INVESTIGATING THE POTENTIAL OF USING *MORINGA OLEIFERA* HUSKS FOR BIOETHANOL PRODUCTION

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ABSTRACT

This study is mainly focusing on ethanol production from *Moringa oleifera* husks, which is an agricultural waste with no appreciable value to industries or competitive use as food. Such waste presents high concentration of carbohydrates, thus it can be viewed as a potential raw material for bio ethanol production. It was good to introduce to others the advantages or uses of husks instead of being thrown away or burnt and to prove zero waste from this plant. Hence, the objective of this research was to evaluate the feasibility of ethanol production by fermentation of *Moringa oleifera* husks by *Saccharomyces cerevisiae.* Batch fermentation studies were performed with different yeast concentration. The methods used included acid hydrolysis with 5% H_2SO_4, followed by the fermentation with *Saccharomyces cerevisae.* Yeast concentration was evaluated at 1, 3 and 5 g/L and fermentation temperature was kept constant at 30^0C. After that, a simple fractional distillation was done to get the final product from the fermented broth. The yield of ethanol was analysed by using gas chromatography analyzer in terms of ethanol concentration (g/L). Ethanol yield from *Moringa oleifera* husks was maximum at 12^{th} hour with ethanol concentration of 4.9747 g/L. This maximum yield was from the fermented broth with yeast concentration of 1 g/L. It was observed that fermentation yield decreased with an increase in yeast concentration and fermentation time. However, the results indicated that the agricultural waste such as *Moringa oleifera* husks could be considered as a potential lignocellulosic material for production of fermentable sugars related to bio ethanol production.

ABSTRAK

Kajian ini terutamanya memberi tumpuan kepada pengeluaran etanol daripada *Moringa oleifera* sekam, yang merupakan sisa pertanian yang tidak mempunyai nilai kepada industri atau penggunaan kompetitif sebagai makanan. Sisa seperti ini mengandungi kepekatan karbohidrat yang tinggi, oleh itu ia boleh dianggap sebagai bahan mentah yang berpotensi untuk pengeluaran bio etanol. Hal yang demikian adalah baik untuk memperkenalkan kepada orang lain tentang kelebihan dan kegunaan sekam selain daripada dibuang atau dibakar. Oleh itu, objektif kajian ini adalah untuk menilai kemungkinan pengeluaran etanol oleh penapaian *Moringa oleifera* sekam oleh *Saccharomyces cerevisiae*. Kajian penapaian telah dijalankan dengan kepekatan yis berbeza. Kaedah-kaedah yang digunakan termasuk hidrolisis asid dengan 5% H_2SO_4 dan diikuti oleh penapaian dengan *Saccharomyces cerevisae*. Kepekatan yis telah dinilai pada 1, 3 dan 5 g / L dan suhu fermentasi malar pada 30^0C. Selepas itu, penyulingan dilakukan untuk mendapatkan produk akhir. Hasil etanol dianalisis dengan menggunakan analisis kromatografi gas dari segi kepekatan etanol (g / L). Hasil etanol daripada *Moringa oleifera* sekam adalah maksimum pada jam ke-12 dengan kepekatan etanol 4.9747 g / L. Hasil maksimum ini adalah dari kepekatan yis 1 g / L. Hal ini diperhatikan bahawa hasil penapaian menurun dengan peningkatan dalam kepekatan yis. Walaubagaimanapun, keputusan menunjukkan bahawa sisa pertanian seperti *Moringa oleifera* sekam, boleh dianggap sebagai bahan lignoselulosa yang berpotensi untuk pengeluaran gula beragi yang berkaitan dengan pengeluaran bio etanol.

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LIST OF ABBREVIATIONS

CH_3CH_2OH	Ethanol
CO_2	Carbon dioxide
EPA	Environmental Protection Agency
FFA	Free Fatty Acid
H_2SO_4	Sulphuric acid
KOH	Potassium hydroxide
NaOH	Sodium hydroxide
NAS	National Academic Sciences
US	United State

1 INTRODUCTION

1.1 Background of Study

The energy crisis is one of the most problems facing the sustainability of human civilization. This scene is an extremely complex one with various issues demanding resolution including rising oil cost, environmental, technical, sustainability and security of supplies among others. In addition, demand for petroleum-derived fuels has increased substantially over the past few decades (Goh et al., 2009). As a result, the search for a long-term solution for a reliable and infinite source of energy supply in the future has been a tremendous challenge.

Biomass is one of the alternatives for a reliable and infinite source of energy supply. It has been considered as a second-generation feedstock for production of renewable biological sources. Apart from that, bio-ethanol is one of biomass products. Nowadays, there is a growing interest in using ethanol bio fuel as an alternative transportation fuel. Bio ethanol is nowadays produced from edible sources such as sugar cane and corn which compete with human food (Abbas and Ansumali, 2010). In this direction, it is a challenge for bio ethanol production to use feedstock that would not compete with human food.

There are several plant species such as *Jatropha curcas, Croton megalocarpus, Aleurites moluccana* and *Moringa oleifera* that has potential for the production of bio-energy which completely would not compete with human food (Sangwan and Kibazohi, 2011). It is found that there is less study about the potential of vegetable oil production from *Moringa oleifera*. Hence, the need for more studies on it may help to increase the production of renewable biological products.

Seeds of *Moringa oleifera* have high potential for vegetable oil production. It has been identified among the most promising non-edible oil-bearing seeds for biodiesel production (Martin et al., 2010). Therefore, it is not possible for *Moringa oleifera* to be used in ethanol bio fuel production. For certain research in producing biodiesel, several residues such as glycerol, press cakes and husks are generated. The husks, generated during de-husking of the seeds for obtaining the kernels, generally have low economic value and it is mainly disposed or burnt. The chemical composition of the husk has been studied and it is found that the husks contain sugars or materials that can be converted into sugars which can produce ethanol by undergoing several processes. Hence, by using the seeds of *Moringa oleifera* for biodiesel production, the husks left over can be used for the ethanol bio fuel production. In some cases, the husks are used as solid fuel or as raw materials for activated charcoal production. Furthermore, it can be considered as renewable biological sources for ethanol production which is biodegradable, non-toxic, and not associated with environmental pollution costs.

1.2 Problem Statement

Unlike fossil fuels, bio ethanol is a renewable energy source produced through fermentation of sugars. Ethanol has already been introduced on a large scale in Brazil, U.S and some European countries. It is expected to be one of the dominating renewable bio fuels in the transport sector within the coming 25 years. A dramatic increase demand for bio ethanol production was lead to this study. The major problem with bio ethanol production is the availability of raw materials for the production and also the production cost. Therefore, sugar-based feedstock will be used as the feedstock. As no one has gone through the investigation of *Moringa oleifera* seeds husks as a feedstock to produce ethanol, the writer try to find out whether this seeds husks can be used as a feedstock to produce ethanol or not. In addition, as there are many uses and benefits of *Moringa oleifera* plant, the writer tries to prove of zero waste from this plant.

1.3 Objective of the Study

The objective of this study is to investigate the possibility of producing ethanol from *Moringa oleifera* seeds husks.

1.4 Scope of the Study

For this research, the scope will be limited to investigate the production of ethanol and also to find the alternative fuel from the waste of *Moringa oleifera* seeds husk, which is left over after the use of seeds in many industries such as in producing biodiesel and coagulant for water treatment.

2 LITERATURE REVIEW

2.1 Fossil Fuels as a Source of Energy

Energy is the backbone of the world economy. Fossil fuels, particularly petroleum fuels have been and still are the major sources of fuel for powering, among others, transportation vehicles, agricultural machinery and power generation. The rate of petroleum reserve discovery is declining while energy demand keeps increasing. Fossil fuel is a limited resource, the global oil peak production was predicted around 2010, to be followed by a peak or plateau of gas, hence a need to prepare for the transition to declining oil and gas supplies (Campbell, 2005).

The United States desperately needs a liquid fuel replacement for oil in the future. The use of oil is projected to peak about 2007 and the supply is then projected to be extremely limited in 40-50 years (Duncan and Youngquist, 1999; Youngquist and Duncan, 2003; Pimentel et al., 2004). Hence, the search for a long-term solution for a reliable and infinite source of energy supply in the future has been a tremendous challenge. Alternative liquid fuels from various sources have been sought for many years.

2.2 Biomass as a Renewable Biological Energy Sources

The renewable energy sector is seeing increased growth especially because of the challenges to overcome the depleting of fossil fuel resources and also the changing governmental legislations aimed at improve energy security and curb greenhouse gas emissions. Recently, much interest has been directed towards bio fuels from biomass. Most notably was the call by the US government seeking alternative energy sources citing bio fuels to reduce its reliance on fossil fuels (The White House, 2007). In addition, US being heavily dependent on oil imports and by far the largest consumer of petrol in the world (The Economist, 2007). Other countries are already well underway into their bio fuels programmes like Brazil in which around 40% of the total fuel used in cars is bio ethanol (Wikipedia, 2008). Countries like Sweden and India are seeing strong government support in developing their bio fuel programmes with various legislations providing gasohol mandates and consumer incentives (European Commission, 2005).

Biomass energy is defined as any organic materials that can be burned and used as a source of fuel (Biomass Energy, n.d.). Biomass is one of the alternative ways for a reliable and infinite source of energy supply. One of the major advantages of biomass energy as a source of fuels is its renewability. With approximately 140 billion metric tons of biomass produced every year and growing, we could see biomass fuels replacing fossil fuels in the near future as they deplete, further increasing demand for a cheap, efficient renewable fuel source (Biomass Energy, n.d.).

2.3 Ethanol

Ethanol also called ethyl alcohol or grain alcohol is a clear, colourless liquid with a characteristic, agreeable odour. In dilute aqueous solution, it has a somewhat sweet flavour, but in more concentrated solutions it has a burning taste. Ethanol, CH_3CH_2OH, is an alcohol, a group of chemical compounds which molecules contain a hydroxyl group, (–OH), bonded to a carbon atom (Shakhashiri, 2009). Figure 2.1 shows the ethanol structures.

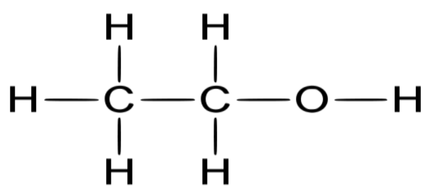

Figure 2-1: Ethanol Structure

Ethanol has been made since ancient times by the fermentation of sugars. All beverage ethanol and more than half of industrial ethanol is still made by this process. Simple sugars are the raw material. Zymase, an enzyme from yeast, changes the simple sugars into ethanol and carbon dioxide. The fermentation reaction, represented by equation (1).

$$C_6H_{12}O_6 \longrightarrow 2\ CH_3CH_2OH + 2\ CO_2 \text{ ------------------------ } (1)$$

It is actually a very complex, and impure cultures of yeast produce varying amounts of other substances, including glycerine and various organic acids. In the production of beverages, such as whiskey and brandy, the impurities supply the flavour. Starches from potatoes, corn, wheat, and other plants can also be used in the production of ethanol by fermentation. Table 2.1 shows the physical properties of ethanol (Ethanol, 2012).

Table 2-1: Physical properties of Ethanol

Properties	**Criteria**
Molecular formula	C_2H_6O
Molar mass	46.07 g mol $^{-1}$
Exact mass	46.041864814 g mol $^{-1}$
Appearance	Colourless liquid
Density	0.789 g/cm^3
Melting point	-114 ^oC, 159 K, -173 ^oF
Boiling point	78 ^oC, 351 K, 172 ^oF

2.4 Ethanol Bio fuel Production

Bio ethanol is a domestically produced liquid fuel made from renewable plant resources known as biomass. Bio ethanol can be produced from any biomass that contains sugars or materials that can be converted into sugars such as grains that contain starch. Traditional biomass is estimated to contribute around 11% of the world’s energy demand (Energy Information Administration, 2006). The increasing trend in the world’s petroleum consumptions translates into similar increasing trend in ethanol demand (Abbas and Ansumali, 2010). This is because ethanol is a desirable fuel additive as it allows fuel to burn more cleanly and lowers greenhouse gas emissions. A worldwide interest in the utilization of bio ethanol as an energy source has stimulated studies on the cost and efficiency of industrial processes for ethanol production (Tanaka, 2006). The top ten bio ethanol producers are presented in Table 2.2 (Balat et. al, 2008).

Table 2-2: The top ten bio ethanol producers (billion gallons)

Country	2004	2005	2006
USA	3.54	4.26	4.85
Brazil	3.99	4.23	4.49
China	0.96	1.00	1.02
India	0.94	0.45	0.50
France	0.22	0.24	0.25
Germany	0.07	0.11	0.20
Russia	0.20	0.20	0.17
Canada	0.06	0.06	0.15
South Africa	0.11	0.10	0.10
Thailand	0.07	0.08	0.09

However, ethanol contributes to air pollution problems when burned in automobiles. Major air and water pollution problems are also associated with the production of ethanol in the chemical plant. The Environmental Protection Agency (2002) has issued warnings to ethanol plants to reduce their air pollution emissions or to be shut down. Therefore, it is our responsibility to find an alternative for a better and much safer ethanol production that will never gives any negative impacts to the environment and people as well.

Nowadays, bio ethanol is produced by using ready and cheaply available agricultural raw materials such as sugar cane beet, maize husk, rice husk and many more. In addition, current studies focused on the production of bio ethanol from these raw materials using *Sacharomyces cerevisie* as fermentation agent. Results obtained indicate that, as the concentration of glucose increases, ethanol concentration also increases (Cheng et al., 2007). Fermentation was carried out for the hydrolyzed samples using *Sacharomyces cerevisie* at room temperature for seven days of incubation. Primary distillation of fermented samples was carried out in Rotary vacuum evaporator at 80^{o}C. Amount of ethanol production from different raw materials was presented in Table 2.3 (Prasad et. al, 2009).

Table 2-3: Volume of ethanol produced from different raw materials

Sample No.	Sample	Volume of extract before distillation (mL)	Volume of extract after distillation (mL)	Volume of Bioethanol (mL)
1	Potato	250	221	29
2	Sweet potato	250	216	34
3	Cassava	250	219	31
4	Fruit extract	250	211	39
5	Boiled rice water	250	226	24
6	Rice husk	250	233	17
7	Rice straw	250	238	12
8	Wood bark	250	241	9
9	Sugar cane beets	250	203	47
10	Waste paper	250	232	18
11	Saw dust	250	239	11
12	Coconut pith	250	245	5
13	Groundnut waste	250	231	19
14	Leaf litter	250	243	7
15	Maize husk	250	231	19

From Table 2.3, it shows that further work can be carried out to evaluate the economic potential of bio ethanol production from biomass. The result also shows that ethanol production from cellulosic biomass is becoming increasingly popular.

Furthermore, corn is one of the examples of agricultural raw material for ethanol production, but cannot be considered to provide a renewable energy source. From previous research, stated that U.S. corn production causes more total soil erosion than any other U.S crop (Pimentel et al., 2005; National Academic Sciences, 2003). In addition, corn production uses more herbicides and insecticides than any other crop produced in the U.S. Thereby, causing more water pollution than any other crop (NAS 2003). Therefore, it is of great importance to find another resource for ethanol production. From Table 2.3, it can be noticed that rice husk and other wastes is one of the cellulosic feedstock for ethanol bio fuel production on the ground of its abundance.

Rice husk which is part of the rice paddy is a by-product of the rice milling process. Global production of rice husk is very significant and this presents an attractive opportunity to utilise such waste material for further processing particularly for the conversion into bio ethanol (Abbas and Ansumali, 2010). In addition, its composition is very suitable for the production of ethanol as it composed of sugars that can be converted into ethanol. Compositions of main constituents of rice husk are tabulated in Table 2.4.

Table 2-4: Composition of main constituents of rice husk

			Composition (%)		
Constituents	1*	2*	3*	4*	Average
Cellulose	35	35.62	28.7	34.4	33.43
Hemicelluloses	25	11.96	17.7	29.3	20.99
Lignin	20	15.38	18.4	19.2	18.25
Ash/silica	17	18.71	17.0	17.1	17.45

1*: Usha and Ghose TK (2007)

2*: Saha et. al (2005)

3*: Park et. al (2004)

4*: Williams and Nugranad (2000)

From Table 2.4 above and by looking to the composition of rice husks, it can be concluded that the husk contains high concentration of glucose. This for sure can produce high concentration of ethanol. Besides that, by comparing this with *Moringa oleifera* husks, it is found that the chemical content of the *Moringa oleifera* husk is quite similar to the rice husk which is composed of cellulose, hemicelluloses, xylan, arabinan, protein, and ash. The most important contents are cellulose and hemicelluloses which are sugars that can be converted into ethanol through several processes.

2.5 *Moringa oleifera Seeds Husks for Production of Ethanol*

Moringa oleifera is a member of Moringaceae family, grows throughout most of the tropics, it is drought-tolerant and can survive in harsh and fertile land (Francis et. al, 2002). The plant starts bearing pods 6-8 months after planting and reaches an average of 3.0 tons of seed per hectare per year (Francis et. al, 2002). This plant has been identified as the most promising non-edible oil-bearing seeds for biodiesel production in Cuba. During the biodiesel production several residues such as press cake, husks and glycerol are generated. Previous research revealed that the husks of *Moringa* can be considered as potential substrates for ethanol production due to its high cellulose content which is approximately 30% (Martin et. al, 2010). The investigation of the chemical composition of the husks aims to assess their potential as feedstock for ethanol production. Figures 2.2, 2.3, 2.4 and 2.5 show the *Moringa oleifera* tree, pods, seeds and husks, respectively.

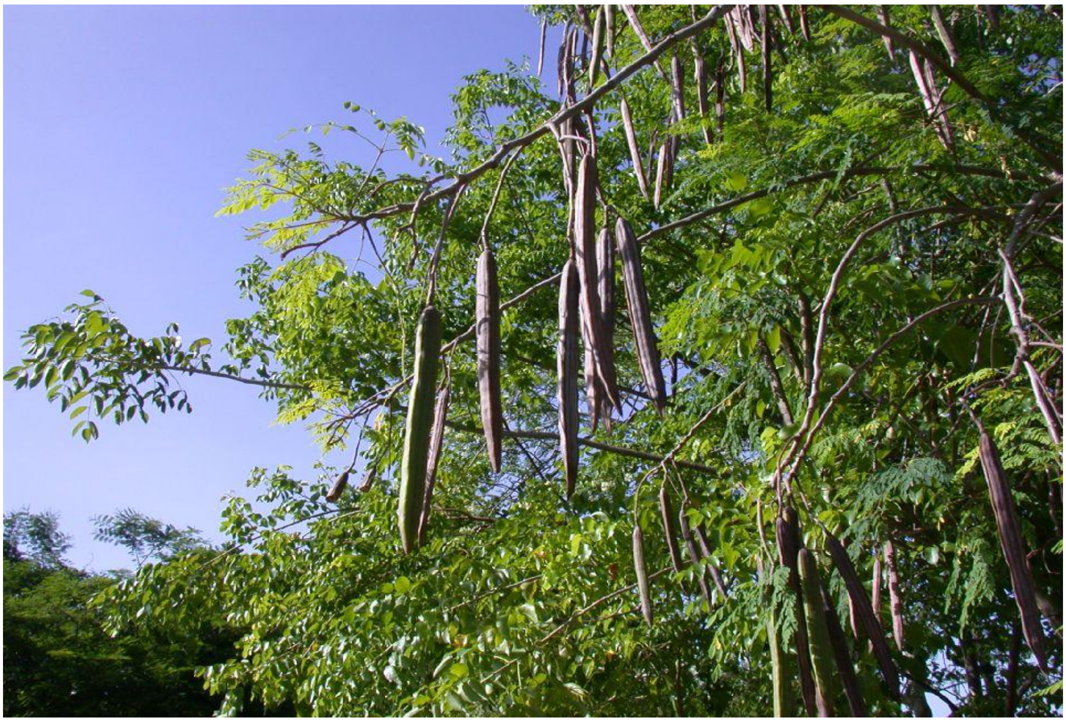

Figure 2-2: *Moringa oleifera* tree

(Source : http://www.plantsystematics.org/imgs/kcn2/r/Moringaceae_Moringa_oleifera_1287.html)

Figure 2-3: *Moringa oleifera* pod

(Source : http://www.themoringa.com/moringa-oleifera-tree/moringa-fruits)

Figure 2-4: *Moringa oleifera* seeds

(Source : http://www.alibaba.com/productfree/106503767/Moringa_Oleifera_Seeds.html)

Figure 2-5: *Moringa oleifera* seeds husks (manually de-husked)

Figure 2-6: Moringa oleifera seeds husks (mechanically de-husked)

Table 2.5, 2.6 and 2.7 shows *Moringa oleifera* physico-chemical properties, yields of vegetable oil and its fatty acid composition, and oil properties respectively.

Table 2-5: *Moringa oleifera* physico-chemical properties

Properties	**Criteria**
Density (kg/m^3)	877.2
Kinematic viscosity at 40 ^{o}C (mm^2/s)	4.830
Cetane No (^{o}C)	67.07
Heating value (MJ/kg)	-
Cloud point (^{o}C)	13.3
Cold filter plugging point	13
Flash point	-
Oxidation stability (h), 110 ^{o}C	2.3

Sources: Barnwal and Sharma (2005), Sanford et. al (2009), and Ramos et. al (2009)

Table 2-6: *Moringa oleifera* yields of vegetable oil and fatty acid composition

Properties	**Criteria**
Oil content (%)	35-40
Palmitic (Hexadecanoic,C16)	6.5
Stearic (n-Octadecanoic,C18)	6.0
Oleic (C18:1)	72.2
Linolenic (C18:2)	1.0
Arachidic C20 (Eicosanoic)	4.0
Any specific fatty acid	Elcosenoic acid 2.0, C22:0, 7.1

Sources: Van Gerpen et. al (2004) and Lewis (2007)

Table 2-7: *Moringa oleifera* oil properties

Properties	***Moringa oleifera* oil**
Specific gravity	0.907
Dynamic viscosity (cP) at 20^{o}C	92.6
Acid value (mgKOH/g)	1.194
Free fatty acid,FFA (%)	0.6
Saponification value	192.3

Source: Kafuku and Mbawara (2010)

Previous research by Abbas and Ansumali (2010) stated that the global quantity of rice husk is shown to be a very attractive biomass for conversion into ethanol bio fuel. The global potential production of bio ethanol from rice husk is estimated herein and found to be in the order of 20.9 to 24.3 Giga Litres per annum, potentially satisfying around one fifth of the global ethanol bio fuel demand for 10% gasohol fuel blend. According to the research done by Martin et. al (2010), the composition of the *Moringa* husk is similar to the rice husk which showed in Table 2.8 and it is good to do further study on the *Moringa oleifera* seeds husks as a potential feedstock for the ethanol production. This study can contribute to the new renewable biological sources of energy that may overcome the challenges of depleting fossil fuel resources in the world.

Table 2-8: Comparison of chemical composition between *Moringa oleifera* husk and rice husk

Type of husk **Constituents**	***Moringa oleifera* husk**	**Rice husk**
Cellulose	29.1	35.62
Hemicelluloses	-	11.96
Lignin	39.3	15.38
Ash	4.1	18.71

Based on the comparison of chemical composition for both *Moringa* husk and rice husk, it can be seen that *Moringa* husk has similar characteristics and quantity of chemical content with rice husk even in a small amount. As *Moringa oleifera* husk is not well been investigated as candidates for bio ethanol production, this research will study further more about its potential as a sources of glucose for production of ethanol bio fuel. Rice husk as the renewable feedstock of ethanol production will be the guideline or reference for this research.

Pimentel and Patzek (2005), reported that several physical and chemical factors limit the production of liquid fuels such as ethanol using plant biomass materials. For instance, it includes an extremely low fraction of the sunlight reaching America captured by plant. On average the sunlight captured by plants is only about 0.1% with corn providing 0.25% of sunlight. These low values are in contrast to photovoltaics that capture from 10% or more sunlight than plant biomass. Meanwhile, in Malaysia, this problem does not exist. Therefore, it is an advantage in Malaysia to cultivate *Moringa oleifera* crop and use it for producing renewable energy. In addition, for biodiesel production, there are two problems which are the relatively low yield of oil crops ranging from 1500 kg/ha for sunflower to about 2700 kg/ha for soybean, Sunflower averages 25.5% oil whereas soybeans average 18% oil (Pimentel and Patzek, 2005). The oil extraction processes for all oil crop is highly energy intensive. Therefore, these crops are poor producers of biomass energy. *Moringa oleifera* can be used and replaces this kind of crop in producing biomass energy as with 3000kg/ha with average weight of seeds and 66% husks of the seeds weight is the highest among other crops (Pimentel and Patzek, 2005). Table 2.9 shows the comparison of percentage of chemical content of the husks for different crop (Martin et. al, 2010).

Table 2-9: Chemical content of different husk, % (w/w)

	Moringa	**Trisperma**	**Castor**	**Candlenut**
Cellulose	29.1	14.6	8.1	10.7
Xylan	9.1	9.0	10.4	10.2
Arabinan	1.6	0.0	1.8	0.5
Acetyl group	0.6	1.1	0.6	0.8
Klason lignin	39.3	62.9	45.2	51.6
Unidentified	1.0	6.5	23.8	13.9
Protein	15.2	3.7	6.2	2.3
Ash	4.1	2.2	3.9	10.0

Table 2.9 shows that *Moringa oleifera* husk has high cellulose content which is a source of glucose for fermentative production such as ethanol. Hence, this study may find alternative biofuel from the waste or husk of *Moringa oleifera* in order to overcome the energy supply shortage.

2.6 Ethanol Yield and Production Process

The overall process involves the following steps : a) pretreatment of the *Moringa oleifera* seeds husk, b) hydrolysis of the pretreated material to release reducing sugars and, c) fermentation of the released sugars, to produce ethanol.

Pre-treatment deals with breaking the crystalline structure of the lignocellulose and removes the lignin to expose the cellulose and hemicellulose molecules to facilitate the cellulose hydrolysis by either acids or enzymes. This is important because hydrolysis, which is the next step, can be affected by porosity of lignocellulosic biomass, cellulose fiber crystallinity, lignin and hemicellulose content (Vaithanomsat et. al, 2011). Depending on the biomass material, either physical or chemical pre-treatment methods may be used. Chemical pre-treatment of cellulosic materials is done by using chemicals such as dilute acid, alkali, organic solvent, ammonia, sulphur dioxide, carbon dioxide or other chemicals to make the biomass more digestible by the enzymes. Karimi et. al (2006) reported about chemical pre-treatment method by using dilute H_2SO_4 for ethanol production from rice husk. Therefore, this method can be used to pre-treatment the *Moringa* husk.

The pre-treatment is followed by the hydrolysis of the raw materials. The acid hydrolysis of the cellulosic substrate is an excellent alternative to the enzymatic hydrolysis. Two acid hydrolysis processes are commonly used which are hydrolysis by either dilute acid or hydrolysis by concentrated acid. Most common acid used is sulphuric acid. Megawati et. al (2010) have utilized dilute sulphuric acid (1-9%) for the hydrolysis of rice husk for ethanol production. The major advantage of dilute acid hydrolysis is that it is quicker than concentrated acid hydrolysis and hence can be used as a continuous process. The hydrolyzed material is finally fermented with the help of suitable microorganism to produce ethanol. Different strategies have been employed for the fermentation depending on the raw material and the microorganism (Mishra et. al, 2011).

Since these three steps have been used to produce ethanol from rice husk and since *Moringa* husks have similar chemical contents with rice husk, production of ethanol from *Moringa oleifera* seeds husks by the conversion of cellulose into reducing sugars and fermenting the sugars into ethanol can be done by following the same procedure.

3 METHODOLOGY

3.1 Chemicals, Materials and Apparatus

Several chemicals, materials and apparatus were absolutely needed to run the experiment. Chemicals used were Sulphuric Acid (H_2SO_4) and Sodium Hydroxide (NaOH). Meanwhile, the raw material used was *Moringa oleifera* seeds husks and being fermented using enzymes, *Saccharomyces cerevisiae.* Apart from that, the glassware used were conical flask, beaker, measuring cylinder, glass road, micro centrifuge tube, vial, syringe and filter syringe. Besides, to run the experiment, several equipments were used such as autoclave, stackable incubator shaker, micro centrifuge, vacuum rotary evaporator and gas chromatography.

3.2 Collection and Preparation of Moringa oleifera Seeds Husk

Seeds of *Moringa oleifera* were selected from manually-collected ripened fruits and the seeds were mechanically de-husked.

3.3 Hydrolysis Process

Two hundred and fifty grams of *Moringa oleifera* seeds husks were weighted into five conical flasks, 50 gram in each flask. A volume of 500 ml of 5% H_2SO_4 was measured and poured into each flask. The flasks were covered with cotton wool and wrapped in aluminium foil to prevent vaporization of acid due to heat. After that, the flasks were heated for three hours in the water bath and then autoclaved for 30 minutes at 121^{o}C. The flasks were then allowed to cool at room temperature, then the solution of *Moringa oleifera* husks and 5% H_2SO_4 were filtered through whatman filter paper. The pH value of filtrates was adjusted to 5 or 6 for neutralized the solution to enhance the growth of enzymes during the fermentation process. It was neutralized by adding required amount of sodium hydroxide (NaOH).

3.4 Fermentation Process

The fermentation was carried out for the filtrate of *Moringa oleifera* husks collected from hydrolysis process in order to convert the released sugars into ethanol. The conversion process being accomplished by the enzymes released by *Saccharomyces cerevisiae*. Detoxification of the hydrolyzed material is not necessary as most of the toxins are detoxified due to the pre-treatment and hydrolysis process (heat and chemicals). The fermentation process was done in three closed conical flasks at constant temperature of 30 ^{o}C, agitation rate at 180 rpm, but with different yeast concentration for each flask in a stackable incubator shaker of 1 g/L, 3g/L and 5 g/L, respectively. The process was continued for three days and the samples were taken from each conical flask for every 12 hours. Samples taken were then analyzed by using gas chromatography to analyze ethanol yield.

3.5 Distillation Process

After three days of fermentation process, the ethanol was collected by using the vacuum rotary evaporator. During the process, temperature of 78^{o}C (boiling point of ethanol). Few mL of the distillate was taken for gas chromatography analysis. All data collected were tabulated and discussed in the next chapter.

3.6 Ethanol Determination by Gas Chromatography

3.6.1 Instrument set up and Analytical Procedures

Gas chromatography Agilent Technologies (6890 Series) equipped with a flame ionization detector (FID) was set up to determine ethanol concentrations.

- The column used was HP-INNOWax Polyethylene Glycol (30 m x 250 µm x 0.25 µm nominal
- Oven temperature 70 °C
- Injector and detector temperature 250 °C
- Initial temperature 70 °C held for 4 min, rate of increase 20 °C/min until 120 °C min held for 2 min)
- The carrier gas was helium at a flow rate of 45 ml/min.