The Effectiveness of Vegetable Oil in producing Biodiesel by Esterification Reaction using *R. oryzae* as a biocatalyst

By

Ms. Vidhi Harin Juthani

Dissertation
Submitted in partial satisfaction of the requirements for the degree of
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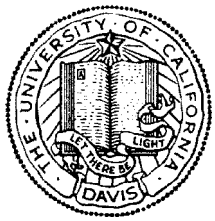

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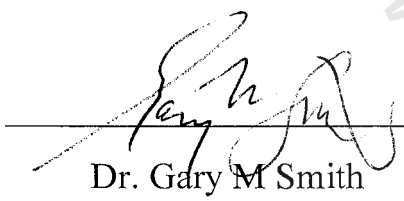

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ABSTRACT

Today, the world is faced with more challenges than one can count and it keeps increasing each passing day. Fuel crisis makes it to the top few on the list and to overcome that challenge, scientists have been carrying out research for a few decades now. Biodiesel is a great alternative to the conventional fossil fuel and has been proven so from time to time. But even to this day, using biodiesel by itself in an automobile engine is not feasible due to its instability and low performance characteristics in cooler regions. There are different ways to approach the production of biodiesel like using strong base or strong acid catalyst that were being used initially when biodiesel was in its primary days. This option gave good results, but proved to be toxic to the system, has issues with retrieving the catalyst and thus turned out to be very expensive. A new approach of using biocatalyst was further explored and lipases were the best options to break down TAG' s present in oil lipids. Initially, commercially purified lipase enzymes from different algae were researched on and *Candida antarctica* (Novozym 435) was considered best in the lot. But, culturing the algae, lipase extraction, purification of lipase added extra cost on the final biodiesel product and thus made it a very expensive alternative. Alongside, a different approach of using whole-cell intracellular catalysts was being studied and *Rhizopus oryzae* IFO4697 was a very good option for biodiesel production. This particular study focuses on process optimization of producing biodiesel using refined soybean oil and methanol as substrates for transesterification reaction using *R. oryzae* whole-cell lipase as a catalyst for the reaction. Soybean oil was chosen primarily because of its abundance in the North American region and it is relatively cheaper. *R. oryzae* IFO4697 was chosen because prior research had proven its efficiency in catalyzing methanolysis reaction for other oils and the fact that we could use the cell directly into the reaction system, without lipase extraction or purification made it very cost effective. During the experiments, methanol: oil ratios were varied to study the effect of methanol on methyl ester yields, lipase concentration was a variable to know the minimum quantity of lipase required to achieve maximum methyl ester yields and *tert*-butanol was added to the reaction system to help make methanol more soluble and negate its effect on lipase activity. Hence, to study how efficiently butanol function, that was also a variable. The results showed that methanol at a molar ratio of 3:1 worked best for the system, lipase concentration of 13% gave the highest methyl ester yields and butanol did not produce the desired effect.

CHAPTER 1

INTRODUCTION

Global warming, greenhouse gases, greenhouse effect, climate talks, alternative fuel sources are few words on the list of popular words in the world today. Several climate talk conferences in the world have proven of no help to save this planet. An expectation of these ever persisting problems disappearing from the face of earth is a mission impossible, but to try and minimize it is something that man can fathom. Man has always tried to find an alternative to his problem, a pen replaced the perishable pencil or an airplane replaced the long hour trips by road. Likewise, an alternative fuel source is an imperative discovery for the world. Vegetable oils, hydrogen power, nuclear energy, hydra power or the most sought after fuel resource of current time's biodiesel. Every country is after biodiesel in order to improve its economy. The Middle Eastern countries till date are ruling the world because of their oil reserves. The world realized that these resources are dwindling dramatically and there is a need for an alternative source.

Biodiesel is the product of a transesterification reaction between a vegetable oil or animal fat with an alcohol to get alkyl esters or biodiesel B100 according to the biodiesel standards ASTM D6751 or European standard EN14214 (Knothe, 2009). The difference in emissions between the conventional petrodiesel and biodiesel are drastically different with the latter being more environmental friendly. The Unites States of America is a leading producer of biodiesel and the government invests a lot in research and development of a more efficient fuel source. Each year the government funds many private and university laboratories to get better methods or a more cost effective result. Success of biodiesel production will mark the success of the nation's economy and will be a little closer to getting out of the long time recession. Following is a table of ASTM standards for biodiesel. This ensures the credibility of biodiesel produced in the country.

Propert	Fest meth..d		L. nits
Flash point (closed cup)	ASTM D43	93 min'	
Alcohol control			
One of the followin: must he met:			
. Methanol content	EN 14110	0.2 max	volume
2. Flash point	ASTM D93	30.0 min	
Water and sediment	ASTM D2709	0.050 max	
Kinematic. viscosit.. 40 C	ASTM	1.9–6.0	mm²/s
Sulfated ash	ASTM	0.020 max	mass
Sulfur'	ASTM D5453	0.0015 max (S15) max (S500)	mass (ppm)
Copper strip corrosion	ASTM Dl	No. 3 max	
Octane number	ASTM	-7 nun	
Cloud point	ASTM D2500	Tepo	
Cold soak filterability	ASTM D7501	360 max[c]	
Carbon residue	ASTM D4530	0.050 max	? mass
Acid value	ASTM D664		nit! KOH/g
Free glycerin	ASTM D6584		mass
Total glycerin	ASTM D6584		may,
Oxidation -stability	EN 14112	3.0 nun	
Phosphorous content	D495 I	0.'101 max	mass
Ilium and potassium. combined	EN 1453	5 max	ppm
ium and magnesium. combined	14538	5 max	ppm
Distillation temperature. Atmospheric equivalent temperature. recovered	ASTM D1160	max	

[a]For all Tables: nun refer, minimum and max refer, to maximum
[b]The limits are for Grade S1.5 and Grade S500 biodiesel. with S15 and S500 referring to maximum allowable sulfur content (ppm)
[c]B100 intended for blending into petrodiesel that is expected satisfactory performance at fuel temperatures at or below −12 C shall. comply with a cold soak filterability limit of 200 s

Table 1: **ASTM** D6751 **biodiesel** fuel standards (Moser, 2011)

1.1 Statement of Problem:

World economies are dwindling due to lack of natural resources and oil is one that tops the charts almost everywhere. Keeping in mind the simple principle of economics of demand and supply, the demand for oil and fuel is exponentially higher than its natural supply. When such a situation arises in the interest of earth, it is better to find alternative methods for the

planet to live. Hence it is imperative that science comes up with a solution that can suffice people's energy needs with sufficient supply.

1.2 Scope:

This research was carried out in a laboratory at University of California, Davis, USA under the kind supervision of Prof. Gary M Smith. All the necessary instruments, materials and reagents were provided for the research by Prof Smith and the university. The set of reactions being carried out were transesterification in the presence of a biocatalyst, followed by the separation of fatty acid methyl esters (FAMEs). These were further analyzed using gas chromatography technique.

1.3 History of Biodiesel:

Biodiesel as a term was invented in 1988 but the use of vegetable oil as a source of fuel dates back to the 1900 (Songstad, 2011). It all began with Rudolf Diesel's invention of diesel engine. Peanut oil was the first vegetable oil used as a source of fuel in World's Fair in Paris when a diesel engine demonstration was held for the first time in 1900 and the engine ran so smoothly that only a few people were even aware of the fact (Knothe, 2001). World War II was a warning bell for the fast developing nations to use a different source of fuel instead of the fast decreasing oil reserves or fossil fuels. Initially, many people were most likely skeptical about the whole issue of using vegetable oil as a fuel source. But eventually more people believed in it and modification to vegetable oil led to the existence of today's biodiesel. In the United States of America, changes were made in their law books in the Clean Air Act Amendments of 1900 and the Energy Policy Act of 1992 which commanded the use of clean or alternative fuels in trucks and buses (Knothe, 2001). With advancement in urbanization, the need for fuel is ever increasing. There are more cars than people on the road and more fuel is used in production plants of various products with increase in demand of goods for living. Biodiesel has a significant place in the world to meet these demands. In addition to the growing urbanization, climate is playing a vital role in reminding mankind of how greatly it is damaging the earth's atmosphere.

Global warming is practically the answer to most of the questions subjected to deteriorating world climate and intensifying natural calamities. Once again, biodiesel comes in play.

1.4 Alternative Methods of Fuel Production:

The direct use of vegetable oils or oil blends is probably considered to be unsatisfactory and impractical for both direct and indirect- injection type diesel engines. This is due to certain parameters like high viscosity, acid composition and free fatty acid content of these oils. Some others also contribute to the problem, like gum formation due to oxidation and polymerization during storage and combustion, carbon deposits and lubricating oil thickening. The efforts were carried out in finding derivatives of these oils to make it a more suitable fuel and that their characteristics can be compared closer to the conventional fuel (Fukuda, 2001). When triglycerides are replaced for diesel fuels, not only high viscosity but low volatility and the polyunsaturated character hinders the efficiency of the triglycerides. As triglycerides are mainly fatty acids, the point of evaporation is too low. Thus, low volatility and as the derivatives of oils may be converted to diglycerides or monoglycerides, the saturation can be lost in the process. Unsaturated fatty acids have low stability.

Several processes are known to have helped overcome this problem to a certain degree. There are three main processes: pyrolysis, micro – emulsification and transesterification (Fukuda, 2001) All these processes alter the composition of the fuel in a way that gives it more stability. According to the need of the engine, these processes can be altered and better products can be achieved.

1.4.1 Pyrolysis:

Pyrolysis refers to a chemical change caused by the application of thermal energy in the presence of an air or nitrogen spurge (Fukuda, 2001). This process is carried out at high range of temperatures ~ 350 - 400°C in a pyrolysis reactor. The sample oils are subjected to this reactor and flash/rapid pyrolysis occurs. Different fractions of oil separate out at different phases. Various compounds like alkanes, alkynes, alkenes, *etc.,* fractionate out in form of vapor. These vapors are further subjected to water-cooled heat exchangers. Consequently, these vapors come out as liquid streams. One forms an aqueous fraction while one comes out as organic fraction.

These streams are separated by decantation and the organic phase is further distilled. Further, the organic fractions are analyzed using techniques like gas chromatography, FITR and are matched with ASTM standards (Lima, 2003).

1.4.2 Micro – emulsification:

Micro-emulsification as the name suggests is a means to stabilize a mixture of immiscible liquids. This method can also be applied to the production of bio fuel to reduce the number of free fatty acids in the oil. It is important to choose the right co-surfactant (Wang, 2008). In a laboratory, Ziejewski *et al.* prepared an emulsion of 53.3% (v/v) alkali-refined and winterized sunflower oil, 13.3% (v/v) 190-proof ethanol and 33.4% (v/v) 1-butanol. This was a nonionic emulsion with the following properties: viscosity, 6.31×10^{-6} m^2/s at 40°C; cetane number, 25; sulfur content, 0.01%; free fatty acids; 0.01% and ash content, less than 0.01%. Better results were achieved when the proportions of 1-butanol were increased. But when an endurance test was performed, irregular injector needle sticking, heavy carbon deposits, incomplete combustion and an eventual increase of lubricating oil viscosity were reported when a similar experiment was carried out by Schwab with soy bean oil (Schwab, 1987). Thus, it can be observed that even this technique of producing fuel has critical limitations which make it a little impractical to put in to use. Micro-emulsification is though quite successful in the pharmaceutical industries (Fukuda, 2001).

1.4.3 Transesterification:

This technique is the most common method to produce biodiesel today. As mentioned earlier, this is a process where animal/plant fat is converted into alkyl esters in the presence of alcohol and a catalyst. This catalyst could either be a strong base, strong acid or a biocatalyst such as a lipase. The type of catalyst used depends on the type of raw material and its properties. There are a few basic variables in the reaction such as reaction temperature, reaction time, ratio of alcohol to oil source, amount of catalyst, the type of oil used and the type of catalyst (Marchetti, 2005). Conventionally, oil used in the production of biodiesel is vegetable oil (soybean, rapeseed, palm, corn) but ideally, these oils are not very favorable. The reason is that a lot of land is used for growing these crops and in turn the final product becomes expensive and

not easily available. It also affects the primary purpose of growing these crops in the first place and that is food. A basic transesterification reaction is displayed in the following figure:

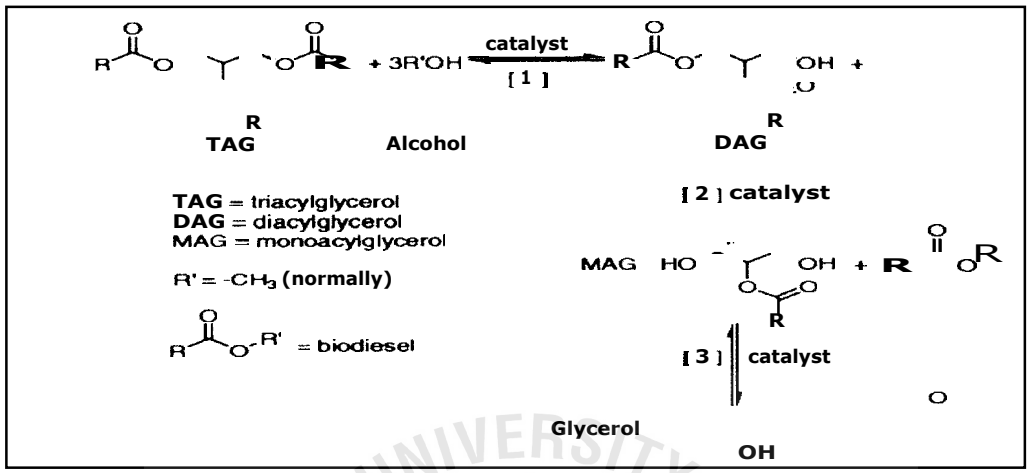

Figure 1: Transesterification of triacylglycerols to yield fatty acid alkyl esters (biodiesel) (Moser, 2011)

There are basically three different types of catalysts:

i. Alkali Catalyst:

Alkali catalysts such as potassium hydroxide (KOH) or sodium hydroxide (NaOH) are used for transesterification. This helps in converting the triglyceride to an alkoxy molecule. A pretreatment is required in this reaction to avoid saponification. An alkali catalyst is preferred over an acid catalyst because it is less toxic and less waste management is needed making it a more desired choice in the industries (Marchetti, 2005).

ii. Acid Catalyst:

An acid catalyst is used in cases where the free fatty acid content of the oil is greater than 2%. It is slower than an alkali catalyst, but gives higher yields. The most common acid catalyst used is concentrated sulfuric acid (Marchetti, 2005). This type of catalyst also generates toxic waste which is difficult to get rid of or involves more processing which increases the cost of fuel.

iii. Lipase Catalyst:

Lipase is an enzyme that hydrolizes lipids. This type of biocatalyst is probably not popular in the conventional production of biodiesel, but is being researched extensively and will soon be

used in practice. So far lipases were believed to be used as catalysts for hydrolysis, alcoholysis and acidolysis. But now they're found to aid in esterification and transesterification too (Marchetti, 2005). Since animal and plant fats both contain lipids; they act as substrates for lipase to break down and aid esterification and transesterification.

The source to get lipase is mostly microorganisms that produce either intracellular lipase or extracellular lipase. If the lipase is synthesized inside the cell, it needs to be extracted and immobilized before adding it to the transesterification reaction. On the other hand, if the microorganism is synthesizing extracellular lipase, the whole cell can be used as a catalyst without extracting the enzyme. This makes the process easier to handle and cost efficient. In addition, it is easier to regenerate the enzyme and more quantities can be cultured in less space. A higher concentration can be added if necessary without the risk of toxicity. Also, the separation of biodiesel becomes easier (Marchetti, 2005).

1.5 Lipase as a Catalyst:

Since alkali catalyst can be expensive and non-reusable, and glycerol disposal could be difficult, and acid catalyst is corrosive to the biodiesel reaction system, lipase is a more preferred option. The enzyme used in the process is either extracted from the microorganism and immobilized or used as a whole cell catalyst as mentioned above. The source of lipases could be bacteria or fungi. The type of enzyme used in a reaction greatly depends on the raw material being used, its fatty acid content, temperature of the reaction, stability of the enzyme, pH of the reaction and alcohol resistance (Fjerbaek, 2009). Some common sources for lipase are *Penicillium citrium, Candida lypolytica, Candida rugosa, Candida antarctica, Aspergillus niger, Aspergillus oryzae* and *Rhizopus oryzae* (Song, 2008).

Conventionally lipases were probably extracted from the cells, separated, purified and immobilized to be used in transesterification reaction to produce biodiesel. This is a greener way but not a cheaper way to produce biodiesel. Instead recent developments in this field lead to the usage of whole cell biocatalyst for the production of biodiesel the fuel. This doesn't only make it easier but also cuts out costs for the purification process and is an environmental friendly way too. To use whole cell catalyst, a source that can translocate its lipase directly in the reaction to hydrolyze the lipids is more feasible.

In the recent past, many researchers have found that *Rhizopus oryzae* is one of the most promising sources of lipase for alternative fuel production through transesterification (Ban, 2001), (Ban, 2002), (Li, 2007). These researchers showed that lipase activity of *R. oryzae* was good and competitive enough as compared to other purified intracellular lipases or commercial lipases. Hence, cost cutting could be achieved using whole cell catalyst in place of the commercial ones. Its activity depended on free fatty acid content, water content in the reaction, amount of phospholipids, temperature and methanol concentration (Ban, 2002) (Li, 2007).

1.6 Mechanism of *Rhizopus Oryzae* Lipase (ROL)

Rhizopus oryzae is a mycelial filamentous fungus that is well known as the causative agent of zygomycosis. It is not a very serious pathogen and hence was not extensively used until recently, it became popular as a whole cell biocatalyst for transesterification reaction to produce biofuel (Hama, 2006). Acyl migration is an important factor in the transesterification reaction because it determines the breakdown of triglycerides to its subsequent smaller fractions to diglycerides and monoglycerides. In this particular case *R.oryzae* has a 1(3) – positional specificity to catalyze this reaction (Li, 2010) (Oda, 2004) (Antczak, 2008). In the methanolysis process, 2 – MG and 1,2 – DG is converted to its corresponding isomer 1-MG and 1,3- DG respectively. This is the result of *R.oryzae*'s 1(3) – positional specific activity of lipase by acyl migration which helps in yielding more methyl esters and improving the efficiency of the reaction. This mechanism is a substantial parameter that determines the success of the reaction and the quality of the product (Li, 2010). Li and his team have proposed a mechanism to support the working of ROL. Following figure shows the mechanism:

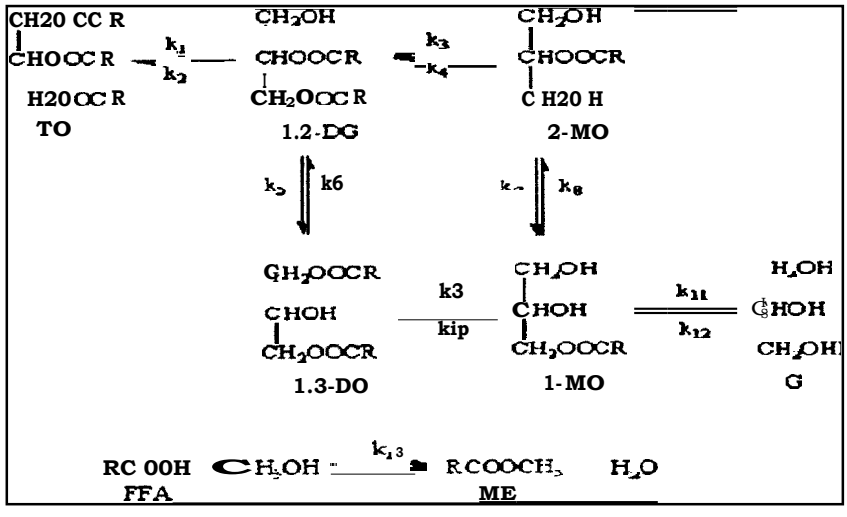

Figure 2: Mechanism of Rhizopus oryzae lipase in methanolysis (Li, 2010)

The figure clearly explains the conversion of triglycerides to diglycerides and eventually to monoglycerides. Further reaction with alcohol leads to the production of methyl esters. This gives an overall picture of how it works. Hama (2006) goes on to find and explain the genetics of this reaction. They performed Western blot analysis and found that *R. oryzae* cells produce two lipases of molecular weight 34kD and 3 1kD also called ROL 34 and ROL 31 respectively. They found that in the interior of the cells, ROL 34 was attached to the cell wall while ROL 31 could be bound to cell wall or membrane. They went on to determine that most of the lipase localization occurs in the cell wall and it varies because of the size of the lipase due to a change in the amino acid sequence (Hama, 2006). This gives an insight on what affects the localization and how it can be modified (if required) to increase efficiency. The difference in the amino acid sequence between ROL 34 and ROL 31 determines the processing site for lipase precursor. The difference lies in their N-terminal sequences which are D-D-N-L-V and S-D-G-G-K for ROL 34 and ROL 31 respectively. Hama (2006) went on to show that substrate related compounds affect the localization of ROL. So if the growth medium contained olive oil or oleic acid, it could affect lipase secretion in the medium. They found out that if olive oil or oleic acid was present in the medium, emission of lipase into the medium was inhibited, while the presence of fatty acids caused a reverse effect and lot of lipase was found in the medium. It is probably good to have olive oil in the growth medium in order to prevent the lipase from leaking out in the growth medium. This can eventually affect methanolysis since a higher concentration of lipase in the immobilized cells would lead to a better transesterification reaction. Hence, if the importance of ROL 34 and ROL 31 is known, the right inducers can be added to get a higher intracellular activity of the enzyme (Hama, 2006).

1.7 Benefits and Limitations of Biodiesel

It is already known that biodiesel is an important tool in sustaining earth's natural resources. People have been trying to optimize the process and up-scaling it to achieve better results. Biodiesel has been known to have a substantial effect on the emission of green house gases (GHG) and it keeps the environment a lot greener. A research carried out on biodiesel suggests that it gives out about 93% more energy to carry out processes than fossil fuels, it reduces the emission of the green house gases by 41% when being compared to the conventional

diesel and it also lowers other air pollutants (Hill, 2006). The authors here are concluding from their research that even though the cost of biodiesel is a few cents more than the regular diesel, if we take the total net emission costs into consideration, then biodiesel will cost a lot lesser than diesel. Reduction in green house gases would lower air pollution and will help us fight through the crisis pertaining to air pollution like health hazards, impaired hearing, heart problems and others. Also, technically biodiesel is miscible with regular petro-diesel in any ratio making it easier to use. It also has low viscosity, is a good lubricant, a high flashpoint, easily degradable in nature, negligible sulfur content and very low emissions as seen earlier in the table (Moser, 2011). Moreover, decrease in the emission of sulfur dioxide, nitrogen oxides, carbon monoxide would probably result in lesser acid rain. This could save architectural mishaps such as buildings to collapse as well as other environmental issues. It could improve overall health of people and make them more efficient to work. This would in turn get more profit than actually spending those few extra cents for a gallon on biodiesel.

Every coin has two sides and so does biodiesel. According to Moser, major disadvantages of biodiesel are its high susceptibility to oxidation, high cost for feedstock, not good for storage, lower volumetric energy content and it does not work too well in low temperatures. Biodiesel is hygroscopic in nature which affects the quality of fuel when it comes in contact with humid air. Also, the rules and regulations of its production are not standardized outside the US and European lands which make it difficult to use since it causes corrosion, blockages, filter clogging and similar issues. Biodiesel has a lower volumetric energy which means more fuel is needed to travel the same distance, giving a lower mileage. A more frequent oil change is necessary while using biodiesel since it has a tendency to dilute engine oil. Overall, the engine, system and the infrastructure need changing which can add to the overall cost of fuel (Singh, 2009). All of this is in comparison to petrodiesel which has other disadvantages to it. But the problems of biodiesel can be reduced by either mixing it with petrodiesel or adding antioxidants in the process (Moser, 2011).

Ideally, producing oil for transesterification without having to use food crops would change the approach towards alternative fuel and the entire issue of using food crops for fuel would not exist. There has been an on-going research on the subject of producing biodiesel from microalgae which looks very promising. It is still in its early stages but if this becomes successful, it will benefit in more than one way.

1.8 Objectives of this research

- To measure the effectiveness of soybean oil in the production of biodiesel
- To measure the efficiency of *Rhizopus oryzae* as a biocatalyst
- To minimize the cost of production and make biodiesel a more common commodity
- To test the efficiency of Gas Chromatography as a medium to verify the process of transesterification

CHAPTER 2

LITERATURE REVIEW

2.1 Importance of Soybean oil for biodiesel production:

Many crops have been used to produce oil for food with corn oil, sunflower oil, canola oil, olive oil and soybean oil being the most popular ones. Soybean or *Glycine max is a* very popular crop all around the world and its importance increased even more ever since it has been known to produce biodiesel. The history of the crop dates back to around 1700 – 1100 B.0 in Northeastern China as the evidence suggests but it was initially domesticated in around 2500 — 2300 B.0 where as in the USA, the first documented use of soybean was made in 1765 in what is now known as Georgia (Hartman, 2011). The crop is used as a meal or to produce oil. As is known, it is rich in proteins and soy protein chunks are a popular ingredient in Southeast Asian cuisine. This has been known for a while but what really ticked off soybean production is biodiesel. Here's a chart that shows the increase in soybean production in major soybean producing countries and overall world production by Hartman (2011)

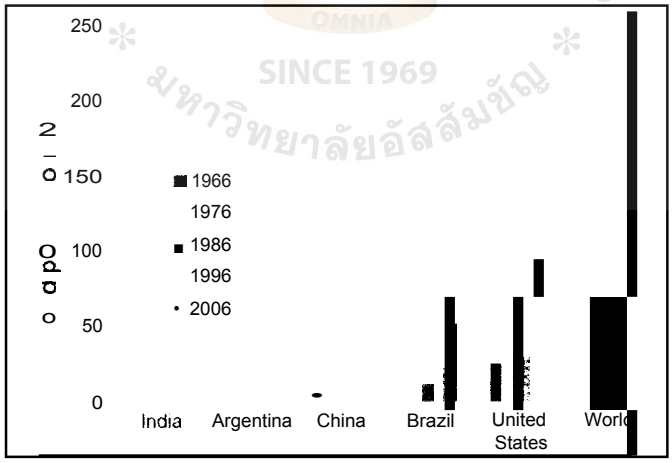

Figure 3: Volume of soybean production in the highest soybean producing countries and total world production in million metric tons (MMT) from 1966 to 2006. Data has been taken from FAO statistics (Hartman, 2011)

It is evident from Figure 3 that soybean production has increased drastically over the years and with its increasing significance, more research has been done to prove its worth. Not only has the overall production increased in the world, but also a major percentage increment has been observed compared to other staple crops such as wheat, rice, maize and more (Hartman, 2011). This indicates that supply of soybean oil is higher in comparison to other oils and thus expected price to be lower which in turn keeps the final cost of biofuel in check. Especially, in the United States of America the production has increased substantially which makes it easier to access. That motivated us to choose soybean oil as a source of triglyceride for transesterification in our research.

Also, the chemical composition of soybean oil favors the production of biodiesel. A higher degree of polyunsaturated fatty acids will give a higher methyl ester yield which makes the process more efficient. Following table shows a comparison of fatty acid composition of different oils used in biodiesel production:

Fatty acid		Palm	Olive	Peanut	Rape	Soy bean	Sunflower	Grape	110. Sunflower	Almond	Com
taunt	a 2:0	0.1	0.0	C.0	10	00	0.0	0.0	0.0	D.0	0.0
Myristic	0 4:0	0.7	0.0	C.1	3.0	00	0.0	0.1	0.0	0.0	0.0
Palmitic	C16:0	36.7	11.6	8.0	4.9	11.1	6.2	6.9	4.6	10.4	6.5
Palmitoleic	(16:1	0.1	1.0	C.0	3.0	01	0.1	0.1	0.1	0.5	0,6
Stearic	C18:0	6.6	3.1	1.8	1.6	36	3.7	4.0	3.4	2.9	1.4
Oleic	018:1	46.1	75.0	513	33.0	24.9	25.:	19.0	618	77.1	555
Linoleic	(18:2	8.6	7.8	28.4	20.4	53.0	63.1	691	27.5	7,6	252
Unolenic	(18:3	03	0.6	O	7.9	61	02	0.3	0.1	0.8	0,1
Arachidic	(20:0	0.4	0.3	C.9	3.0	03	03	0.3	03	0.3	0.1
Gadoleic	C20:1	02	0.0	2.4	3.3	03	0.2	0.0	0.0	0.0	0.1
Behenic	02:0	0.1	0.1	3.0	3.0	00	0.7	0,0	0.7	0.1	0.0
Erucic	02:1	0.0	0.0	C.0	23.0	03	0.1	0.0	0.0	0.0	0.1
Lignoceric	C24:0	0.1	0,5	1.8	10	01	02	0,0	03	0.2	0.1
Neuronic	04:1	0.0	0.0	C.0	7.0	00	0.0	0.0	0.0	0.4	0.0

Table 2: Fatty Acid composition of different oils used in biodiesel production (Ramos, 2008)

This table gives an overview of fatty acid quantities in ten different refined oils used to produce biodiesel. According to the author and his group of researchers, they found that oils with more polyunsaturated fatty acids gave relatively low cetane numbers but high iodine values and even though their oxidation stability was low, the cold flow properties were better than oils with

low polyunsaturated fatty acids (Ramos, 2008). Soybean oil is amongst the top oils with high polyunsaturation. But most of the researchers talk about cold flow properties being a major setback in commercializing biodiesel and in case of soybean oil; it has better cold flow properties. Even though the cetane number of soybean oil is lower than its competitors, it is still higher than the specified standards which do not pose a serious issue. Hence, we chose to use refined soybean oil as our substrate.

2.2 Factors affecting Biodiesel production:

The final quality of biodiesel is based on many factors during its process. Each parameter we choose is crucial to the quality and price of biodiesel. Conventionally, selling biodiesel alone is much more expensive than petro-fuel. Hence, there are different blends of biodiesel with petro-fuel like B10, B20, B50 where is the numbers 10, 20, 50 depicts the percentage of biodiesel present in the blend of fuel. This will help lowering the emissions as well as can help keep a check on price. Out of the many different factors affecting the reaction as shown below in figure 4, we'll discuss the most crucial ones like lipase selection, alcohol selection and molar ratio of substrates that determine the final quality and price of biodiesel. The following figure depicts the factors affecting biodiesel very efficiently:

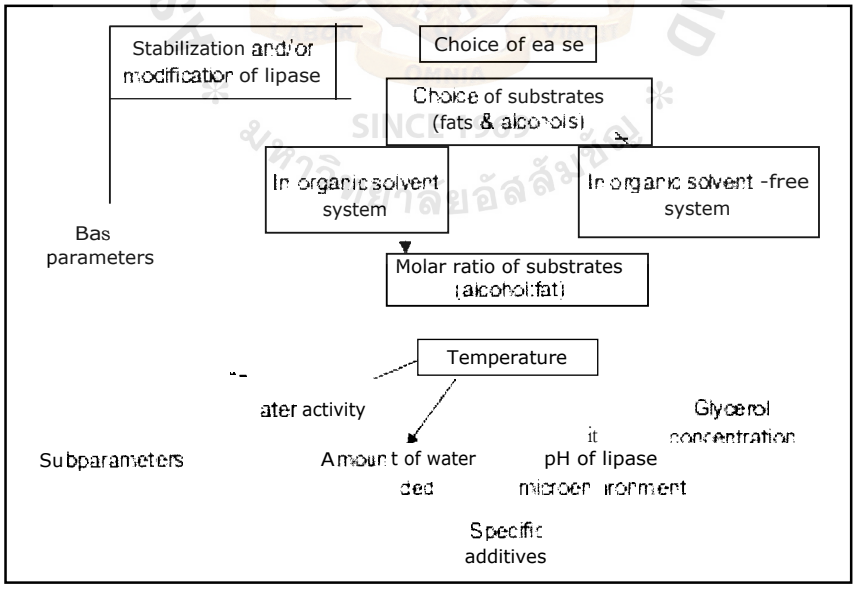

Figure 4: Factors affecting the final quality and quantity of biodiesel by enzymatic transesterification (Antczak, 2008)

Factors:

2.2.1 Selection of Lipase:

Many different microorganisms were screened that were capable of producing lipase in their system. Many of the lipases available in the market are extracted and purified from their respective cells like *Pseudomonas fluorescens, Candida antartica, Mucor miehei, Candida rugosa* and some more (Antczak, 2008). But buying purified lipases is an expensive affair which eventually affects the final cost of biodiesel. Hence, we chose to use a whole-cell biocatalyst like *Rhizopus oryzae* which releases its lipase in the system which can directly catalyze the transesterification reaction. This not only cuts cost but also saves on time.

2.2.2 Type of alcohol used:

Many different types of alcohol like ethanol, methanol, propanol, isopropanol, n-butanol and isobutanol can be used for transesterification. But amongst all these ethanol and methanol have been the cheapest and more readily available choices and hence the most popular alcohols used in transesterification reaction (Antczak, 2008). In this particular study, we also used methanol because it was the cheapest option as well as easily available. Even though methanol and ethanol are the most used ones, they are also the stronger than most of other longer aliphatic competitors. These two alcohols have higher enzyme denaturing capabilities which make it difficult for the reaction to go on for long without adding more enzyme to the reaction. The rate of enzyme denaturation influences the final yield and the velocity of the enzyme catalyzed reaction (Antczak, 2008). During the transesterification reaction, water is released and the presence of water in the reaction alongside methanol can increase the speed of lipase denaturation. To avoid this, we added 3 A molecular sieve 2 hours into the reaction to absorb any water released and hence saving the lipase from getting denatured. This ensured a complete reaction while the lipase was still active as well as a cost effective reaction with the use of methanol as the alcohol to yield methyl esters.

2.2.3 Molar ratio of the substrates used in the reaction:

The two important substrates in the transesterification reaction are alcohol and lipid. Excess alcohol is important to ensure the completion of reaction and a higher yield of acyl esters. But as mentioned earlier, a higher concentration of alcohol can cause denaturation of enzyme and can reduce the final yield. This happens especially when the alcohol is insoluble in the oil, and forms droplets in the reaction. The size of these droplets depends on the stirring speed of the reaction (Antczak, 2008). It has been shown that batchwise addition of alcohol can also solve this problem (Li, 2007). In our study, we added methanol in total of 4 parts with 60 minute time interval between each. This would give enough time for lipase to catalyze the reaction without methanol denaturing it. A research could help figure out the most optimum molar ratio of alcohol and triglycerides based on which alcohol and which oil is being used in the reaction. It is important to know the optimum ratio to get the least time in completion of reaction and the highest yield of methyl esters.

2.3 Properties of Biodiesel:

Biodiesel has properties similar to other diesel fuels but is greener in nature and lowers the emission substantially. Because it has characteristics similar to that of diesel, it becomes a strong contender to replace the conventional diesel fuel today. Many parameters affect the efficiency of biodiesel like, initial boiling point, cetane number, kinematic viscosity and others specified in Table 2 below. Out of the numerous attributes of biodiesel, we chose a few of those and discussed them further.

2.3.1 Kinematic Viscosity:

When transesterification converts TAG' s to methyl or ethyl esters, it reduces the molecular weight to one-third of the TAG and thus reduces the viscosity by a factor of eight and this increases the volatility slightly (Singh, 2009). Viscosity of the fuel is important to the engine, because that determines the spray characteristics of the fuel and in turn establishes the combustion properties of the engine. If the viscosity is high, it will cause problem in fuel exhaust and may even create clogs. For biodiesel, the kinematic viscosity should be between 1.9 to 6.0mm^2/s at 40°C. This held true for most of the biodiesel-diesel blends measured by Fernando *et al* (Fernando, 2007). Singh, also reports that biodiesel has about 10-11% oxygen by weight,

which enables a higher combustion as compared to the other diesel fuels. This helps the engine's performance on the longer run.

2.3.2 Cetane Number:

Cetane number of a fuel is the time between injection and ignition and it basically measures the ignition performance of the fuel. Ideally, the least delay means the quality of fuel is the best. Cetane number is inversely proportional to the time delay. Biodiesel has an average cetane number which ranges from 48 to 56, which is higher than the conventional diesel fuel that has a cetane number of around 50 (Candeia, 2009) (Singh, 2009). Cetane number is affected by the presence of residual methanol in the fuel as well as the structure of fatty acid alkyl esters (FAAE). The degree of unsaturation, chain length and branching can all affect the final cetane number. Presence of high residual methanol can decrease the cetane number of the fuel which negatively affects its functionality. Cetane number is also influenced by the chain length with a longer chain giving a higher cetane number and it reduces with increasing unsaturation (Candeia, 2009). This shows that biodiesel is a better alternative to the conventional fuels. Even though the cetane number is high, performance issues of cold flow properties in low temperatures still persists and Singh suggests, using tertiary fatty amines and amides can help in solving this problem. Also, biodiesel – diesel blends is a workable option and it helps in all aspects till a more substantial form of biodiesel is out in the market.

2.3.3 Flash Point:

The flash point of a fuel is the temperature at which the fuel becomes a mixture that can ignite when exposed to a flame or a spark (Candeia, 2009). It is directly proportional to the methanol content in the fuel and is very crucial to the efficiency of the fuel. A high flash point directly refers to more safety in handling the biodiesel, and according the regulation ASTM D93, the minimum requirement is 130°C and that of soybean FAME is 168°C and FAEE is 170°C which not only comply by the standards but are much higher than that of petro diesel which is only 53°C (Candeia, 2009). The measure of a flash point is proportional to the completion of the reaction and the presence of TAG' s that did not undergo transesterification or the presence of mono – alkyl esters. This shows that if a biodiesel – diesel blend is being used, a higher

percentage of biodiesel will ensure a higher flash point and this in turn guarantees a safer fuel (Candeia, 2009).

2.3.4 Cloud Point:

Cloud point of a fuel is defined as the temperature at which a cloud of wax crystals starts to form in the fuel or liquid when it is cooled under defined conditions as stated in ASTM D 2500 (Fernando, 2007). In case of biodiesel, cloud point depends on the initial substrates used, the structure of oil and presence of saturated esters in biodiesel. This is an important parameter that needs to be considered in case of biodiesel especially since it has been seen that biodiesel does not perform very well in low temperatures (Fernando, 2007), (Candeia, 2009) (Singh, 2009). ASTM D 2500 regulations do not specify a limit for cloud point but it directs the manufacturers to specify the cloud point to its customers. When different samples of biodiesel were measured for cloud point, they showed a higher cloud point than regular diesel which makes it difficult to use in cold regions (Fernando, 2007).

2.3.5 Sulfur Content:

Total sulfur count in the fuel emissions is an important factor attributing to the efficiency of the fuel since high sulfur is a critical air pollutant and is responsible for acid rain and other environmental hazards. For biodiesel, sulfur content is measured by ASTM D 5453 and according to the regulations; the maximum limit for sulfur content is 0.0015% mass of sulfur in biodiesel (Fernando, 2007). According the research carried out by Southwest Research Institute, sulfur content in all biodiesel samples were within the specified limits, so much so, that B100 has sulfur content of less than 1ppm. This makes biodiesel a better fuel alternative than the conventional fuel.

The following table shows the difference between the various parameters of biodiesel and diesel in accordance to the ASTM limits:

Fuel property	Diesel	Biocliesel
Fuel Standard	ASTM	ASTM PS 121
Fuel composition	C10-C21 HC	C12-C22 FAME
Lower Heating Value (Btu/gal)	131295	117,093
Viscosity, @ 40 C	1.34.1	1.9-6.0
Specific Gravity kg;l 50 F	0.85	0.88
Density, lb/gal 4' 15 C	7.079	7328
Water, ppm by wt.	161	0.05. max
Carbon)	87	77
Hydrogen (wt.%)	13	12
Oxygen, by d if. wit	0	11
Sulfur (wt.%)	.05 max	0,0 - 0.0024
Boiling Point (C)	188=343	182-338
Flash Point	60-80	100-170
Cloud Point (	15 to 5	-3 to 12
Pour Point C)	-35 to 15	-15 to 10
Cetane Number	40-55	48-65
Stoichiometric Air/Fuel Ratio	15	13.8
BOCLE Scuff	3,600	-7,000
HFRR Jim	685	314

Table 3: Comparison of different parameters between diesel and biodiesel (Singh, 2009)

This table shows that overall biodiesel is a better fuel in comparison to petro diesel but it cannot be used alone due to its limitation with the current day technology. Hence, a good biodiesel—diesel blend can be the best option considering the rate at which the earth's natural and non-renewable resources are being used up.

2.4 Relevant Research Reviews:

There have been numerous researches, short term studies, reviews and other pilot scale projects that have been done on biodiesel. Through years, with advancement in technology and knowledge available to scientists today, biodiesel has only seen progress in recent years. Different substrates, different catalysts and different co-substrates have helped researchers achieve a viable product and they've been successful in delivering a very vital alternative to fossil fuel. Following are a couple of researches that have been carried out in this field that are relevant to the current study.

2.4.1 Enzymatic production of Biodiesel from Jatropha oil: A comparative study of immobilized-whole cell and commercial lipases as a biocatalyst (Tamalampudi, 2007).

In this research Tamalampudi and his group are set out to know if *Rhizopus oryzae* lipase is a more effective catalyst than the best commercial catalyst available in the market, Novozym 435. Their objective of this research was to know which one of these two lipases are more efficient in transesterification and if they are cost effective. To test this, they used Jatropha oil, which is non-edible oil available from the seeds of *Jatropha curcas*. Biodiesel is a more expensive commodity than petro diesel and the major cost of biofuel goes into the feedstock oil. Since Jatropha is non-edible and abundant in nature, Tamalampudi *et al* chose jatropha oil as a feedstock. Conventionally, strong base catalysts like NaOH were used to transesterify oils, but glycerol was difficult to recover, it was hard to separate base from the product and wastewater treatment was posing problems to biodiesel production. Hence, inclusion of an immobilized biocatalyst gave promising future prospects. But, commercially available lipases are a very expensive choice and it renders enzymes like Novozym 435 useless in case of biodiesel production. Instead, immobilized-whole cell biocatalyst like ROL would eliminate the laborious steps of purification and can prove cost effective.

Tamalampudi *et al* used Jatropha oil with a saponification value of 210 and water content of 1.5%w/w, Novozym 435 (*Candida antarctica* lipase B immobilized on macro-porous acrylic resin) which has an activity of > 10,000 U/g and *R.oryzae* IFO 4697 as their basic substrate and catalysts respectively for alcoholysis reaction. They used air-lift bioreactor to cultivate ROL using reticulated polyurethane foam with over 97% voidage and 50ppi specifications for immobilization. Alcoholysis was carried out in 50m1 screw-capped vessels on a reciprocal shaker. Each tube had essentially, 5g jatropha oil, 3:1 molar ratio of alcohol: oil and 0.2g of lipase. After the methanolysis reaction, samples were analyzed using GC – 18A Gas chromatograph connected to a DB-5 capillary column. They used different alcohols like methanol, ethanol, n-propanol and n-butanol to check for the lipase activities for each alcohol and the other variable was different alcohol: oil molar ratio. After the experiments, the researchers found that methanol was the most effective alcohol amongst the others and ROL showed more activity for all the alcohols used in comparison to Novozym 435. When they compared the resistance of each lipases used to methanol, they found that ROL was more

susceptible to denaturation than Novozym 435 and this could be due to the different bases used for immobilization. In case of Novozym 435, it is acrylic resin while that for ROL is polyurethane foam. According to Tamalampudi *et al,* foam would adsorb more methanol than the resin which could negate lipase activity. Next, they wanted to test the effect of the weight of lipases on the transesterification reaction and they found that ROL activity remained constant for up to 6wt.% while that of Novozym 435 remained only till 2wt.%. This suggests that Novozym 435 is needed in much lesser quantities than ROL. Furthermore, they wanted to test for the efficiency of these lipases in regards to time and water content in the reaction mixture. As a result they found that ROL reached 80% ME yield after 60 hrs in comparison to 75% ME yield after 90 hrs for Novozym 435. Also, water content of up to 5% w/w did not decrease ROL activity as opposed to a substantial decline in activity of Novozym 435 after 90hrs of 0% water content. ROL still showed some activity from 5-10% water content. This shows that ROL is a better lipase to use keeping in mind that most vegetable oils have some water content. About reusing the same lipases, it was found that both the lipases showed activity above 90% up until the fifth batch but it is easier to separate ROL from the reaction mixture due to its 4mmx4mm size as compared to <1 mm diameter for Novozym 435.

This research shows that ROL is a better catalyst in comparison to the best commercial catalyst used like Novozym 435 in terms of cost, lipase activity in presence of water and batch methanol addition can eliminate the negative effect on ROL's activity. Hence, overall our choice to use ROL in our research with soybean oil is supported by this research.

2.4.2 Comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors (Du, 2004).

This research is focused on process optimization by using different acyl acceptors to negate the impact of methanol on transesterification. Du *et al* used crude and refined soybean oil as their substrates along with Novozym 435 as their catalyst and methyl acetate as the different acyl acceptor. The objective of this study was to find out if a different acyl acceptor other than methanol could affect on the overall methyl ester yield, duration of the reaction and overall activity of the lipase. Along with that, they also wanted to know the difference between using crude soybean oil and refined soybean oil for final FAME yield.

In order to carry out the experiments, Du *et al* used crude soybean oil and refined soybean oil as their substrates, along with methyl acetate as an acyl acceptor and Novozym 435 as the lipase catalyst. Initially, they carried out the experiments without adding any methyl acetate in 50m1 shaking flasks at 40°C adding soybean oil, 4% Novozym 435, and 1 molar equivalent of methanol. After sample analysis, the residual activity of lipase was determined as the percentage of methyl ester yield at specified time as compared to the maximum yield obtained which was 100% when 3:1 molar ratio of methanol: oil was used. Similar experiments were carried out, now adding methyl acetate along with Novozym 435. Now, only 30% of Novozym 435 was used w/w of soybean oil and 12 molar equivalent of methyl acetate was added. Du *et al* also wanted to know the difference in lipase activities of pretreated lipase with methyl acetate and non-pretreated lipase. Hence, they left the lipase with methyl acetate for 100h before carrying out the aforementioned experiments. All the samples were then analyzed using a GC-14B gas chromatograph connected to a HP-5 capillary column. It was found that methanol affected the activity of the lipase substantially with 1:1 molar ratio which is not sufficient to complete the reaction. In such a case, methyl acetate helped, by being the acyl donor and had no negative effect on the lipase activity. The optimum molar ratio of methyl acetate to oil was 12:1 and the results showed anything higher or lower resulted in a reduction of methyl ester yield. Along with the effect of methyl acetate on lipase activity, Du *et al* also wanted to know the difference between crude soybean oil and refined soybean oil on final ME yield. It was found that, crude soybean oil on its own had a reduced ME yield as compared to refined soybean oil due to the presence of unwanted lipids in crude oil. But when methyl acetate was used, the yield was 92% in both the cases and the researchers attribute this result to methyl acetate's capability of dissolving the unwanted lipids in the crude oil. Additionally, presence of methyl acetate also lead to the preservation of lipase activity even after 100 cycles which means it could cut costs and reduce the price gap between biodiesel and conventional fuel.

This research shows some promising ideas to improve the current biodiesel production on a large scale. This also proves that our use of refined soybean oil instead of crude as a substrate worked better for us, since we did not use methyl acetate as an acyl acceptor to know the behavior of ROL in soybean oil.

CHAPTER 3

MATERIALS AND METHODS

3.1 Attempted growth of *Neochloris oleoabundans*:

To date, there are so many micro- algae that contain relatively high amount of lipids but the lack of substantial lipid extraction and high productivity costs has made it difficult for use (Li, 2008). Li suggests that *Neochloris oleoabundans* is a fresh water species which is capable of producing up to 80% triglycerides and most of them are saturated fatty acids with 16-20 carbons. This is the most favorable algae amongst its competitors and if high lipid extraction hurdle is crossed than this could save money and time. Microalgae have photosynthetic capabilities with high growth rates and can be grown in relatively smaller area as compared to the vast fields for crop culture (Li, 2008).

This process was carried out in our laboratory too on a lab scale model. We also used *Neochloris oleoabundans*, UTEX 1185 from University of Texas, Austin and employed a similar method as Li. The growth medium used was Soil Extraction medium which consisted of 0.15g $K_2HPO_4.3H_2O$, 0.15g $MgSO_4.7H_2O$, 0.05g $CaCl_2.2H_2O$, 0.35g KH_2PO_4, 0.05g NaCl, 2.86mg H_3BO_3, 1.81mg $MnCl_2.4H_2O$, 0.22mg $ZnSO_4.7H_2O$, 0.079mg $CuSO_4.5H_2O$ and 0.039mg $(NH_4)_6Mo_7O_{24}.4H_2O$ per 1 liter medium. In addition to the growth medium, 5mM sodium nitrate ($NaNO_3$) was also used as a nitrogen source. For every 1 ml of media, 2µl of $NaNO_3$ was added to the medium. Agar slants were used for the initial growth of microalgae. Later it was transferred to a cubicle for a higher yield.

According to certain conditions needed for *N.oleoabundans* to grow, a wooden cubicle was made with 12 fluorescent lamps attached, 2 fans on either side controlled the temperature and it needed 12hr light and 12 hrs darkness which was maintained using an automated timer. The optimum temperature for growth is 30°C ± 2 and agitation was achieved by bubbling using sparger. CO2 gas tank was attached to a water tank and a microfiltration cartridge (0.47µm) and then to the cubicle using a fritted glass dispersion tube.

3.2 Microorganism and media:

After a brief research on what microorganism will best suit as a catalyst in the transesterification reaction, *Rhizopus oryzae* IFO 4697 was chosen. The reason being its ability to produce lipase directly on site. This proves cost effective and time saving.

The experiments were carried out using filamentous *Rhizopus oryzae* IFO 4697 aquired from NBRC (NITE Biological Resource Centre), Osaka, Japan as the catalyst. The fungus needed to get rehydrated before using it as a culture.

A rehydration fluid was made and a few drops were added to the dry cell culture. 5g Peptone, 3g Yeast Extract (pH 7), 1g $MgSO_4.7H_2O$ in 1L distilled water was used to make the rehydration fluid. Once the *R.oryzae* cells got rehydrated, they were cultured on PDA plates for 72 hrs.

Inorder to immobilize the fungal cells to aid catalysis in the reaction, Biomass Support Particles(BSP's) were used. In this experiment, we used polyurethane foam with 97% voidage and 50 pores per inch to carry out the experiment. The foam was supplied by Crest Foam Industries Inc., NJ, USA. 5 x 5 cm cubes were cut to serve as a BSP.

To grow *R.oryzae* incorporated with BSP, shake flask cultivation method was implemented and a basal medium was used. Basal medium consisted of 1L tap water, 70g peptone, 1.2g $NaNO_3$, 1.2g KH_2PO_4, 0.5g $MgSO_4$. 7H20 and 30g soybean oil (Safeway brand) as a carbon source.

3.3 Shake Flask Cultivation:

For the growth of *R.oryzae* spores, 100m1 of the basal medium was transferred to a 500m1 Sakaguchi flask. About 5 flasks were used. A larger flask is used to allow some room for splashing. Add about 80 BSP's to each flask and autoclave at 121°C for 15min at 15psi. Innoculate each flask with 2 loopfull spores and incubate these flasks on a reciprocal shaker at 35°C and 130rpm for 72hrs.

Shake flask cultivation method was employed to keep the whole-cell catalyst incorporated BSP's suspended in the medium at all times. This will ensure overall process efficiency and help achieve more fatty acid methyl esters by the end of the process.

3.4 Lyophilization Treatment:

After 72hrs, wash the BSP's with water and store the flasks at -80°C for 24hrs for the cells to coagulate in the BSP. After 24 hrs, the BSP's are lyophilized for 6hrs in a VirTis benchtop K freeze dryer.

3.5 Methanolysis Reaction:

This stage is where the actual transesterification reaction occurs and di- , tri- glycerides get converted to Fatty Acid Methyl Esters (FAME). It is important to maintain a constant environment throughout the reaction to minimize error.

Methanolysis reaction was carried out at 30°C and 130rpm for 96hrs in 100m1 flasks. The flask contained soybean oil (Safeway Inc. brand), methanol, butanol, *R.oryazae* catalyst in BSP's and phosphate buffer pH 6.8. All the reagents varied according to the reaction ratio set up.

Molecular seive 3 A supplied by Alfa Aesar, MA, USA was used to absorb water from the reaction and was added after 2 hrs into the reaction. This will ensure that water will not affect towards completion of the reaction.

100µl samples were taken in small 2m1 vials at 0, 4, 24 and 96 hr intervals. The time span will show the progress and completion of transesterification reaction. Unknown Samples were stored at -80°C freezer to be used later for data analysis.

To make the phosphate buffer at pH 6.8:

Sodium phosphate monobasic – 6.95g in 250m1 dH_2O

Sodium phosphate dibasic – 13.412g in 250m1 dH_2O

Take 127.5ml of Sodium phosphate monobasic solution and 122.5m1 of sodium phosphate dibasic solution. Mix the two and meausre the pH.

Three variables were used in the experiments: Methanol, Lipase and butanol. Soybean oil formed 5g of the reaction mixture and phosphate buffer was a constant 3% (w/w) of soybean oil.

Methanol Variation: This was based on methanol to oil ratio. It helps in determining the optimum amount of methanol needed to achieve maximum transesterification in the reaction.

Methanol:Oil ratio variations: 2.5:1, 3:1, 3.5:1, 4:1 and 4.5:1. Methanol was added step wise at 0, 1, 2 and 3 hours to minimize the negative effect of methanol on lipase activity which will be explained later.

Lipase variation: ROL was used as a catalyst and was also varied based on the weight of soybean oil. This helps in determining the optimum amount of lipase needed to achieve the best conversion of tri and diglycerides to methyl esters.

The variation was (% w/w) of Soybean oil: 7, 10, 13, 16 and 19

Butanol variation: Butanol gives stability to the reaction and nullifies the negative effect of methanol on *R.oryzae* cells. Varying the amount of butanol will help establish the most favorable quantity of butanol needed in the reaction to achieve hightest transesterification. Variation was based on butanol: oil ratio.

Butanol: Oil ratio variations: 0:1, 0.75:1, 1.5:1. 2.25:1 and 3:1

All the experiments were duplicated for better and more accurate results.

Calculations:

For Methanol and Butanol, ratio was based on the weight of soybean oil as mentioned above. The following formula is used to calculate this ratio:

$$\text{Amount of Methanol (g)} = \frac{190 \times 32 \times \text{weight of oil (g)}}{56 \times 1000}$$

Where,

190 = Acid value of soybean oil (determined by titration with KOH)

32 = Molecular weight of Methanol

56 = Molecular weight of KOH

Similarly, for amount of Butanol, replace 32 with 74.1(molecular weight of Butanol) and we get the amount of butanol needed in the reaction set up.

3.6 Gas Chromatography Analysis:

The collected samples at 0, 4, 24 and 96 hrs stored at -80°C were then used to analyse the product using Gas Chromatography. HP 6890 Series Aus GC Version A.03.05 was used to analyse the products. Column used was DB-225 with the following program:

GC Program:

Column Flow rate: 2ml/min

Split Ratio: 20:1

Inlet temperature: 270°C

Initial temperature: 165°C

Detector temperature: 280°C

Hydrogen flow rate: 35ml/min

Air flow rate: 375ml/min

Nitrogen flow rate: 25ml/min

Temperature 1(°C)	Temperature 2(°C)	Rate (°C/min)	Hold time (min)	Total time (min)
165	192	8.25	-	
192	197	3.75	-	
197	235	16.5	10	
235	240	50	3.5	13.5

Table 4: Gas Chromatograph program for data analysis

Sample Preparation for GC Analysis:

C17:0 from Nu-Chek Prep Inc., MN, USA was used as internal standard. Density of C17:0 is 0.853g/cc. 300mg of C17:0 in 7.635m1 of hexane gave a stock solution of 60mg/g hexane. A

higher concentration was used to give a more pronounced peak for the ease of peak differentiation.

Similarly, C18:1. C18:2 and C18:3 standards were also ordered from Nu-Chek Prep Inc., MN, USA to get a common factor to find the amount of fatty acid methyl esters in each of our uknown samples. The same concentration of 60mg/g hexane was used.

The conversion factor thus being:

$\frac{60mg/g \times 100\mu l}{100\mu l + 50\mu 1} = 40mg/g$ [100µ1 is the amount of a respective C18:1 or C18:2 or C18:3 standard and 50µ1 is the amound of internal std]

Once the standards were prepared and the stock solution made, each unknown sample was taken in a 2m1 vial and 50µ1 of C17:0 internal standard was added with the unknown sample. All the samples were then centrifuged for 10min at 130K rpm and the upper layer was used for GC. From the 150 µ1 of total solution, 100111 was taken for GC analysis.

3.7 Experimental Design:

This research was carried out with the objective of process optimization of producing biodiesel from soybean oil and a biocatalyst. Following table shows an experimental design planned in carrying out the experiments:

Substrates	Soybean Oil (g)	Molar ratio of Methanol:Oil (w/w)	Molar ratio of Butanol : Oil (w/w)	Phosphate buffer (% wt. of oil)	ROL catalyst (% wt. of oil)
	x	**2.5:1**	**1.5:1**	**3**	**7**
	x	**3:1**	**1.5:1**	**3**	**7**
	x	**3.5:1**	**1.5:1**	**3**	**7**
	x	**4:1**	**1.5:1**	**3**	**7**
	x	**4.5:1**	**1.5:1**	**3**	**7**
	x	4:1	1.5:1	3	7
	x	4:1	1.5:1	3	10

	x	4:1	1.5:1	3	13
	x	4:1	1.5:1	3	16
	x	4:1	1.5:1	**3**	**19**
	x	4:1	**0:1**	**3**	7
	x	**4:1**	**0.75:1**	3	7
	x	4:1	**1.5:1**	3	7
	x	4:1	2.25:1	3	7
	x	4:1	3:1	3	7

Table 5: Experimental design illustrating the variables used and its quantities

This table gives an overview of the way the experiments were performed for process optimization. This forms a base to carry out further research in this area knowing which combination gives the maximum methyl ester yield. Further changes can be made and a better process can be designed to achieve a higher yield and make the process cost effective.

3.8 Study Period:

Time period: September 2008 - September 2011

- Planning and screening for different substrates and lipases took us about 4-5 months
- Performing the experiments with different variables took about 10 months
- Data analysis and collection went on for another 4-6 months
- Documentation and thesis was written in the next year

CHAPTER 4

RESULTS AND DISCUSSION

The purpose of this research was to find how effective soybean oil and *R.oryzae* are for the production of biodiesel and process optimization.

Conventionally a simple titration using potassium hydroxide (KOH) or other substitues was used to measure the acid value of biodiesel as a measure to test the quality of the product. This method gives a clear indication of the ratio of di- or tri- glycerides being converted to free fatty acids, rather than methyl esters. The following formula would give the acid value of the unknown sample indicating the % of hydrolysis.

$$\text{Acid value} = x \text{ cc} \times 56 \text{ mgKOH}/y \text{ g of sample}$$

$$\% \text{ FFA} = \frac{x \text{ cc} \times 25.64 \text{ g/mol} \times 1}{\text{Weight of Sample (g)}}$$

This is an effective way to know the conversion of fats to esters but not very accurate to give specifics into the process. Today, the more acceptable and a method with more accuracy is using Gas Chromatography. GC basically measures the final quantities of methyl esters of all the fatty acids present in soybean oil. In this case, the three most important fatty acids are oleic acid, linoleic acid and linolenic acid. The results in this chapter will show the graph between the quantities of the methyl esters of these fatty acids present in the biodiesel with reference to time.

As we saw earlier, different sources of oil can be used to produce biodiesel. One of the sources is algal oil and an attempt to produce algal oil was made in the laboratory. *N. oleoabundans* was used to produce algal oil for the transesterification process. But, after several trials, we could not get sufficient yield of *N.oleoabundans* to use it for oil extraction to produce biodiesel and we left the experiment at that. But there have been many successful attempts in achieving oil for analysis by other researchers like Li, Tornabene *et al,* Sheehan *et al,* and Beal *et*

al. An article on algal biofuels on the department of energy of United States of America's website states the advantages of having algal biofuel but it also states that the current technology is not efficient enough to produce biodiesel at competitive prices using algal oil. They say, based on current situation, algal biofuel would probably cost about $8/gallon in contrast to $4/gallon of soybean biodiesel. If the method becomes a success in producing substantial lipid rich oil for biodiesel production, many hurdles will be crossed and alternative fuel will have a very promising future.

In this research as mentioned earlier gas chromatography was employed to not only measure the overall conversion but also how much of each fatty acid in soybean oil was converted to fatty acid methyl esters.

4.1 Effect of Methanol on methyl ester yield:

Methanol acts at the primary acyl group acceptor in the transesterification reaction and is thus very critical to the process of producing biodiesel. Typically, in a transesterification reaction, triglycerides get hydrolyzed to di- or mono-glycerides and free fatty acids and these further react with methanol to form methyl esters giving fatty acid methyl esters (FAME) or biodiesel (Moser, 2011).

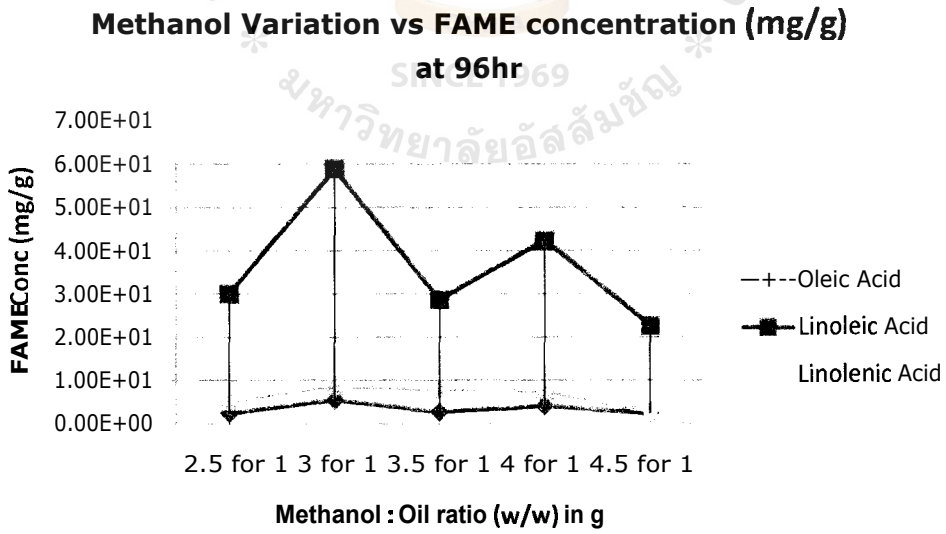

Figure 5: Effect of methanol in the production of biodiesel

The above figure gives an overview of the optimum molar ratio of methanol: oil in case of biodiesel production using soybean oil and R *oryzae* lipase as a catalyst in the transesterification reaction. The objective of this study was process optimization and from the result it is determined that the optimum molar ratio of methanol: oil is 3:1 as the methyl ester yield at 3:1 is about 60mg/g hexane. Since, linoleic is the most predominant fatty acid in soybean oil, the highest methyl ester yield of linoleic acid is taken into consideration for determining the optimum molar ratio. Experiments were performed over a span of 96 hours with each sample analyzed at 0hr, 4hr, 24 hr and 96 hr. This would indicate if transesterification continued for a longer period of time and according to the results, the highest yield of methyl esters was shown at 96hr. Hence, we used 96 hr as the base result. This could be reduced by taking a sample analysis somewhere between 24hr to 96hr, and if the results showed minor difference from 96hr, it would have been better to stop the reaction at that particular time. This would save cost and time for large scale production.

Methanol negatively impacts lipase activity and presence of excess methanol can cause denaturation of the enzyme (Tamalampudi, 2007) (Du, 2004). More than 1:1 molar ratio of methanol: oil can lower lipase activity but it is important to add excess methanol in the reaction to ensure completion of transesterification. From figure 5, it is evident that 2.5:1 molar ratio showed significantly lower methyl ester yield in comparison to 3:1 ratio. Also, a higher methanol to oil ratio showed a significant reduction in methyl ester yield contributing to the fact that excess methanol dissolves the n-glycerides present in the oil and makes it unavailable for transesterification. Addition of an organic solvent could stabilize the lipase activity because it increases solubility of methanol and thus keeping the lipase active for a longer time (Antczak, 2008). To reduce the impact of methanol on lipase, a step-wise addition of methanol was employed. Methanol was added in 4 parts total at 0hr, 1 hr, 2hr and 4hr to help lipase restore its activity. After the experiments, we realized more gaps in the time period to add methanol would have facilitated a higher methyl ester yield since lipase would have had more time to catalyze the reaction before more methanol was added to denature the enzyme. This change in process could help in future. The slight rise in levels of methyl ester at molar ratio 4:1 could be the result of noise and no significant effect of methanol or lipase. Additionally, 3 A molecular sieve was added to adsorb the excess water from the reaction mixture. When free fatty acids are present in the oil, transesterification reaction can lead to release of water as a byproduct. This water in

excess can aid in denaturation of lipase enzyme affecting the efficiency of lipase. Consequently, molecular sieve helps in adsorbing excess water and maintaining lipase activity constant. It was added after 2hrs into the reaction mixture. The effect of methanol on transesterification reaction is crucial in production of biodiesel and for this reason it is important to study it further to minimize the molar ratio and optimizing product yield.

4.2 Effect of lipase concentration on methyl ester yield:

Transesterification reaction on its own is an extremely slow reaction process and thus requires a catalyst to minimize the activation energy of the reaction. In this particular study, a biocatalyst in form of *Rhizopus oryzae* lipase was used. As mentioned earlier, a biocatalyst is a more suitable choice for biodiesel production. Lipase is a more expensive option in comparison to a strong acid or a strong base catalyst in terms of direct comparison. Even though, overall cost after considering, waste removal, wastewater management plant and other purification costs adds up to the total cost of production, making enzymatic transesterification a more viable option. Hence, the amount of lipase needed to catalyze the reaction and achieve highest possible methyl ester yields is important for process optimization.

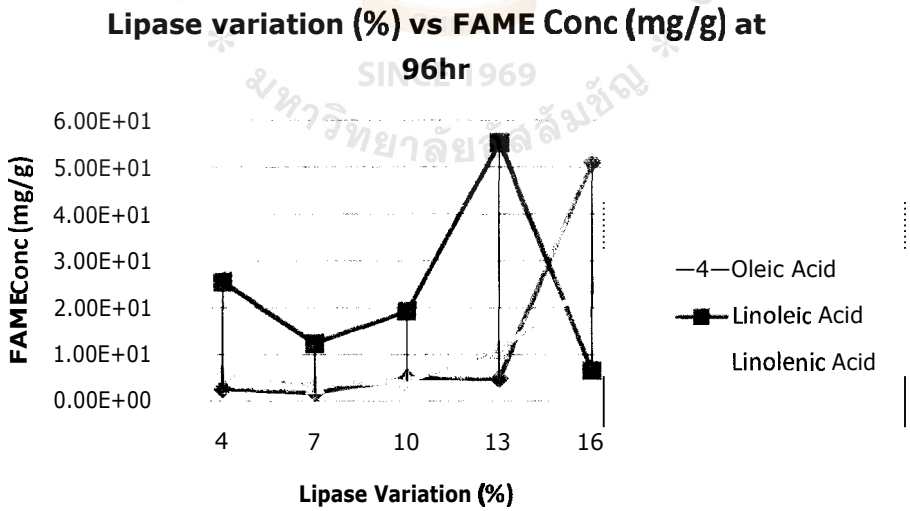

Figure 6: Effect of lipase concentration on methyl ester yield

Figure 6 gives an overview of the optimum concentration of lipase required to achieve maximum methyl ester yield. From the figure it is evident that 13% lipase concentration (w/w) gave the maximum methyl ester yield of almost 55mg/g and which is significantly higher than 4,7,10 or 16% at 25mg/g hexane, 12mg/g hexane or 19mg/g hexane respectively. In this case, increase in lipase concentration gave a higher methyl ester yield which shows that ROL was an efficient catalyst in methanolysis reaction. ROL has a 1(3) - positional specificity in catalyzing the reaction as mentioned earlier. It breaks down 2 – monoglycerides and 1,2 – diglycerides to its corresponding isomers 1-monoglycerides and 1,3- diglycerides respectively. Since ROL has a 1(3) – positional specificity, it helps in achieving higher methyl ester yield (Li, 2010) (Hama, 2002). Thus it is important to choose the right enzyme for transesterification.

But at 16% methyl ester yield dropped significantly which is only about 8mg/g, suggesting that for amounts greater than 13%, the amount of triglycerides are not enough for the lipase to catalyze and also the interfacial tension between oil and liquid phase could be the limiting factor to produce methyl esters any further. When 16% lipase was added to the reaction mixture, most of the triglycerides were hydrolyzed right away to mono glycerides and free fatty acids, but there wasn't enough methanol to convert these free fatty acids to methyl esters and when more amounts were added, the water plus the excess methanol could have denatured the lipase limiting its activity any further indicating the significant drop in the methyl ester yield. Since, linoleic acid is the most important fatty acid in soybean oil, the sharp increase in methyl esters of oleic acid at 16% is not significant and the amounts don't really matter in the quality of biodiesel. Phosphate buffer helps in maintaining the pH of the reaction mixture which helps in maintaining lipase activity. Lipase enzyme as a catalyst in methanolysis is a vital factor contributing to the final product since the methyl ester yield and biodiesel cost depend on it. The cost of enzyme is a major concern in this field of research and thus using an intracellular whole-cell catalyst like ROL is a good option.

4.3 Effect of Butanol on methyl ester yield:

Tert-butanol was added to the reaction mixture to stabilize the lipase activity of *Rhizopus oryzae*. Butanol helps in making methanol more soluble and thus it does not allow methanol to

have its negative effect on *R.oryzae* enzyme (Li, 2006). Li and his group used refined, crude and acidified rapeseed oils to produce biodiesel using ROL in a *tert*-butanol system. They found out that stability of *R.oryzae* could be substantially enhanced and relatively higher yields of methyl esters be achieved. (Li, 2007).

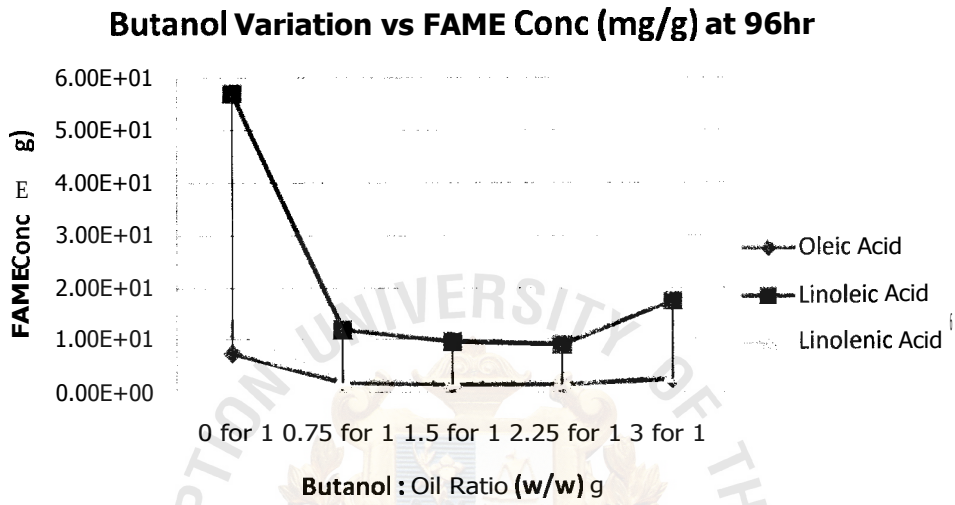

Figure **7: Effect of tert-butanol on methyl ester yield**

Looking at figure 7, it is evident that in case of soybean oil, butanol had negative effect on methyl ester yield. Highest results were achieved when no butanol was added to the system which was about 57mg/g hexane of methyl ester. This indicates that *R.oryzae* whole-cell catalyst behaves differently in different oil systems. Presence of water can affect the functionality of butanol in the system. It was observed that water till about 3% of oil weight enhaced the function of butanol and helped in stablizing ROL (Li, 2007) and this was confirmed by Antczak that presence of water is must in case ethanol, propanol, isopropanol, butanol or isobutanol were used in the transesterification reaction. (Antczak, 2008). In case of oils with high free fatty acid content, water is a product of the transesterificaiton reaction and excess water can denature the enzyme (Li, 2007). In this particular study, 3 A molecular sieve absorbed the excess water from the system and that is probably why, butanol could not produce the desired effect. Instead, in absence of water, butanol diluted the reactants resulting in decrease of methyl ester yields. It can be deduced from the results here that the amount of molecular sieve added was more than necessary which made the reaction system more arid than necessary, resulting in butanol not

being efficient. Hence, lesser quantity of molecular sieve should be used or from current results it is apparent that even omitting butanol from the reaction would be plausible and cost effective on the final product.

The slight increase in methyl ester yield at molar ratio of 3:1 could be just some noise as the increase is not very substantial and it still holds the probability of butanol not being effective in this case true. Butanol as an organic solvent to help negate the negative effect of methanol on ROL can be very useful but other parameters need to be taken into account to make sure highest FAME yields are achieved. ROL behaves differently in different systems and factors affecting the efficiency of transesterification reaction need to be adjusted according to the fatty acid composition of each oil as well as the water content in the reaction system to achieve highest methyl ester yields.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions:

This research was carried out with the objective to optimize the process of transesterification reaction using soybean oil and methanol as the substrates and *R.oryzae* IFO 4697 whole-cell as the catalyst. Following conclusions were deduced from the experiments and this study:

- Refined soybean oil is a good source to produce biodiesel and is abundantly available all over the world. Its production has gone up substantially with increase in world biodiesel production
- *Rhizopus oryzae* IFO 4697 whole-cell is a very efficient catalyst for transesterification in terms of time and cost as well as gives high fatty acid methyl ester yields.
- It is easier to use a whole-cell intracellular catalyst as compared to commercial extracellular catalyst because it turns out to be more cost effective and easier to handle. Commercial enzymes like Novozym 435 and other alike, need to be first cultured in the microorganisms, extracted from them, purfied to the highest levels and only then are available for use. In contrast to that, ROL is cultured and directly used in the system.
- Methanol is the most efficient, easily accessible and cheapest substrate available to accept the acyl group. The molar ratio yielding highest methyl esters was 3:1 for this particular study.
- Lipase content plays a vital role in competence of the transesterification reaction in terms of time and cost. Lipase makes up one major cost concern in biodiesel production and finding the minimum lipase amount needed to get maximum yield is important. For this particular study, 13% lipase was required to get the best results.
- Butanol is known to stabilize the enzyme catalyst in transesterification reaction by making methanol more soluble. But in this particular study, presence of molecular sieve

made the reaction arid and it did not allow butanol to perform. Instead, butanol made the reactants more soluble than necessary giving very low methyl ester yields. Hence, in this particular study, experiment without butanol worked better.

5.2 Recommendations:

After carrying out the research for more than a year and achieving the results we did, there are certain changes I can recommend to probably get better results and a more efficient alternative fuel to meet today's fuel needs.

- Using algal oil instead of refined vegetable oil will be highly effective in terms of cost and time. Even though we failed in our attempt on algal oil, if the right equipments are used and appropriate conditions are available, oil extracted from algae and used for transesterification reaction can save a lot of money. Firstly, the growth rate of algae is exponentially faster than that of crops, making it easier to culture. It does not require a lot of space in comparison to crop fields and it is easier to extract oil from algae than have a multi-step process to extract oil from crops. Secondly, world food crisis can also be addressed since no plant or vegetable oil will be used for biodiesel production. It is only a matter of time and more advanced technology in science that fungal oil will make it big in to the biodiesel market and scientists will come up with more stable and a better biodiesel product than current day options available.
- Stepwise addition of methanol is good but the time period between two steps should be substantial enough to allow lipase to catalyze the reaction efficiently without letting methanol denaturing the lipase. In this study the time period was not long enough and that may have reduced the methyl ester yields.
- Butanol is an effective solvent that helps in making methanol more soluble and thus stabilizing the lipase but other parameters need to be adjusted before introducing butanol into the system. Different substances other than butanol can be used to help transesterification reaction and something more useful for soybean oil can be found to help gaining better results.

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APPENDIX

Data Analysis:

Calculating a standard factor f_i, for each fatty acid C18:1, C18:2 and C18:3

C18:n (n=1,2,3)	C17:0	C18/C17	Factor (f) = C18/C17 40mg/g
C18:1	A_{C17}	x1	f1
C18:2	A_{C17}	x_2	f2
C18:3	AC17	x_3	f3

Where A = peak area of the C17:0 internal standard

Table 6: Calculation of standard factors to know final methyl ester yield

Factors for C18:1, C18:2 and C18:3

C18:n Peak Area (pA*S)	C17:0 Peak Area (pA*S)	Ai: C18:n/C17:0	FACTOR (C18:n/C17)/40mg/g
C18:1			
3.07E+04	1.12E+04	2.75E+00	**6.87E-02**
C18:2			
8655.68945	1.10E+04	7.88E-01	**1.97E-02**
1.83E+04	1.34E+04	1.36E+00	**3.41E-02**

Table 7: Factors for each fatty acid

Data analysis for each experiment:

Methanol Variation

Experiment 1 Molar ratio of Methanol: Oil

Where,

1.1 = Molar ratio 2.5:1, 1.2 = Molar ratio or 3:1, 1.3 = Molar ratio of 3.5:1, 1.4 = Molar ratio of 4:1 and 1.5 = Molar ratio of 4.5:1

Experiment 1.1 Molar ratio 2.5:1

Reactants	Quantities
Oil	3g
Methanol (2.5:1)	0.814g (0.203g/time period)
Butanol (1.5:1)	1.13g
Phosphate buffer (3%)	0.09g
Lipase (7%)	0.21g

Table 8: Experiment 1.1 with methanol: oil molar ratio 2.5:1

Experiment 1.2 Molar ratio 3:1

Reactants	Quantities
Oil	2.98g
Methanol (3:1)	0.97g (0.24g/time period)
Butanol (1.5:1)	1.12g
Phosphate buffer (3%)	0.09g
Lipase (7%)	0.20g

Table 9: Experiment 1.2 with methanol: oil molar ratio 3:1

Experiment 1.3 Molar ratio 3.5:1

Reactants	Quantities
Oil	3g
Methanol (3.5:1)	1.14g (0.285g/time period)
Butanol (1.5:1)	1.13g
Phosphate buffer (3%)	0.09g
Lipase (7%)	0.21g

Table 10: Experiment 1.3 with methanol: oil molar ratio 3.5:1

Experiment 1.4 Molar ratio 4:1

Reactants	Quantities
Oil	2.99g
Methanol (4:1)	1.298g (0.32g/time period)
Butanol (1.5:1)	1.127g
Phosphate buffer (3%)	0.089g
Lipase (7%)	0.209g

Table 11: Experiment 1.4 with methanol: oil molar ratio 4:1

Experiment 1.5 Molar ratio 4.5:1

Reactants	Quantities
Oil	3g
Methanol (4.5:1)	1.465g (0.366g/time period)
Butanol (1.5:1)	1.13g
Phosphate buffer (3%)	0.09g
Lipase (7%)	0.21g

Table 12: Experiment 1.5 with methanol: oil molar ratio 4.5:1

Results from Gas Chromatogram for each fatty acid C18:1, C18:2 and C18:3

For C18:1

Time	C18:1				
	1.1	1.2	1.3	1.4	1.5
	3.49E-01	5.85E-01	8.93E-01	0.00E+00	0.00E+00
4	4.35E+00	1.61E+00	1.46E+00	1.94E+00	1.42E+00
24	1.62E+00	2.73E+00	1.87E+00	2.20E+00	1.64E+00
96	2.43E+00	5.50E+00	2.75E+00	4.17E+00	2.46E+00

Table 13: Gas Chromatogram results for C18:1 for methanol variation

For C18:2

Time (hr)	C18:2				
	1.1	1.2	1.3	1.4	1.5
0	2.52E+00	2.70E+00	0.00E+00	0.00E+00	0.00E+00
4	3.53E+01	1.39E+01	9.51E+00	1.38E+01	9.37E+00
2	2.26E+01	2.18E+01	1.28E+01	1.56E+01	1.45E+01
96	3.01E+01	5.90E+01	2.87E+01	4.25E+01	2.26E+01

Table 14: Gas Chromatogram results for C18:2 for methanol variation

For C18:3

Time (hr)	C18:3				
	1.1	1.2	1.3	1.4	1.5
0	3.57E-01	9.57E-01	1.39e	7.80E+00	0.00E+00
4	8.44E+00	2.35E+00	2.18E+00	3.14E+00	2.03E+00
24	3.85E+00	5.33E+00	2.14E+00	3.23E+00	2.60E+00
96	5.40E+00	8.95E+00	8.07E+00	7.77E+00	2.95E+00

Table 15: Gas Chromatogram results for C18:3 for methanol variation

Chromatogram for Molar ratio of Methanol: Oil, 3:1 at 96 hr

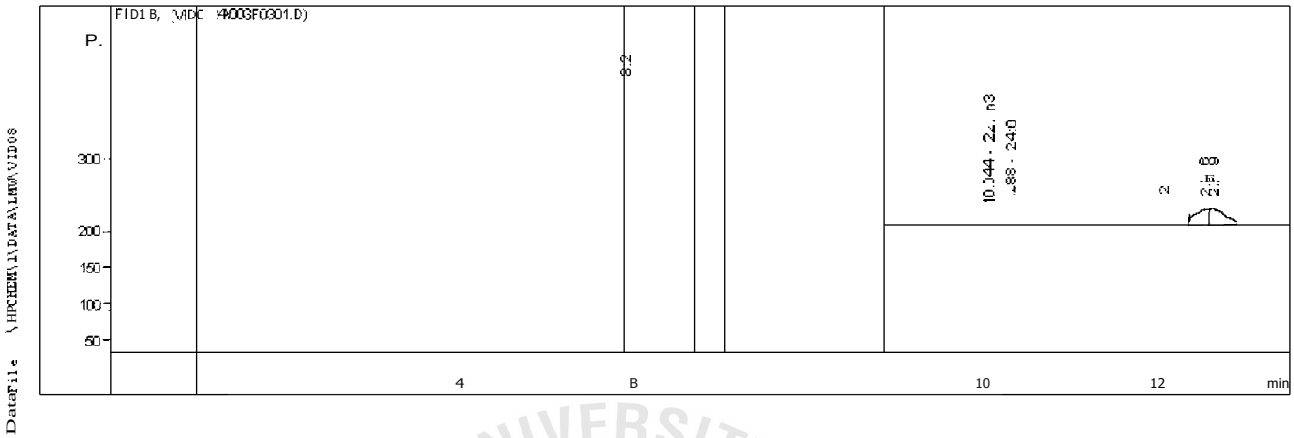

Figure 8: Chromatogram from the GC run for experiment 1.3 at 96hr for methanol: oil ratio 3:1

Lipase Variation

Experiment 3 Varying lipase by percentage of oil

Where,

3.1 = 4%, 3.2 = 7%, 3.3 = 10%, 3.4 = 13% and 3.5 = 16% (w/w of oil)

Experiment 3.1 Lipase concentration 4%

Reactants	Quantities
Oil	2.99g
Methanol (4:1)	1.3g (0.32g/time period)
Butanol (1.5:1)	1.127g
Phosphate buffer (3%)	0.089g
Lipase (4%)	0.12g

Table 16: Experiment 3.1 with 4% lipase concentration (w/w) of oil

Experiment 3.2 Lipase content 7%

Reactants	Quantities
Oil	3.0g
Methanol (4:1)	1.3g (0.32g/time period)
Butanol (1.5:1)	1.13g
Phosphate buffer (3%)	0.09g
Lipase (7%)	0.21g

Table 17: Experiment 3.2 with 7% lipase concentration (w/w) of oil

Experiment 3.3 Lipase content 10%

Reactants	Quantities
Oil	3.0g
Methanol (4:1)	1.3g (0.32g/time period)
Butanol (1.5:1)	1.13g
Phosphate buffer (3%)	0.09g
Lipase (10%)	0.3g

Table 18: Experiment 3.3 with 10% lipase concentration (w/w) of oil

Experiment 3.4 Lipase content 13%

Reactants	Quantities
Oil	2.98g
Methanol (4:1)	1.29g (0.32g/time period)
Butanol (1.5:1)	1.127g
Phosphate buffer (3%)	0.089g

Lipase (13%)	0.387g

Table 19: Experiment 3.4 with 13% lipase concentration (w/w) of oil

Experiment 3.5 Lipase content 16%

Reactants	Quantities
Oil	2.98g
Methanol (4:1)	1.29g (0.32g/time period)
Butanol (1.5:1)	1.127g
Phosphate buffer (3%)	0.089g
Lipase (16%)	0.477g

Table 20: Experiment 3.5 with 16% lipase concentration (w/w) of oil

Results from Gas Chromatograph for each fatty acid C18:1, C18:2 and C18:3

For C18:1

	C18:1				
Time (hr)	3.1	3.2	3.3	3.4	3.5
0	0.00E+00	0.00E+00	1.79E+00	3.23E-01	0.00E+00
4	1.75E+00	1.63E+00	1.28E+00	2.70E+00	2.88E+00
24	1.86E+00	1.93E-01	2.50E+00	3.90E+00	2.21E+00
96	2.64E+00	1.78E+00	5.00E+00	4.81E+00	5.11E+01

Table 21: Gas Chromatogram results for C18:1 for lipase variation

For C18:2

	C18:2				
Time (hr)	3.1	3.2	3.3	3.4	3.5
0	0.00E+00	0.00E+00	1.04E+00	9.28E+00	0.00E+00
4	1.15E+01	1.12E+01	9.84E+00	2.25E+01	2.46E+01
24	1.33E+01	9.04E+00	2.26E+01	4.50E+01	2.93E+01

96	2.56E+01	1.24E+01	1.93E+01	5.54E+01	6.65E+00

Table 22: Gas Chromatogram results for C18:2 for lipase variation

For C18:3

Time (hr)	C18:3				
	3.1	3.2	3.3	3.4	3.5
0	0.00E+00	2.50E+00	1.47E+00	1.40E+00	0.00E+00
4	2.58E+00	2.66E+00	1.71E+00	4.10E+00	4.32E+00
24	3.58E+00	4.26E+00	3.81E+00	9.56E+00	6.27E+00
96	6.04E+00	3.61E+00	4.30E+00	1.11E+01	2.50E+01

Table 23: Gas Chromatogram results for C18:3 for lipase variation

Chromatogram for Lipase, 13% at 96 hr

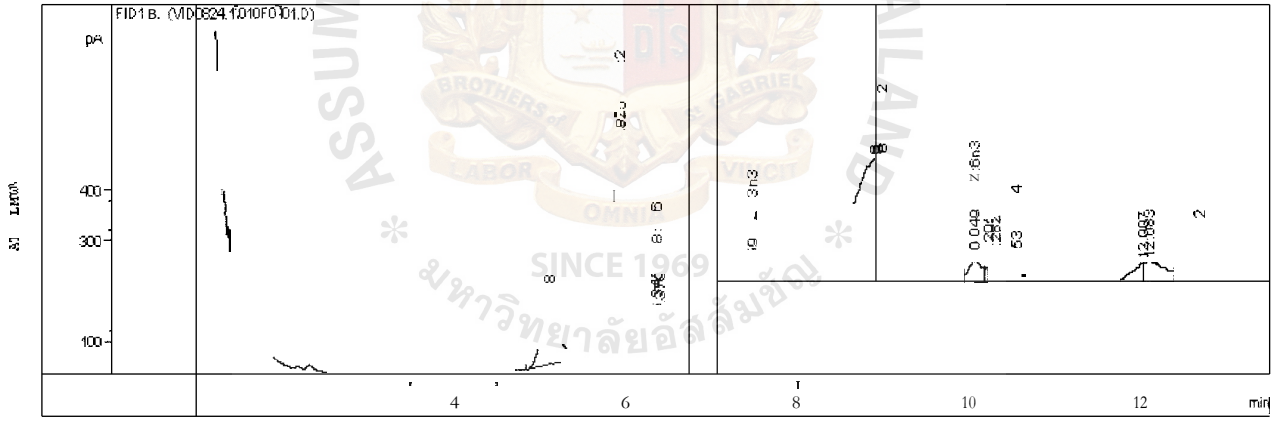

Figure 9: Chromatogram from the GC run for experiment 3.4 at 96hr for lipase concentration of 13%

Butanol Variation

Experiment 6 Molar ratio of Butanol: Oil

Where,

6.1 = Molar ratio 0:1, 6.2 = Molar ratio 0.75:1, 6.3 = Molar ratio 1.5:1, 6.4 = Molar ratio 2.25:1 and 6.5 = Molar ratio 3:1

Experiment 6.1 Butanol: Oil 0:1

Reactants	Quantities
Oil	4.98g
Methanol (4:1)	2.16g (0.54g/time period)
Butanol (0:1)	0g
Phosphate buffer (3%)	0.149g
Lipase (7%)	0.348g

Table 24: Experiment 6.1 with butanol: oil molar ratio 0:1

Experiment 6.2 Butanol: Oil 0.75:1

Reactants	Quantities
Oil	4.95g
Methanol (4:1)	2.15g (0.537g/time period)
Butanol (0.75:1)	0.933g
Phosphate buffer (3%)	0.148g
Lipase (7%)	0.34g

Table 25: Experiment 6.2 with butanol: oil molar ratio 0.75:1

Experiment 6.3 Butanol: Oil 1.5:1

Reactants	Quantities
Oil	4.98g
Methanol (4:1)	2.16g (0.54g/time period)

Butanol (1.5:1)	1.87g
Phosphate buffer (3%)	0.149g
Lipase (7%)	0.348g

Table 26: Experiment 6.3 with butanol: oil molar ratio 1.5:1

Experiment 6.4 Butanol: Oil 2.25:1

Reactants	Quantities
Oil	5.0g
Methanol (4:1)	2.17g (0.54g/time period)
Butanol (0:1)	2.83g
Phosphate buffer (3%)	0.15g
Lipase (7%)	0.35g

Table 27: Experiment 6.4 with butanol: oil molar ratio 2.25:1

Experiment 6.5 Butanol: Oil 3:1

Reactants	Quantities
Oil	4.99g
Methanol (4:1)	2.167g (0.54g/time period)
Butanol (3:1)	3.76g
Phosphate buffer (3%)	0.149g
Lipase (7%)	0.349g

Table 28: Experiment 6.5 with butanol: oil molar ratio 3:1

Results from Gas Chromatograph for each fatty acid C18:1, C18:2 and C18:3

For C18:1

	C18:1				
Tittle (he)	6.1	6.2	6.3	6.4	6.5
0	4.65E-02	7.87E-02	9.12E-02	6.16E-02	0.00E+00
4	6.60E+00	2.32E+00	9.36E-01	8.22E-01	1.71E+00
4	6.21E+00	2.13E+00	1.29E+00	1.29E+00	1.59E+00
96	7.39E+00	1.87E+00	1.53E+00	1.43E+00	2.41E+00

Table 29: Gas Chromatogram results for C18:1 for butanol variation

For C18:2

	C18:2				
(ha	6.1	6.2	6.3	6.4	6.5
0	0.00E+00	5.80E-01	8.06E-01	5.74E-01	0.00E+00
4	4.96E+01	1.62E+01	5.78E+00	4.86E+00	1.07E+01
24	4.96E+01	1.41E+01	7.68E+00	7.38E+00	9.77E+00
96	5.70E+01	1.19E+01	9.57E+00	8.98E+00	1.74E+01

Table 30: Gas Chromatogram results for C18:2 for butanol variation

For C18:3

	C18:3				
Tithe (hr)	6.1	6.2	6.3	6.4	6.5
0	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
4	7.78E+00	2.85E+00	1.08E+00	1.21E+00	2.36E+00
24	1.11E+01	2.66E+00	2.32E+00	2.12E+00	2.09E+00
96	**1.22E+01**	**2.65E+00**	**2.31E+00**	**2.35E+00**	**3.49E+00**

Table 31: Gas Chromatogram results for C18:2 for butanol variation

Chromatogram for Molar ratio of Butanol: Oil at 0:1, 96hr

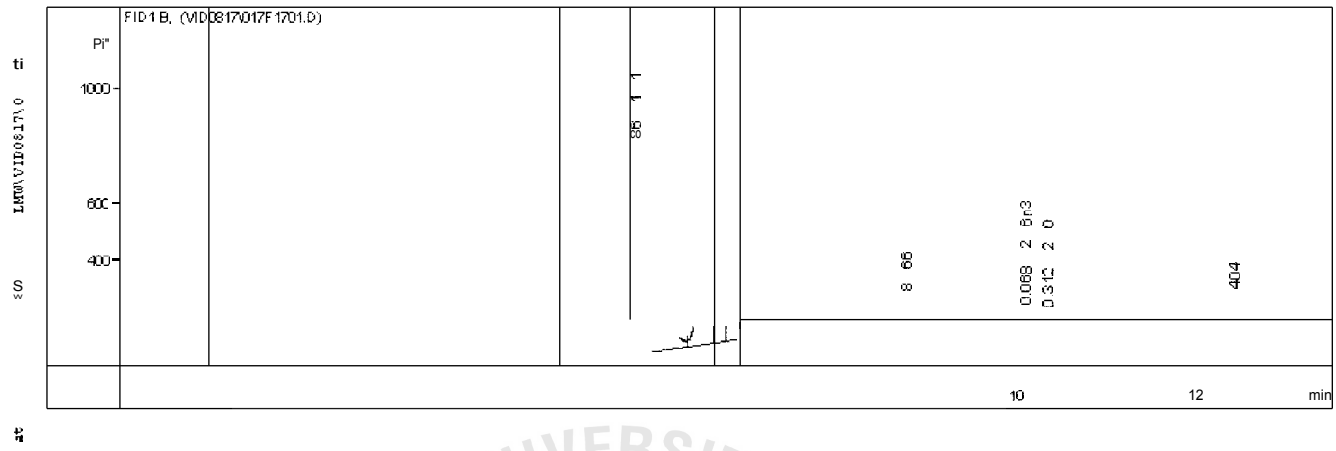

Figure 10: Chromatogram from the GC run for experiment 6.1 at 96hr for butanol: oil molar ratio 0:1