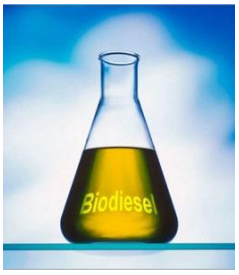
TÉCNICO LISBOA



Supported Enzymes as Catalysts for Biodiesel Production

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MUITO OBRIGADO

“I dedicate this thesis to my mother as gratitude. She was my first teacher in my life and the person who admired me and gave strength, courage and blessing throughout my life until the day she left me. “

Nomenclature

AE	Alkyl esters
AOT	Bis (2-ethylhexyl) sodium sulfosuccinate
BCA	Bradford Protein Assay
DG	Diglycerides
EDTA	Ethylenediaminetetraacetid acid
EIA	Energy Information Administration
Eq.	Equation
EU	European Union
FA	Fatty acids
FAO	Food and Agriculture Organization
FFA	Free fatty Acid
F-T	Fischer-Tropsch
GHz	Giga Hertz
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl-p-D-thiogalactopyranoside
MG	Monoglycerides
NG	Natural gas
NOx	Nitrogen oxides
OD	Optical density

OECD	Organization for Economic Cooperation and Development
PAGE	Polyacrylamid Gel Elctrophoresis
PNPB	P-N itrophenylbutyrate
TG	Triglyceride
USA	United States of America

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Abstrato

Biodiesel está a emergir como um substituto relevante para os combustíveis para transportes à base de petróleo. Quando olhamos para o nível atual de produção de biodiesel a partir de óleos, tanto comestíveis como não-comestíveis , vemos que não será capaz de atender à procura a um nível que permita substituir os combustíveis convencionais, sem que a produção de biodiesel passe a uma escala de massa a um preço competitivo. Foi demonstrado no passado recente que biocatalisadores têm um enorme potencial para a produção de biodiesel e, assim, a catálise enzimática está a atrair cada vez mais atenção ao longo das últimas décadas. As vantagens da catálise enzimática quando comparada com os catalisadores alcalinos tradicionais, são a sua capacidade de actuar sbre todos os substratos e uma elevada especificidade para o produto, uma actividade muito elevada e a capacidade de catalisar as reacções desejadas de forma eficiente em condições muito suaves, tais como a temperatura, a pressão, o meio aquoso ,e o pH neutro , com referência às suas ambiente nativo.

As lipases suportadas sobre zeolitos têm sido utilizados para catalisar a transesterificação de triglicéridos com eficiência, proporcionando catalisadores muito estáveis. Foi também demonstrado no passado, a partir de pesquisas por várias pessoas, que lipase é um catalisador promissor para a produção de biodiesel. Esta tese abrange o estudo que se destina a avaliar a possibilidade de utilização da enzima imobilizada sobre zeólitos como catalisadores para a produção de biodiesel por transesterificação de triglicerídeos com um álcool de cadeia curta. Um conjunto de catalisadores contend a enzima suportada sobre um zeólito foi preparado e cineticamente caracterizado pela hidrólise de um compost padrão (p- nitrofenil butirato), reação essa que nos permitiu verificar a atividade da enzima suportada. Os catalisadores, que mostraram ser bastante ativo, foram usados. para produzir biodiesel em pequena escala utilizando um triglicerídeo e butanol.

Abstract

Biodiesel is emerging as a significant replacement for petroleum based automotive fuels. When we look at the present level of the production of biodiesel from both edible and non-edible oils, we see that it will not be able to cater for the demand required to replace conventional fuels without producing biodiesel in mass scale at a competitive price. It has been shown in the recent past that biocatalysts have tremendous potential in the production of biodiesel and, thus, enzymatic catalysis is getting more attention over the past decades. The advantages of enzymatic catalysis with reference to traditional alkaline catalysts are substrate and product specificity, very high activity and the ability of catalyzing the desired reactions efficiently in very mild conditions such as temperature, pressure, aqueous medium, and neutral pH, with reference to their native environment.

Zeolite supported lipases have been shown to catalyze the transesterification of triglycerides efficiently, providing very stable catalysts. It has shown in the past, from the research by various people, that lipase itself is a promising catalyst for the production of biodiesel. This thesis work covers the study of assessing the possibility of using immobilized enzyme on zeolites as catalysts for the production of biodiesel by transesterification of triglycerides with alcohol. A set of zeolite supported immobilized enzyme catalysts has been prepared and kinetically characterized by the hydrolysis of a synthetic substrate (*p*-nitrophenyl butyrate) reaction This allowed us to check for supported enzyme activity. The catalysts, which were found active, were used to produce biodiesel in a small scale using a triglyceride and butanol.

1.Introduction

Biofuels can play a vital role in transportation sector in future. Oil is depleting but yet no large scale replacement for oil-based transportation has emerged, except for a limited number of electric vehicles. Due to growing demand and high rate of increase in the number of cars, from 1 billion in 2010 to an expectable 2.5 billion on the road by 2050, it is the duty of researches and engineers to find an alternative fuel for the vehicles. The biggest challenge is the production of biofuels in mass scale to cater for the demand. A variety of feed stocks with different technologies have been tested and tried over the past two to three decades to achieve this objective but still the task is huge. Much research is being done all over the world on biofuels to produce them in large scale as well as to improve the quality needed to replace fossil fuels.

Biofuels as a renewable energy source are essential elements of the world's energy system. Biofuels are important because they help in solving two of the most important challenges associated with energy use, the overwhelming dependency and increase in demand of fossil fuels and the emissions associated to their use. We can use biofuels for people to live independently of fossil based fuels. For example in a small village it would be a remarkable achievement if the total power requirements could be achieved using energy resources available locally by managing and utilising them wisely. Hence it is vital to find solutions to increase the production of biofuels because the main issue of replacing fossil fuel with biofuels is not having capability to cope with the present demand. A lot of research has already been done on this purposes and it has been observed that by using a variety of biomass resources with recent technological developments it is possible to produce biofuels in mass scale.

Electrical power generation has many options such as wind, biomass, solar, geothermal and wave energy. Yet energy for transportation is highly dependent on liquid fuels, except for electric cars. It is therefore important to study methods to increase the rate of the production of biofuels. Biofuels has so far gone up to three phases, referred to as first generation, second generation and third generation biofuels. A variety of feedstock has been used over the years to produce biofuels but one has yet to realize how to replace fossil fuels in the future.

First generation biofuels started with the use of edible oil such as soya beans, corn and sugar cane, etc. Biodiesel was produced by transesterfication of these edible oils and bioethanol was produced using sugar cane by fermentation and distillation. Second generation biofuels are produced from a sustainable feedstock which is defined as a feedstock that does not affect food price, land usage, biodiversity and GHG emissions. Lignocellulostic biomass derived from non-food sources such as grasses and trees can also be used to produce bioethanol, using enzyme catalysts, or to produce other biofuels by a variety of processes including liquefaction, pyrolysis or gasification. Non edible seeds such as Jatropha can also be used to produce biodiesel using transesterfication. But all these processes require an uninterrupted supply of feedstock in large quantities to produce biofuels in mass scale. One of the major problems associated with the use of non-edible feedstock is that, although they do not use food-sources, there may still be some competition for agricultural land in its production.

Finally, third generation biofuels appear as using alternative sources such as micro algae based biofuels, hydrogen, syngas and synthetic biofuels. Algae have the capacity to grow fast and double their mass in 24-48 hrs and require less land. Also one of the biggest advantages of algae is that it is not a seasonal feedstock like most of the others. It can grow throughout the year and if produced in mass scale a nonstop feedstock supply is possible. It also has a variety of other advantages such as being able to grow in waste waters and lands which are not suitable to grow agricultural products thus saving water and nutrients because it can absorb nutrients in municipal and industrial waste waters. Also they can grow near power plants so that the algae can act as a carbon sequester for the power plant. Algae are also rich in oil content and it is possible to produce biodiesel as well as bio gasoline, which can be used in petrol engines. There is a possibility of producing bioethanol as well from the residues after esterification and if the residues are allowed to digest in an anaerobic digester it is possible to produce biogas as well. It is also possible to use residues under enzymatic digestion to produce bioethanol. Also residues after esterfication or fermentation can be used as solid fuels for boilers and after anaerobic digestion they can be used as fertilizers. However the biggest advantage of the use of algae is that they require less land and less fresh water. Hence land usage and water consumption for biofuels can be minimized. But there is still the need to improve the methods to extract oil from algae and control its growth. The fact that there is a high content of water in the biodiesel produced from algae is also a drawback.

Whatever the feedstock being used for biodiesel production, the type of catalyst used plays a major role in the process. As a consequence of the drawbacks of homogeneous catalysts heterogeneous catalytic processes are being developed for the production of biodiesel by many researches over the years and it has been shown promising results. The biggest advantage of the use of heterogeneous catalysts is that the catalyst can be reused several times and it reduces the cost effectively.

1.1 Motivation

Biodiesel is mainly used as an alternative fuel for diesel engines. Ever-increasing petroleum price and its eco friendliness is a driving force to pay attention to increase production rate of biodiesel. Global biodiesel production has reached over 6 billion litres a year and represents 10% of the entire biofuels production [1]. The method of production of Biodiesel is the transesterification of edible or non-edible oils such as jatropha, rapeseed, soya bean, corn, sunflower, and palm oil. It has been shown that in 2007, about 7% of global edible oils have been spent on biodiesel production [2]. It is important to produce biodiesel in mass scale to cope with the growing demand, but at the same time the attention must be given to utilize more non edible oils which do not give any threat to food scarcity.

Production of biodiesel in mass scale, while cutting the cost, is a challenge for researchers. Homogeneous alkali-catalysis has been widely used in production of biodiesel, but the drawbacks are the requirement of complicated downstream processes including the removal of inorganic salts from the product, the recovery of salt-contained in glycerol, and the wastewater treatment. The conventional homogeneous process requires low

contents of water and free fatty acids to prevent the generation of soaps. Due to these drawbacks, attention has been given to the search for an alternative method using heterogeneous catalysts [3]. Application of enzyme gives promising results with possible solutions to the problems encountered before as it allows easy recovery of biodiesel and glycerol [4]. It makes use of the versatile lipase activity that provides simultaneously catalytic activity for the conversion of triglycerides and fatty acids by transesterification and esterification [5].One of the most promising aspects of using enzymes as catalysts is that they can be operated at relatively low temperatures and atmospheric pressure, which greatly helps to reduce energy consumption. However, lipases are catalysts that operate in liquid phase and, thus, present the disadvantages of homogeneous catalysis and potentially increases the cost of the process. This, however, can be overcome by immobilising the enzyme on a suitable support to allow its recovery.

1.2 Objectives

1 Zeolite-supported lipases have been shown to be able to catalyse the transesterification of triglycerides [6] in an efficient way and providing very stable catalysts. Lipase itself has also been shown to be a promising catalyst for the production of biodiesel. The objective of this work is to assess the possibility of using lipase supported on zeolite as catalysts for the production of biodiesel by transesterification of triglyceride with butanol in isooctane.

2. The hydrolysis of a synthetic substrate is used to arrive at a suitable kinetic characterisation of the activity of the catalysts. Accurate kinetic information is also important to allow the analysis of the reactor type and reaction conditions adequate for the practical use of this type of catalysts for the production of biodiesel.

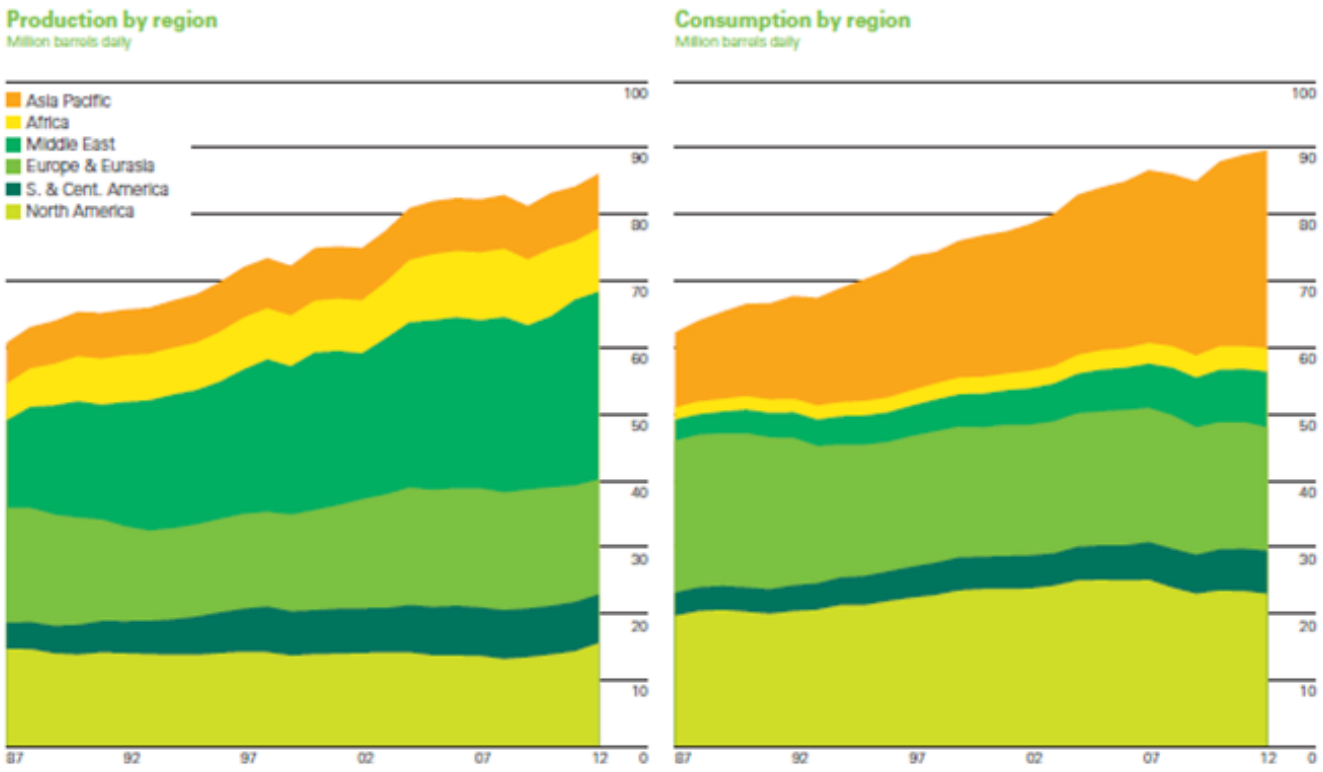
1.3 Thesis Outline

Chapter 1 presents Abstract, Introduction, Motivation and Objectives of the thesis work. Chapter 2 presents a literature review on the topic, focused on information previously available about the different processes to produce biodiesel and production technologies applied. Chapter 3 presents the materials and methods used in the experimental work and the experimental set-up is described. Chapter 4 gives an analysis of all the results obtained in experiments and detailed description of the work. Chapter 5 presents the conclusions of the present work while Chapter 6 presents the suggestions for future work.

2. Literature Review

2.1 World Energy Production and Consumption

International Energy Outlook 2013 *(*IEO2013*)* predicts that world energy consumption will be increased by 56 percent during periods between 2010 and 2040. More energy will be consumed by countries outside the Organization for Economic Cooperation and Development known as non-OECD due to their high demand and long-term economic growth and expected to increase by 90% but increase expected from OECD countries will be 17%.

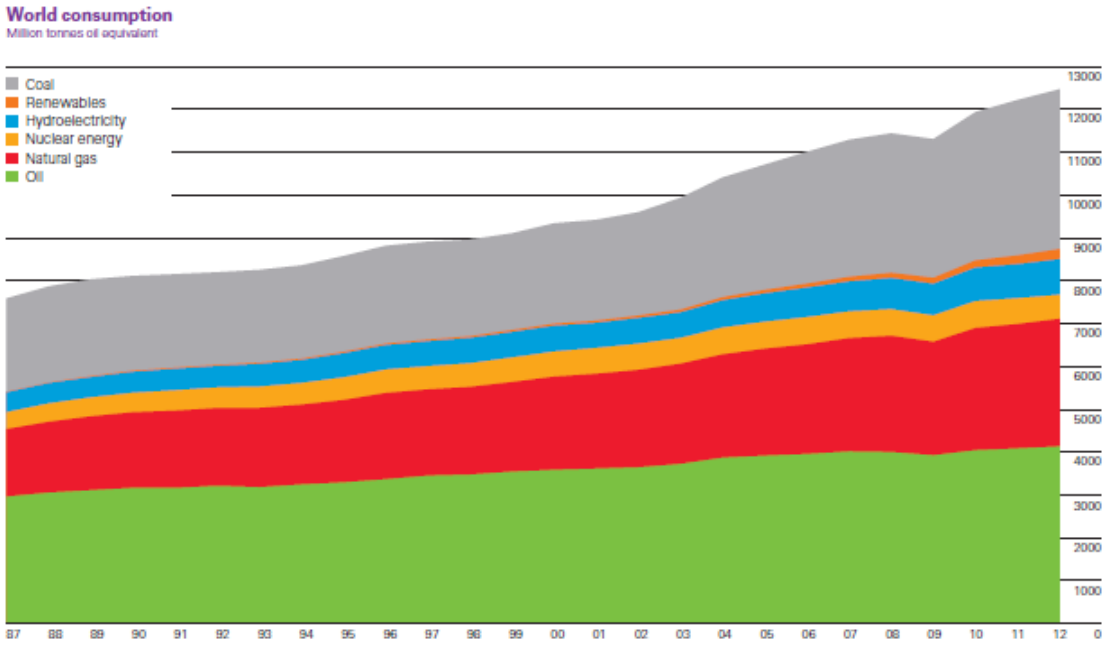


World oil production increased by 1.9 million b/d in 2012, more than double the growth of global consumption. US output grew by 1 million b/d, the largest increase in the world and in the country's history. The recovery in Libyan production drove robust growth in African output. Global oil consumption grew by just 990,000 b/d, with declines in Europe and North America offsetting gains elsewhere.

Figure 1[7]

Renewable energy and nuclear power are the world's fastest-growing energy sources at present, 2.5% increase per year. At the same time, fossil fuels continue to dominate representing 80% world energy till 2040. Natural gas (NG) is the fastest-growing fossil fuel according to the outlook. Natural gas consumption increases by 1.7 percent per year globally due to increasing supplies of tight gas, shale gas, and coal bed methane. As a

consequence of increases in China's consumption of coal, it is growing faster than petroleum and it is expectable that it continues to grow faster than liquid fuels at least until 2030. Worldwide energy related carbon dioxide emissions too could rise from about 31 billion metric tons in 2010 to 45 billion metric tons in 2040[8].



World primary energy consumption grew by a below average 1.8% in 2012. Growth was below average in all regions except Africa. Oil remains the world's leading fuel, accounting for 33.1% of global energy consumption, but this figure is the lowest share on record and oil has lost market share for 13 years in a raw. Hydraulic output and other renewable in power generation both reached record shares of global primary energy consumption (6.7 and 1.9% respectively).
Source: BP Statistical Review of World Energy June 2013

Figure 2[7]

2.2 Current Situation

By considering the future growth rate and the way the world population is increasing the size of the world population will be nearly 8 billion around year 2040. It clearly indicates the abrupt rate of increase of world energy consumption. This demand is still based on fossil fuel availability because about 80% of the total primary energy supply consists of fossil fuels. Fossil fuels, coal and gas are still leading energy supplies in the world. However, as concluded in the Agricultural Outlook from OECD-FAO, biomass may increase more rapidly.

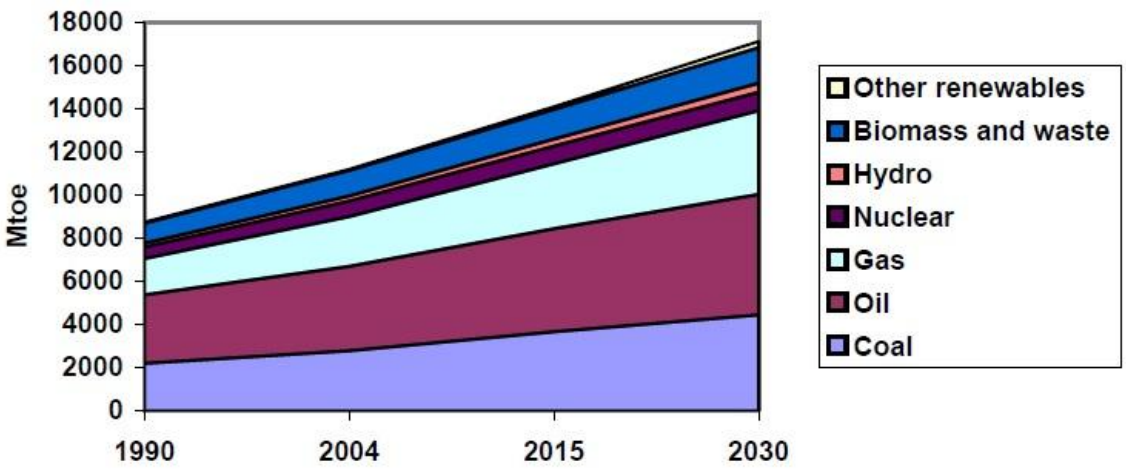
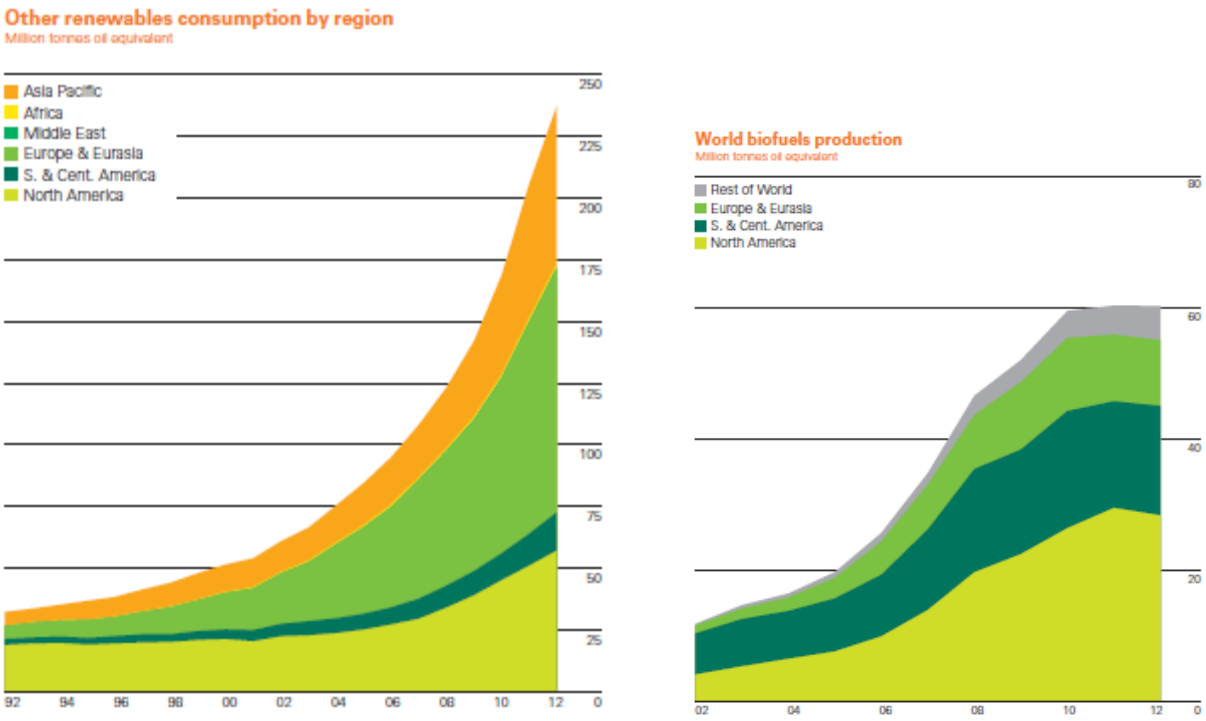


Figure 3.Past pattern and future energy consumption forecast[9]

2.3 Future Trends

The transport sector is increasing dramatically and consumes at present about 25% of the world energy supply and according to present development, the main source in transport fuel still expected to be oil till at least 2030. This oil dependency creates many issues;

As a result of limited fossil fuel resources the duration of availability of these resources are constantly changing depending on the present consumption and future growth. Reserves-to-production ratios for oil, coal and natural gas are about 40 years, 150 years and 60 years, respectively. The use of fossil fuels also implies a large dependency on a limited number of supplier countries. There is also a growing concern about increased CO_2 levels such as global warming caused by human activities. Alternative vehicle liquid fuels like ethanol are rarely commercially available at present, except in countries like Brazil, and the implementation of improved vehicle technologies a is n its very beginning. Hence renewable liquid transportation fuel must be given proper attention.[10]



Renewable energy in power generation grew by an above average 15.2%. Europe and Eurasia delivered the largest growth increment and continues to hold the largest regional share of the global total accounting for 41.7% of the world total. Renewable energy accounted for a record 4.7% of global power generation with an 8.2% share in Europe &Eurasia. World biofuels production declined by 0.4% in 2012, the first decline since 2000. Increased output in South America and Asia Pacific was outweighed by declines in North America and Europe. Global ethanol output declined by 1.7% the second straight annual decline. Biodiesel production grew by 2.7% and has doubled in the last years and now makes up 31% of total biofuels supply.
(Source: BP Statistical Review of World Energy June 2013)

Figure 4[7]

2.4 Summary of world energy

Following world energy statistics shows the ever increasing demand for energy and the place where biofuels stand by 2010.

Table 1 Summary of world energy **[11]**

Global energy consumption growth	5.6%
Chinas world energy share	20.3%
Global oil production growth	+3.1%
Global NG production growth	+7.4%
Global coal production growth	6.3%
Chinas share of global Coal	48.2%
World total renewable energy growth	1.8%
World total renewable energy production	13.8%
World oil price (crude)/barrel	100$
World oil production increased by	1.8 million barrels /day

World oil consumption increased by	2.7 million barrels /day
World oil import/export	53.5 million barrels /day
Natural gas reserves available	187.1 trillion cubic meters
Natural gas reserves Sufficient for	58.6 years
Natural gas consumption	3169 billion cubic meters
Natural gas production	2990.9 million tons oil equivalent
Coal power resources	860938 million tones
Coal power resources sufficient for	118 years
Coal power price	90-100 $ per tone
World coal production	3731.4 million tons of oil equivalent
World coal consumption	3555.8 million tons of oil equivalent
Nuclear power growth	2%
Nuclear power consumption	626.2 million tons of oil equivalent
Hydro power growth	5.3%
Hydro power consumption	775.60 million tons of oil equivalent
Other renewable consumption	158.60 million tons of oil equivalent
Biofuels production	59.26 million tons of oil equivalent
World primary energy consumption	12002.4 million tons of oil equivalent
World oil contribution	33.6%

From the statistics of Fig. 3 & 4, and Table 1, it can be clearly seen that the task of producing biofuels to fill the gap or replace fossil fuel based oil is huge and that there are enormous challenges ahead for the researches. On the other hand it is clearly indicated that there is a huge potential in the world if proper technology is used and managed so that biomass based biofuels can be produced globally to cater for the demand in the future. World biofuels production trend can be seen from the table 2.

Table 2 Summary of world biofuels production **[12]**

Total Biofuels Production (Thousand Barrels Per Day)					
	2007	**2008**	**2009**	**2010**	**2011**
North America	472.8301	666.4791	769.3396	916.6467	1004.829
Canada	15.4	16.7	22.1	26.4	32.7
United States	457.3301	649.6791	747.1366	889.8467	971.7288
Central & South America	425.8854	533.6738	533.4864	588.0659	519.152
Argentina	3.9	14.1	23.5	38.1	50.34
Brazil	395.6761	486.3488	477.5284	527.1348	438.058
Colombia	4.8	5.8	11.3	12	15
Guatemala	2.9	2.9	3.01	3.01	4.01
Jamaica	4.852	6.423	6.9	2	3
Paraguay	1.1	1.7	2.2	2.3	2.22
Peru	0.9	0.7	1.2	2.2	2.7
Trinidad and Tobago	3.167	4.26	2.797	0.0001	2.2
Europe	153.8	198.05	233.18	255.243	250.491

Austria	5.5	5.7	8.6	8.2	8.7
Belgium	3.2	5.8	10.6	13.5	15.2
Czech Republic	2.2	2.8	5	6	6
Denmark	1.4	1.8	1.71	1.8	2.8
Finland	1.35	2.5	4.5	5.9	4.2
France	28	50.4	58	55	51.4
Germany	63.8	65	58	62	65.3
Greece	1.8	1.3	1.4	2.4	2
Ireland	0.4	0.6	1.4	1.3	1.1
Hungary	0.7	5.1	5.1	6	5.8
Italy	10.2	14.1	16.6	16.5	12.2
Netherlands	1.9	2.2	5.4	9.5	13.6
Romania	0.7	1.3	0.7	1.7	1.9
Serbia	0.5	1	1.5	1.5	1.5
Poland	2.9	7	9	11	10.4
Portugal	3.5	3.3	4.9	6	5.5
Turkey	1	1.1	1	1	1.1
Slovakia	1.4	3.6	4	4	3.2
Latvia	0.5	0.9	1.2	1.1	1
Spain	10.5	10.3	22	24	20
Sweden	3.7	4.5	6.5	7.5	8.4
United Kingdom	8.3	6.7	5.3	9	9
Eurasia	1.37	3.2	5.1	4.48	3.67
Lithuania	0.8	1.7	2.4	2.5	1.9
Middle East	0	0	0	0.1	0.1
Asia & Oceania	49.222	75.573	93.7671	99.8105	118.1705
Australia	2.1	3.4	5.2	7.9	9.1
China	30.7	39.4	43	43	46.8
India	4.7	5.2	7	7	8
Taiwan	0.1	0.4	0.7	0.5	1.5
Vietnam	0	0	0.044	0.6	1
Indonesia	1.2	2.2	6.2	8.1	20.1
Korea, South	1.7	3.2	5	6.5	6.3
Japan	0.102	0.103	0.203	1.3	1.3
Malaysia	2.5	4.5	4.5	2	1
Philippines	0.6	1.11	2.5	3.4	3
Sri Lanka	0	0	0	0	0
Taiwan	0.1	0.4	0.7	0.5	1.5
Thailand	4.2	13.4	17.4	18.5	19.1
Africa	0.2	0.345	0.54	1.02	0.79
World	1103.307	1477.321	1635.413	1865.366	1897.202

*Countries producing less than one thousand barrels per day are removed from the list except Sri Lanka.

2.5 Future Targets

The European Union (EU) has set targets for the future energy policies expressed in the Green paper from 2006: An European strategy for sustainable, competitive and secure energy. Strategic targets to be reached by the year 2020 have been defined at the meeting in the European Council in March 2007.

20% savings of EU energy consumption compared to projections as estimated by the Commission in its Green paper on Energy Efficiency.

20% renewable energy in overall EU energy consumption.

10% share of biofuels in overall EU transport petrol and diesel consumption. The binding character of this target is appropriate but subject to production being sustainable and second-generation biofuels becoming commercially available.[10]

2.6 Biofuels Conversion Processes and Technologies

Biomass is applied in different energy sectors: heat and electricity production and transportation fuel. The process diagram shown in Figure 5 shows various possible technological conversion approaches from biomass to bio combustibles .

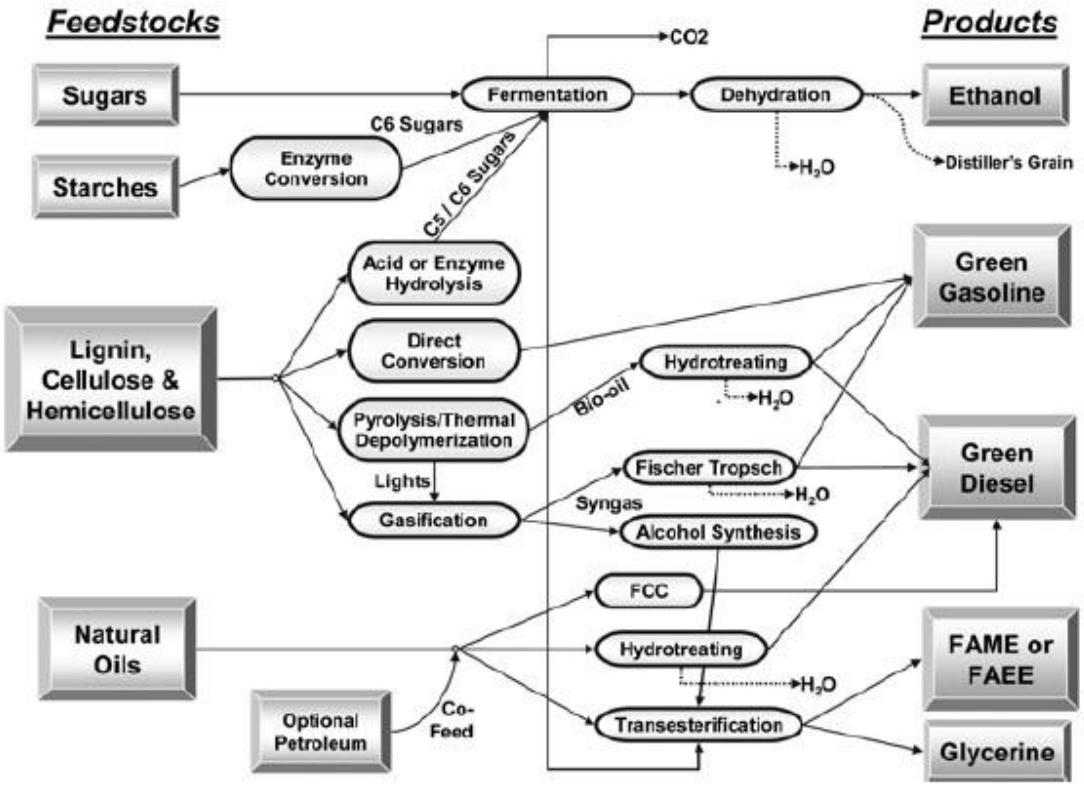


Figure 5 Bioenergy conversion technologies[13]

2.6.1Transesterification

Transesterification has been used in the biodiesel production process since its beginning. It is required to store stock of enough supply to feed to the production line throughout its production to be fed into the system at required rate. The seeds will first be separated from dust and rust particles to send through crushers or screw driven oil press to extract oil. After the extraction of oil it will be fed into a reactor and mixed with alcohol (methanol or ethanol are commonly used) and a catalysts and stirred to produce the ester from the oil in the process of transesterification. The mixture containing the esters, glycerol and water will pass through a centrifuge to separate glycerol and water. It is then required to wash the product in few more stages to remove soap and finally remove the water from the ester to get the final product of biodiesel. One of the biggest challenges of this process is to remove the free fatty acids (FFA) fully from the biodiesel and increase the rate of production. Over the years and after much research the technology has been improved to reduce the esterification time and FFA formation. Bio refineries will be an option to produce biodiesel from edible or non-edible oil in continuous process in relatively large quantities.

2.6.2 Fermentation and distillation

Bio ethanol is produced by sugar cane and other sugar based biomass. Sugar cane is more popular in countries like Brazil and USA to produce bio ethanol. In this process sugar has to be first extracted from the feedstock and then yeast is added. After fermentation it has to be distilled to extract methanol or ethanol. The process is efficient and if enough feedstock is available it is possible to produce bio ethanol that can be mixed with gasoline and in future there is the possibility to use existing fossil fuel refineries, with some modifications, to produce bio ethanol as well.

2.6.3 Enzyme based bio ethanol

Second generation biofuels aimed to use feedstock which are not used for food production. Forest residues like wood, straw and grass, and any feedstock of green biomass, they all can be used to produce bio ethanol, bio methanol, biodiesel or even biogas by using a biomass feedstock. In this process all residues should be cut and unwanted wood parts removed followed by crushing. Lignocelluloses, cellulose wood or grass can be used in this process. After the preparation and treatments with several stages of hydrolysis biomass can be converted to cellulose and hemicelluloses and it is then allowed to convert to sugars by enzymatic catalyst digestion. After that the sugars can be fermented and distilled to produce bio ethanol. The remaining residue can be used directly as a solid fuel for the ethanol plant or it can be used to produce biogas by allowing it to go through an anaerobic digestion. Also it is possible to use final residue to make wood pallets to burn as a fuel.

2.6.4 Microalgae based biofuels

Microalgae are the latest development in biofuels production as a more efficient way to produce biofuels in mass scale. Land use and high consumption of water are major issues in all previous biofuels production processes. At present the world land area is about 13.5 billion Ha and 8.3 billion Ha of land is already occupied for agriculture, forest and other purposes. Still ~~0~~5 billion Ha of land are available for use in the production of feedstock for biofuels. But if we use feedstock of microalgae it is possible to produce full capacity of USA demand of biofuels in 200 km^2 of land [14]. The biggest advantage is that it will need less labour; it can use waste water or sea water and less land to grow the algae. Any other feedstock needs fertilizers to grow but algae can extract nitrogen from municipal waste and if grow near power plants, exhaust CO_2 gases can be used to grow algae. It is also possible to produce biodiesel, bio ethanol or bio gasoline and biogas as well using algae as feedstock. Compared to other feedstock, algae contain a large amount of oil, up to 70% of lipids, but presenting some difficulties in extraction. If extraction methods can be improved algae will probably be one of the best ways to produce biofuels in mass scale.

2.6.5 Biocatalyst for Biodiesel Production

It has been shown recently that enzymes also can play a vital role in biodiesel production. In this context, biotechnological routes such as enzymatic processes are very promising because of its high selectivity, mild operative conditions, easy product recovery and catalyst recycling. One of the enzymes that is found to be capable of catalyzing transesterification is lipase, which is obtained from microorganisms like Rhizomucor

miehei (formerly called Mucor miehei), Rhizopus oryzae, Candida antarctica, Chromobacterium viscosum, Thermomyces lagunisous, Pseudomonas fluorescencs and Pseudomonas cepacia. Biocompatibility, biodegradability and environmental acceptability of the biochemical procedure are the desired properties in industrial applications. The optimum temperature for activity of various lipases used for the transesterification of oils ranges between 30 ^{0}C and 50 ^{0}C, clearly indicate it require less energy for the conversion. But the process has yet to be implemented in an industrial scale due to some drawbacks like enzyme inhibition by the alcohol, exhaustion of enzyme activity and high cost of production of enzymes. Research work has been undergoing recently to overcome these drawbacks. Various alcohols have been tested for the alcoholysis reaction including methanol, ethanol, propanol, isopropanol, 2-propanol, *n*-butanol and isobutanol. Methanol and ethanol have been mainly used for industrial biodiesel production since they are the cheapest alcohols and are produced in the largest scale[15].

2.7 Conventional Technologies

2.7.1 Technologies used in Transesterification

Transesterification of triglyceride (TG) can be done using either homogeneous or heterogeneous catalyst. If the catalyst is in the same phase during transesterification, it is called homogeneous catalytic transesterification. If the catalyst remains in different phase (e.g. solid, immiscible liquid or gaseous) the process is called heterogeneous catalytic transesterification. The homogeneous catalyst works very efficiently but it is difficult to recover after the esterification process and hence it is not possible to reuse the catalyst. Water required for washing the methyl ester and later separation of water and soap formed during esterification, is a process that costs a lot of time and money. If a heterogeneous catalyst can be used, the catalyst can be reused after reaction and, as it does not dissolve in the reaction mixture, less washing will be required. This saves lot of water as well as time and energy.

The transesterification is the reaction by which the oil a mixture of triglycerides, reacts with an alcohol to form alkyl esters and glycerol, where a catalyst is usually added to improve the reaction rate and yield. Figure 6 shows the chemical reaction involved in the transesterification.

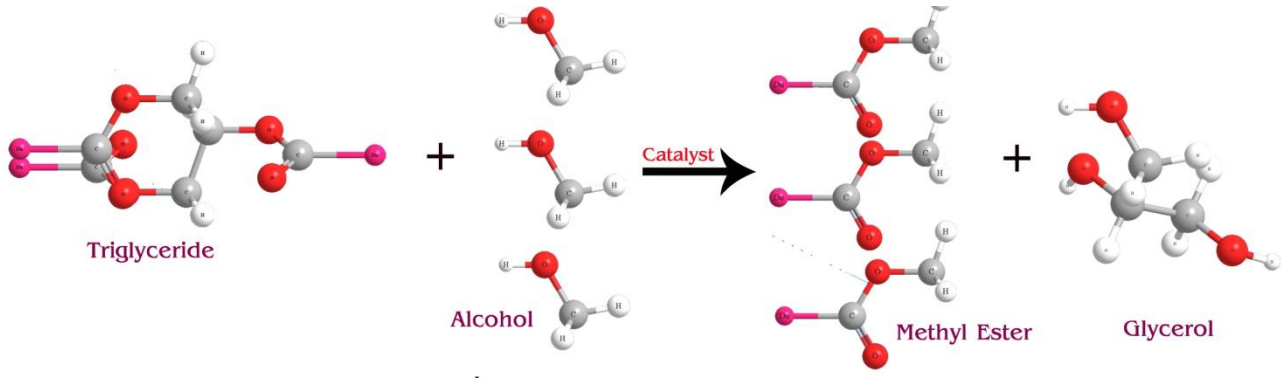


Figure 6 Chemical reaction in transesterification [32]

2.7.2 Homogeneous catalytic transesterification

Homogenous catalysts are categorized mainly into two that is basic and acidic catalyst such as sodium methoxide, potassium methoxide, sodium hydroxide, potassium hydroxide. The homogenous transesterification process requires a high purity of raw materials and thorough separation of product, by-product, and catalyst at the end of the reaction. As a result it affects high production cost of biodiesel. Homogeneous catalytic transesterification is described in detail on a process flow diagram of Figure 7 below.

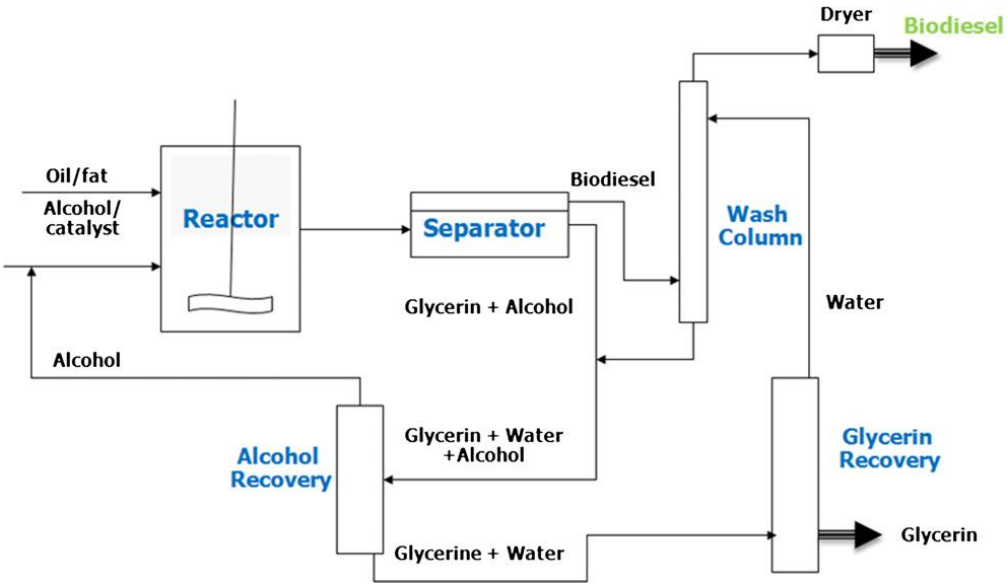


Figure 7 Homogeneous transesterification [16]

Transesterification is the conversion of triglycerides to diglycerides, and then it is followed by the conversion of diglycerides (DG) to monoglycerides (MG) and finally the mono glycerides to glycerol, producing one methyl ester molecule from each glyceride in each step. The factors affecting the transesterification process are temperature, time, pressure, alcohol to oil ratio, type of catalyst and its concentration, mixing speed and the feedstock type.

Catalyst selection is an important aspect to reduce the production cost of biodiesel. At present, commercial biodiesel is mainly produced by transesterification using a homogenous catalyst. The amount of free fatty acid (FFA) present in the oil is another factor to be decided on catalyst type. Better conversion is given in a relatively short time of oils having lower amount of FFAs by using Base-catalysts and, oils having higher FFAs require the use of acid-catalyst for esterification. For example homogeneous catalysts are sodium hydroxide, potassium hydroxide or sulphuric acid, and heterogeneous ones are CaO and MgO[16].

2.7.3 Homogeneous base catalytic transesterification.

Biodiesel is mainly produced using homogeneous base catalyst, such as alkaline metal alkoxides and hydroxides, or sodium or potassium carbonates. It is observed that sodium hydroxide or potassium hydroxide is more frequently used with concentration ranging from 0.4% to 2% w/w as a catalyst in the process of basic methanolysis. The reasons behind the use of Homogeneous base catalysts in the industries are:

moderate operation condition

high conversion efficiency

high activity

availability

cost effectiveness.

2.7.4 Homogeneous acid catalytic transesterification.

Another way of processing triglycerides for biodiesel production is to use an acid catalyst. It is possible to economically compete with base catalytic process using virgin oil, especially when base catalytic process uses low-cost feed stocks. Sulphuric acid or hydrochloric acid are preferably used as acid catalysts. In this process, transesterification starts by mixing the oil directly with the acidified alcohol, hence the separation and transesterification occur at once with the alcohol acting concurrently as a solvent and esterification reagent. The excess alcohol significantly reduce the reaction time [16].

2.7.5 Heterogeneous catalytic transesterification

Homogeneous catalyst act in the same phase as the reaction mixture, but heterogeneous catalysts act in a different phase from the reaction mixture. Due to the existence of in a different phase, heterogeneous catalysts have the capability of easy separation and reuse. The high energy consumption and cost incurred in the process of separation of the homogeneous catalyst from the reaction mixture drives the search for the development of heterogeneous catalysts. On the other hand heterogeneous catalyst does not form soap. It also helps to eliminate many steps of washing, recovery of catalyst, ensuring higher efficiency and profitability of the process

while lowering its production costs. It makes it also possible to implement continuous production by using a fixed bed reactor. Schematic diagram of a heterogeneous catalytic transesterification process is shown in Figure 8.

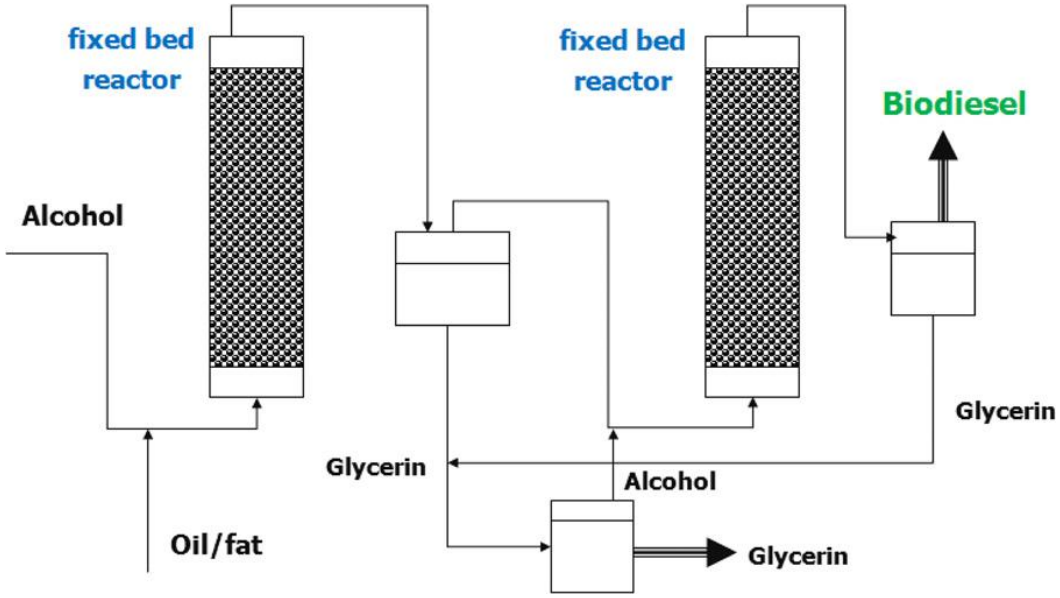


Figure 8 Heterogeneous transesterification [16]

2.8 New Technologies

2.8.1Ultra Sound Technology

Ultrasound is defined as a sound of frequency between 20 kHz and 100 MHz. It compresses and stretches the molecular spacing of the medium while it passes through the medium. Molecules will be continuously vibrated while cavities are created, if a large negative pressure gradient is applied to the liquid. At the same time micro fine bubbles are formed and undergo sudden expansion to an undesirable size, causing to collapse violently. The energy is generated concurrently by chemical and mechanical effects. It could be useful for transesterification of triglycerides with alcohol using low frequency ultrasonic irradiation as it provides the mechanical energy for mixing and activation energy for initiating the reaction. The advantages of this technology is that its simplicity, shorter reaction time, less energy consumption when compared to the conventional mechanical stirring and efficient molar ratio of methanol to triglycerides. A layout diagram of the ultra sound technology is shown below.

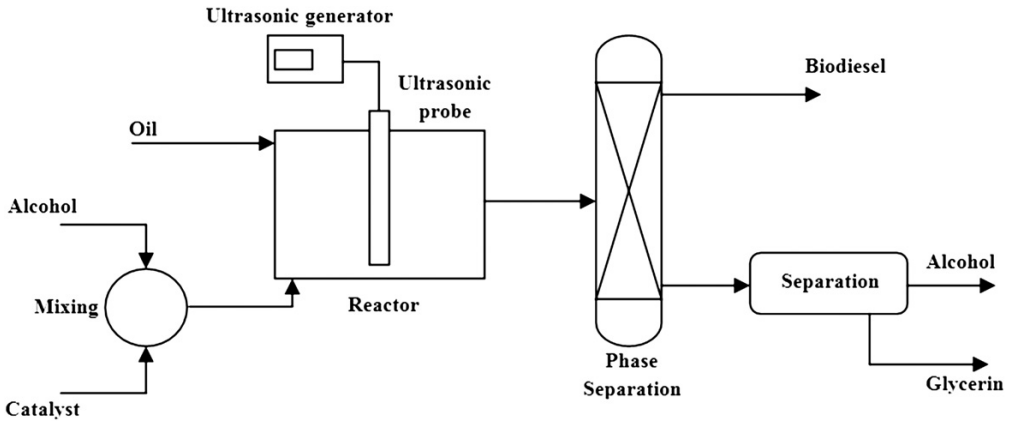


Figure 9 Ultrasonic biodiesel production process[17]

In the transesterification reaction ultrasound provides the mechanical energy for mixing and the required activation energy for initiating the reaction and it helps to shorten the reaction time as well as to increase the biodiesel production rate. It provides the way to produce biodiesel in continuous process as shown in fig 10.

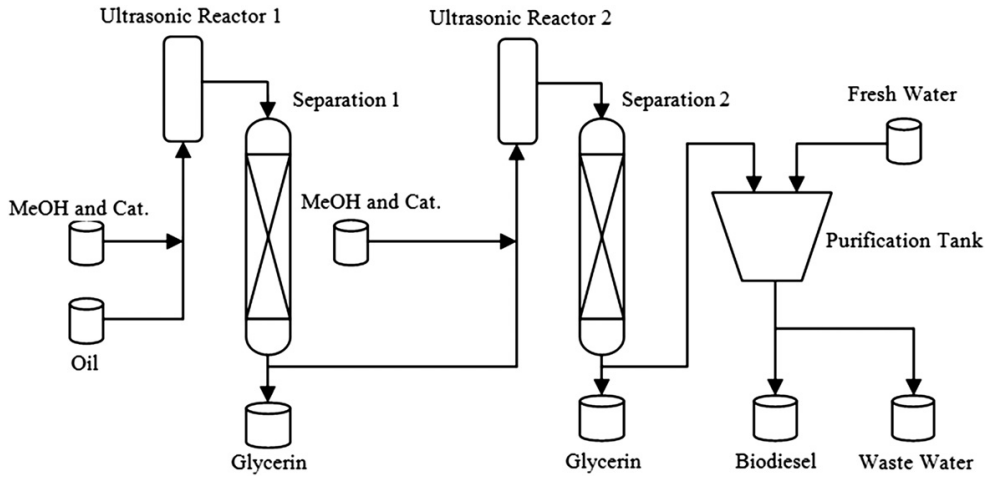


Figure 10 Ultrasonic biodiesel continuous flow process[17]

2.8.2 Microwave Technology

Microwave is an electromagnetic radiation in the range of wavelengths from 0.01 to 1 m and corresponding frequency range of 0.3 to 300 GHz. Radar transmissions uses the wavelengths between 0.01 and 0.25 m while telecommunications use the remaining wavelengths. Most microwave reactors for chemical synthesis and all domestic microwave ovens operate at 2.45 GHz frequency, which corresponds to a wavelength of 12.25 cm. This is to avoid any interference with telecommunications and cellular phone frequencies. Microwave irradiation is a well-established method of accelerating and enhancing chemical reactions due to its capability of delivering

the energy directly to the reactant. Hence, the heat transfer is more effective compare to conventional heating while it takes a shorter time to complete the reaction which leads to increase the rate of biodiesel production immensely. The continuous-flow production of biodiesel using a commercially available scientific microwave apparatus gives a quick and easy way to produce biofuels [18].

2.8.3 Fischer-Tropsch Synfuels

The need to produce biodiesel arises because there were no liquid fuel except petroleum based liquid fuels for transportation. But there is another opportunity to compete with fossil fuel based biofuels using biomass based synthetic fuel. It is called Fischer Tropsch Synfuels where it uses feedstock converting into commercially viable liquid fuels. The most common feedstocks used to produce Fischer-Tropsch synfuels are natural gas**,** coal and biomass**.** Most promising art of this innovative techonology is that any kind of biomass can be converted in to biofuels using this method.

This synthetic liquid fuels, use feedstock that can be converted directly into liquid fuels, skipping the syncrude step. The most common feedstocks used to produce Fischer-Tropsch (F-T) synfuels are coal, any form of solid biomass and natural gas. In this process of conversion, the feedstock is subjected to very high heat (over 1000 C^0) and high pressure to produce a mix of carbon monoxide and hydrogen is called syngas**.** In this process of conversion it makes Fischer-Tropsch liquid fuels much cleaner compared to fuels produced from crude oil. Impurities such as heavy metals can easily be removed after the gasification process by filtering the syngas using proper carbon captures. The fig.11 shows the conversion process.

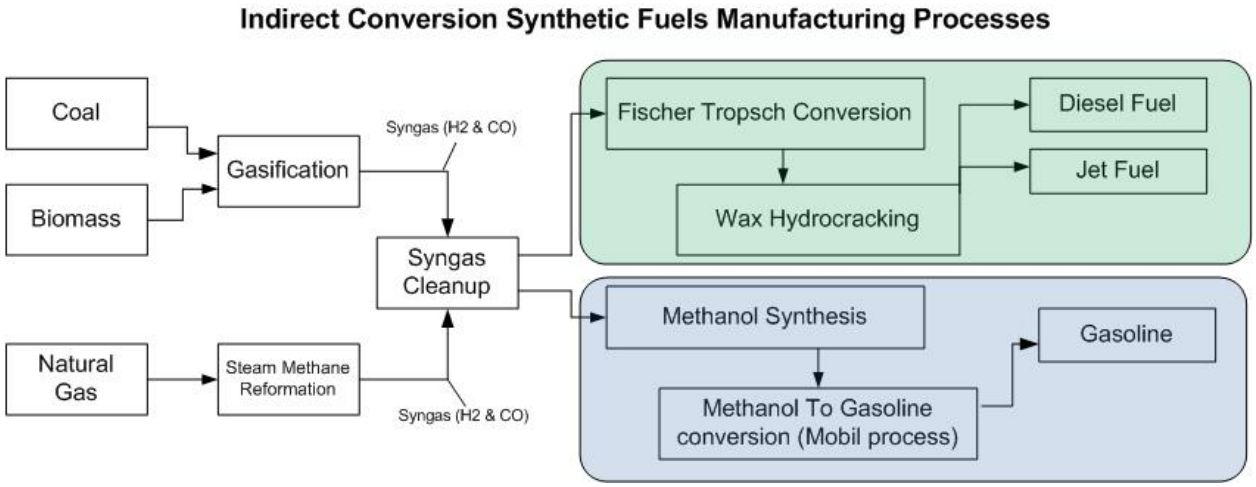


Figure 11 Synthetic Fuels Manufacturing Processes

After producing the syngas, it is condensed and put under high heat and pressure, and a catalyst is introduced into the process, usually a compound containing either iron or cobalt. The catalyst initiates a chemical reaction between H_2 and CO, creating long chains of hydrocarbons thus producing variety of hydrocarbon structures. These hydrocarbons can be cooled and condensed into liquid form. Any form of fossil fuel based liquid fuels such as diesel fuel or gasoline, jet fuels or even industrial lubricants can be produced by this conversion of biomass by pyrolysis process.

Fischer-Tropsch liquids burn much cleaner and produce environment friendly liquid fuel with fewer emissions, and contain less nitrogen oxide than traditional fuels and less carbon monoxide. The fig 12 below shows the future potential for syngas based liquid fuels in the US and it clearly shows how big the potential for this innovative technology if improved cost effectively to compete with fossil fuels. (It is stated in hydrocarbon engineering June 2007 about 1.3 Gigatons of unused biomass is available inside the United States that could be used to produce synfuels)[19].

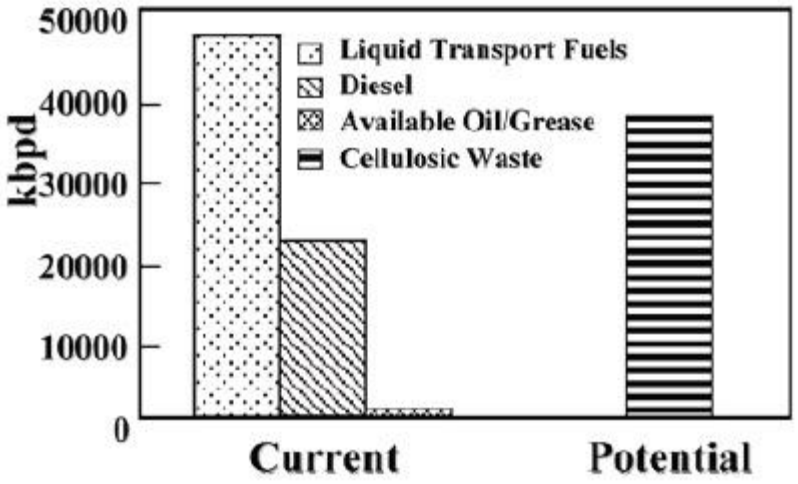


Figure 12 Biorenewable feedstock availability in US[19]

2.9 Enzymatic transesterification

2.9.1 Cutinase

The transesterification reaction of oil with alcohol yielding alkyl esters, which are the components of biodiesel, and glycerol, can be catalyzed by lipolytic enzymes, in particular by cutinase. The ability to degrade short and long chain triacylglycerols (TAGs), in addition to the ability to degrade cutin is the advantage of cutinase [20]. Cutinase can be obtained from different sources, such as the phytopathogenic fungus *Fusarium solani pisi*, and is an example of a small carboxylic ester hydrolase that bridges functional properties between lipases and esters. Although cutinase has been named due to its ability to degrade cutin polymers [21], at the same time they are able to convert a large variety of short and long chain triacylglycerols (TAGs). The enzyme belongs to the family of serine hydrolyses which contain α/hydrolase fold [22].Cutinase is a 197 amino acid long molecule, with a molecular weight of approximately 22 kDa and an isoelectric point of 7.6.

2.9.2 Cutinase Stability

The detergent area is the largest application of industrial enzymes. Stability of enzyme is affected by temperature, presence of surfactants, solvents, pH, induced stress and binding co-factors. The temperature is the most important parameter above all because it can cause irreversible protein unfolding and consequently enzyme deactivation [23]. Thermal and operational cutinase stabilities have been assessed in several studies [24]. Even after setting these parameters to obtain optimal performance, it has shown that the yields achieved are not suitable. Hence protein engineering is a commonly used method to improve enzymes yields.

The wild-type is rapidly inactivated by anionic surfactants in the case of cutinase, [25]. Many single mutations and post-translation modification have been performed on cutinase to investigate activity improvements [26].The stability of the enzyme could not be assessed by this test with that objective even though some have shown improvements in activity. In the presence of anionic surfactant bis (2-ethylhexyl) sodium sulfosuccinate (AOT), Brissos and co-workers screened all 19 possible amino acid exchanges at each 214 positions, for improved stability [27]. Three were selected from these mutants, since they have shown improvements in stability in the presence of AOT, namely S54D, 1153Q and T179C. These names are assigned according to the amino acid mutated. The active site, decreases the distance between helices A and F far from the S54D mutation, which occurs in the a-helix A [28]. The 1153Q mutation changes from a hydrophobic residue to a less hydrophobic amino acid, a weak spot regarding stability [29]. A cysteine is introduced with the objective of filling space near the active center and next to another cysteine in the T179C mutant involved in a disulphide bridge. The stability of AOT presence is due to the mutations which prevent the hydrophobic crevices, that are the cause of the enzyme unfolding induced by the surfactant [30].

2.9.3 Industrial Applications of Cutinase

Cutinase has been presented as a versatile enzyme showing several interesting properties for applications in industrial products and processes as a lipolytic enzyme.

Cutinase also has important applications in the dairy industry for the hydrolysis of milk fat, in house hold detergents, polymers and surfactants , oleochemical industry, synthesis of structured triglycerides, synthesis of ingredients for personal-care products, and the synthesis of pharmaceuticals and agrochemicals containing one or more chiral centers. Transesterification of triglycerides or selective esterification of alcohols can be achieved at low water activities. [31].

2.10 Enzyme Immobilization

The role of enzyme catalysis has given more attention over the past decades, due to its high potential to use as a biocatalyst. Catalyst stability is a key factor for successful industrial bioprocesses. Concerns with enzyme stability in the presence of organic solvents, extreme pH values, surfactants, or high temperatures have shown the path to researches to provide substantial improvements for many biocatalysts. Enzymatic thermal stability is

a desirable property, due to reaction rates increases exponentially with temperature, up until enzyme denaturing reaches prevalent. The most preferably used methods to overcome stability issues are the use of various immobilization techniques [32].

Lipases immobilization by adsorption: In physical adsorption enzyme fixation is performed through hydrogen bonds, salt linkages, and Van der Waal's forces. The process works in mild conditions. Interaction of the enzyme with the support, particle size, the surface area, the molar ratio of hydrophilic to hydrophobic groups, and the chemical composition of the carrier determine the amount of enzyme bound and the enzyme behaviour after immobilization [32]

Lipases immobilization by entrapment and/or encapsulation: Entrapment involves capture of the enzyme within a matrix of a polymer, although enzyme encapsulation refers to the formation of a membrane-like physical barrier around the enzyme preparation [33]. During the process of immobilization, the matrix is formed and the enzyme entrapped in a gel matrix can be further encapsulated. Enzymes immobilized by entrapment or encapsulation are more stable compared to physically adsorbed enzymes.

Some of the advantages and disadvantages of immobilized enzymes are given below.

Advantages

- Stability increases according to the strength of the bond created between enzyme and support (van der Waals, ionic, covalent...)
- The possibility of using continuous reactors. Packed and fluidized bed reactors, membrane reactors and stirred tanks with catalyst recycling possibilities.
- Solid catalysts can be recovered easily and reused.
- Heterogeneous catalysts can be easily separated from the reaction mixture by filtration, centrifugation or settling.
- The chemical and physical properties of the support may change the reaction microenvironment for the enzyme.

Disadvantages

- Activity can be reduced due to the strength of the bond created between enzyme and support (van der Waals, ionic, covalent,…)
- Cost of the support, immobilization process, the manpower, time required for the process could affect the final cost of biodiesel.

Over hundreds of enzyme immobilization procedures have been developed in the past but they all are mainly grouped into three categories such as support binding, entrapment and cross linking [34].

2.10.1 Zeolites

In this thesis, the enzymes have been used in free form and as heterogeneous catalysts to catalyse ester hydrolysis and transesterification. They have been immobilized by physical adsorption on zeolite NaY-LZY-52.

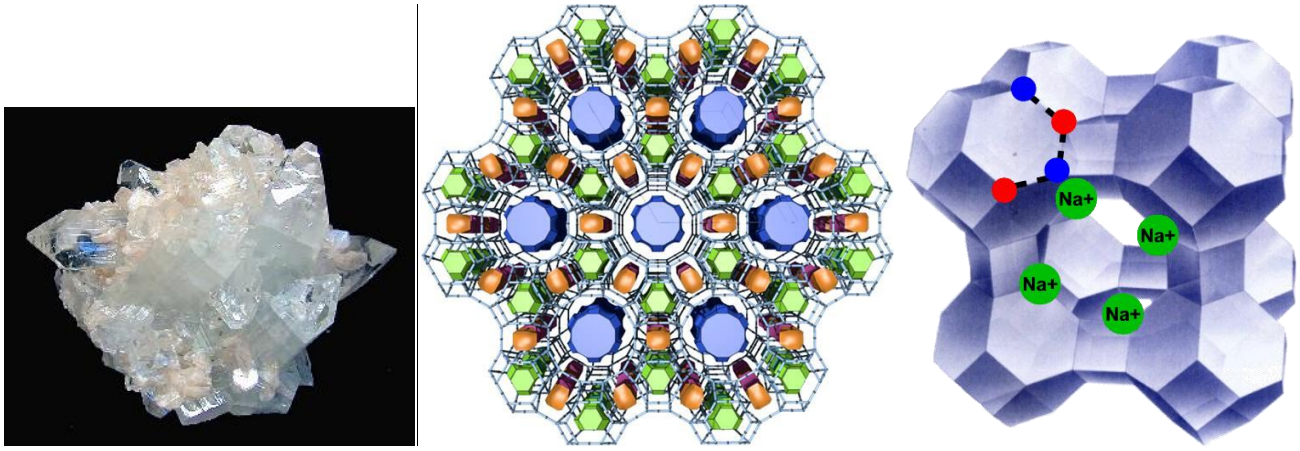


Figure 13 Some Images of Zeolites [35]

Zeolites are porous aluminum silicates that have been used as an industrial catalysis for over many years. On the other hand they are also used as molecular sieves, in detergents, adsorbents, and supports for other catalysts. Out of over 40 known natural zeolites, they can be fit into suit different needs in all their respective applications, namely catalysis, by changing properties such as strength, pore size and shape, acid or basic elemental composition or structural properties [36]. The number of known artificial zeolites exceeds the natural one and is now in excess of 150[37]. The formula of Zeolites are represented by Mx $(AlO_2)x$ $(SiO_2)y.zH_2O$. Counter cations (M) result from the replacement of Si_4^+ ions in the crystal lattice by Al_3^+ and the most common are Mg_2^+, Ca_2^+, K^+ and Na^+ [38].

Zeolite Y (faujasite or FAU) has been used for enzyme immobilization. The structure of faujasite is composed by sodalite cages arranged in a tetrahedral shape (Figure 16c). The junction of these units produces a three-dimensional structure with super cages of 12 Å connected by channels delimited by 12 oxygen atom rings and with 7.4 Å of diameter (Figure 1.7b). The sodalite cages and hexagonal prisms between them are not accessible while supercages are accessible to molecules of great dimensions (large pore zeolite), [31].

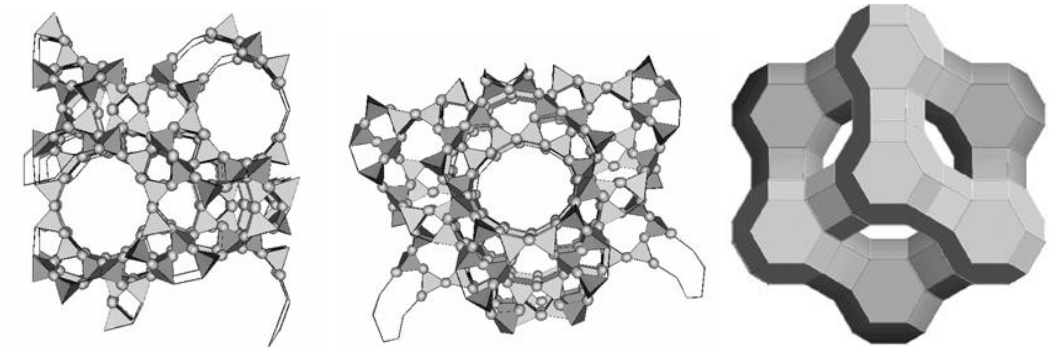


Fig16(a) Fig16(b) Fig16(c)

Figure 14 Different structures of Zeolites **[31]**

2.10.2 Zeolite as enzyme supports

The concept of supporting enzymes on zeolites has been first found in a paper by Webster [36] where they described the advantages of trapping an enzyme in zeolite pores and the application of molecular shape selectivity concepts.

Supporting enzymes on zeolites is a relatively easy and very versatile technique, due to the adaptable properties of zeolites in addition to the other benefits of heterogeneous catalysts, including the ease of separation from the reaction medium and the increased stability [37]. Many papers have been published in the past on immobilization procedures and its applications of zeolite supported enzymes.

2.10.3 The behavior of zeolite

Zeolites are cation exchangers because their crystalline frame work bears negative charges. Studies have been reported that the activation effect on enzymes in non-aqueous media as a result of their tuneable solid-state acid-base buffer properties being able to change the protonation conditions of the enzyme[38].

The support may selectively remove water molecules produced in the reaction and store them within the micropores during an ester synthesis reaction in organic medium with a lipase immobilized on a zeolite which helps driving the equilibrium towards the ester. At the same time the adaptable hydrophobicity of the zeolite partly modulates the reaction microenvironment surrounding the enzyme molecules adsorbed at the external zeolite surface, by maintaining the water activity at suitable levels for optimal enzymatic activity [39]. Although zeolites have surface areas of around 500 to 1000 m^2g^{-1}enzyme loadings are limited because the enzyme is limited as enzyme molecules are too bulky to enter the zeolite pores. Porcine pancreatic lipase has been immobilized in the mesopores of molecular sieves MCM-41[40]. The problem of immobilized enzyme is that interactions between the enzyme and the zeolite are weak.The reverse reaction is naturally catalysed by the same zeolite-supported lipase, if water is available in the catalyst pores. In case of a water consuming process in the system outside the zeolite, a series of equilibrium driven physico-chemical processes would induce the controlled release of water molecules from the micropores of the zeolite.

As a consequence of enzymes being spontaneously soluble in aqueous media a hydrophilic support is usually preferred in lipase immobilization, Hence, while maximizing the area available for lipase attachment, it also assures the presence of the essential water layer which surrounds the biocatalyst thus helping maximum performance. The drawback of using hydrophilic supports for lipases is the decrease of accessibility of the substrates, especially hydrophobic, limiting the reaction rate [41].

2.9 Advantages of biodiesel as diesel fuel

The main advantages of biodiesel as an automotive fuel are liquid nature and easy transportation, also less risky in transporting due to less flammability compare to petroleum fuels, readily availability, renewability, higher combustion efficiency, lower sulfur and aromatic content [42] higher cetane number and higher biodegradability [43], domestic origin thus reducing the foreign exchange on importing crude oil, biodegradability, high flash point and inherent lubricity [44].

2.10 Disadvantages of biodiesel as diesel fuel

The disadvantages of applying biodiesel as a transportation fuel, in comparison to diesel, are lower energy content, higher viscosity, higher cloud point and pour point, higher nitrogen oxides (NOx) emissions, high production cost and higher engine wear, lower engine speed and power, injector choking and engine compatibility.

2.11 Economic advantages

Economic advantage of biodiesel can be listed as follows:

Reduces greenhouse gas emissions.

Helps to reduce a country's dependency on crude oil imports.

Helps agriculture by providing a new labour and market opportunities for domestic crops.

It has a great opportunity to use biodiesel replacing petroleum fuels in agricultural machineries making agriculture industry more sustainable.

Enhances the lubricating property[45].

The feedstock is the major economic factor in relation to the production cost of biodiesel which is about 80% of the total operating cost. In addition the costs include labor, methanol and catalyst costs. Biodiesel usually costs the double of the price of petroleum diesel but varies depending on the base stock, geographic area, variability in crop production from season to season, the price of the crude petroleum and various other factors. Nowadays biodiesel cost is 1.5–3 time higher than the fossil based diesel cost in developed countries.

3. Materials and Methods

In this chapter the laboratory set up, equipments and experimental methods will be described. In respect of the production of biofuels and according to the thesis outline is given more attention on the enzyme extracts characterization, how to quantify protein, how to measure enzyme activities, and assay of the substrates and products resulting from the transesterification of triolein with butanol in reversed micelles of AOT/isooctane.

3.1 Materials

3.1.1 Microorganism

The over expression of recombinant *Fusarium Soloni pisi* cutinase cloned in the plasmid pMac5-8 has been performed with host *Escherichia coli* WK-6 strain a gift from Corvas International (Ghant, Belgium).

3.1.2 Chemicals

Surfactant bis(2-ethylhexyl)sodium sulfosuccinate, AOT (98 %), triolein (65 %), L-butanol (99 %) were obtained from Sigma. Isooctane (99.5 %) is purchased from Fluka. All eluents (acetonitrile, 2-propanol and n-hexane) of HPLC grade obtained from Lab Scan and sodium phosphate buffer (Na_2HPO_4 and NaH_2PO_4) is of analytical reagent grade from Merck. Most triglycerides are unsymmetrical, being derived from mixtures of fatty acids, but in contrast triolein is a symmetrical triglyceride which represents 4-30% of olive oil.

3.2 Analytical methods

3.2.1 Protein Concentration assay

Protein concentration has been determined by the microplate procedure by using Pierce Bradford Protein Assay Reagent kit (BCA). Standard BCA calibration curve has been drawn after preparing the diluted albumin (BSA) standards according to the PIERCE, BCA Protein Assay Reagent Kit. [54] Protein samples have been prepared with different dilutions in buffer. BCA solution is prepared in accordance with reagent kit assay (One part of B: 50 parts of A, A is Pierce BCA Protein Assay Reagent A and B is Protein Assay Reagent B). 25µl of protein solution is filled in each well and 200 µl of BCA solution is added later. Plate was mixed on a plate shatter for 30 seconds and afterwards incubated for 30 minutes at 37 ^{o}C temperature. The absorbance was read at 562 nm in a microplate reader (Molecular Devices, Spectra max Plus) and Softmax Pro software is used to quantify protein in samples. A calibration curve in figure 17 shows a linear zone until the 2000 µg/mL.

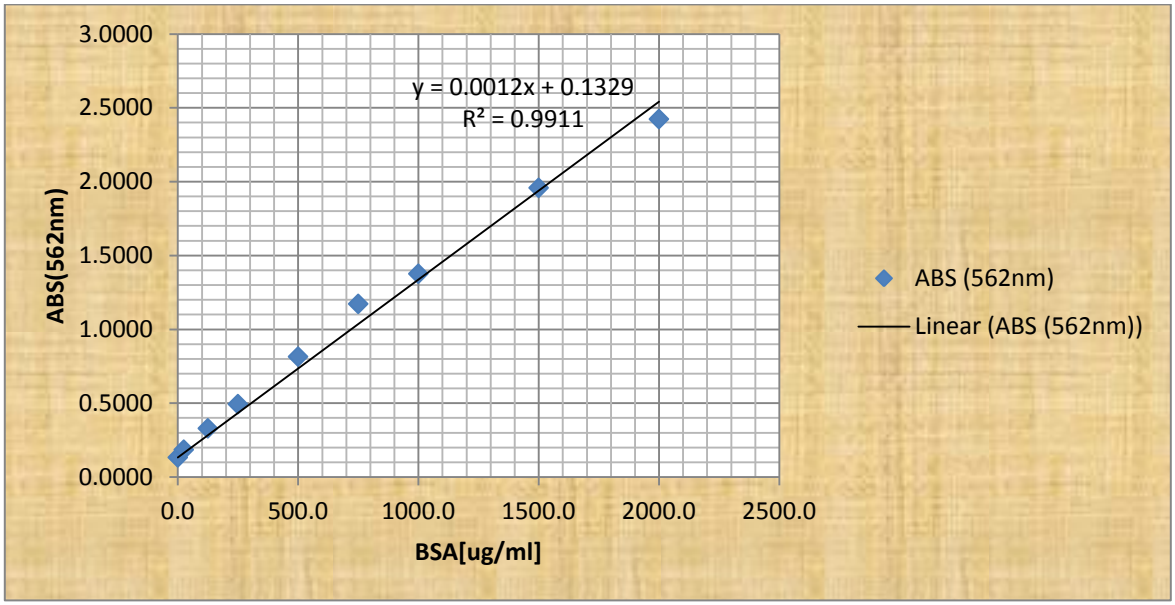


Figure 15 Protein calibration based on Pierce Bradford Protein Assay Reagent kit (BCA).

3.2.2 Cutinse Activity Assay

The activity of cutinase can be determined through a spectrophometric method followed by the hydrolysis of *p*-nitrophenylbutyrate (*p*-NPB) at 400 nm. This ester is hydrolyzed by cutinase following a Michaelis-Menten kinetics, and one unit of enzyme activity (U) corresponds to the amount of enzyme required for the production of 1 µmol of *p*-Nitrophenol (*p*-NP) per minute which is characterized by a yellow colour [46]. As the *p*-NPB is temperature sensitive, it is necessary to keep the substrate in freezer before use, in order to prevent natural occurring degradation [47]. This assay is used in order to determine the activity of the cutinase obtained throughout the enzyme production process.

Enzyme activity assay is determined using *p*-NPB in a 70 mM concentrated stock solution in pure acetronitrile. 15 µl of enzyme extract, previously diluted in Tris buffer, is added to reaction mixtures composed by 1470 µl of 20 mM Tris.HCl with pH 8 and 15 µl of *p*-NPB 70 mM solution inside the glass optical cell (3 ml) mixed by a small magnetic stirrer submitted to magnetic agitation of 700 rpm (Electronic Stirrer Model 300 Rank Brothers Ltd). The cell holder is kept at temperature of 30^{o}C by circulating warm water from a thermostatic bath (Thermomix MM,B.BRAUN) also at 30 ^{o}C. The reaction rate is determined by monitoring spectrophotometrically the hydrolysis of *p*-NPB and formation of *p*-NP at 400 nm (ε = 15,400M^{-1}cm^{-1}) at 30^{o}C for one minute. Dilutions of the enzyme samples in Tris Buffer were made in order to work in linear variation of the concentration of p-NP, i.e., intensity of its yellow colour (Hitachi U-2000 Spectrometer) using a software program (Hitachi Hyper Terminal software) to record the variation of absorption at 400 nm against time with respect to the amount of enzyme in this reaction mixture.

The solution of immobilized enzyme activities is also measured by using the same spectrometer and the same methodology but the results were not so good as expected due to disturbance of solid particles in the absorbance reading. This effect is especially significant when the IMEs preparation has low activity and high

amount of zeolite is necessary to be present inside of the optical cell. Anyway, activity of the IME can be measured with this methodology but with low accuracy.

Another alternative to measure the activity of immobilized enzyme (IME) solution is recommended to use UV-diode array spectrometer with band fitting technique.

3.3 UV/V Diode array spectrophotometry

The activity of the enzyme immobilized on the zeolite support has also been tested by using UV/V diode array spectrometry on a spectrometer (Ocean Optics USB 2000) equipped with a deuterium halogen light source (Micropack DH-2000-BAL), a Fiber Optic Cuvette Holder (Quantum Northwest TC 50F), a temperature control unit (Quantum Northwest TC 125), and a computer running the software Spectra Suite (Ocean Optics). The assays took place inside an optical cell with a volume of 1.5 ml, at a constant temperature of 30 ^{o}C, and with magnetic stirring. A stock solution of *p*-nitrophenylbutyrate (*p*-NPB) of 3.5 mM in acetronitrile was prepared and kept at 0 ^{o}C. The glass optical cell was filled with 1470 µl of phosphate buffer of 50mM, pH 8.5. A dark spectra (corresponding to the signal obtained from the sensors in the absence of light) and reference spectra were captured. Then immobilized enzyme support solution was added and the corresponding spectrum acquired. The activity measurements started by adding 15 µl of *p*-NPB stock solution and the acquisition of spectra along the time for a certain amount of time, around 20 min.

3.2.3 Analysis of Biodiesel using HPLC

Substrates and biodiesel standards, and their presence in samples taken from the transesterification reaction media are monitored at specific times mainly at zero and at end of reaction, and quantified by using High Performance Liquid Chromatography(HPLC).

50 µl of standards at specific concentration or reaction samples is added 1 µl of acetic acid at 58.5 mM pH 3 to correct pH value and to stop the enzymatic reaction. After that, samples have been dissolved in 949 µl of n-hexane and centrifuged (Sigma 201M) to remove any precipitate. The supernatant has been analyzed by high performance liquid chromatography (HPLC) using a Choromolith Performance RP-18 end capped (100mmx4.6mmx2µm) column. HPLC apparatus (ELITE Hitachi La Chrom) equipped with an auto sampler (ELITE Hitachi La Chrom L-2200), a HPLC pump (ELITE La Chrom L-2130) and a UV detector (MERCK Hitachi L-2400) at 205 nm has been used. The flow rate has been maintained at 1 ml/min with injection volume of 20 µl of standards or reaction samples both previously diluted with n-hexane. In this methodology there are three mobile phases: phase A consist with acetonitrile 100%, phase B consist with water 100% and phase C consist of n-hexane and 2-proponol (4:5 v/v).

The products that could be present in the transesterification reaction mixture includes: Triglycerides (TG) with three combinations of oleic (O) and linoleic (L) acid namely OOO, OOL and OLL; diglycerides (DG) with three combinations of the same fatty acids and couples on sn-1,2 and sn-1,3 positions in the glycerol backbone, namely OO^{-}, $O^{-}O$, LO^{-}, $O^{-}L$ and LL^{-}; monoglycerides (MG) of O and L acids in sn-1 and sn-2 positions of the

glycerol backbone, namely O^{--}, $O^{-}L^{--}$ and $-L^{-}$; alkyl esters (AE), in particular, from butanol of the O and L fatty acids, and reaction solvent (isooctane) and surfactant (AOT) [14]. In case of hydrolysis, the free oleic and linoleic acids will also appear in the mixture and they are also detected in the HPLC analysis.

3.3 Methods

3.3.1 Cell growth and enzyme production and purification

Protein production and purification have been carried out adapting the procedure in the literature [50].

3.3.1.1 Inoculum

Sterile petri dish with LB media supplemented with sterile ampicillin (150 µg ml^{-1}) in agar solid cultures were inoculated with *E. coli* WK-6 frozen cells, from the storage stocks at -80 ^{o}C and incubated at 37 ^{o}C.

3.3.1.2 Pre-fermentation medium

The composition of the medium for pre-fermentation was 20 g L^{-1} of LB medium (from Becton and Dickson) with initial pH 7.5. The medium and shake flaks were previously sterilized by autoclaving at 121 ^{o}C during 20 min.

250 ml shake flask with a volume of 50 ml of the corresponding pre-fermentation media, supplemented with sterile ampicillin (150 µg ml^{-1}), have been inoculated with cells of inoculum and have been left at 37 ^{o}C in an orbital shaker (Agitorb 200) at 250 rpm over 3 hrs.

3.3.1.3 Fermentation medium

The culture medium has been composed by 250 ml of TB medium by dissolving the chemical compounds: 12 gL^{-1} Bacto™ Triptone and 24 gL^{-1} Bacto™ Yeast Extract from Becton Dickinson, 5 ml L^{-1} Glycerol (from Acros), 3.81 gL^{-1} KH_2PO_4 (from Merck) and 12.51 gL^{-1} K_2HPO_4 (from Panreac), necessary to adjust and keep the pH to 7.1. The medium and shake flaks were previously sterilized by autoclaving at 121 ^{o}C, during 20 min. 1 M $MgSO_4.6H_2O$ (from Riedel-de Haen) solution was prepared and autoclaved separately to avoid the formation of precipitates, and then have been added aseptically after cooling, to obtain a concentration of 20 mM in the fermentation medium. The fermentation medium was used for the production of cutinase when the cells achieve high saturation density.

In order to start fermentation with an optical density (OD at 600nm) of 0.2, a certain volume of the cells cultured in pre-fermentation medium have been transferred to 2 l shake flask with an initial volume of 250 ml of the fermentation culture medium, supplemented with ampicillin to a final concentration of 150 µg/ml. The fermentations have been performed in the orbital shaker at 25 ^{o}C and 250 rpm. At OD (600 nm) approximately 1.0, the sterile inducer IPTG (isopropyl-β-D-thiogalactopyranoside) (from Bioline) was added to a final concentration of 0.1 mM, with a reinforcement of ampicillin (125 µl of a solution of 100 mg/ml to 250 ml of fermentation media). The sterilization of the thermolabile IPTG and ampicillin were perfomed by filtration using sterile Milex-Gp filter unit (0.22 µm pore diameter, from Millipore).

3.3.1.4 Dialysis

The dialysis membrane (UHP-43, 50ml) is filled with the enzyme extract, dipped in 50 mM phosphate buffer, pH 8.5 at 30 ^{o}C , and left with weak stirring over 48 hrs while changing buffer 3 times.

3.3.1.5 Lyophilisation

Lyophilisation is performed in a Chirst Alpha 2-4 lyophilizer from B. Braun Biotech International, coupled to a Pfeiffer type duo 008B vacuum Pump, over 2 days. Dried lyophilized enzyme is weighted and stored at -20 ^{o}C.

3.3.1.6 Enzyme Immobilization

During this work different batches of immobilized enzymes were prepared, according to the following scheme:

3.4. Immobilization Procedures

In IME1, 50ml of cutinase is washed and purified using phosphate buffer solution of pH 8.5, 50mM using a dialysis membrane (UHP-43, 50 ml). Two reactors one at room temperature (RT) and the other at 4^{o}C are used. To 5 ml of free enzyme solution is added 0.1g of Zeolite NAY-LZY-52 (from Union Carbide) and kept on a stirrer at 300 rpm over 24 hrs. After immobilization, the product has been centrifuged over 10 minutes at 5300 rpm (eppendorf Centrifuge 5810R) to separate solid and supernatant. The activities and protein in the supernatant, and activity of immobilized enzyme solid support are measured in all steps of the enzyme immobilization procedure. This immobilized enzyme solid support (IME1) has been washed twice with phosphate buffer of 50mM, pH 8.5 and then separated again the supernatant and immobilized solid support by centrifugation.

In IME2, 50 ml of cutinase is taken and kept in the dialysis membrane (UHP-43) with 50mM, pH 8.5 phosphate buffer over 24 hrs to remove water and impurities and to make a concentrated enzyme solution. The solution is reduced to 25 ml. Two 5 ml samples has been taken and added 0.1 g of zeolite to each, respectively one has been kept on the reactor at 4^{o}C and the other at RT (~ 30^{o}C) while stirring at 300 rpm (IKAMAG REO DREHZAHL ELECTRONIC) over 24 hrs. Both immobilized enzyme solutions have been centrifuged at 5300 rpm over 10 minutes (eppendorf Cenrtrifuge 5810R) to separate solid and supernatant. But this time, the immobilized solid support (IME2) has not been washed. The activities and protein of the supernatants and activity of immobilized enzyme solid solutions have also been measured.

In IME3, the previous free enzyme preparation was concentrated by lyophilisation and then activity was measured observing less activity, i.e., lyophilisation led to loss of cutinase activity. 25mg of lyophilized enzyme powder is dissolved in 5 ml of buffer of 50mM, pH 8.5 and added to 0.1g of Zeolite and vortex for 1 minute. Similarly 5ml of original free enzyme was also added to 0.1 g of zeolite and vortex for 1 min to control the effect of lyophilisation in the cutinase immobilization on zeolite. Both these samples has kept under vacuum dry (Yamato WP-Handy Aspirator) over 48 hrs. It has taken out from the machine and placed in an incubator to dry over one night. These immobilized and dried enzyme preparations were used to control the trace of water necessary to keep the enzyme active in isooctane and minimize the hydrolysis of triolein during the biodiesel production.

In IME4, 9 ml of free enzyme with activity of 706 U/ml and 3 ml of supernatant with activity of 835U/ml has been mixed to form a one solution of enzyme and added 3 ml of Buffer to make a total volume of 15ml. Then $(NH_4)_2 SO_4$ of 35% (w/v) ratio is added (5.25g) inside the cold room at 4ºC until it fully dissolve while stirring at 600 rpm (IKAMAG REO). This is done to obtain additional enzyme purification. After 2 hrs of stirring at 4^{o}C, it is centrifuged at 6000 rpm (eppendorf Centrifuge 5810R) for 10 minutes to separate enzyme precipitate and supernatant. Then the precipate was dissolved using 2 ml of buffer. This enzyme extract is added to 0.2 g of zeolite by vortex during 30 seconds to perform the cutinase immobilization at room temperature. The solution is kept in a vacuum dryer (Yamato WP-15) over 3 hrs and after removing the remaining liquid it was kept in an incubator (Concessus SA, Memmert) at 30 ^{o}C for 24 hrs. The weight of immobilized enzyme is measured and recorded 200mg and took 10mg from it and added 1 ml of buffer to check (IME4) activities. The 190 mg of the rest is used for biodiesel production.

3.5 Preparation of reversed micelles for biodiesel production

Cutinase is dissolved in 400 mM phosphate or Tris buffer pH 8.0. It is further microencapsulated in 150 mM AOT in isooctane with molar ratio of water: surfactant W_o as 2.7 by the injection method. This method consisted in adding drop wise the aqueous solution with cutinase to the AOT / isooctane solution while strong vortex mixing for 15 s.

For biodiesel production in the reversed micelles solution was previously dried with molecular sieve and then a total reaction volume of 5 ml in closed in a glass reactor (10 ml) with magnetic stirrer. The conditions buffer molarity, pH, AOT concentration and W_o were chosen taking into account the desired optimization [49]. The biodiesel production starts by adding the alcohol and triolein substrates to in the reversed micelles. The alcohol initial concentration is defined at 390 mM and the substrates molar ratio of alcohol to fatty acid chains of 1.6 has been used. The reaction started by adding alcohol with strong vortex mixing of 15 s.

The transestrification is performed in a batch stirred reactor at 35 ^{o}C maintained by an incubator from AGITOR B 160 E, ARALAB while the magnetic stirring is kept at 900 rpm using Thermo Scientific VAR 10 MAG Tele system. All biodiesel production reactions have been performed over 48hrs.

The description of each transesterification used on the biodiesel production was following:

Reaction 1 (R1):

Control of buffer – This was prepared adding 72 µl of Tris. HCl buffer (400mM, pH8), 0.74g of Triolein and 566 µl of n-Butanol to 10 ml of AOT / isooctane during 15 s in vortex.

Biocatalyst Free Enzyme [Ext1] - This was prepared adding 72 µl of enzyme solution (Protein 4 µg/ µl, Activity of 706 U/ml), 0.77g of Triolein and 566 µl of n-Butanol to 10 ml of AOT / isooctane during 15 s in vortex.

Reaction 2 (R2):

Control of Buffer- This was prepared adding 25 µl of Tris. HCl buffer (400mM, pH8), 0.36g of Triolein
Control of buffer – This was prepared adding 72 µl of Tris. HCl buffer (400mM, pH8), 0.74g of Triolein and 566 µl of n-Butanol to 10 ml of AOT / isooctane during 15 s in vortex.
Biocatalyst Free Enzyme [Ext1] - This was prepared adding 72 µl of enzyme solution (Protein 4 µg/ µl, Activity of 706 U/ml), 0.77g of Triolein and 566 µl of n-Butanol to 10 ml of AOT / isooctane during 15 s in vortex and 283 µl of n-Butanol to 5 ml of AOT/ isooctane during 15 s in vortex.
Control of Zeolite – This was prepared adding 25 µl of Tris buffer (400mM, pH8), 0.36g of Triolein, 10mg of Zeolite(NaY,LZY,52) and 283 µl of n-Butanol to 5 ml of AOT/ isooctane during 15 s in vortex.

Biocatalyst IME1[Ext2] - This is prepared adding 25 µl of buffer (400mM, pH8), 0.36g of Triolein, 25 µl Immobilized suspension of enzyme at 4^0C (Protein 3.9 mg/ml, Activity 17.2U/ml),and 283 µl of n-Butanol to 5 ml of AOT/ isooctane during 15 s in vortex.

Reaction 3 (R3):

Control Zeolite - This was prepared adding 25 µl of buffer (400mM, pH8), 0.36g of Triolein , 20mg of Zeolite (NaY,LZY,52) and 283 µl of n-Butanol to 5 ml of AOT /isooctane during 15 s in vortex.
Biocatalyst IME2 [Ext3]– This was prepared adding 25 µl of buffer (400mM, pH8), 0.36g of Triolein , 80mg of Immobilized Enzyme Zeolite (NaY,LZY,52) support(Protein 3.6mg/ml, Activity 55.7 U/ml) and 283 µl of n-Butanol to 5 ml of AOT /isooctane during 15 s in vortex.
Biocatalyst IME3 [Ext4]– This was prepared adding 25 µl of buffer (400mM, pH8), 0.36g of Triolein , 80mg of Immobilized Enzyme Zeolite (NaY,LZY,52) support(Lyophilized enzyme of 1.6mg/ml, Activity 20.2 U/ml) and 283 µl of n-Butanol to 5 ml of AOT /isooctane during 15 s in vortex.

Reaction 4 (R4):

Biocatalyst IME4[Ext5] - This was prepared adding 25 µl of buffer (400mM, pH8), 0.36g of Triolein, 190mg of Immobilized enzyme Zeolite support (NaY,LZY,52) (Protein 9.5mg/ml, Activity 213 U/ml) and 283 µl of n-Butanol to 5 ml of AOT /isooctane during 15 s in vortex.

4 Results and discussion

4.1 Cutinase extracts used in immobilization and transesterification

Table 3 resumes the characteristics (protein and activity) of the cutinase extracts obtained after production and concentration used in the immobilization of the enzyme on the zeolite or as free enzyme on the transesterification reaction in reversed micelles.

Table 3 Identification and characterization of enzyme extracts.

Enzyme source	Identification	Enzyme Activity (U/ml)	Protein enzyme extract (mg/ml)
Dialysed	Ext1	706	4.0
Dialysed	Ext2	17.2	3.9
Dialysed	Ext3	55.7	6.0
Lyophilized enzyme	Ext4	21.2	1.6
Concentrated Enzyme	Ext5	213	9.5

The extracts Ext1, Ext2 and Ext3 were only dialysed against the buffer for 48 hours without any other purification or concentration step while the Ext4 was lyophilized and the powder was later dissolved in buffer. In the Ext5 was obtained by dissolution of the precipitate resulting from the addition of ammonium sulphate to original enzyme extract at 4ºC. The concentration of protein is similar in all 5 enzyme extracts but the values of activity were quite different and those one with high activity saturated the zeolite with high amount of adsorbed cutinase.

Table 4 Identification of the biocatalysts (IMEs) used in the biodiesel production and respective characteristics of supernatants and biocatalyst activity.

Immobilized enzyme supports	Enzyme source	Supernatant Activity (U/ml)	Supernatant Protein (mg/ml)	Biocatalyst Activity /U/g)
IME 1	Ext2	17.2	3.9	4410.3
IME 2	Ext3	55.7	6.0	9283.3
IME 3	Ext4	21.2	1.6	13250
IME 4	Ext5	213	9.5	22421

Table 4 resumes the characteristics of the supernatants after enzyme immobilization on zeolite for the different source of enzyme extracts. Activity of the supernatant occurs because not all of the enzyme is immobilised on the support during the immobilisation procedure and, since adsorption is reversible, some of the immobilised enzyme is likely to desorbs in the washing steps. The high activity of the supernatant for IME1 was due to saturation of solid support and almost all leaking of adsorbed enzyme activity during the washing steps. Thus it is natural that the IME1 does not present great activity.

The low supernatant activity of the IME2 was due to enzyme adsorption and low activity in the original enzyme extract (Ext3), in spite of, the IME2 has not been washed after immobilization.

The low activity of extract 4 and respective supernatant after obtain the IME 3 is due to the loss of activity during the lyophilisation step; in fact lyophilisation induces changes in the enzyme structure that lead to the partial loss of activity; about hall the activity, in relation to the original enzyme, is expected to be lost. Additionally, the supernatant based immobilized samples shows high activity and same protein content. And thus it is an indication of not efficient immobilization.

The high activity in supernatant used in IME4 is due to high activity of the enzyme extract (Ext5). The IME4 was dried at 30^{o}C to eliminate the excess of water before it was used it in the biodiesel production.

4.2 Calibration of HPLC for analysis of alkyl glycerides and Biodiesel Production

The figures 16 to 20 show the HPLC chromatograms obtained in the identification of substrates, alcohol, and products involved on the biodiesel production. The baseline changes present 3 characteristics plateaus, first one between 5.5 and 9 min, the second one between 12 and 15 min, and third one between 18 and 21 min and they are in agreement with the different phases of the efluent gradient.

Figure 16 Typical chromatogram for the injection of a sample of triolein in reversed micelles reaction media.

Figure 16 presents the chromatogram obtained by injection of triolein dissolved in the reversed micellar system used in the transesterification and there are four characteristic peaks with retention time between 17 and 21 minutes corresponding to the different combinations of oleic and linoleic acids in glycerol backbone structure (trilinolein LLL at ~18.0 min; triglyceride OLL at ~18.6 min; triglyceride OOL at ~19.6 min; and triolein OOO at ~20.0 min). The first peaks with retention time between 2 and 3 min are due to the presence of the diluent (n-hexane) and isooctane, and some free fatty acids in original triolein.

Figure 17 – Typical chromatogram type for the injection of a sample of Butanol in reversed micelles reaction media.

The Figure 17 presents the chromatogram of the injection of butanol dissolved in reversed micellar system used in the transesterification that shows some perturbation in the plateau between 8 and 10 min while the first peaks between 2 and 3 min correspond to the butanol, the diluent (n-hexane) and isooctane.

Figure 18 – Typical chromatogram type for the injection of a sample of Glycerol diluted only in n-hexane.

The figure 18 presents the chromatogram of the injection of glycerol diluted only in n-hexane that shows there is no significant peak in the different plateau due to the fact that glycerol does not present significant absorption at 205 nm and additionally also does not show any disturbulance of the baseline and the small peaks at retention time near of 2 min belong only to diluent, n-hexane.

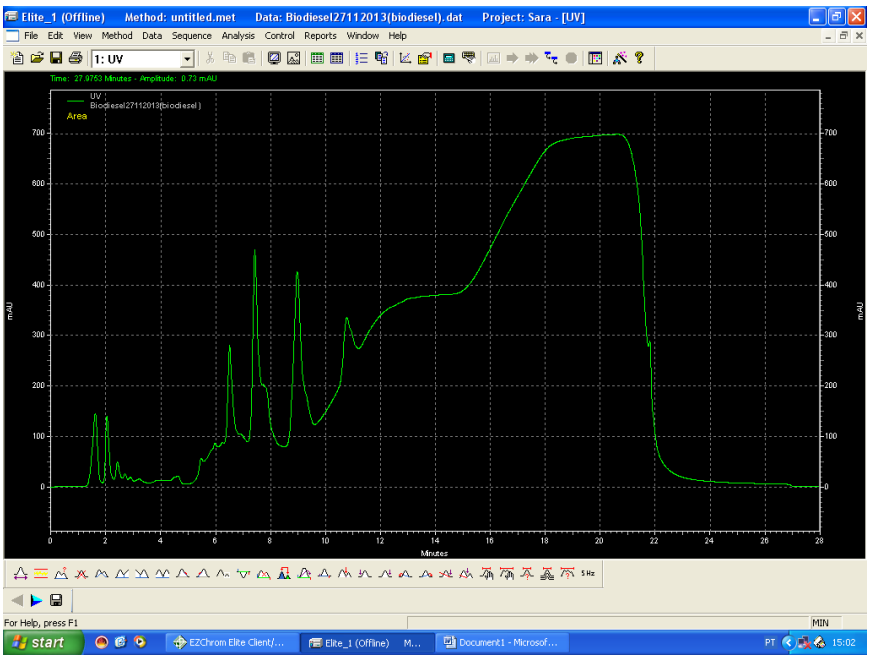


Figure 19 – Typical chromatogram for the injection of a sample of butyl oleate in reversed micelles reaction media.

The Figure 19 shows the chromatogram of the injection of the standard of butyl oleate with initial concentration of 400 mM in reversed micelles. From this chromatogram it is possible to see that butyl oleate also presents 3 significative peaks retention time between 6.5 and 10 min and another near of 11 min due to the different esters resulting from the transesterification of butanol with oleic and linoleic acids and other fatty acids. The peaks near of retention time of 2 min belong also to the diluent used, n-hexane and isooctane.

The injection of MGs and DGs derivates from triolein hydrolysis appear for retention time between 3.0 and 5 min and in second distinguish plateau for retention time between 12 and 16 min, respectively[30].

The resume of the substrates, intermediates and products resulting from transesterification and hydrolysis can be well identify according specific time zones in the Figure 20.

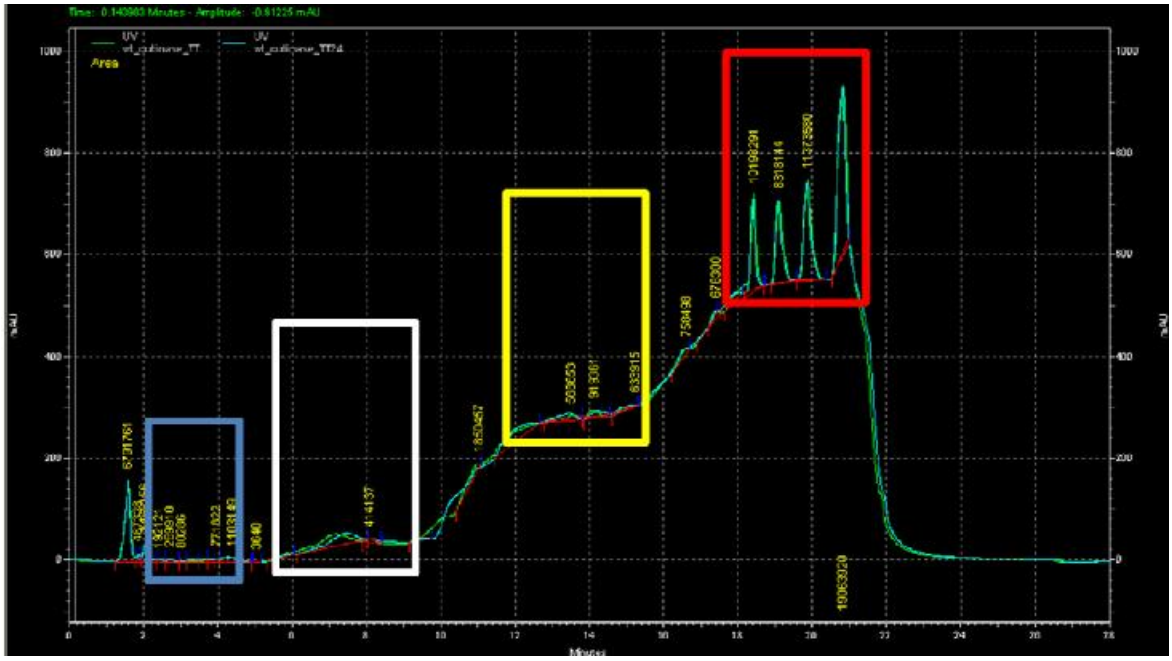


Figure 20 Resume of typical chromatogram and the time zone of substrates, intermediates and products. Blue rectangle – time zone of MG detection, White rectangle – time zone of Alkyl Esters detection, Yellow rectangle – time zone of DG detection, Red rectangle – time zone of TG detection.

The blue rectangle represents the usual retention time zone where the peaks corresponding to the monoglycerides (MG) are detected, the white rectangle time zone for alkyl esters (AE) detection, the yellow rectangle the time zone for diglycerides (DG) detection and the red rectangle the zone for triglycerides (TG) detection. It is expected that in the beginning of the reaction, time zero, there exists a high concentration of TG, a low concentration of DG and MG and no alkyl esters present.

4.3Biodiesel production

Biodiesel Reactions

The different experiments performed on the biodiesel production and the respective chromatograms identifications are presented in the table 5 and Figures between 21 and 28.

Table 5 Resume of the biodiesel production experiments.

Reaction	Reference	Chromatograms
R1	Control	Figure 21 a
R1	Free Enzyme – Ext1	Figure 21 b
R2	Control	Figure 22 a
R2	Pure zeolite#1 (10 mg)	Figure 22 a
R2	IME1, immobilized Enzyme at 4^0C with Ext2	Figure 22 b
R3	Pure zeolite#2 (20 mg)	Figures 23 a – b
R3	IME2, immobilized Enzyme at 30^0C with Ext3	Figures 24 a – b
R3	IME3, immobilized Enzyme at 30^0C with Ext4	Figures 25 a – b
R4	IME4, immobilized Enzyme at 30^0C with Ext5	Figures 26 a – b

The transesterification of triglycerides was performed in closed small scale reactors with total volume of 10 ml. Control experiments were performed in four different ways with: just buffer, pure zeolite, free enzyme, lyophilized enzyme; all experiments, including the ones with immobilized enzyme were carried-out using optimal conditions previously defined [30].

The control experiments of R1, R2 and R3 (Figures 21a, 22a, 23a) performed with buffer and zeolite alone do not show any significant alteration of the chromatograms for zero and 48 hours reaction and consequently, indicate that no degradation occurs of the triolein neither has there been any partial hydrolysis of triolein with formation of DGs or MGs and transesterification as there is no peak corresponding to the alkyl esters resulting from the reaction of one of the triglycerides of the triolein with butanol.

a)

b)

Figure 21 Chromatogram for the injection of samples from R1: a) time zero b) after 48 hours.

a)

b)

Figure 22 Chromatogram for the injection of samples from R2: a) time zero b) after 48 hours.

a)

b)

Figure 23 Chromatogram for the injection of control samples from R3: a) time zero b) after 48 hours.

a)

b)

Figure 24 Chromatogram of the injection of IME2 samples from R3: a) time zero b) after 48 hours.

a)

b)

Figure 25 Chromatogram for the injection of IME3 samples from R3: a) time zero b) after 48 hours.

a)

b)

Figure 26 Chromatogram for the injection of IME4 samples from R4: a) time zero b) after 48 hours.

From the analysis of the different reactions and chromatograms it is possible to conclude the following:
In R1, the production of biodiesel with free enzyme with very high activity (Ext1) no significant consumption of the triglycerides occurred even after 48 hours (Figure 21b) as the area of the 4 peaks between 18 and 22 min is almost the same in relation to zero time (Figure 21a) and neither significant amount of butyl esters (<10%) and diglycerides (<5%). The reason for this low transesterification yield is due to the high amount of water $W_o \geq 4$ present in the reversed micellar system.

In R2, the cutinase from Ext2 was immobilized in the zeolite but also in this case there is not significant consumption of the triglycerides after 48 hours (Figure 22 b) and neither butyl esters or diglyerides formation. The reason for this apparent failure on biodiesel production is due to low activity of the immobilized enzyme preparation (IME1) and the high amount of water $W_o \geq 5.4$ present in the reversed micellar system.
In R3, cutinase preparations (80 mg) previously lyophilized were used, the first one powder of free enzyme (IME 2) and the second one immobilized on zeolite (IME3) to control the amount of water in the reversed micellar system. Both enzyme preparations IME 2 and IME 3 show now an interesting transesterification yield (50 and 10%) and simultaneously formation of diglycerides (15 and 5%), respectively. The interesting results are due to a better control of amount of water in micellar system $W_o = 2.7$ already optimized by Badenes, 2010[30]. These results can be improved by using cutinase extracts with higher activity.

In R4, the IME4 was obtained from cutinase extract Ext5 with very high activity. After immobilization the IME4 the enzyme solution was removed by centrifugation and then completely dried at 30ºC before use in biodiesel production and kept with W_o at 2.7. By comparison of the Figure 26 a) and b) is possible to conclude that the transesterification yield is almost 100% as there was total consumption of triolein and more important without mono and diglycerides formation.

With this work is possible to conclude that reversed micellar system based on AOT and Isooctane and cutinase immobilized in zeolite can be an interesting biocatalyst on the biodiesel production.

5 Conclusions

From the results presented we can conclude that cutinase immobilized on zeolite NaY-LZY-52 is a promising catalyst for the production of biodiesel from triglycerides. Enzymatic biodiesel production offers many advantages over the chemical catalysis that has been used over a years in practice. It has shown that it is more efficient due to enzyme specificity and selectivity. It greatly helps to consume less energy as it works under very mild conditions such as low temperature and pressure. It also helps to reduce water waste and produce more environmentally friendly product. Recovery and reuse of the enzymes to catalyze several batches makes the enzymes more economically feasible. Repeated use of the enzymes, enhancement of their thermal and operational stability, effective control of the reaction parameters, etc. are the benefits of the use of catalytic transesterification using immobilization of lipases. It also greatly helps to reduce the production cost of biodiesel. The possibility of using continuous reactors, packed or fluidized bed, membrane reactors and stirred tanks with catalyst recycling are added advantages of using immobilized cutinase in biodiesel production.

,

6 Future Work

In the current work the catalysts were only tested in small batch reactors and, although the results seem promising, a lot of work is needed before it is possible to evaluate the potential of these catalysts. The following studies are suggested:

Kinetic studies of the catalytic activity;
Perform the reaction with different alcohols. For these preliminary testes iso-butanol was used but the reactions is usually carried-out with methanol or ethanol which are cheaper and more readily available. The choice of iso-butanol was made so as to ensure that the catalyst stability is not impaired by the alcohol since it is known that cutinase can suffer some deactivation by shorter chain alcohols and the behaviour with these has to be tested.

Testing of other supports. Although previous studies have indicated that NaY-LZY-52 is one of the best zeolites in terms of supporting cutinase, this was done for a limited set of reactions and other zeolites might be tested for biodiesel production under these conditions.

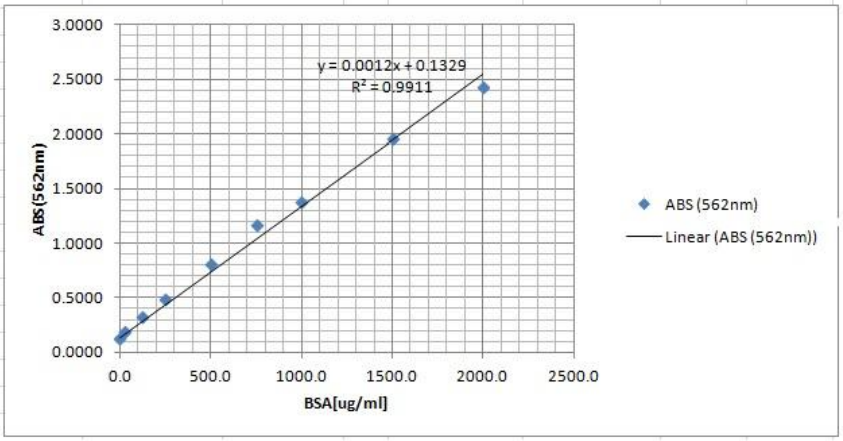
It is also important to test with other oils such as Jatropha, rapseed and soya bean. Triolein is used in this experiments because it is already known exact amount of olive oil content in the sample but if used any other form at this moment it has to be tested those contents in the given sample before use.

Annex

Experimental Analysis

BCA Analysis of Protein

22/07/2013	Protein Concentration_BCA		
	Calibration curve **in water**		
[BSA] ug/mL	ABS (562nm)		
0.0	0.1329		
25.0	0.1853		
125.0	0.3297		
250.0	0.4940		
500.0	0.8154		
750.0	1.1726		
1000.0	1.3768		
1500.0	1.9585		
2000.0	2.4241		
blank	0.1329		
y=	0.0012	x +	0.1329



BCA Sample			Abs(562nm)	Median					
A	2.4073	2.4241	2.4427	2.4241					
B	1.9585	1.9281	2.0267	1.9585					
C	1.4541	1.3111	1.3768	1.3768					
D	1.1674	1.1726	1.1809	1.1726					
E	0.8154	0.7941	0.8213	0.8154					
F	0.4940	0.5139	0.4713	0.4940					
G	0.3519	0.3297	0.3227	0.3297					
H	0.1853	0.1882	0.1807	0.1853					
I	0.1405	0.1329	0.1296	0.1329					
Protein Samples					Correct Median	Total Protein	FD	Protein[ug/ml]	Protein [mg/ml]
1 (Free Enzyme)	0.3777	0.3744	0.3815	0.3777	0.2448	204	20	4080	4.08
2 (4C after Immo)	2.4068	2.5258	2.5220	2.522	2.3891	1990.916667	2	3981.833333	3.981833333
3 (after Imo Sup1)	1.0110	1.1110	1.1121	1.111	0.9781	815.0833333	2.00	1630.166667	1.630166667
4 (after Imo Sup 2)	2.2207	2.2937	2.2743	2.2743	2.1414	1784.5	2.00	3569	3.569

Activity

Cutinase Activity

One unit of estereolytic activity = amount of enyme required to convert 1 umole of PNPB to p-nitrophenol for 1 min

Extinction coefficient of p-nitrophenol =1,54x10E4 M cm 15400

Cuvette	
Components	Volume (ul)
Tris-HCl 20mM buffer	1470
Sample	15
p-NPB	15
Total	1500

60

V sample extract (mL) 1.5

V sample assay activ (n 0.015

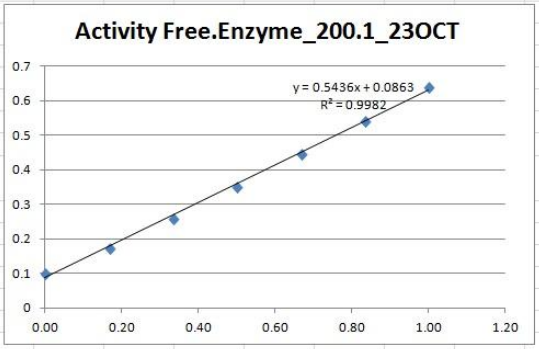
Cutinase

Activity Free.Enzyme _200.1.23OCT

Activ (U/mL)

Time (min)	Abs (400nm)	SLOPE	Concentration (M)	Activ (µm/mL)	Activ (U/mL)	Dilution 1:200
0.00	0.1					
0.17	0.173					
0.33	0.259					
0.50	0.351	0.54	3.53E-05	3.53E-02	3.530	706.030
0.67	0.445					
0.83	0.54					
1.00	0.639					

Recation1_R2

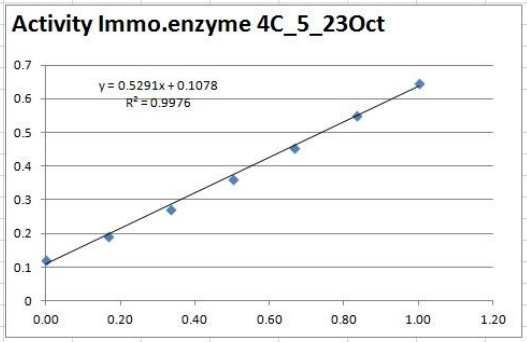


Activity Immo.enzyme 4C_5_23Oct

FD 1:5

Time (min)	Abs (400nm)	SLOPE	Concentration (M)	Activ (µm/mL)	Activ (U/mL)	
0.00	0.123					
0.17	0.192					
0.33	0.274					
0.50	0.364	5.29E-01	3.44E-05	3.44E-02	3.436	17.1776438
0.67	0.456					
0.83	0.551					
1.00	0.646					

Reation2_R3

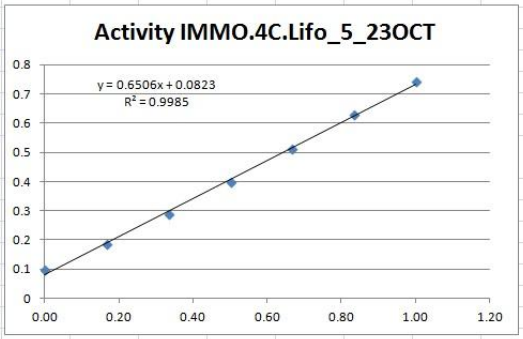


Activity IMMO.4C.Lifo_5_23Oct

FD 1:200

Time (min)	Abs (400nm)	SLOPE	Concentration (M)	Activ (µm/mL)	Activ (U/mL)	
0.00	0.097					
0.17	0.187					
0.33	0.29					
0.50	0.398	6.51E-01	4.22E-05	4.22E-02	4.22	21.122449
0.67	0.512					
0.83	0.628					
1.00	0.741					

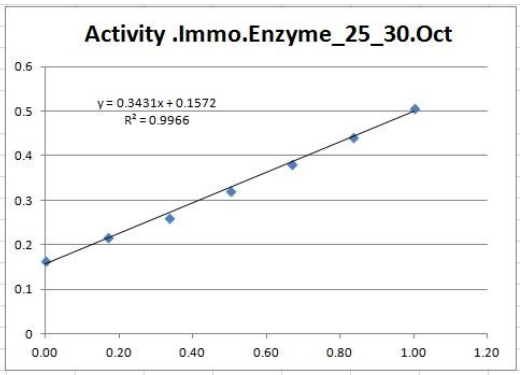
Reation 3_R3



FD 1:25

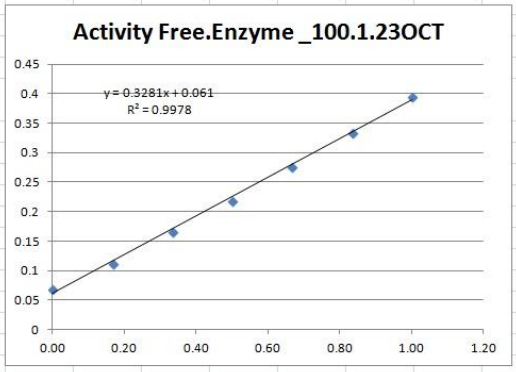
Activity .Immo.Enzyme_25_30.Oct

Time (min)	Abs (400nm)	SLOPE	Concentration (M)	Activ (µm/mL)	Activ (U/mL)	
0.00	0.166					
0.17	0.217					
0.33	0.261					
0.50	0.323	3.43E-01	2.23E-05	2.23E-02	2.23	55.6934137
0.67	0.383					
0.83	0.442					Recation 3_R2
1.00	0.509					



Activity Free.Enzyme _100.1.23OCT FD 1:100

Time (min)	Abs (400nm)	SLOPE	Concentration (M)	Activ (µm/mL)	Activ (U/mL)	
0.00	0.07					
0.17	0.112					
0.33	0.167					
0.50	0.219	3.28E-01	2.13E-05	2.13E-02	2.13	213.033395
0.67	0.277					
0.83	0.335					Recation4_R1
1.00	0.395					



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