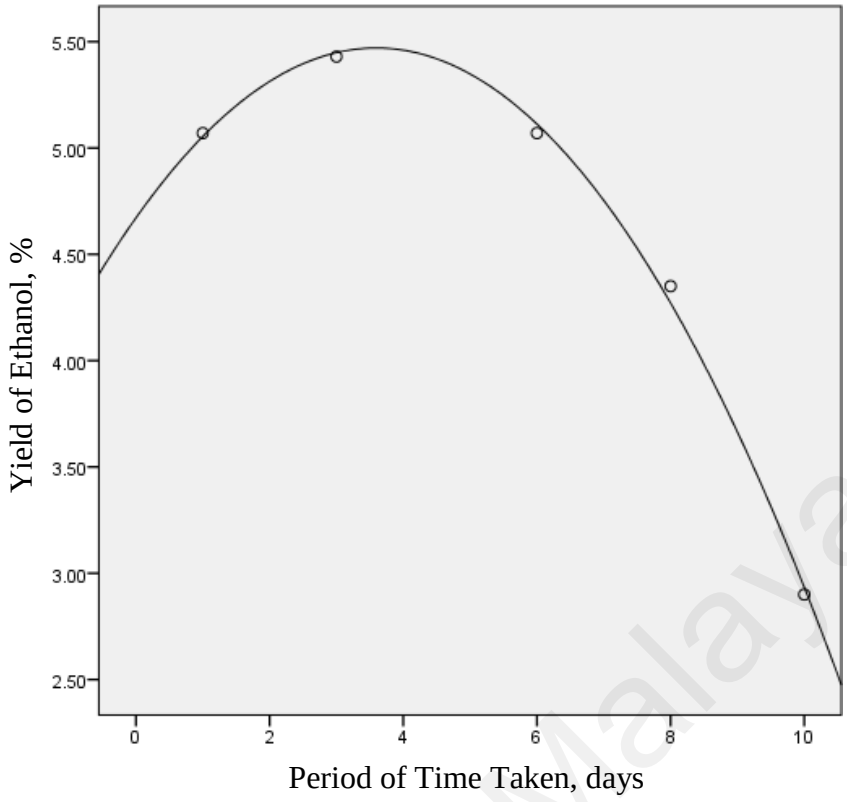

Figure 4.1: Yield of ethanol for the first evaluation

On the other hand, the strength of relationship between period of time taken for fermentation process and yield of ethanol was strong negative linear relationship. This is because value of correlation coefficient, *r* was –0.831 where, according to Bluman (2014), it was near to value of –1. Next, the critical values were obtained. The *t* value was then obtained at –2.587. Since *t* value was not fallen in critical region, the null hypothesis was accepted. Hence, there was no significance relationship between days of fermentation and yield of ethanol. When the value of *r* was not significant, the equation of regression line followed by standard error of estimate was not been able to determined.

4.2.1.2 Optimum Amount of Glucose

Next computation was made on yield of ethanol in percentage for the second evaluation. The results obtained were then being tabulated in Table 4.2. Based on Table 4.2, it was proved that 20 g of glucose is the optimum amount of glucose as it produced the highest yield of ethanol which was 3.65%. On the other hand, the amount of tap water used was varied because it was based on similar ratio of 1:5 for both amount of glucose to amount of tap water so that both amount of samples were constantly added.

Table 4.2: Yield of ethanol for the second evaluation

Amount of Glucose, g	Amount of Tap Water, mL	Yield of Ethanol, %
5	25	0.72
10	50	0.36
15	75	3.12
20	100	3.65
30	150	2.11

However, 3 days of fermentation, 5 g amount of yeast and 25 mL amount of warm water were applied in both the first and second evaluation to produced 5.43% and 3.65% optimum yield of ethanol respectively. Thus, 5.43% still became optimum yield of ethanol rather than 3.65%. Nonetheless, the first evaluation was act as controlled variable in order to determine the optimum days of fermentation and applied the optimum days in other experiments of evaluation.

Meanwhile, there was slightly difference between both evaluation studied was because small amount of glucose used in the first evaluation was less inhibited by yeast. On the other hand, graph in Figure 4.2 was plotted and cubic fit line was drawn by using SPSS according to Table 4.2. Figure 4.2 shows the graph plotted from 10 g until 30 g amount of glucose. Based on Figure 4.2, Periyasamy et al. (2009) stated that the yield of ethanol increased with increasing amount of sugar but it would decline after the maximum yield of ethanol is achieved. Maximum yield of ethanol is obtained at a certain amount of sugar. This is because the yield of ethanol would be inhibited with more increasing amounts of sugar.

The statement which is reported by Periyasamy et al. (2009) also agreed with Klis et al. (2004) where yeast is growing with increasing amount of glucose. Firstly, there is low amount of ethanol produced at early growth of yeast. This is because ethanol is utilized by glyoxylate cycles, tricarboxylic acid and mitochondrial electron transport chain in yeast cell. Then, yield of ethanol is increased as ethanol produced from glycolysis occurred in yeast cell after the biochemical of cell is changed into respiratory metabolism from fermentative metabolism.

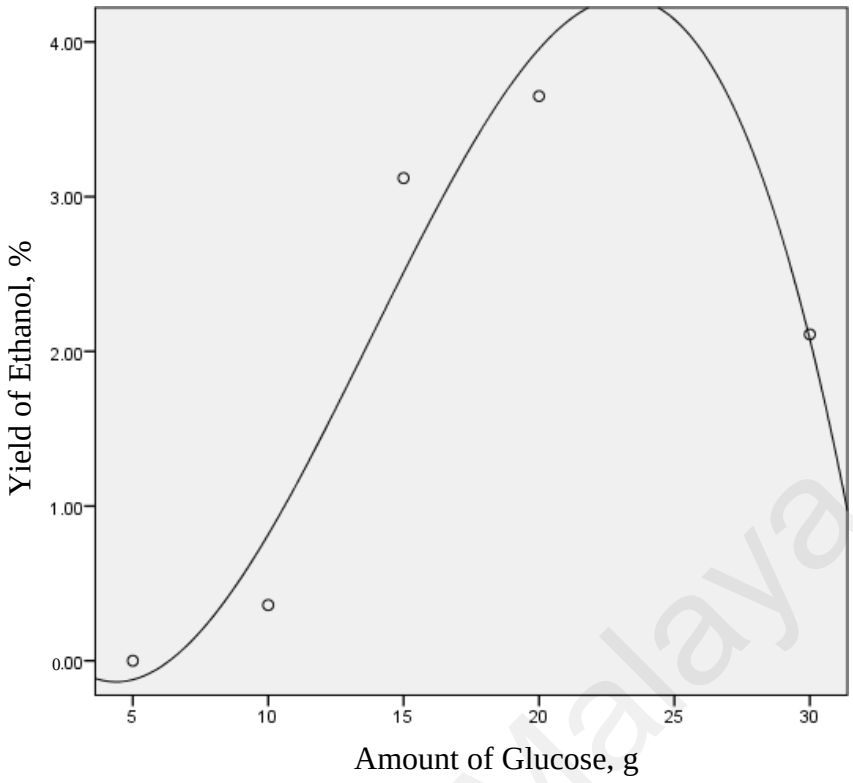

Figure 4.2: Yield of ethanol for the second evaluation

Meanwhile, value of *r* was 3.145 which proved that, according to Bluman (2014), there was weak or no linear relationship between amount of glucose and yield of ethanol as it was far from value of +1. Next, the critical values were obtained. Then, *t* value was obtained at 0. Since *t* value was not fallen in critical region, the null hypothesis was accepted. Hence, there was no significance relationship between amount of glucose and yield of ethanol. Thus, there is no determination in equation of regression line including the standard error of estimate.

4.2.1.3 Optimum Amount of Yeast

After the second evaluation was done, the yield of ethanol in percentage for the third evaluation was being computed. The results of computation were then being organized in Table 4.3.

Table 4.3: Yield of ethanol for the third evaluation

Amount of Yeast, g	Amount of Warm Water, mL	Yield of Ethanol, %
1	5	0.69
2	10	1.38
3	15	1.78
4	20	0.89
5	25	0.00

Table 4.3 shows the yield of ethanol for the third evaluation. It was indicated that the highest yield of ethanol is achieved by using 3 g of yeast which was 1.78%. Thus, 3 g of yeast would be the optimum amount of yeast. On the other hand, both 3.65% optimum yield of ethanol for the second evaluation and no yield of ethanol for the third evaluation were achieved by using 3 days of fermentation, 20 g amount of glucose and 5 g amount of yeast. Nevertheless, 5.43% optimum yield of ethanol for the first evaluation was obtained by using less amount of glucose with similar days of fermentation and amount of yeast. These results were varying because high amount of glucose used in the second and third evaluation would more inhibited by yeast rather than using low amount of glucose used in the first evaluation which was fully utilized by yeast.

Meanwhile, there was no ethanol produced when using 5 g amount of yeast in the third evaluation compared by the second evaluation with same parameters. The reasons were because there were some errors during conducting the experiment and the result was still had to record in order to observe the trend in graph between growth of yeast and yield of ethanol. Graph in Figure 4.3 was then being plotted and quadratic fit line was drawn by using SPSS based on Table 4.3. Based on Table 4.3 and Figure 4.3, Periyasamy et al. (2009) stated that the yield of ethanol would increase when the amount of yeast is increased. After that, the yield of ethanol started to decline. This is because high number of yeast would be competitive in converting glucose into ethanol. Hence, high number of yeast would produce less amount of ethanol.

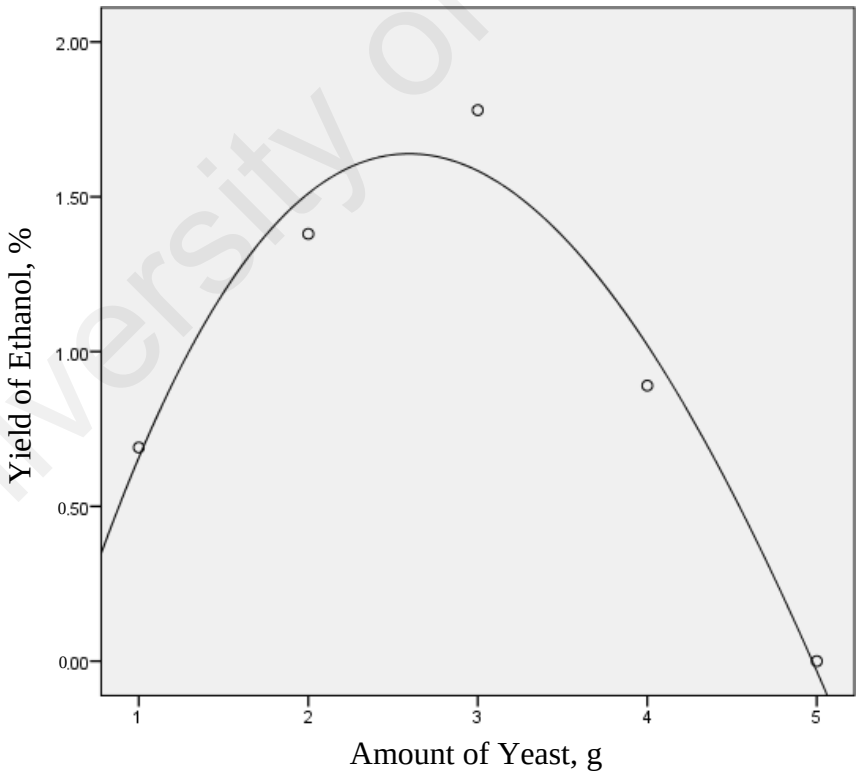

Figure 4.3: Yield of ethanol for the third evaluation

On the other hand, the strength of relationship amount of yeast and yield of ethanol was also weak or no linear relationship. This is because, according to Bluman (2014), the value of correlation coefficient, *r* was –0.433 where it was near to value of 0. Next, the critical values were obtained. Then, *t* value was obtained at –0.832. Since *t* value was also not fallen in critical region, the null hypothesis was also accepted. Hence, there was also no significance relationship between amount of yeast and yield of ethanol. When the value of *r* was not significant, the equation of regression line followed by standard error of estimate was not been able to determined.

4.2.1.4 Optimum pH Range

The fourth evaluation was the next evaluation where the yield of ethanol in percentage was computed. The results of ethanol yield were then being arranged in Table 4.4.

Table 4.4: Yield of ethanol for the fourth evaluation

pH Range	Yield of Ethanol, %
3.0 – 3.9	4.14
4.0 – 4.9	4.24
5.0 – 5.9	3.55
6.0 – 6.9	2.07
7.0 – 7.9	1.78

Based on Table 4.4, it is stated that the highest yield of ethanol was achieved at 4.24% when the pH range was between 4.0 –4.9. Hence, the pH range between 4.0 – 4.9 would be the optimum pH range. Meanwhile, graph in Figure 4.4 was also plotted and quadratic fit line was drawn by using SPSS according to Table 4.4. Based on Table 4.4 and Figure 4.4, according to Periyasamy et al. (2009), bioethanol production would increase from a low pH to a high pH. However, ethanol reached its peak when pH is at 4.0 as yeast are not suitable in the lowest pH which is less than pH 4.0 and highest pH which was more than pH 5.0.

Then, ethanol production started to decrease as the pH increased. This is because there was low activity of yeast. The explainations are also agreed by Lin et al. (2012) where the maximum yield of ethanol reached below pH 4.0. The yield of ethanol was then declined when it reached above pH 5.0. Hence, pH range between 4.0 – 5.0 would be the maximum yield of ethanol. The decrease of ethanol production was because glucose was utilized by yeast and converted it into secondary product. The study in determination of optimum pH range showed that the secondary product was butanol.

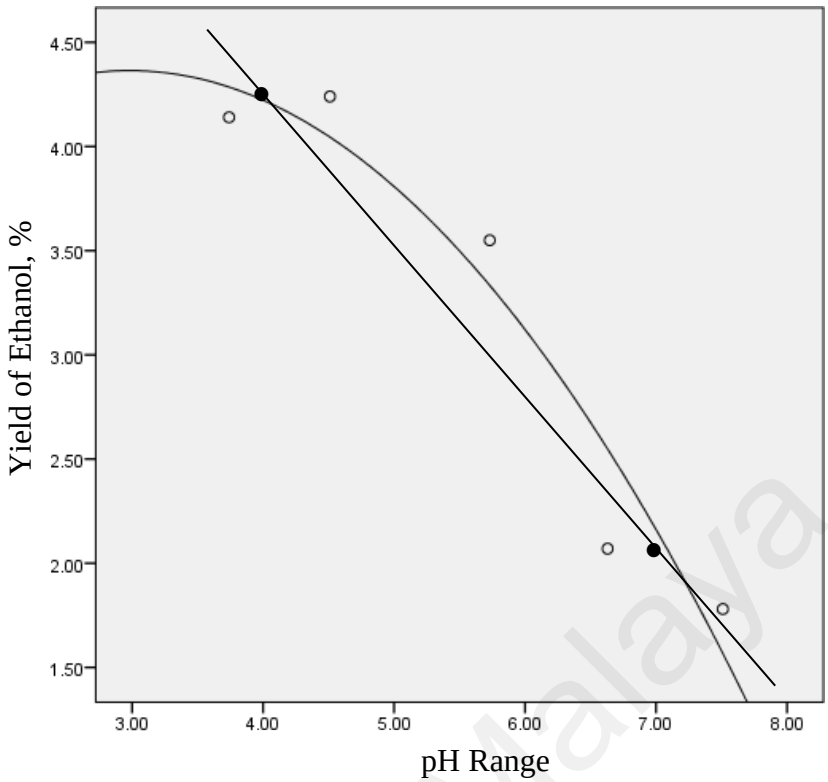

Figure 4.4: Yield of ethanol for the fourth evaluation

Meanwhile, value of *r* was –0.947 which showed that relationship between pH range and yield of ethanol was strong negative linear relationship. This is because, according to Bluman (2014), the value of *r* was closer to –1. This relationship was the strongest than relationship in the first, second and third evaluations. Next, the critical values were obtained. Then, the *t* value was obtained at –5.106. Since *t* value was fallen in critical region, the null hypothesis was rejected. Hence, there was significance relationship between pH range and yield of ethanol. Thus, it is the only significance relationship than the first, second and third evaluation.

Thus, the equation of regression line could be determined because value of *r* was significant. After using formula in Figure 3.8, the equation of regression line at $y' = 7.186 - 0.717x$ was obtained. When value of *x* was equal to pH 4.0, then, value of *y'* was equal to 4.318% for yield of ethanol while value of *y'* was equal to 2.167% for yield of ethanol if the value of *x* was equal to pH 7.0. Then, the line was connected between plotted point (4.0, 4.318) and (7.0, 2.167) as showed in Figure 4.4. After that, the standard error of estimate or standard deviation of observed values about the predicted values could be able to determine where its value of error computed was 0.436.

4.2.2 Determination in Yield of Ethanol Obtained Cellulose Which is Extracted from *Leucaena leucocephala* Seeds (CLLS) and Non-Extracted *Leucaena leucocephala* Seeds (LLS)

The yield of liquid still could be obtained directly from analysis done by using GC/MSD although there were no products obtained after purification step for the real study of cellulose which is extracted from *Leucaena leucocephala* seeds (CLLS) and non-extracted *Leucaena leucocephala* seeds (LLS) and hence, it was unable to compute manually the yield of liquid by using formula in Figure 3.3. Thus, both quantitative and qualitative analyses for the real study were done simultaneously by using GC/MSD and achieved the results showed in Figure 4.5.

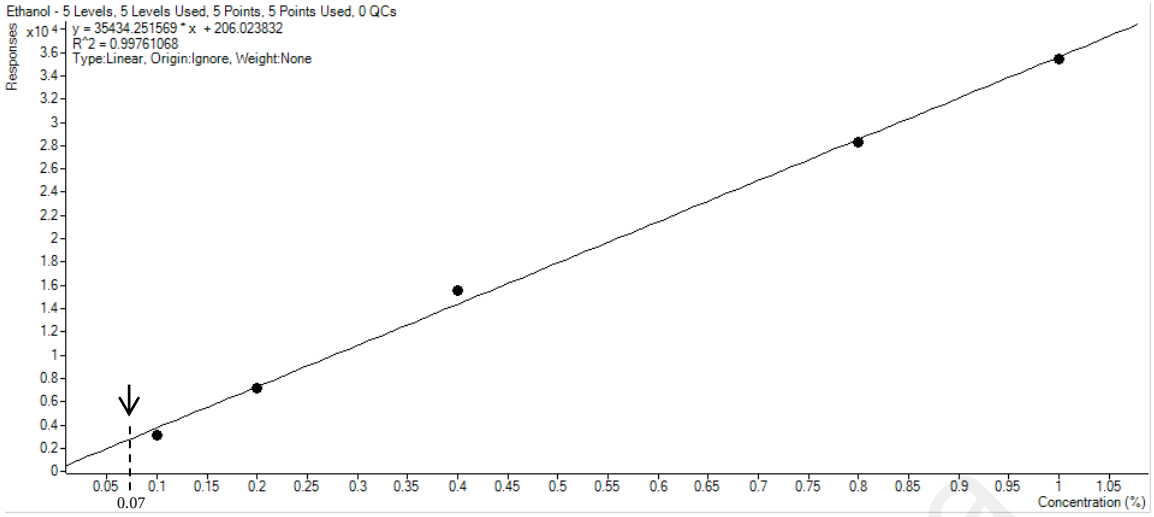

Figure 4.5: Calibration curve for yield of ethanol obtained from amount of CLLS and LLS

Graph in Figure 4.5 was plotted by using Gas Chromatograph (GC) software to generate calibration curve. The graph showed that both amount of CLLS and amount of LLS obtained the same yield of ethanol at 0.07%. The similar yield of ethanol was because CLLS and LLS were originated from the same *Leucaena leucocephala* species. The yield of ethanol from both CLLS and LLS was obtained by using GC/MSD analysis with same optimum parameters as experiments of evaluation which were 3 days of fermentation, 20 g amount of CLLS and LLS respectively, 3 g amount of yeast and pH range between 4.0 – 4.9. The yield of ethanol from cellulose obtained from *Leucaena leucocephala* seeds was lower than the yield of ethanol from free cell of corn stem ground tissue which was at 4.91%. The study on corn stem was employed by Vučurović, et al. (2009).

On the other hand, there was no linear relationship between CLLS and LLS with yield of ethanol because value of *R* was equal to 0.000 as both study achieved same yield of ethanol. Since $\alpha = 0.05$ and there were $4 - 2 = 2$ degrees of freedom, numerator while there was $4 - 2 - 1 = 1$ degree of freedom, denominator, the critical value obtained from Table 3.2 was 199.5. Since, the value of *F* was obtained at 0.000, the null hypothesis was accepted. Hence, there was no significance relationship between CLLS and LLS with yield of ethanol. When the value of *R* was not significant, the multiple regression equation *y'* was also not been able to determined. However, the yield of ethanol for amount of CLLS and amount of LLS are lowest than the first, second, third and fourth evaluations. The lowest yield of ethanol is because the yeast, *Saccharomyces cerevisiae* has no ability to degrade cellulose components. Hence, ethanol cannot be obtained directly from cellulose (Cho et al., 1999). However, the statement stated by Cho et al. (1999) is argued by Sun and Cheng (2002) including Szczodrak and Fiedurek (1996) as they reported that conversion of cellulose into ethanol directly is feasible if the cell surface of yeast is modified.

4.2.3 Relationship between Experiments of Evaluation and Yield of Ethanol

On the other hand, optimum yield of ethanol from the first, second, third and fourth evaluation as well as the real study on both amount of CLLS and LLS were tabulated as shown in Table 4.5.

Table 4.5: Overall evaluation experiments for the optimum yield of ethanol

Parameters / Evaluation	Days of Fermentation	Amount of Glucose, g	Amount of Yeast, g	pH Range	Yield of Ethanol, %
First	3	6	5	None	5.43
Second	3	20	5	None	3.65
Third	3	20	3	None	1.78
Fourth	3	20	3	4.0 – 4.9	4.24
Real Study	3	20	3	4.0 – 4.9	0.07

Based on Table 4.5, yield of ethanol was decreased until 3 g amount of yeast was used with no pH were applied in the third evaluation. Then, it was continued decreased in real study with the same paramaters which was used in the fourth evaluation. However, yield of ethanol increased sharply for the fourth evaluation. Nonetheless, the highest optimum yield of ethanol achieved for the first evaluation. Scatter graph in Figure 4.6 was then plotted based on Table 4.5.

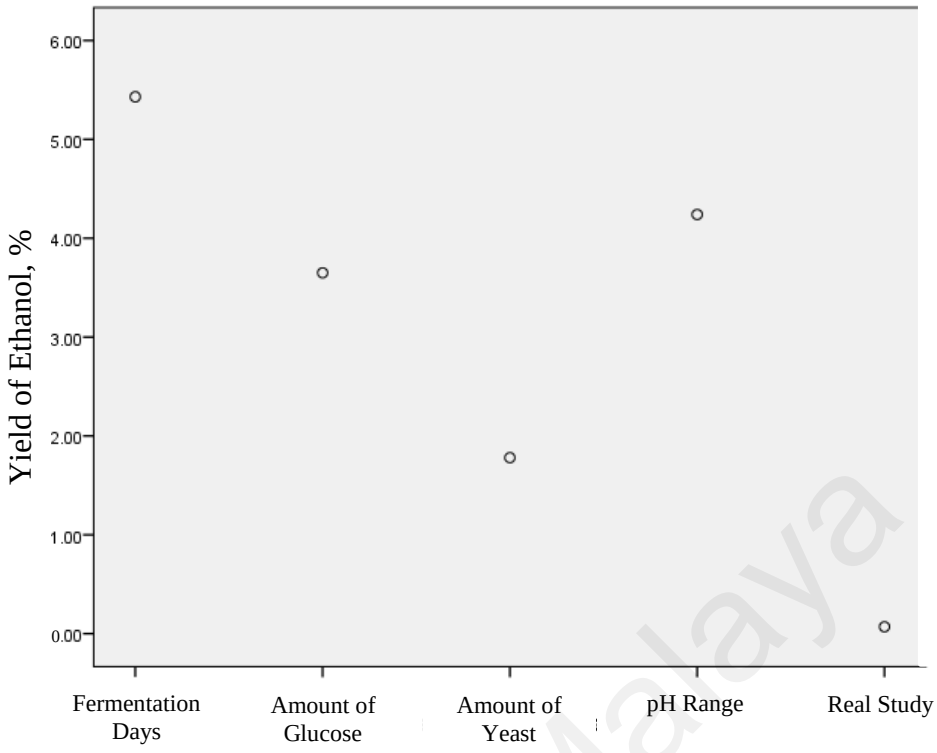

Figure 4.6: Relationship between optimum yield of ethanol and experiments of evaluation

Based on Figure 4.6, the quadratic fit line was drawn on graph by using SPSS and proved that optimum yield of ethanol was decreased from evaluating days of fermentation until real study CLLS and LLS. Hence, the relationship between CLLS and LLS with yield of ethanol was the weakest or no linear relationship than the first, second, third and fourth evaluations. This is because cellulose obtained from *Leucaena leucocephala* seeds could not be able to convert directly into ethanol (Cho et al., 1999). However, yield of ethanol from evaluation in days of fermentation until the real study would increase if high amount of yeast is used with its modified DNA and cell surface as well as high amount of cellulose obtained from *Leucaena leucocephala* seeds with low pH.

Meanwhile, there was no linear relationship between experiments of evaluation as well as real study with yield of ethanol because value of multiple correlation coefficient, *R* was equal to 0.000. Since $\alpha = 0.05$ and there was $5 - 4 = 1$ degrees of freedom, numerator while there was $5 - 4 - 1 = 0$ degree of freedom, denominator, the critical value obtained from Table 3.2 was null value. Since the value of *F* was obtained at 0.000, the null hypothesis was accepted. Hence, there was no significance relationship between experiments of evaluation including of real study with yield ethanol. Thus, there is no determination in the multiple regression equation.

4.3 Qualitative Analysis

After distillation, the liquid samples from all experiment of evaluation except for the real study was tested by using GC/MSD where this chromatograph was employed to prove the presence of ethanol. Based on Figure 4.7, the retention time of ethanol for the first evaluation by using GC/MSD was approximately at 1.586 which the highest than 2-methyl-1-propanol and methyl-silane in descending order where both compounds showed retention time at 2.081 and 3.117 respectively. However, there was an unknown retention time for unknown compound before retention time of ethanol was being detected. Besides that, GC/MSD also proved that there was presence of ethanol for the second evaluation by measuring retention time (Figure 4.8) which was at 1.548 while the retention time for both methyl-glyoxal and isopropyl alcohol was 1.737 and 2.051 respectively where isopropyl alcohol had the lowest retention time.

Furthermore, the presence of ethanol for the third evaluation was also proved by GC/MSD and displayed retention time (Figure 4.9) at 1.529. On the other hand, the retention time at 2.053, 1.760 and 1.447 were displayed by isopropyl alcohol, 2-methyl-1-propanol and unknown compound respectively in ascending order but these results were still the lowest than retention time of ethanol. In addition, GC/MSD also proved that there was presence of ethanol obtained from the fourth evaluation as this chromatograph showed the retention time of ethanol at 1.525 (Figure 4.10). This retention time was the highest than the unknown compound which was at 1.446 retention time and 1-3 butanol, 3-methyl which was at retention time 2.060 in descending order.

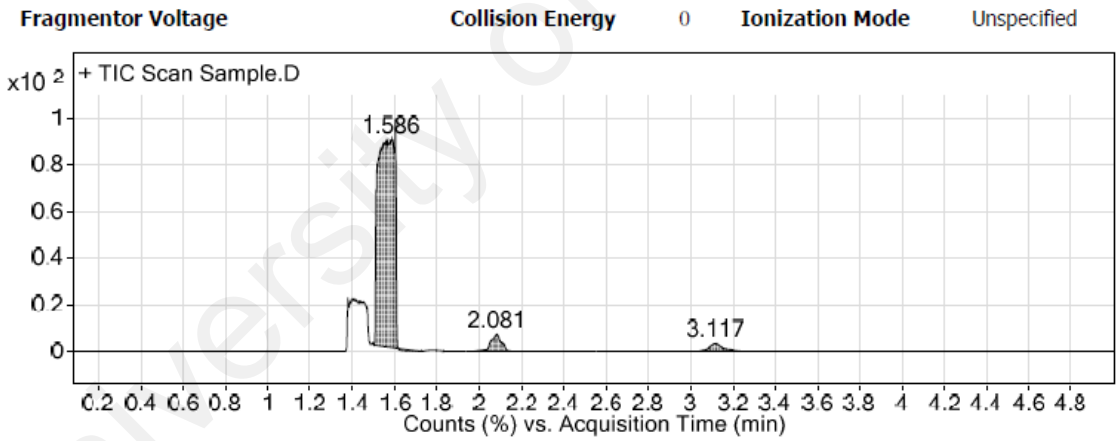

Figure 4.7: Retention time of ethanol for optimum days of fermentation

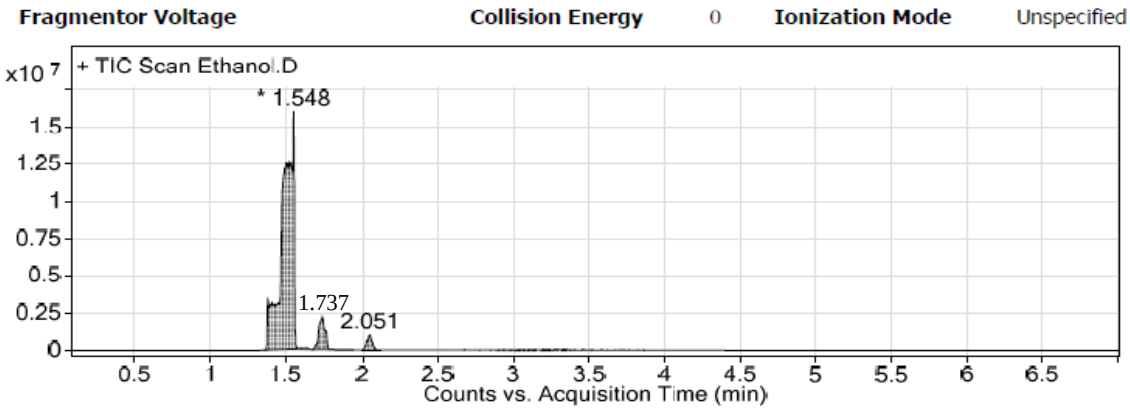

Figure 4.8: Retention time of ethanol for optimum amount of glucose

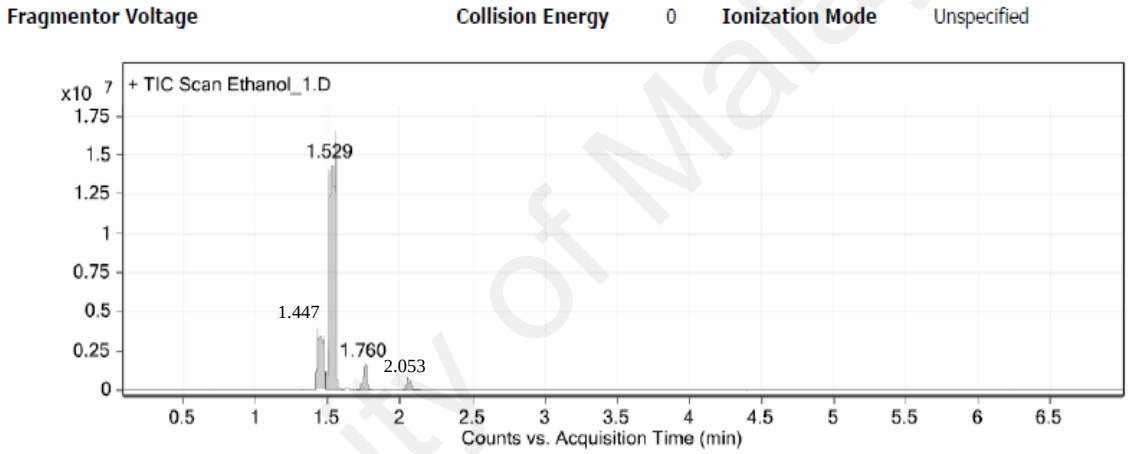

Figure 4.9: Retention time of ethanol for optimum amount of yeast

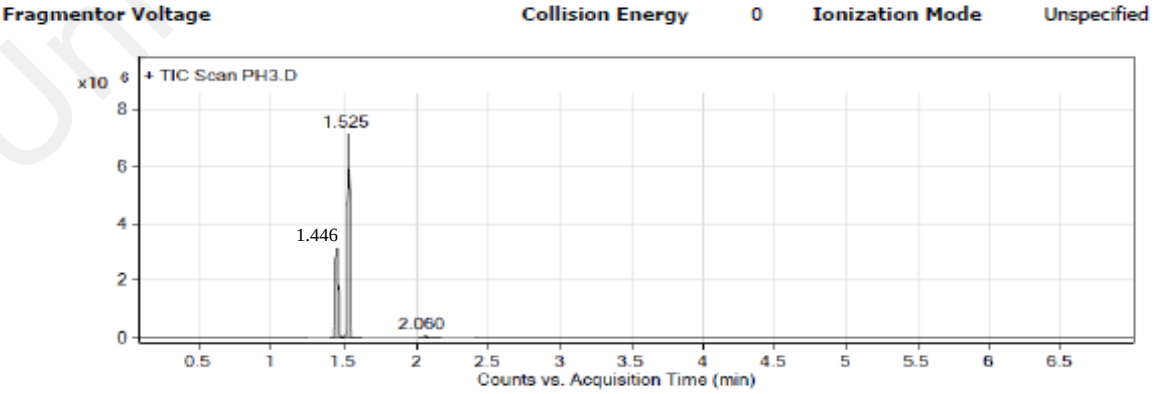

Figure 4.10: Retention time of ethanol for optimum pH range

CHAPTER 5: CONCLUSION

The aim of the study is to determine the optimum parameters for the conversion of cellulose obtained from *Leucaena leucocephala* seeds into bioethanol. The parameters studied which are known as independent variables are days of fermentation, amount of glucose, amount of yeast and pH range. These parameters are firstly needed to evaluate in determining the optimum results before applying them in both real study of cellulose which is extracted from *Leucaena leucocephala* seeds (CLLS) and non-extracted *Leucaena leucocephala* seeds (LLS). The highest yield of ethanol which is known as dependent variable would be the optimum parameters. The results obtained for optimum parameters in the experiments of evaluation are 3 days of fermentation, 20 g of glucose, 3 g of yeast and pH range between 4.0 – 4.9.

Meanwhile, the results obtained for yield of ethanol from both CLLS and LLS are similar which is at 0.07% by using optimum parameters with the help of non-genetically engineered DNA and non-modification on cell surface of *Saccharomyces cerevisiae*. There is low yield of ethanol because cellulose obtained from seeds of *Leucaena leucocephala* is converted directly into ethanol without modify the surface of yeast cell and DNA of yeast. The statement on using non modified yeast is agreed by Szczodrak and Fiedurek (1996) including Sun and Cheng (2002). The low yield of ethanol from CLLS and LLS is also because a weak relationship between both CLLS and LLS with yield of ethanol.

This statement is proved by using SPSS analysis where none of multiple linear correlation coefficient, *R* is achieved. However, before study on CLLS and LLS is carried out, the strongest relationship between pH value and yield of ethanol in evaluation of pH range is achieved as its linear correlation coefficient, *r* is at –0.947. It is the strongest relationship than –0.831, 3.145, –0.433 and 0.000 for evaluation in days of fermentation, amount of glucose, amount of yeast and real study with yield of ethanol respectively. This is because, according to Bluman (2014), the closer the value of *r* with –1 or +1, the stronger the relationship between one independent and one dependent variable while the closer the value of *R* with +1, the stronger the relationship between multiple independent variables and one dependent variable.

The study is continued and showed that the relationship between pH value and yield of ethanol in evaluation of pH range is the only significant while there is no significance relationship in other evaluations including the real study as, according to Bluman (2014), the value of *t* value is at –5.106 which is fallen in critical region. The results on the strongest and the most significance relationship in evaluation of pH range concluded that the highest yield of ethanol would achieved by using amount of glucose instead of cellulose obtained from *Leucaena leucocephala* seeds with controlling of pH range because according to Cho et al. (1999), cellulose could not be able to convert directly into ethanol.

Nonetheless, the hypothesis made on this study in obtaining the highest yield of ethanol from CLLS and LLS by using the optimum of fermentation days, amount of glucose, amount of yeast and pH range was argued with the study implemented as there is the weakest relationship between CLLS and LLS with yield of ethanol than other experiments of evaluation. Another reason is the ethanol could not be able to obtained directly from both extracted and non-extracted of *Leucaene* seeds (Cho et al., 1999). The way to overcome this problem is by hydrolyzing cellulose into glucose initially (Sreenath and Jeffries, 2000) before ethanol produced from fermentation process (Sun and Cheng, 2002; Mussatto et al., 2008).

Although the yield of ethanol from both extracted and non-extracted *Leucaena leucocephala* seeds which is at 0.07% and lower than corn stem which is studied by Vučurović et al. (2009) where its yield of ethanol is at 4.91%, ethanol produced from lignocellulose of Leucaena seeds still could be potentially become an alternative fuel (Hahn-Hägerdal et al., 2006) as it could be a renewable source which has an ability to replace non-renewable sources (Gray et al., 2006) with the recommendations. The recommendation for future work would be a study on conversion from cellulose obtained from seeds of *Leucaena leucocephala* into ethanol directly without modify the surface of yeast cell and DNA of yeast. This statement is agreed by Szczodrak and Fiedurek (1996) as well as Sun and Cheng (2002) but with modification made on cell surface of the yeast.

It is also recommended that the future work had to use recombinant DNA or genetically engineered of yeast or *Saccharomyces cerevisiae* as stated by Sun and Cheng (2002) or microorganisms to convert both hexoses and pentoses into ethanol which is stated by Dien et al. (2003) as well as Jeffries and Jin (2004). In the meantime, the seeds of *Leucaena leucocephala* species contained cellulose which is surrounded by hemicellulose and hence, *Leucaena leucocephala* has lignocellulose components. Thus, the minimal in degradation of sugar during the completely breaking down of hemicellulose and cellulose including the competently in fermentation of sugars are necessary in order to produce high amount of ethanol (Hahn-Hägerdal et al., 2006).

Furthermore, the amount of cellulose obtained from *Leucaena lecucocephala* seeds and amount of yeast were also needed to being amplified at scale industry with maintaining low pH. The modification DNA of yeast and surface of yeast with the big scale of all parameter were then applied in fermentation process for evaluation in days of fermentation, amount of glucose, amount of yeast, pH range including real study on CLLS and LLS. Hence, these recommendations with, according to Hahn-Hägerdal et al. (2006), the combination between cellulose and other sugars-based ethanol would succeed in the industrial scale for production of fuel ethanol.

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