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A GREEN INTEGRATED PROCESS FOR BIODIESEL PRODUCTION

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Por tudo, ao meu Pai

"**Failure** is only the opportunity to begin **again** more intelligently"

Henry Ford

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RESUMO

Neste trabalho experimental desenvolveu-se um processo contínuo de produção de biodiesel, a partir de óleo virgem e óleo usado, usando dióxido de carbono supercrítico como solvente e utilizando catálise enzimática. A produção de biodiesel através da reacção de transesterificação de óleo de girassol com metanol, utilizando a Lipozyme TL IM® demonstrou tratar-se de um processo com um elevado potencial nas seguintes condições p = 20 MPa; T = 313,15 K; tempo de residência: 20 segundos e razão molar óleo: metanol: 1:24. O processo integra, igualmente, uma fase de fraccionamento. A separação a p=12 MPa e 333,15 K permite um elevado enriquecimento da fase gasosa em biodiesel.

Usando apenas a Lipozyme TL IM® a conversão do óleo usado em biodiesel revelou-se inferior à obtida com óleo virgem, no entanto, a combinação de duas enzimas, Lipozyme TL IM® e Novozyme 435®, revelou ser uma opção viável, atingindo-se um rendimento de 98,9% nas seguintes condições: p=20 MPa; T = 313,15 K; tempo de residência: 30 segundos e razão molar óleo: metanol: 1:24. Uma vez que este rendimento é muito elevado, foi descrito um processo simples de fraccionamento para a recuperação do glicerol e metanol.

Foi desenvolvido um estudo de engenharia para o processo de produção contínua de biodiesel utilizando óleo usado com a elaboração de balanços de massa e de energia. Através do aproveitamento do calor libertado durante a compressão de CO_2, conclui-se ser necessário fornecer ao sistema 89,75 MJ / h. Um estudo de viabilidade económica foi desenvolvido através do qual se determinou um valor de investimento total de 21.728.718 € e um lucro anual de 3.359.731 €.

ABSTRACT

The aim of this thesis consists on the development and design of a continuous process for Biodiesel production under supercritical carbon dioxide conditions using an enzymatic catalyst. Biodiesel production trough transesterification reaction of sunflower oil with methanol, using Lipozyme TL IM® proved to be a high potential process, with a conversion of oil into biodiesel of 98,6%, under the follow conditions: p=20 MPa; T=313,15 K; residence time: 20 seconds and molar ratio oil: methanol: 1:24. The continuous biodiesel production process integrates a fractionation stage. A separation at p=12 MPa and 333,15 K allows a high biodiesel gas phase purity of 93%.

Using Lipozyme TL IM® with waste cooking oil resulted in a lower biodiesel yield than when using virgin oil. The combination of two enzymes, Lipozyme TL IM® and Novozyme 435® proved to be a viable alternative with a reaction yield of 98,9% under the follow conditions: p=20 MPa; T=313,15 K; residence time: 30 seconds and molar ratio oil: methanol: 1:24. Due to this high reaction yield, a simple fractionation process was described to recover glycerol and methanol.

An engineering study was made including mass and energy balances elaboration for a continuous biodiesel production using waste cooking oils. Taken advantage from the heat released from the CO_2 compression it is only necessary to provide to the system 89,75 MJ/h. An economic viability study was also made achieving a total investment value of 21.728.718 € and an annually profit of 3.359.731 €.

Nomenclature

GHG- greenhouses gases

FAAE: fatty acid alkyl esters

TAG: triglycerides

FFA: free fatty acid

FDH: formate dehydrogenase

MDH: methanol dehydrogenase

SFC: supercritical fluid

Sc-CO_2: supercritical carbon dioxide

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Energy Crisis: Need for alternatives

❖ CONSUMPTION OF PRIMARY ENERGY FUELS

Throughout human history, the development of societies has been accompanied by an increase in growing energy needs, particularly after the Industrial Revolution. These energy requirements have been achieved mostly through the combustion of various materials such as oil, coal and natural gas (Figure 1). These materials are considered natural sources or fossil fuels and therefore non-renewable.

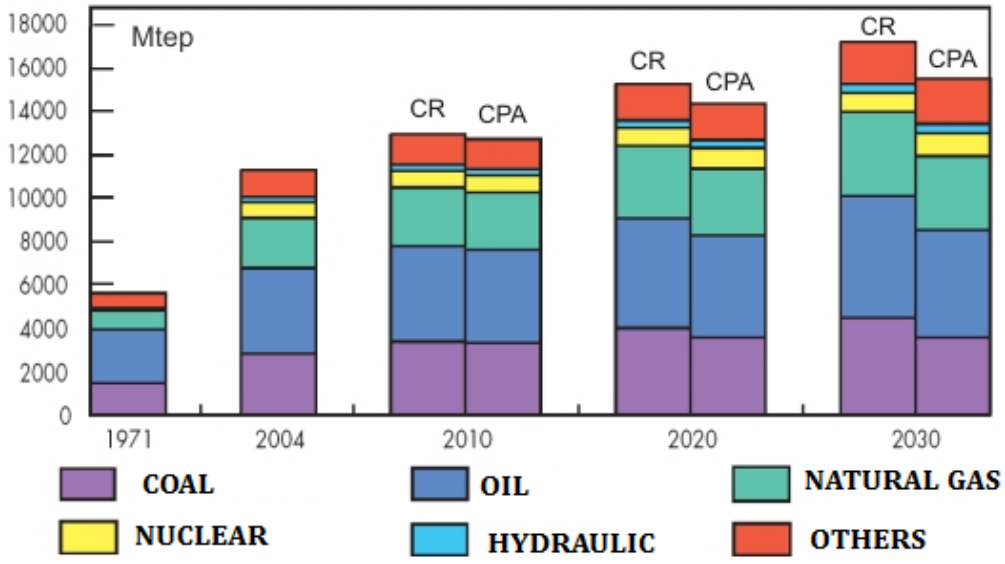

Figure 1: Projections for growth of worldwide primary energy consumption until 2030 under a baseline scenario (CR) based on current policies and alternative policy scenario (CPA) (Mtep: million tons oil equivalent) [1].

According to the current increasing rate of world population, shown in Figure 2, it is estimated that existing oil reserves will be exhausted in the next 40 to 100 years. It is estimated that the currently existing oil reserves allow only the extraction equivalent to 1.2 trillion barrels of oil according to the National Petroleum Agency (ANP). With the exponential development of underdeveloped areas and the

increase in world population, it is essential to changing energy sources because they will not follow this growth and its energy consumption per capita.

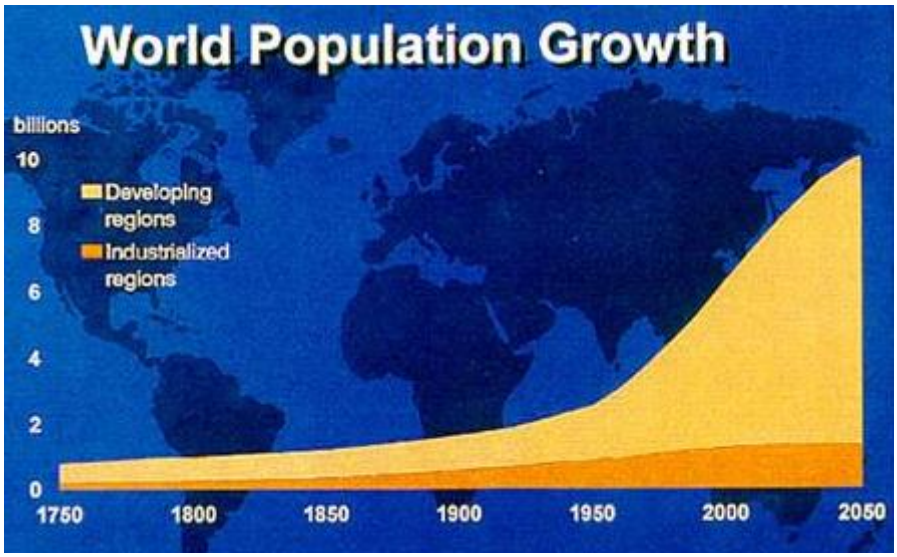

Figure 2: Estimated increase in global population until 2050 [2].

Given the conditions necessary for the formation of crude oil, this is only available in specific locations around the world and the vast majority of reserves are already discovered. Thus, a possible discovery of new oil fields is already highly unlikely and, secondly, the large oil reserves values indicate its collapse. From the oil fields, mainly in the Saudi Arabia region as shown in Figure 3, with lower capacity discovered recently, the vast majority are at depths too high to turn the oil extraction feasible. Thus, we only have the stock of the large oil fields already discovered and the reserves of small fields whose capabilities allow the supply of oil just for a few more years.

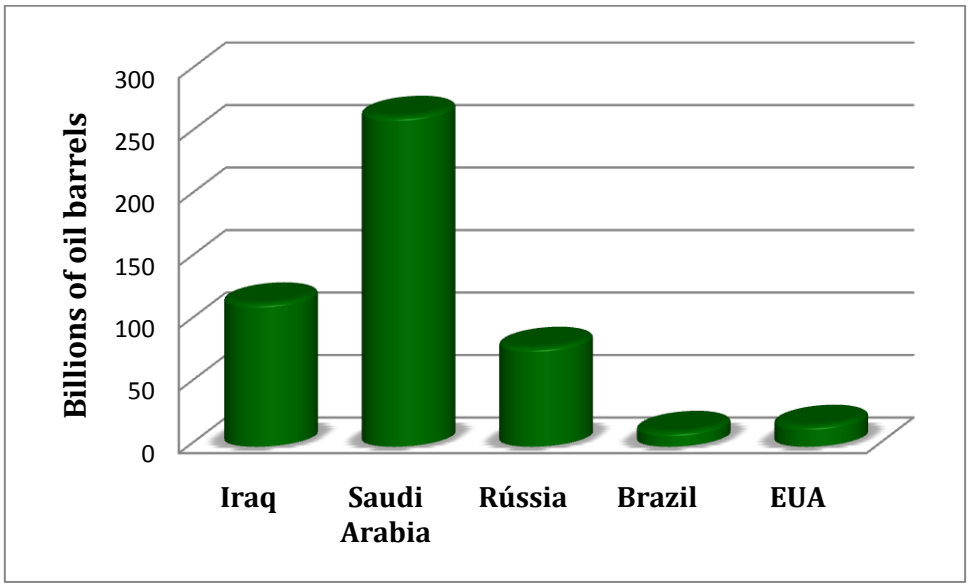

Figure 3: Estimates of oil reserves in billion barrels per region [2].

More than a third of the world's crude oil comes from large fields of which is easier to be extracted. However, for decades, the discovery of new giants (defined as the oil fields from which is expected an extraction of 500 million to 5.000 million barrels) and the average production per field has been decreasing. The largest producing field remains a megagiant (defined as the oil fields of which is expected to extract more than 50.000 million barrels) located in Saudi Arabia, discovered in the 1940s in which average production has been decreasing since its discovery (Figure 4).

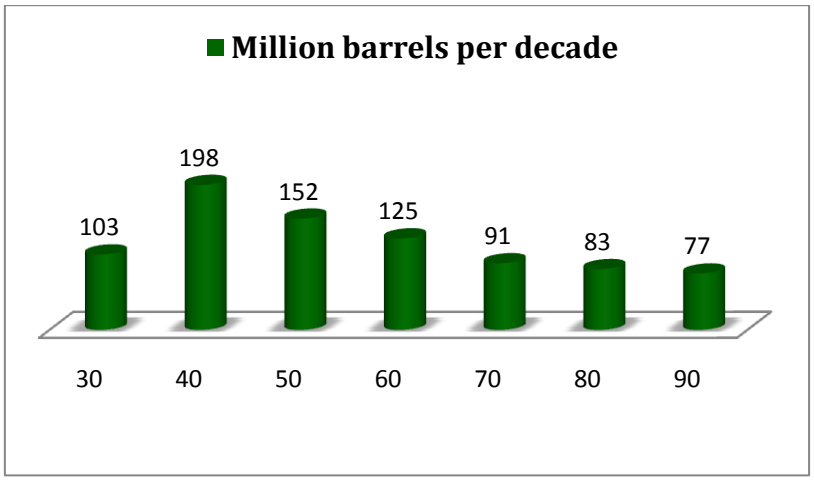

Figure 4: Average production for current giant oilfield [3]

The daily production will decrease as the extraction becomes increasingly more difficult due to the lower amount that can be extracted. Knowing the data information about the daily production of millions of barrels and oilfields actual capacity, it is possible to estimate the duration of the reserves in years (Figure 5).

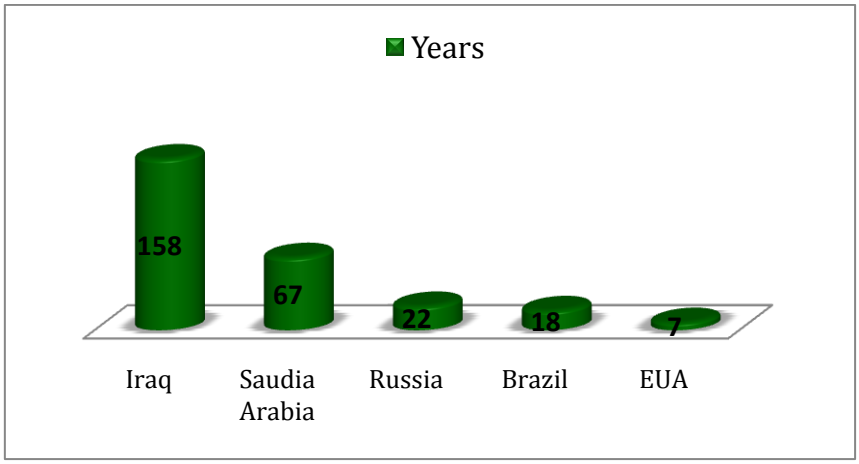

Figure 5: Estimated maximum duration in years of oil reserves by region [3]

Among the non-renewable energy resources, coal is first on the availability and perspective of life and, in the long run, will be the most important world energy since is expected to meet global needs over 219 years [4]. As it is possible to observe in Figure 6, these reserves are mainly located in the USA and Eastern Europe regions.

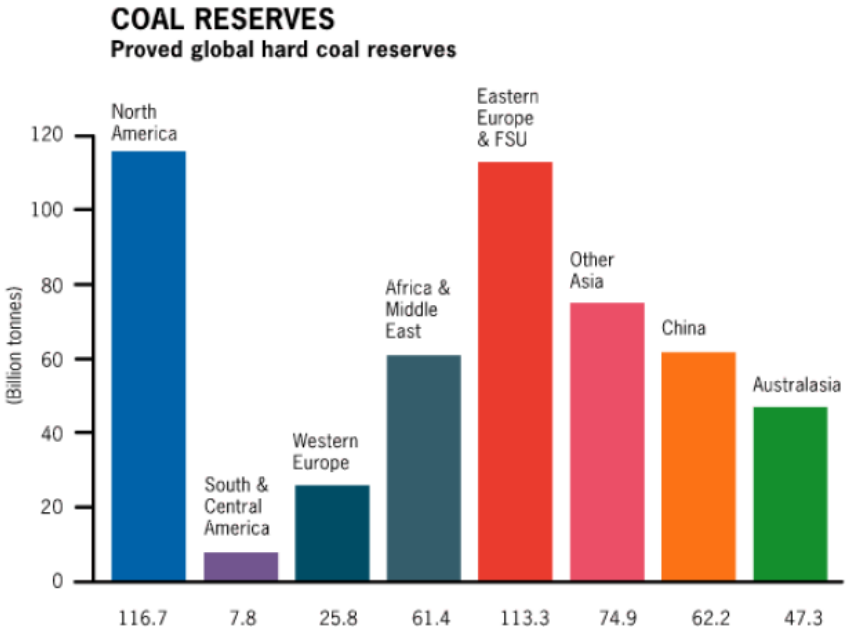

Figure 6: Graphical representation of the distribution of known coal natural reserves natural [5]

The burning of coal causes the release of large quantities of pollutants such as carbon dioxide, nitrogen oxides and dust with negative impacts on air quality, greenhouse effect and human health. Given the current state of climate change which is now a reality quite dramatic, reducing the consumption of natural coal becomes a measure to be taken immediately.

Natural gas is currently the third largest primary energy source in the world. However, as all the other fossil fuels, is expected a decline of natural gas availability over the coming years since it has an estimated useful life of just more 65 years [4].

Therefore, an urgent change of energy sources favoring the use of alternative energies is need changing to renewable sources such as solar, hydro, wind or biomass. Portugal is a country poor in energy resources of fossil origins, depending substantially on imports of primary energy consumed. As a consequence of energy dependence, the country consumes significant resources on energy importation. However, the national potential of renewable energy is high and Portugal has a greater opportunity to further develop this technologies.

❖ WORLDWIDE CONSUMPTION OF LIQUID FUELS

Studies conducted by the EIA (Energy Information Administration) for the first quarter of 2009 indicate a worldwide oil consumption of 77 million barrels per day [6], which means that it can be estimated considering that this value remains constant, an annual consumption of 12.243 billion liters per year. Knowing that a barrel corresponds to 129 kg, we can estimate a global consumption of 3.63 trillion tons in 2009. Since the trend of world energy consumption is increasing to meet all our needs, we can assume that in the next years this value will be increasing until it reaches a point where the supply is not sufficient to meet demand. Moreover, the costs of primary energy consumption will also increase due to the decline of its availability. In mid-January 2008, the price of a barrel of oil reached values never seen before and unaffordable by society (Figure 7).

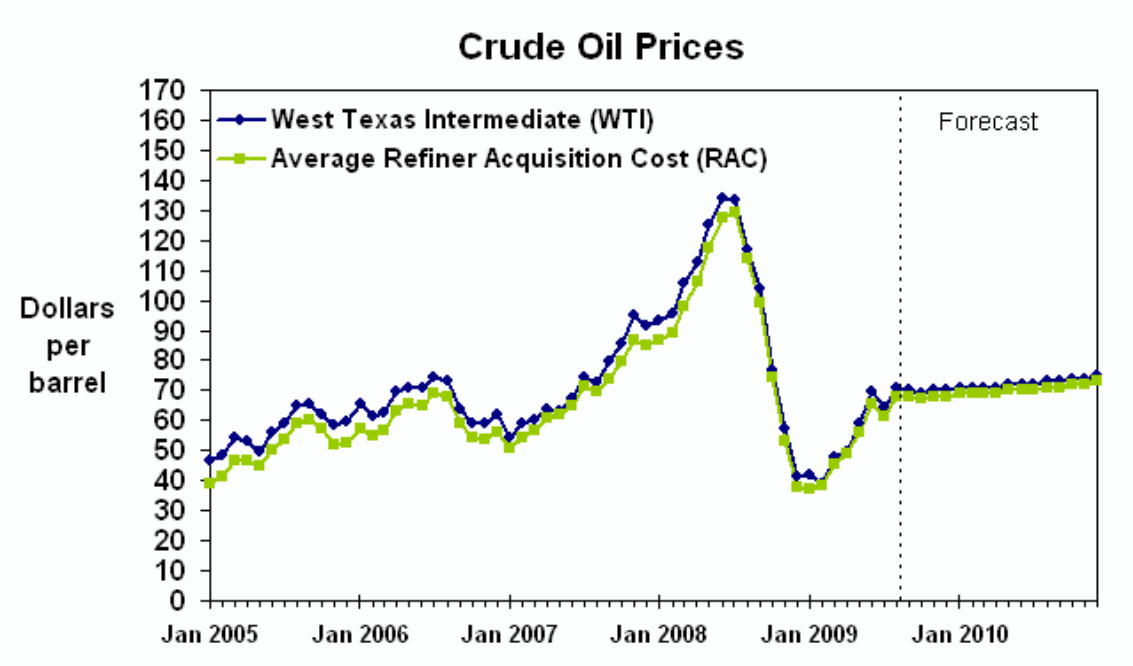

Figure 7: Oil barrel price evolution in 5 years [6].

Considering the liquid fossil fuels, it is estimated a world consumption of about **4 billion tones** for 2009. Of this amount, **1.3 billion tones** are Diesel and 894 Mton

is gasoline. Thus, from the total amount of liquid fuels consumed annually, ca. **33%** corresponds to the consumption of diesel, **22.3%** of gasoline and the remaining **44.7%** for Jet fuel, Naphta, LPG, among others.

Climate Change: Reversing the present situation

The greenhouse gases (GHG) are gaseous compounds capable of absorbing part of the infrared radiation emitted mainly by the surface hindering their normal reflection to space. Thus, preventing the loss of heat into space, the Earth becomes warmer.

Greenhouse gases differ in their ability to absorb radiation as well as in the retention time in the atmosphere. Although CO_2 is the gas that spent less time in the atmosphere and has a capacity for absorption of radiation lower than the others, given the amount emitted becomes the most important and harmful to humanity.

Global warming and its consequences are already a reality in modern civilization and its irreversibility is scientifically proven - even if we stop today all our CO_2 emissions to the atmosphere, it would take several thousand years before the CO_2 concentration reached again the values of the beginning of the industrial era of 280 parts per million (280 ppm). Because of our daily activity and total dependence on excessive consumption of energy, the concentration of CO_2 in the atmosphere has been increasing at a rate never detected in any other era of our planet and the current value is already 385 ppm [7].

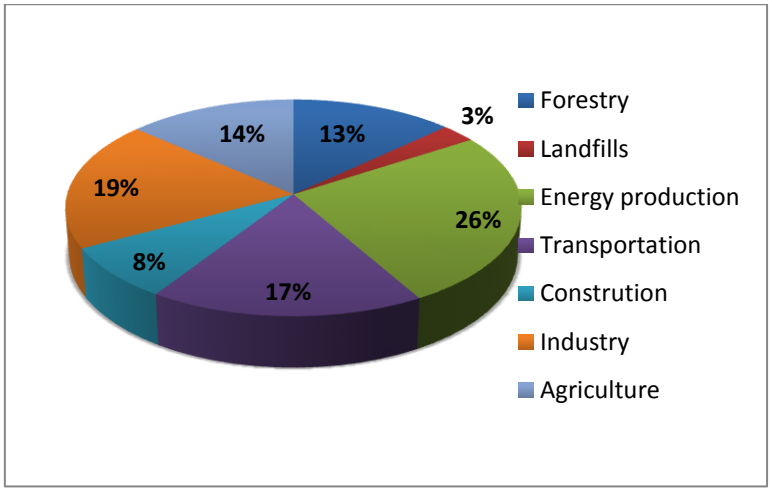

Figure 8: Greenhouse gas emissions by sector.

One of the serious consequences of global warming is the occurrence of many natural phenomena (tornados, earthquakes, floods) with greater intensity and frequency resulting in real material, economical and social disasters. With every natural disaster, the affected country becomes more vulnerable slowing their own development.

As seen in Figure 8, the transport sector is in third place on the quantity emitted of greenhouse gases. Depending on the type of vehicle used and the type of fuel, GHG emissions per vehicle take different values when the full energetic cycle is considered (Table 1).

Table 1: GHG emissions taking into account the load factor and the type of vehicle used [7].

Vehicle	Load factor	CO_2eq/km emissions
Gasoline passenger car	2,5	130-170
Diesel passenger car	2,5	85-120
LPG passenger car	2,5	100-135
Diesel bus	40	20-30
LPG bus	40	25-35
Subway	Occupation of 75%	20-50

The combustion of fossil fuels results in different CO_2 amounts emitted depending on the type of fuel used. Coal has the highest amount of CO_2 emissions per kg of fuel burned while Biodiesel B100 produced by biomass which can sequester CO_2 has the lowest value (Figure 9).

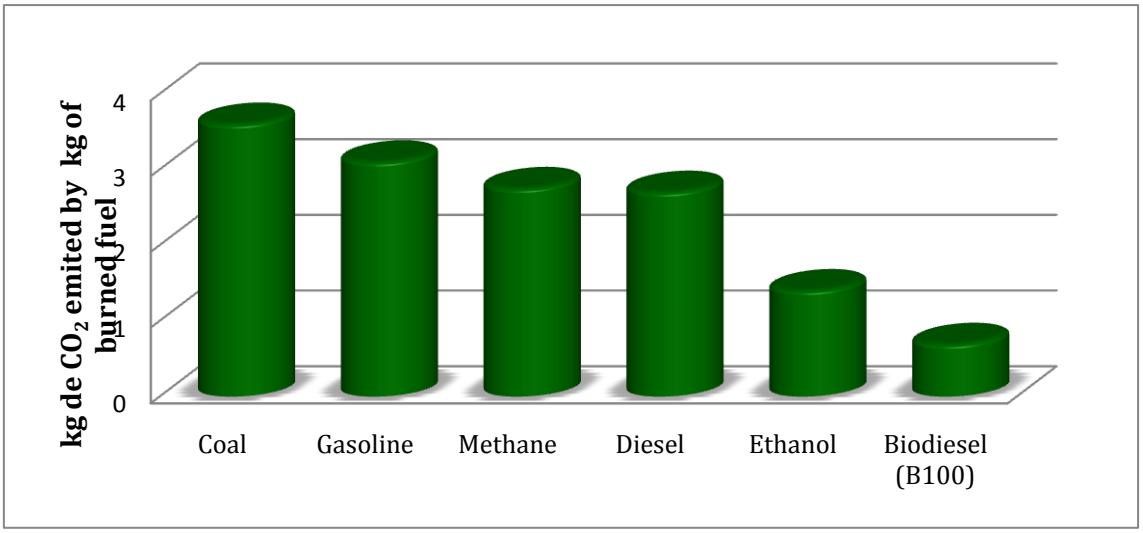

Figure 9: GHG emissions taking into account the load factor and the type of vehicle used [8].

Biofuels include a variety of fuels that have biological origin, and therefore a source of renewable energy. Currently, there are many countries in the world who are betting heavily on the research and the production of these alternatives to fossil fuels. The solution does not rely under a unique alternative implementation. All alternatives must be study and explored. Biodiesel represents one of those options that must be developed and implemented in order to reduce immediately CO_2 emissions and our extreme fossil fuel dependence.

CHAPTER I: STATE OF THE ART

PART I: BIODIESEL

1.1. Biodiesel as an alternative for diesel fuel

In order to face the global crises on energy sector introducing in the market an alternative to the conventional diesel which must be technically feasible, economically competitive, environmentally acceptable, readily available and, in the other hand, reduce significantly the global emissions of greenhouse gases, Biodiesel arises these days like a feasible bet due to its similar main characteristics to conventional diesel.

The use of vegetable oils as alternative fuels has been around for 100 years when the inventor of the diesel engine Rudolph Diesel first tested peanut oil in his compression ignition engine. He said: "*The use of vegetable oils for engine fuels may seem insignificant today but such oils may in course of time be as important as petroleum and the coal tar products of the present time".* However, due to the abundance and low prices of fossil fuels at that time, the non-conventional fuels never took chance to be developed in order to turn them competitive against fossil fuels [9].

Biodiesel has advantages and disadvantages when considered as the same application of conventional diesel. Biodiesel has a viscosity slightly higher than conventional diesel moreover it is non-flammable and non-explosive comparing to petroleum diesel since the flash point of biodiesel is at 423 K against 337 K for conventional diesel .The heating value of Biodiesel is slightly lower (39 to 41 MJ/kg) comparing to petrodiesel (43 MJ/kg) or petroleum (42 MJ/kg) but it is higher than coal (32 to 37 MJ/kg). On the other hand, biodiesel is biodegradable and it significantly reduces toxic and other emissions when burned as a fuel. Currently, biodiesel is a technologically feasible alternative to fossil diesel, but nowadays biodiesel costs 1.5 to 3 times more than fossil diesel which appears to be the primary factor in preventing its more widespread use. [10]

The advantages of biodiesel as diesel fuel are its renewability, higher cetane number resulting in a larger combustion efficiency, lower sulfur and aromatic emissions and higher biodegradability. Biodiesel has got better lubricant properties than fossil diesel. Its higher oxygen content improves the combustion process, leading to a decreased level of polluting emissions. The risks of handling, transporting, and storing biodiesel are much lower than those associated with fossil diesel. The competitiveness of biodiesel relies on the prices of biomass feedstock and costs which are linked to conversion technology. Taking into account subjects such as impacts on the environment, employment and climate changes which are not reflected in the price mechanism of Biodiesel production, Biodiesel turns on the best and competitive alternative to conventional diesel [10].

In order to be used and commercialized in Europe, Biodiesel must be produced under some specifications. Those specifications allow Biodiesel to became similar to petrodiesel and, because of that, it can be used in the same applications without the need of large changes in engine motors or in the equipment associated. The specifications are organized and presented in the European Norm: EN14214. All the Biodiesel produced in Europe must satisfy all the requirements of the European Norm 14214 [11]. The specifications of EN 14214 are described in APPENDIX A.

1.2. Biodiesel from triglycerides: Transesterification reaction

Biodiesel has been defined as the monoalkyl esters of long-chain fatty acids derived from renewable feedstocks such as vegetable oils or animal fats for use in compression-ignition diesel engines [12]. These mixtures of oils and fats need to be chemically altered to fatty acid alkyl esters (FAAE) to be useful as biodiesel fuel for currently used diesel engines. Chemically, these oils or fats are mainly consisted of triglycerides molecules (TAG) of three long chain fatty acids (FFA) that are ester bonded to a single glycerol molecule. These fatty acids can differ by the nature, the length, the number and the position of double bonds in the carbon chain [13].

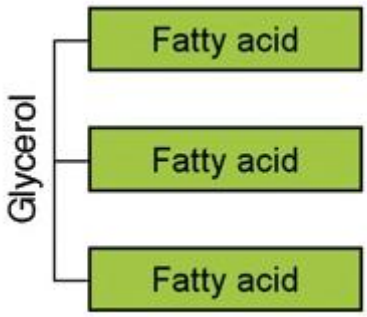

Figure 10: Structure of a triglyceride molecule.

Biodiesel is consisted by alkyl esters of long chain fatty acids and can be synthesized by several processes. Transesterification reaction with small chain alcohols has proved to be the most efficient and promising process [14].

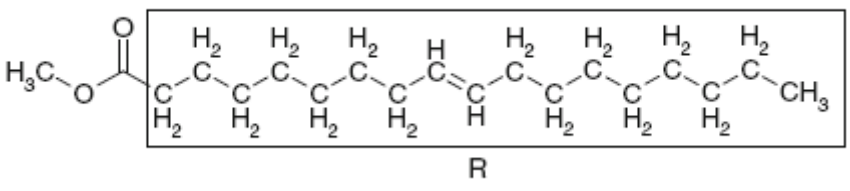

Figure 11: Oleic acid methyl ester with 18 carbons and 1 double bond (biodiesel). R- Group of oleic acid methyl ester.

Vegetable oils shouldn't be used directly in motors engines due to its high viscosity that is about 11-17 times higher than diesel fuel and lower volatilities which causes formation of deposits in engines due to the incomplete combustion and incorrect vaporization characteristics. These problems are associated to physical properties of triglycerides related to its size (large triglyceride molecule) and its higher molecule mass [15]. Thus, triglycerides must be transesterified in order to reduce its viscosity and be able to be applied as a feasible alternative to conventional motors engines.

Through catalyzed transesterification reaction, triglyceride reacts with an alcohol molecule producing FAAE and a glycerol molecule. Transesterification reaction consists of a three consecutive and reversible reactions where the triglyceride is converted stepwise to diglycerides, monoglycerides (intermediate compounds) and finally glicerol in which 1 mol of FAAE is removed in each one of the three steps [16]. Figure 12 describe the overall reaction and Figure 13 the three consecutive reactions.

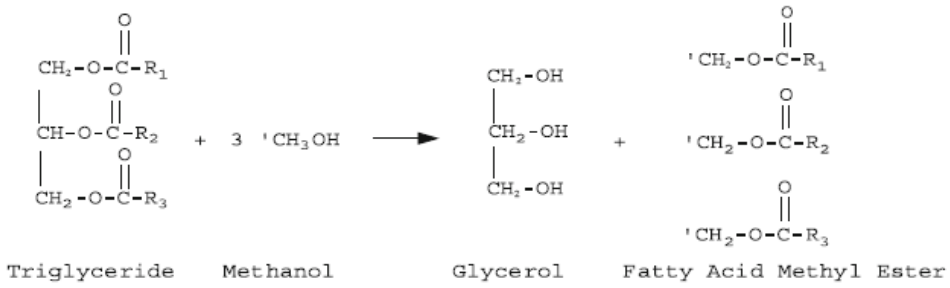

Figure 12: Overall mechanism of transesterification reaction.

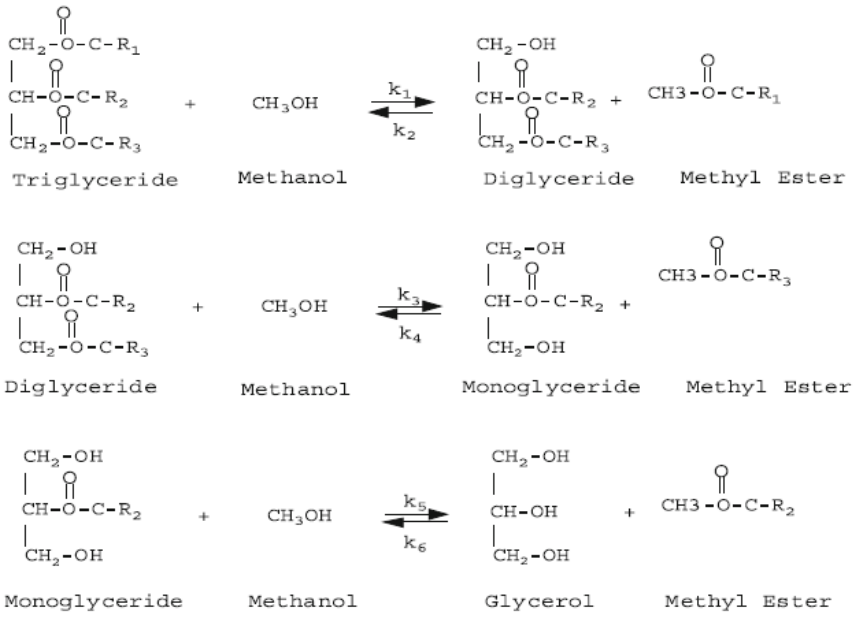

Figure 13: Intermediate steps in biodiesel transesterification [17]

According to the stoichiometric reaction, 1 mol of triglycerides reacts with 3 moles of the alcohol to yield 1 mol of glycerol and 3 moles of Biodiesel (FAAE). However, in order to favor the direct reaction increasing the yield of the alkyl esters, an excess of alcohol is used.

Several alcohols were tested but methanol and ethanol consists in the most promising alcohols to be used in transesterification reaction. Although ethanol represents a renewable source, methanol is, nowadays, the alcohol more used due to the simplicity of the process with lower reaction time, spontaneous separation between glycerol and methyl esters, high reaction yield and is less expensive. [11].

There are three main classes of catalysts that can be applied to transesterification reaction: acid and basic catalysts in homogenous or heterogeneous state and enzymes (biocatalysis). Alkali-catalyzed transesterification is much faster than acid-catalyzed transesterification and is the most often used commercially. Among this, alkaline catalyst such as sodium hydroxide and sodium methoxide are the most applied in conventional industrial processes. However, alkaline catalyst causes the formation of by-products such soaps and sodium salts that must be recovered resulting in high production costs [18].

The process of transesterification is affected by various factors such as molar ratio oil: alcohol, the type of alcohol used, reaction time, temperature, catalyst type and the mixture content [18].

The higher the molar ratio used, the most shifting is the reaction to the products side, theoretically. However, it should be taken into account that an excessive amount of alcohol can cause a loss of activity by the catalyst in the case of the application of biocatalysts and the fact that above a certain value, the amount of FAAE obtained does not increase since the reaction is affected by other parameters also, turning it unnecessary increasing more the molar ratio.

Methanol and ethanol are the alcohols used more frequently. The type of FAAE obtained varies taking into account the type of alcohol used as well as the final properties of the final product since with methanol, fatty acid methyl esters (FAME) are obtained as with ethanol it is obtained fatty acid ethyl esters (FAEE). Ethyl esters obtained by transesterification reaction using karanja oil, when compared with methyl esters obtained by the same reaction, had lower efficiency due to low volatility, slightly higher viscosity and higher density which affects mixture formation of the fuel and thus leads to slow combustion [19].

In case of basic catalysis using methanol, it was reported that the optimum conditions elucidated for the methanolysis of sunflower oil were found to be: methanol/sunflower oil molar ratio 6:1; reaction temperature of 60ºC; and NaOH catalyst concentration of 1.00% (w/w) related to oil mass yielding 97%. [20]. Using alkaline catalysts, the mixture content in FFA and the presence of water produced negative effects since they favor the soap formation which consumes catalyst reducing its effectiveness which result in a low conversion [15].

1.3. Industrial conventional process

Most of the biodiesel is currently made from edible oils by using methanol and alkaline catalyst.

Frequently, catalyst is prepared separately from the reaction mixture in order to favor the mass transfer. Sodium hydroxide is often used because it is cheaper and it is more available than potassium hydroxide. The amount of NaOH used in conventional processes consists, normally, in 1% of the total mass of edible oil. From the mixture between methanol and NaOH is obtained sodium methoxide which will be transferred to the transesterification reaction [21]. The molar ratio oil:methanol used in basic catalysis conventional processes is 1:6. Transesterification reaction is carried out in a mixed batch reactor during 1h30 at T=60ºC yielding 97% [17]. After the reaction, the mixture must settle down between 4h to 8h hours in order to recover glycerin (by-product), methanol that did not react and contaminants that might be formed. In the separator, the mixture glycerin/methanol is recovered at the bottom and the mixture with biodiesel, mono-, di-, and triglycerides and contaminants (small amounts of methanol, NaOH, soaps) is recovered at the top. Methanol recuperation is made with a mixture glycerin/methanol distillation at 65ºC and 1 bar. The Biodiesel will pass through a downstream process with three consecutive distillated water washes followed by a drying process in order to produce Biodiesel according to the norm EN14214. The first wash is done with water and a small amount of HCl in order to neutralize soaps that have been formed during the reaction and the NaOH left. The volume used of water is the double of biodiesel produced. This wash should be done at pH 7 with mechanical agitation with a minimal period of 4 hours. The second and third wash is made only using distillated water at the same conditions. The water used in the 2nd and 3th wash is reused to the 1st and 2nd followed washes, minimizing the water consume per batch. This amount of water must be recovered and a drying process is the last stage of the conventional process. An evaporation at 80ºC and 450 mbar is done and after this stage the biodiesel is stored and later distributed [11].

The FFA content of the edible oil is an important parameter to take into account before starting the transesterification reaction. There are large amounts of low cost oils and fats which often contain large amounts of FFA that cannot be converted to biodiesel using alkaline catalyst. FFA reacts with the basic catalyst added for the reaction and give rise to soap products. Therefore, soap formation decreases the amount of triglyceride reactants and NaOH catalyst in transesterification reaction. Formation of soap not only contributes to a decrease in biodiesel yield, but also results in higher glycerol purification costs if high quality product is needed [22].

An alternative process for this situation was applied. Initially, the FFA can be converted to fatty acid methyl esters by an acid catalyzed pretreatment and in the second step transesterification is completed by using alkaline catalyst to complete reaction [23].

Although chemical transesterification using an alkaline catalysis process gives high conversion levels of triglycerides to their corresponding methyl esters, the reaction has several drawbacks: it is energy intensive, recovery of glycerol is difficult, the alkaline catalyst has to be removed from the product, alkaline waste water requires treatment and FFA and water interfere with the reaction. Enzymatic catalysts like lipases are able to effectively catalyze the transesterification of triglycerides and can overcome the problems mentioned above. In particular, glycerol can be easily removed without any complex process and also the free fatty acids contained in waste oils and fats can be completely converted to alkyl esters [24].

1.4. Worldwide production

Portugal cultivation of non-edible oils is based on cultures of soybean, palm and sunflower. The most promising culture is sunflowers which achieve productivities of 700 kg/ha during culture period. Nowadays, Portugal does not have non edible oil production necessary to meet the goal set by the Kyoto Protocol to incorporate 5,75% of renewable fuels into fossil fuels used in the transport sector until 2010. Thus, actually active industries import raw materials in order to accomplish the factories capacities.

There are 4 active factories in Portugal: Torrejana, Iberol, Prio e Biovegetal. Torrejana is producing 50.000 tons/year although has a capacity for 100.000 ton. Iberol produces 90.000 tons/year and it has the major capacity installed of about 130.000 tons. Prio activity covers Biodiesel production and distribution with a national production estimated at 50.000 tons/year with a capacity of 100.000 tons/year.

Diesel average annual consumption in Portugal is estimated in 5,5 billion liters. In order to incorporate 5,75% of biodiesel into diesel, it is necessary to produced annually 316 million liters or 253.000 tons of Biodiesel. The capacity already installed in Portugal is enough to achieved the production necessary for the incorporation of 5,75%. The government established a goal of 10% of biodiesel incorporation in order to stimulate the biofuels market and attempting to fulfill the commitments undertaken under the Kyoto Protocol until 2010. For that, is necessary an annually production of 352.000 tons of biodiesel. However, due to the importation of the raw materials and the negligible government assistance, Portugal is only producing 140 million tons of Biodiesel, 40% of the total production necessary.

With capacity of 300.000 tons of biodiesel/year already installed, Portugal needs to increase the capacity for more 52.000 tons which represents a market opportunity of **58,5 M€** assuming a sales price for biodiesel of **0,9€/L**.

In 2008, Germany presented the major biodiesel production with a total of 2.819.000 tons produced. Romania is the country with the lower production and Portugal occupied the 6th in the ranking.

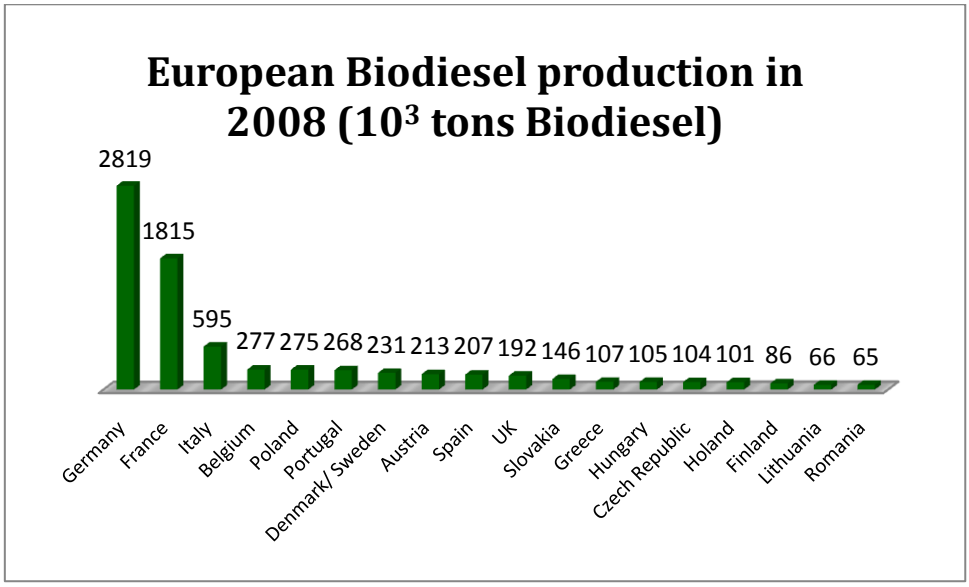

Figure 14: European biodiesel Production in 2008.

In Europe, biodiesel production increased in 2007 16,8% comparing to 2006 and in 2008 it have increased 35,7% relatively the previous year. In Germany, biodiesel is produced from rapeseed oil and it represents the major producer and consumer of biodiesel with more than 1000 gas stations. Biodiesel commercialized in Germany is at form B100 (100% mixture is biodiesel).

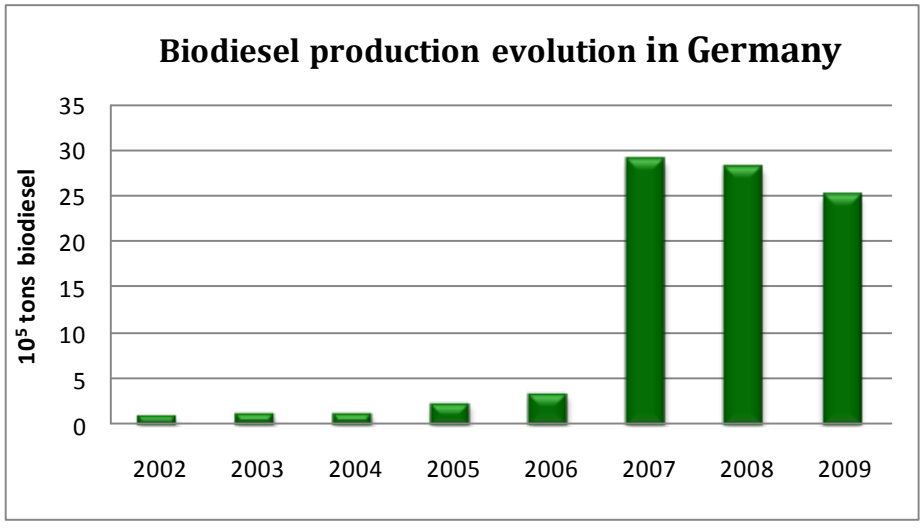

Figure 15: Biodiesel production evolution in Germany.

France represents the second major biodiesel producer with a production capacity of 900.000 tons a year. The biodiesel commercialized in France is at B20 form (20% of Biodiesel mixed with 80% of conventional diesel).

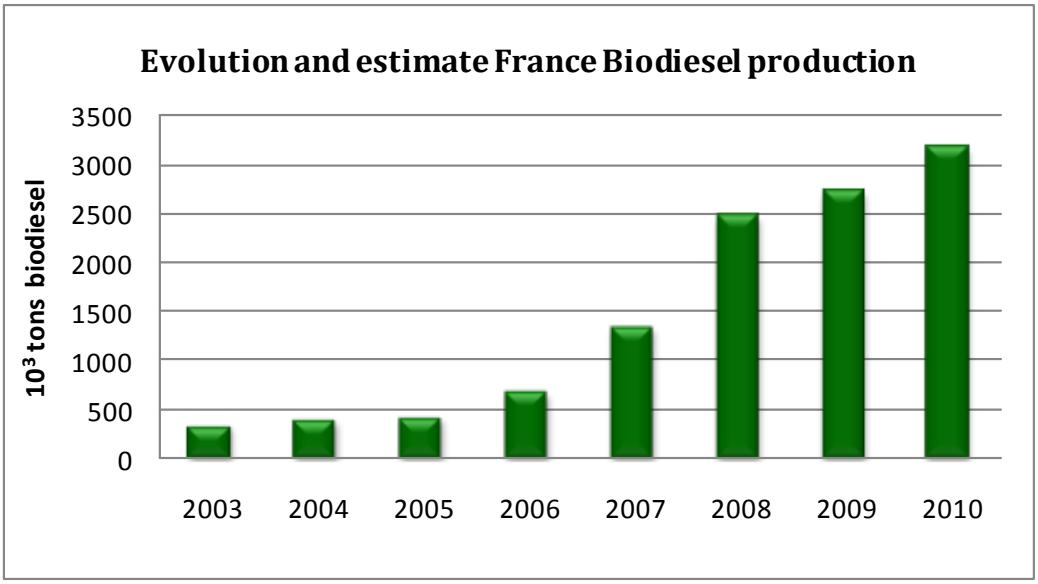

Figure 16: Biodiesel production in France.

The biodiesel programme in EUA is significantly different from Europe programme. Biodiesel is mainly produced from soybean oil and is commercialized as B20. Biodiesel producers follow other specifications which are described at the american norm ASTM D-6751. The production of Biodiesel in EUA began to grow in year 2000.

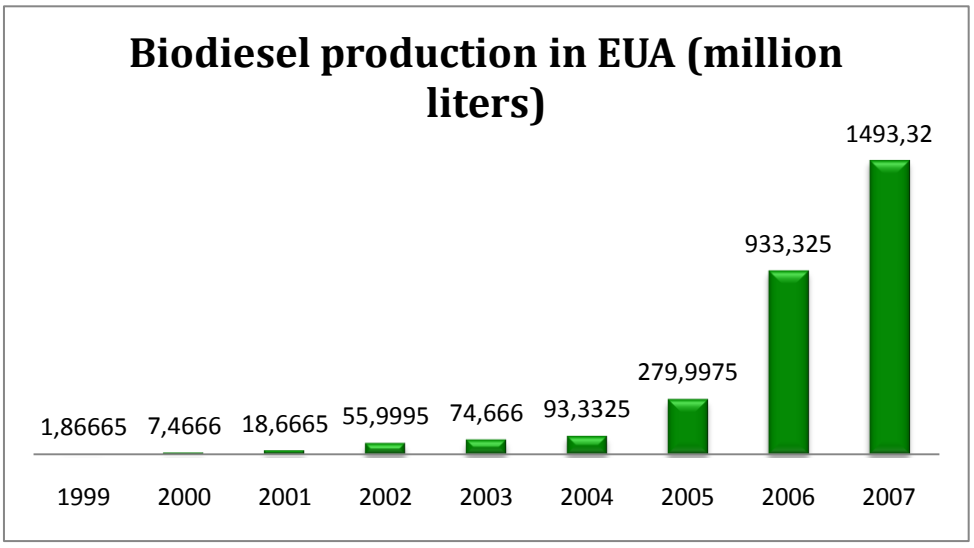

Figure 17: EUA Biodiesel production until 2007.

In South America, the majority of the countries does not have capacity installed for biodiesel production with an exception for Brazil. However, they have ideal climate conditions for oilseeds cultivations such as soybean or palm.

Argentina is the major world soybean exporter but biodiesel is a recent reality. Countries like Chile or Peru are facing with the absence of structures capable of ensuring the production of biofuels. Bolivia and Ecuador are highly dependent on oil and government assistance for the development of biofuels is negligible.

The goal for Brazil to January of 2008 was to achieve a degree of mixture of 2% biodiesel with conventional diesel. This goal was not only met, but exceeded. Nowadays, units of biodiesel production have a capacity to produce 2,7 billion liters a year.

Several projects have been studied and implemented for the production of biodiesel in Asia when oil prices began to shoot. Since three of the four largest producers of palm belong to this continent (Malaysia, Indonesia, Thailand), will be a good bet to invest in biodiesel from vegetable oils in these regions.

1.5. Environmental impact

Comparatively, biodiesel has several advantages over conventional diesel in terms of greenhouse gases emissions (GHG). The most used raw material is vegetable oils produced by crops and since the carbon released during combustion in process were those absorbed from the atmosphere by the crops, Biodiesel has a CO_2 life cycle, theoretically, closed.

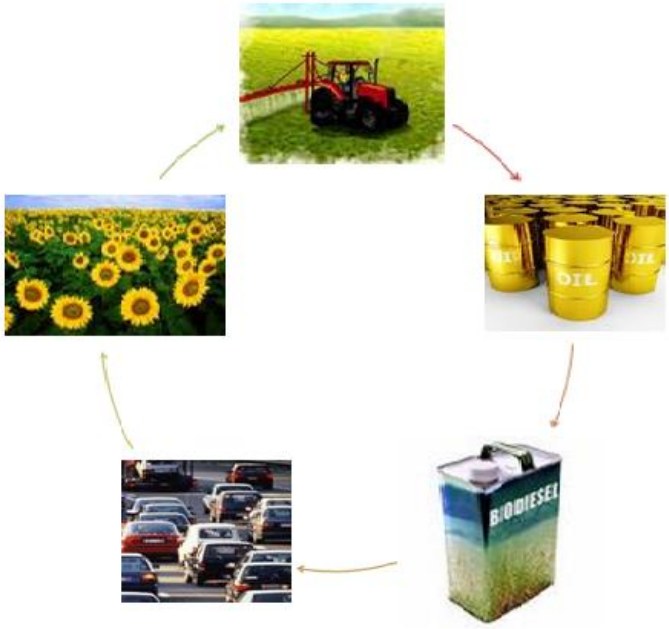

Figure 18: Simple Biodiesel from vegetable oils life cycle representation.

However, for the biodiesel produced by vegetable oils, many other parameters need to be taken into account. A product life cycle has several stages and each stage needs rigorous analysis. In order to demonstrate the environmental impact of the application of Biodiesel in the transportation sector, a life cycle assessment is usually study to analyze greenhouse emissions throughout product life from raw material extraction through production, use and disposal. The energy consumption in each stage is also taken into account. The energy flows during the life cycle are of two types: direct and indirect energy. A direct energy flow is the

energy consumed in the form of fossil fuel, steam and electricity while an indirect energy flow is the energy involved in transportation purposes [25].

Considering sunflower oil as raw material, biodiesel life cycle must have into account three main stages: plantation or agriculture stage, milling stage and finally the transesterification reaction. In the agricultural, several processes are involved including planning, field maintenance, harvesting, collection and replanting. In the agricultural stage the transportation of fertilizers involve the utilization of fossil fuels. Thus, to declare that CO_2 life cycle of Biodiesel from vegetable oil is a closed system, it is necessary to analyze GHG emissions in several stages such as feedstock production, harvesting and collection as well as the conventional transesterification process itself using methanol and basic catalyst.

The conventional process has several emissions associated. For every 1 l of methanol and 1 kg of sodium hydroxide produced, 1.33 kg CO_2 and 0.79 kg CO_2 will be released to the atmosphere, respectively [26].

Waste cooking oils has been proposed as an important possible future source of biodiesel. After uses in cooking, waste oils are not disposed adequately turn it in an appealing raw material to biodiesel production.

The life cycle of the waste cooking oil methyl esters starts from transportation of the waste oil to the biodiesel production plant until use in a diesel vehicle which can be compared with conventional diesel that starts from crude oil extraction, transport of crude oil to the refinery plant until diesel use in the vehicle.

Figure 19: Simple Biodiesel Life Cycle from waste cooking oils.

Considering carbon dioxide, nitrous oxide (N_2O) and methane (CH_4) as greenhouse gases, a study was made in order to compare emissions from biodiesel and diesel combustion in vehicles. Considering the life cycle of both products, the study conclude that a vehicle using biodiesel from waste cooking oils emits 2,35 kg CO_2-eq/km against 32,57 kg CO_2-eq/km from a vehicle moved by conventional diesel. Thus, GHG emissions from the life cycle of biodiesel from waste cooking oils are 93% less than those of conventional diesel production and use [27].

Moreover, Biodiesel does not emit sulfur compounds and reduces particle matter emissions in 47%, carbon monoxide emissions in 48% and 67% in total unburned hydrocarbons comparing to conventional diesel emissions [28].

1.6. Promising alternative biodiesel production processes

The raw material selection for biodiesel production is essential since it represents about 60 to 80% of total cost production. The raw material most used for transesterification reaction consists in vegetable oil obtained from several oilseeds. These oilseeds must to be cultivated, maintained, harvested and collected in large croplands. The culture period can take months and is necessary the occupation of large areas in order to achieved the oil quantity necessary. To face these problems, new studies are being done in order to propose new sources of raw material for biodiesel production.

Microalgae have been discussed as a promising solution for biodiesel production. Microalgae consists in unicellular photosynthetic microorganisms capable of realize photosynthesis in which solar energy is converted in chemistry energy (organic compounds) using CO_2, minerals and water. One type of those organic compounds is exactly triglycerides which represent nutritional reserves for the microalgae and they are similar to triglycerides contained in vegetable oils from oilseeds. Due to its similarity, they can be used for biodiesel production as well.

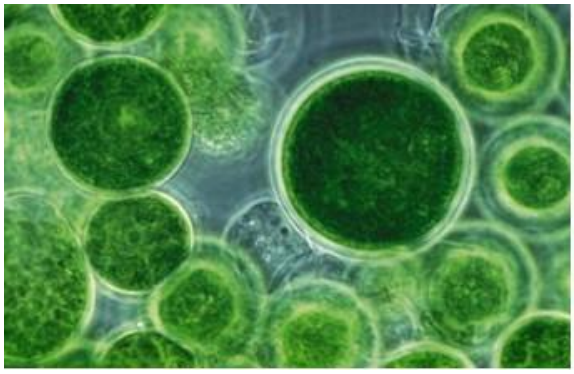

Figure 20: Microalgae.

The quantity of the triglycerides defines the natural microalgae content of lipids. Manipulating growth conditions, the lipid content increase. The growth and maturation of microalgae are made in two distinct stages applying in each step the necessary conditions in order to achieve the best lipid content as possible.

The great advantages of microalgae against oilseeds are:

- Microalgae does not compete with food sector, consequently, does not redirect food products for energy production;
- Higher productivity which allows producing the same quantity of oil but in an area 100 times smaller.
- Lower culture periods. Oilseeds are, normally, collected after cultivation period of months and the microalgae can be collected after only 15 days of culture.
- 30 times higher production capacity per hectare since, at the same area, the oil quantity is much higher than in the same area of oilseeds culture.

Several reactors types have been studied for biodiesel production. The aim of the first stage is just to growth the microalgae in photobioreactors achieving high biomass concentration. At this stage, all the nutrients need to be added as well as CO_2 and solar illumination in order to achieve a feasible growth. The second stage is to mature the microalgae, i.e., increase the lipid content creating stress conditions by depriving microalgae from one of the essentials nutrients to its growth. These stress conditions will induce an increase of the nutritive reserves of microalgae. The nutritive reserves are the triglycerides. The second stage occurs at open systems called raceways ponds.

Figure 21: Several reactors configurations for microalgae production.

The methanol used in biodiesel production is still derived from fossil fuels, specifically from natural gas. Thus, methanol production is dependent on the availability and the price of oil. In order to reduce this dependence and to improve the environmental impact of biodiesel production, the methanol should be obtained from renewable sources. Biomethanol is often obtained by the gasification from biomass such as wood or cellulosic residues. However, biomethanol obtained from renewable resources contains some impurities such as higher alcohols, methyl formate and di-isopropyl ether which interfere in transesterification reaction. Biomethanol can be refined to remove impurities but this process makes it more expensive than crude methanol and this is the reason because methanol is more often used than biomethanol [29]. Further developments of this technology must be done in order to decrease environmental impact of biodiesel production process.

A promising alternative production process of methanol had been studied by Kuwabata *et al* since 1994 and consists in an electrochemical conversion of carbon dioxide to methanol using two types of enzymes. The strategy consists in the application of two distinct enzymes types which will reduce different intermediates in order to obtained CO_2 at the end of the process. The first enzyme is formate dehydrogenase (FDH) that catalyses reduction of carbon dioxide to formate. The second enzyme is methanol dehydrogenase (MDH) and it has the capacity of catalyzing the reduction of formate to methanol *via* formaldehyde. Then, it is possible to electrochemically synthesize methanol from carbon dioxide with utilization of the cooperative works of FDH and MDH [30].

The electrochemical conversion of carbon dioxide to methanol process has a great potential as an alternative process to obtained methanol since it uses a greenhouse gas as a resource turning methanol production into an environmentally friendly process.

PART II: VEGETABLE OILS

2.1. Vegetable oil composition

Fats and oils are primarily water-insoluble, hydrophobic substances in the plant and animal kingdoms that are made up of one mole of glycerol and three moles of fatty acids and are commonly referred to as triglycerides [31]. The proportions of the various acids vary from fat to fat; each fat has its characteristic composition and these oils contain not only triglycerides but they also have free fatty acids and diglycerides [32].

Fatty acids are monocarboxylic acids that have a long chain composed of carbon and hydrogen atoms with a terminal characteristic of organic acids, the carboxyl group. The fatty acids have high molecule weights with linear chains and they can be saturated or unsaturated. The principal saturated acids are lauric acid, palmitic acid and stearic acid. The unsaturated acids more often constituents of vegetable oils are oleic acid, linolenic acid and linoleic acid.

Triglycerides or triacylglycerols are named as the number fatty acids existing in the molecule. Monoglycerides contain one fatty acid, diglycerides contain two fatty acids and the triglycerides have three fatty acids. In each position, these fatty acids can be different in the three or two positions or can be the same fatty acid. This must be indicated in the molecule nomenclature. The distribution of fatty acids in triacylglycerols can happen randomly in positions one and three, that are identical, but not in position two, which is the more sterically hindered [33].

Animal fats and vegetable oils are mainly consisted by fatty acids with carbon chains with 16 to 18 carbon length. All of fatty acids founded in natural fats have carbon chain pair numbers and, when they are unsaturated, they are, normally, in *cis* configuration [10].

Mainly fatty acids constituents of vegetable oils are described in APPENDIX B.

The melting and boiling points of the fatty acids increases linearly with increasing of the chain and are influenced by the presence of branches and substitutes. In unsaturated acids, melting and boiling points are also influenced by the number and position of double bonds. The boiling points and melting points increases as the number of carbon atoms in the carbon chain increase but decrease with increases in the number of double bonds. Thus, unsaturated fatty acids have melting and boiling points lower than the saturated ones. Moreover, the *cis* configuration has always melting points and boiling points lower than the *trans* configuration [18].

The vegetable oil used for biodiesel production must be carefully analyzed before its application in order to check if it has the best composition. Vegetable oils with lower contents of saturated fatty acids and enriched in oleic unsaturated fatty acids (C18:1) are the most feasible for biodiesel production.

2.2. Vegetable edible oils resources

The uses of a triglyceride feedstock for biodiesel production depends on regional availability, yield and production cost. The main differences among these oils are their fatty acid composition which strongly affects on some important properties of the biodiesel such as cetane number, heat of combustion, melting point and viscosity [34].

There are several types of biolipids which can be used to produce biodiesel. These are virgin vegetable oil feedstocks such as rapeseed and soybean oils which are the most commonly used, though other crops such as mustard, palm oil, sunflower, hemp which represents also alternative oils. The other types are waste vegetable oils, animal fats including tallow, lard, and yellow grease and non-edible oils such as jatropha oil, castor oil, tall oil, *etc*. [10].

Table 2: Oil species for biofuel production [10].

Group	Source of oil
Major oils	Coconut (Copra), corn (maize), cottonseed, canola (a variety of rapeseed), olive, peanut (groundnut), safflower, sesame, soybean, and sunflower
Nut oils	Almond, cashew, hazelnut, macadamia, pecan, pistachio and walnut
Other edible oils	Amaranth, apricot, argan, artichoke, avocado, babassu, bay laurel, beech nut, ben, Borneo tallow nut, carob pod (algaroba), cohune, coriander seed, false flax, grape seed, hemp, kapok seed, lallemantia, lemon seed, macauba fruit (*Acrocomia sclerocarpa*), meadowfoam seed, mustard, okra seed (hibiscus seed), perilla seed, pequi, (*Caryocar brasiliensis* seed), pine nut, poppyseed, prune kernel, quinoa, ramtil (*Guizotia abyssinica* seed or Niger pea), rice bran, tallow, tea (camellia), thistle (*Silybum marianum* seed), and wheat germ
Inedible oils	Algae, babassu tree, copaiba, honge, jatropha or ratanjyote, jojoba, karanja or honge, mahua, milk bush, nagchampa, neem, petroleum nut, rubber seed tree, silk cotton tree, and tall
Other oils	Castor, radish, and tung

The most important oilseeds for biodiesel production are rape, palm, soya and sunflower. The Figure 22 shows the world edible oils production with projections for the four most important oilseeds.

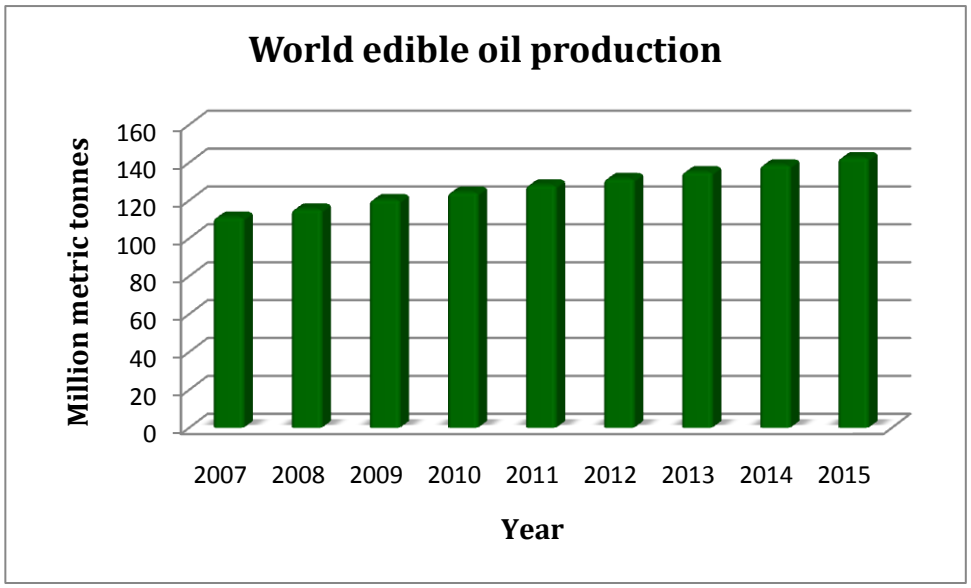

Figure 22: World edible oil production from rape, Palm, soya and sunflower oilseeds [35].

From Figure 22, it can be seen that world edible oil production had been growing an average of 1% a year and this is because of the increasing biodiesel demand and

due to the fact the biodiesel production from edible oils is the only implemented indutrial technology until now making edible oils the only possible raw material.

The oilseeds most used for biodiesel production in Europe are rapeseed, soybean and sunflower. Soybeans are also widely used in the U.S.A.

In the Mediterranean and in Portugal, due to its climatic conditions, sunflowers are widely used for biodiesel production and it represents a good bet for national biodiesel production. In 2008, Portugal had already cultivated 18.000 ha for sunflower production. However, this competes with food sector. Nowadays, it has been investigated the alternative of cultivating oilseeds that do not occupy lands designated for food production. These oils are the non-edible oils such as castor, jatropha, karanja and others.

2.3. Waste cooking oils: Adding value to a residue

Biodiesel is already a reality for many countries and its benefits are already aware for all people. However, conventional production processes is starting to indicate several indirect problems which influence other vital sectors.

With the increase in global human population, more land may be needed to produce food for human consumption. However, the land used for biodiesel production instead of food production is becoming larger and larger. Nowadays, lands that were supposed to be used for cultivation of several types of cereals as well as other type of food were deviated for oilseeds cultivation for biodiesel production. The problem already exists in Asia and in several countries where vegetable oils prices are relatively high. The same trend will eventually happen in the rest of the world.

The major challenges for biodiesel production are its costs and limited availability of fat and oil resources. According to Nelson *et al,* the significant factors that affect the cost of biodiesel are feedstock cost, plant size and the value of the glycerine byproduct [36].

There are two major parameters to take into account for the cost of biodiesel: the costs of raw material (fats and oils) and the cost of processing. The cost of raw materials accounts for 60 to 75% of the total cost of biodiesel fuel against 40 to 25% for processing costs [12].

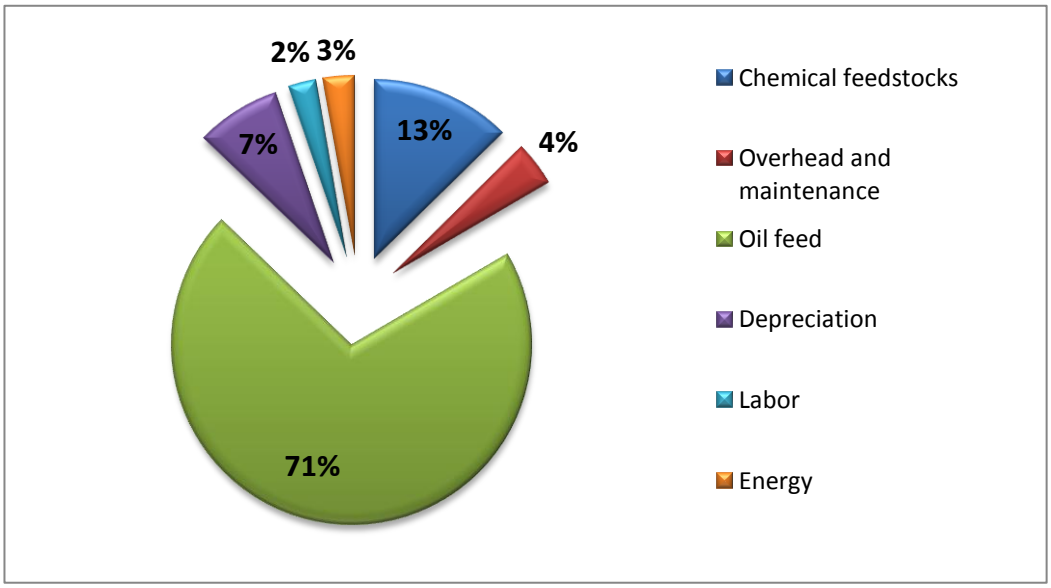

Figure 23: Average Biodiesel production costs distribution [37].

Analyzing the cost distribution, it is easy to conclude that the cost of vegetable oil has a crucial role in the economics of the biodiesel production. New alternatives for the raw materials must be proposed in order to decrease the huge influence of this parameter in biodiesel final production cost. One of those alternatives is the use of **waste cooking oils** which can lower the cost significantly since they represent, nowadays, a residue without any commercial value and the only cost associated is for the collection.

Huge quantities of waste cooking oils and animal fats are available throughout the world, especially in the developed countries. Currently, these waste oils are sold commercially as animal feed. However, since 2002, the European union (EU) has enforced a ban on feeding these mixtures to animals because during frying, many harmful compounds are formed and if the waste cooking oil is used as an additive to feeding mixtures for domestic animals, then it could result in a return of harmful compounds back into the food chain through the animal meat. Hence, the waste

cooking oil must be disposed safely or be used in a way that is not harmful to human beings [38]. However, the disposal of waste cooking oil is problematic due to its possible contamination of the water and land resources. Many developed countries have set policies that penalize the disposal of waste oil though the water drainage. The production of biodiesel from waste cooking oil is one of the better ways to utilize it efficiently and economically.

The amount of waste cooking oil generated in each country varies depending on the use of vegetable oil. An estimate of the potential amount of waste cooking oil collected in the EU is 700.000- 1.000.000 tons/year [39].

The waste cooking oil is categorized by its FFA content. If the FFA content of waste cooking oil is <15% then it is called "yellow grease" otherwise, it is called "brown grease" [23]. According to Zhang *et al*, it is estimated a total yellow grease production of 135.000 tons/year in Canada and E.U.A. and a total of 200.000 tons/year in UK [40].

Food is subject to a different temperatures range during cooking, boiling and frying. Depending on the degree of heating, various physical and chemical changes occur in vegetable oil after frying such as an increase in the viscosity, an increase in the specific heat, a change in the surface tension, a change in color and an increase in the tendency of fat to foam [41]. Studies done by Mittlebach *et al* on frying oil suggested the occurrence of three types of reactions during frying: thermolytic, oxidative and hydrolytic [42]. As combined result of all these chemical reactions, many undesirable compounds are formed during frying. To remove these undesirable compounds in waste cooking oil, in most of the cases a pretreatment needs to be done before the transesterification such as a filtration [43].

Chhetri *et al* had analyzed biodiesel obtained by the transesterification reaction using a waste cooking oil sample from a local restaurant in Nova Scotia, Canada [44]. Biodiesel was analyzed by Gas Chromatography and the results are summarized in the Table 3.

Table 3: Gas Chromatography analysis result of a biodiesel sample obtained by transesterification reaction using waste cooking oil [44].

Fatty acid	Content
Oleic acid (C18:1)	59,7%
Linoleic acid (C18:2)	19,31%
Linolenic acid (C18:3)	6,82%
Palmitic acid (C16:0)	5,18%
Stearic acid (C18:0)	2,1%
Ecosenoic acid (C20:0)	1,21%
Others	4,36%

Although this is only an analysis of an unique waste cooking oil sample from a specific local and not all the waste cooking oils have this composition, this study confirms the application of waste cooking oil as a feasible raw material for biodiesel production due its high content in oleic acid (59,7%) and lower content in saturated fatty acids (8,5%).

Assuming that transport and pretreatment of waste cooking oil are the only additional costs involved in the production of biodiesel from waste cooking oil, the effect of the price of raw material in the biodiesel production costs can be reduced as much as 50%.

Table 3: Comparation between edible oil and waste cooking oil prices [11].

Raw material (oil type)	Average oil price (€) per liter (before Taxes)
Edible oil	0,45
Waste cooking oil	0,22

The data on the requirements of diesel fuel and availability of waste cooking oil may not replace diesel fuel completely. However, a substantial amount of diesel fuel can be prepared from waste cooking oil which would partly decrease the dependency on petroleum-based fuel. In Portugal, the "Federação das indústrias de óleos vegetais, derivados, e equiparados (FIOVDE)" says that in 2007 was produced nearly 150.000 tons of waste cooking oils. Assuming that all the 150.000 tons are collected and used to produce biodiesel, it could be obtained more than 150.000 tons of biodiesel from waste cooking oils which represent 43% of the total biodiesel production to achieve the 10% of mixture between diesel and biodiesel established until 2010. Thus, the use of waste cooking oils produced in Portugal for biodiesel production is not only a feasible alternative as well as the availability of the raw material allows a production of almost 50% of the total biodiesel necessary until 2010.

2.4. Application of vegetable oils and animal fats in diesel engines

Conventional diesel is derived from crude oil consisting mostly of hydrocarbons with carbon chain lengths between 10 until 20 carbons. Compounds with similar structures and properties can be used as a substitute of conventional diesel. One of those compounds are oils and animal fats.

The application of vegetable oils in diesel engines is not new. 100 years ago, Rudolf Diesel had already proposed the use of vegetable oils as a substitute to conventional diesel. However, at the time, vegetable-oil fuels had not been acceptable by the society because they were more expensive than petroleum fuels. With the recent increases in petroleum prices and uncertainties surrounding petroleum availability, vegetable oils have become more attractive recently because of their environmental benefits as a possible fuel and the fact that they are made from renewable resources.

Natural vegetable oils and animal fats usually contain FFA, phospholipids, sterols, water odorants and other impurities. Thus, crude vegetable oil should not be used before a refining process since some of the impurities can interfere negatively in the transesterification reactions as is the case of water and FFA using basic catalysis [18]. Due to their high viscosity, lower volatility and the reactivity of unsaturated hydrocarbon chains, vegetable oils should not be used directly in diesel engines since they reduce its efficiency or, after the engine has been operating on vegetable oils for long periods of time, it can also result in engine malfunction. In order to solve these problems, the refined oil is transesterified [45].

In order to be competitive, vegetable oils have to present similar properties and engine performances. Properties such as heating value, density, viscosity and cetane number must be similar so that vegetable oils can be applied in the same conventional diesel applications.

Table 4: Comparisons of some fuel properties of vegetable oils with D2 fuel (D2 fuel is a diesel engine fuel with 10 to 20 carbon number hydrocarbons) [46].

Fuel type	Heating value (MJ/kg)	Density (kg/m^3)	Viscosity at 300 K (mm^2/s)	Cetane number[a]
D2 fuel	43.4	815	4.3	47.0
Sunflower oil	39.5	918	58.5	37.1
Cottonseed oil	39.6	912	50.1	48.1
Soybean oil	39.6	914	65.4	38.0
Corn oil	37.8	915	46.3	37.6
Opium poppy oil	38.9	921	56.1	–
Rapeseed oil	37.6	914	39.2	37.6

[a] Cetane number is a measure of the ignition quality of diesel fuel.

The heat contents of vegetable oils are 88% of that of conventional diesel. Engines efficiencies using vegetable oils are then slightly lower but this alone does not represent their non availability as fuel. On the other hand, the difference between the gross heat content of any of the vegetable oils is negligible [46]. The density values of vegetable oils are between 912 and 921 kg/m^3, while that of D2 fuel is 815 kg/m^3. The kinematic viscosity values of vegetable oils vary between 39.2 and 65.4 mm^2/s at 300 K concluding that they are all extremely viscous, with viscosities ranging from 9 to 15 times greater than D2 fuel. These major problems are overpass by the transesterification reaction obtaining methyl esters with lower viscosities.

The increase in biodiesel heat content results from a high increase in the number of carbons and hydrogens comparing to conventional diesel. The higher the length of the fatty acid chain the higher is the heat content of the vegetable oil, and the subsequent alkyl ester. Thus vegetable oils with longer fatty acids are more appealing, taking into account that they will result in a biodiesel with higher heat content.

This effect is related with the number of hydrogen atoms in the fatty acid. A longer fatty acid has more hydrogen atoms and consequently higher heat content. This means that oils rich in short or unsaturated fatty acids are not a good option to be used for biodiesel production [47].

However, long saturated fatty acids are solids at room temperatures or near to room temperature turning them unviable to the use in engine motors. Therefore, vegetable oils should contain high unsaturated carbon chains hydrocarbons but and, at the same time, with unsaturations number as low as possible in order to increase the molecule heating value.

PART III: SUPERCRITICAL FLUIDS

3.1 Fundamentals: Carbon Dioxide

The Montreal Protocol was introduced in 1987 in order to reduce a massive wide scale use of organic solvents that represents a serious problem to the environment. Thus, the worldwide industries are forced to adopt new sustainable processes which do not require the use of environmentally damaging organic solvents. Supercritical fluids (SFC) represent a viable alternative since some of its applications could, partially or totally, substitute organic solvents.

Any fluid is considered at supercritical conditions if its temperature and pressure are above the critical point. The critical point of a pure substance represents the highest temperature and pressure at which vapor and liquid phases coexist at equilibrium. At the critical point, it only exists a single supercritical phase because the densities of the two phases become identical and the distinction between them disappears.

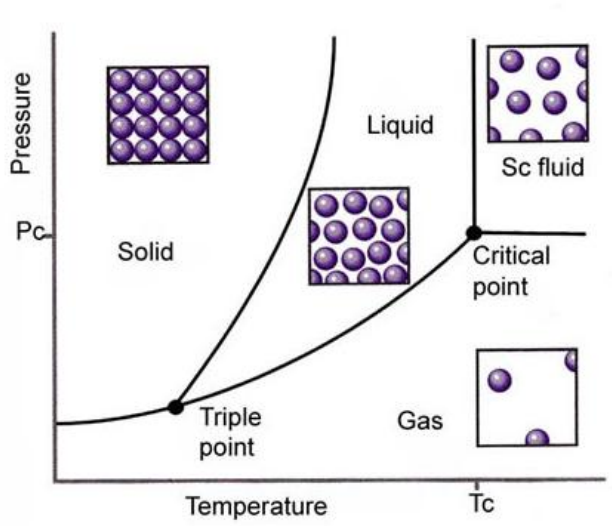

Figure 24: Supercritical conditions region. Pc: critical pressure; Tc: critical temperature

Supercritical fluids, also known as compressed gases, are characterized by having properties between gas and a liquid – gas like viscosities increase mass transfer and solvating properties similar to a wide range of organic solvents [48].

Supercritical fluids have several advantages over organic solvents near at supercritical point conditions including facilitated mixing of compounds resulting in a better heat and mass transfer, fast reaction comparing to other solvents medium and have good scability as well as being simple applied to continuous processes.

The more common components used as supercritical fluids are water, CO_2, acetone, alcohols like ethanol and methanol, alkanes such as methane, ethane and butane or unsaturated hydrocarbons like ethylene or propylene, among others [49].

Table 5: Critical point of most commonly supercritical fluids [49].

	Critical point	
Compound	**Temperature (K)**	**Pressure (MPa)**
Water	647	22,06
CO_2	304,1	7,38
Methanol	512,6	8,09
Methane	190,7	4,6
Propane	369,6	4,25
Acetone	508,1	4,7

Water is considered as the most “greener” solvent since it represents a natural resource with high availability and totally environmentally benign. However, critical conditions are too high to be considered as a solvent to several supercritical processes since the implementation of the energy necessary to achieve the critical conditions represent, necessarily, an increase of production costs. Moreover, at supercritical conditions water is a non-polar compound which results in a decrease of salts solubility. The precipitation of this salts cause corrosion which represents one of the major problems to the implementation of supercritical water.

Although methane has the lowest critical conditions, it represents a highly inflammable compound and, consequently, a dangerous compound to implement in any industrial process.

Propane is often applied as a solvent at supercritical conditions. Propane has suitable critical conditions and is a considered as a good solvent of long chain aliphatic compounds [50]. However, it is considerable as a very dangerous solvent to work with due to its high inflammability. Thus, the propane is not taken into account as a supercritical solvent in an industrial process due to the risks it represents.

However, supercritical conditions processes have some limitations that must be overcome related to the harsh operation environment and their effect on the materials. The corrosion and salt deposition are the two major problems for the most industrial processes besides the fact the high energy necessary to reach supercritical conditions. This can be overcome by choosing the best supercritical fluid that better adapts to the process as well as improving system design with heat and mass recycling.

Carbon dioxide represents, nowadays, the most attractive compound to apply as a supercritical solvent due to its nonflammability, low toxicity and high availability. The cheap and inexhaustible world supply of carbon dioxide originate by our own emissions can be explored to provide a wide range of environmentally friendly and economically attractive sustainable processes solving a major worldwide problem: global warming. Comparing with other compounds like as water, CO_2 presents suitable critical conditions which can be easily applied without turning it economically unattractive. Supercritical carbon dioxide (Sc-CO_2) intrinsic properties which allows, at the same time, gas like high diffusivity and liquid like high density, are responsible for increasing mass transfer which favor reactions rates [51].

The solvent properties of Sc-CO_2 can be easily modified adjusting temperature and pressure conditions which decrease or increase the solubility of a certain compound. The compound solubility in supercritical CO_2 is related to its density controlled by the pressure and temperature applied. Manipulating these two conditions, a decrease of a certain compound solubility occur resulting in a precipitation. This simplifies the downstream cleanup process since the carbon

dioxide can be easily separated from others components and unreacted compounds [52].

3.2. Enzyme catalyzed in Supercritical CO_2

Conventional industrial production process of biodiesel usually applies alkali-catalysis which results in several problems that need to be solved using more energy and utilities. Several oils have high contents of free fatty acids and water that could react with alkaline catalyst forming undesirable saponified compounds responsible for a decreasing of the reaction yield. Furthermore, several purification processes are needed in order to recover the catalyst and the saponified products which turn the process more extensive and complex [17].

In the recent years, lipases are very often applied as biocatalysts as an alternative for the conventional catalysts since they achieved more efficient processes with higher selectivity and fewer environmental problems. Biocatalysis applied to processes under continuous supercritical conditions has several advantages since catalyst lifetime is extended keeping the catalytic system running longer and decreasing the charges number to replace the enzyme. The enzyme is usually used in immobilized form so that it can be reused several times reducing production costs [53].

The transesterification reaction between oil and methanol results in a two phase mixture which required a vigorous stirring to favor the mass transfer. To form a single phase reaction medium, the transesterification reaction can occur under organic solvents medium reducing significantly the reaction time [54]. However, the use of organic solvents must be reduce and, when possible, totally eliminate from industrial processes decreasing the emissions of VOC's that deplete the ozone layer.

Transesterification reaction under supercritical fluids has several notable advantages over the conventional processes. Reactions rates within supercritical fluid media are enhanced due to the higher mass transfer of reactants compared to organic solvents since the high density and diffusivity of the media promote the

collisions between the reactants accelerating the reaction rate [48]. Transesterification of triglycerides (non-polar molecules) with an alcohol (polar molecule) is usually a heterogeneous (two liquid phases) reaction at conventional processing temperatures because of the immiscibility of the non-polar and polar components. Under supercritical conditions, however, the mixture becomes a single homogenous phase which will accelerate the reaction because there is no interphase mass transfer to limit the reaction rate.

Reactions involving SCF media offer the best opportunity for separation of reaction products and the removal of the solvent accomplished through one simple depressurization reducing energy demand compared to processes that have implemented multi-stages in order to recover the final product. So as to remove unreacted products and by-products using supercritical CO_2 it is only necessary to adjust pressure and temperature manipulating the density of Sc-CO_2 in order to precipitate the products that must be recovered avoiding this way the implementation of complex process which required higher investments.

3.3 Separation processes using Supercritical CO_2

One of the most common and successful applications of supercritical CO_2 consists in extraction and separation processes. Intrinsic properties turn Sc-CO_2 at an important solvent to these processes since the separation is easily achieved by just adjusting temperature and pressure conditions. After the product(s) recovery, CO_2 is easily recycled.

The most important parameters to extraction or separation processes are the solubility and phase equilibrium of the systems. With constant temperatures, higher solubilities can be achieved by increasing pressure since with high fluid density the solvent power increases. On the other hand, with constant pressures, increasing temperature decreases fluid density and, consequently, compounds solubility. The vapor pressure of the compounds must be taken into account also since high the temperature, higher vapor pressure of a certain compound increasing its solubility. Manipulating temperature and pressure conditions, the separation between several components can be achieved by changing compounds

solubilities at different conditions of temperature and pressure applied. The separated products are, later, collected at the bottom of the separator. In order to be recycled, the fluid has to be recompressed and heated up to separation conditions [55].

MULTI-STEP SEPARATION

The fractionation process is based on the different solubilities of the compounds to be separated in Sc-CO2. The ideal case is obtained when only the compounds to be recovered are insoluble in Sc-CO2, whereas, all the other components are totally soluble. However, this case is rare and a limited solubility of the other components has to be taken into account and for this reason the separation conditions must to be accurately chosen in order to achieve the maximum difference in solubility among the compounds to be recovered and all the other compounds in the mixture [56].

When a mixture contains several components that have different solubilities at different conditions, it is necessary the implementation of more than one separator. This process is called multi-step separation consists of more than one separator operating at series and each one operates at different conditions in order to recover one or more components. In each separator are applied specific temperature and pressure conditions concerning the solubility of all the compounds.

The aim consists in the implementation of the right conditions that allows the precipitation of the components that must be recovered in that separator. The other mixture components maintains soluble in Sc-CO_2 in order to be recovered in the following separators at different conditions. Joining a few number of separators, is possible to recover several mixture components at the same process just manipulating the operation conditions. In figure XX, an example of an ideal multi-step separation process is described assuming a separation between 3 components, A, B e C.

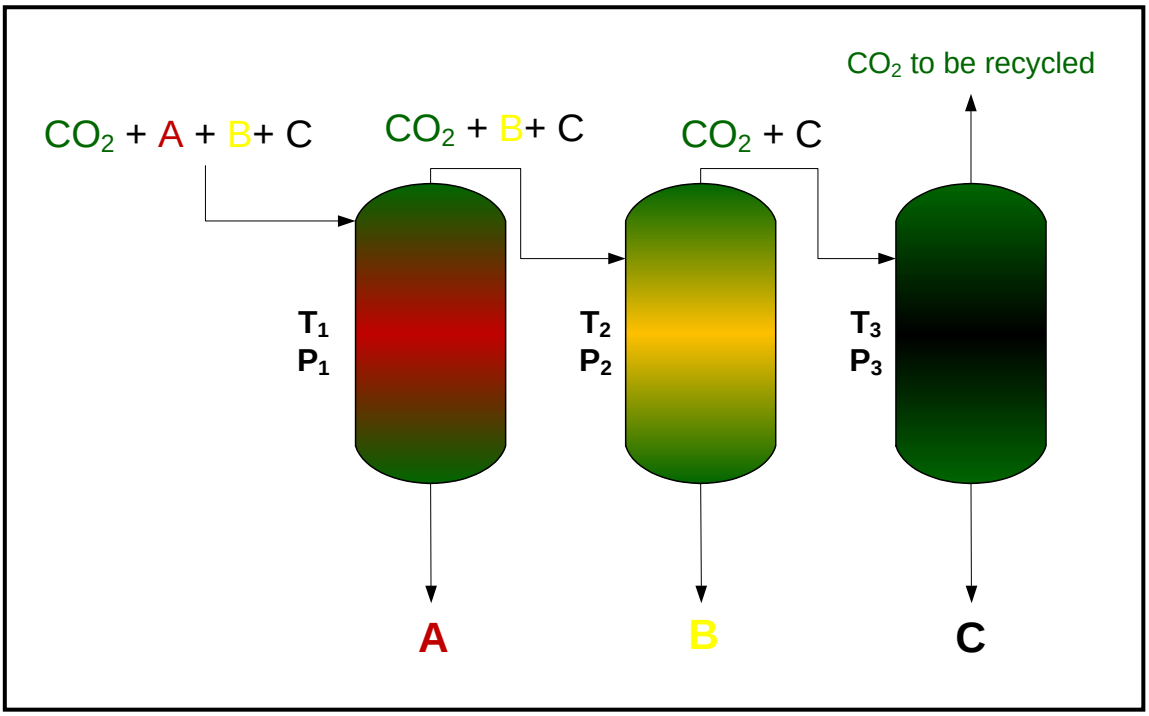

Figure 25: Diagram of a Multi step separation example.

The first separator operates at T_1 and P_1 conditions in which components B and C are soluble in Sc-CO_2 and component A it's not. Thus, at this conditions component A precipitates being recovered at the bottom of separator 1 whereas component B and C are directed to the second separator. At T_2 and P_2, component B is insoluble in Sc-CO_2 precipitating at the bottom of the separator 2 and the mixture CO_2 and component C is directed to the last separator that operates at T_3 and P_3 where component C is not soluble and precipitates. Finally, CO_2 without any component dissolved, is recycled to the process.

Implementing this kind of process, is possible to obtain a downstream stage that does not mean necessarily high costs production or the application of many utilities due to the high potential of supercritical CO_2 which, in the same process, can be used as solvent for the reaction medium and after, as separator agent.

3.4. Biorefinary: An integrated process

The term refinery is defined as production facility consisting by a group of chemical engineering unit processes in which occurs the refining of raw materials into value products. In an Oil refinery that raw material is crude oil and the value products are, among others, liquid fuels as gasoline and diesel oil. A biorefinary has exactly the same propose but is considered as an environmentally friendly factory. Biorefinary term refers to the conversion of biomass feedstock into value-added chemicals and fuels with minimal waste and emissions. Biorefinaries represents the future energy production systems integrating the production of higher value chemicals and commodities optimizing the use of different resources considered as low value materials, maximizing profitability and minimizing wastes.

The process proposed consists in an integrated green process of biodiesel production. The use of supercritical CO_2 and biocatalysis allows the production of a high value product using a raw material considered as a residue which represents a major waste treatment world problem. Besides adding value to a residue, the process integrates a greenhouse gas responsible for the biggest challenge that humanity has ever need to overcome: the global warming. Giving an application to CO_2 it will incentivize the development of capture processes since CO_2 starts having commercial value at the same time that it's removed from atmosphere avoiding catastrophes that, at long term, could result in the ending of humanity. Nevertheless, enzymes are considered as natural catalysts so that they are reproducible resources which can be easily decomposed in natural environmental when they come obsolete. Due to its high selectivity, the production of waste is also minimized.

Economic and production advantages of a biorefinary increases with the level of integrated processes. The use of Sc-CO2 as a solvent for vegetable oils allows the integration of the two main stages of the process: the enzymatic reaction of the dissolved waste oil in a continuous flow fixed bed reactor as well as the

fractionation of the obtained mixture components by their stepwise separation from CO_2 in a series of separators at different temperatures and pressures.

Applying biocatalysts in reactions under supercritical conditions provides a green and sustainable process and joining the power of supercritical carbon dioxide as a lipid solvent and reaction medium with the availability of commercial immobilized lipases, the possibility of a large-scale production of FAME it will result in a construction of a novel *Biorefinary*.

PART IV: ENZIMATIC PROCESSES AT HIGH PRESSURE

4.1. Enzymes: Immobilized Lipases

Enzymes are biological catalysts involved in various biochemical reactions. These catalysts accelerate chemical reactions converting substrates. Like all catalysts, enzymes work by lowering the reaction activation energy (E_a) increasing dramatically the rate of the reaction. On the other hand, enzymes are not consumed nor do they alter the reactions equilibrium [57].

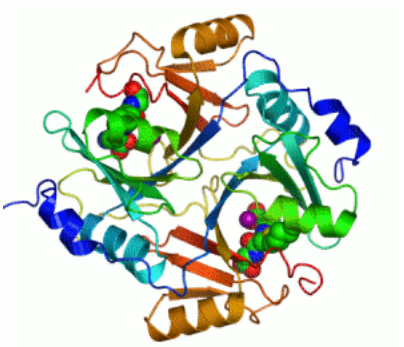

Figure 26: Example of an enzyme structure.

By being proteins, most enzymes are much larger than the substrates and only a small portion of the enzyme is directly involved in the catalysis. The region that contains these catalytic residues, binds the substrates, and then carries out the reaction is known as the **active site**. Since enzymes are rather flexible structures, the active site is continually reshaped by interactions with the substrates as the substrates interacts with the enzyme. The amino acid side chains which make up the active site are molded into the precise positions that enable the enzyme to perform its catalytic function. The active site continues to change until the substrate is completely bound at which point the final shape and charge is determined [58].

Enzymes are classified into the following six major classes based on the nature of the catalyzed reaction [59].

In the third group, hydrolases, are inserted Lipases which are a group of enzymes that catalyze the hydrolysis and synthesis of triacylglycerols. This is the type of enzymes that must be used in transesterification reactions using triglycerides.

Immobilization is the method most used for maintaining the activity of lipases and has generally been used to obtain a reusable enzyme and increases its stability. This enables easy handling, recovery and recycling of the biocatalyst decreasing the production cost [60].

There are 5 main methods of immobilization of enzymes: adsorption, covalent binding, occlusion, encapsulation or entrapment and crosslinking [61].

The immobilization of enzymes is a technique especially suitable to restrict freedom of movement of enzymes and the different methods of immobilization can influence the activity and the average lifetime of the enzyme. This kind of technique improves enzyme life time turning it in a promising alternative to the application of biocatalysts into industrial scale.

Substrate specificity of lipases consists in the capability of distinguishing structural features of acyl chains such as the length, the number, position or configuration of double bonds or the presence of branched groups, as well as the nature of the acyl source: free acid, alkyl ester, glycerol ester, etc. [59]

Differences in catalysis by lipases used for biodiesel synthesis refers to their regiospecificity with respect to the length of hydrocarbon chain of fatty acid. In terms of regioselectivity, i.e., the position of ester linkage, lipases have been divided into three types [62]:

- sn-1,3-specific (hydrolyze ester bonds in positions R1 or R3 of TAG);
- sn-2-specific (hydrolyze ester bond in position R2 of TAG);

- nonspecific (do not distinguish between positions of ester bonds to be cleaved).

Lipases characterized by narrow regiospecificity are believed not to be applicable to biodiesel production and therefore the majority of lipases used for this purpose display both wide substrate specificity and regiospecificity.

4.2 Advantages of enzymatic catalysis vs. acid/basic catalyses

Chemical catalysts used for industrial biodiesel production provide very high yields. Despite this excellent productivity, the overall biodiesel production is relatively limited mainly due to problems related to the recovery of pure glycerol and formation of soaps, mono- and diglycerides during transesterification reaction which worsen biodiesel quality. The multi-step purification of transesterification reaction products comprises steps of neutralization of the catalyst and water washes which increase the production costs. On the other hand, the concentration of FFA in plant oils used for biodiesel production has to be below 0,5% otherwise the reaction yield decrease dramatically. Enzymatic transesterification of triglycerides offers an environmentally more attractive option to the conventional process since some of these drawbacks can be eliminated [9].

Enzymatic transesterification has certain advantages over the chemical catalysis since it allows synthesis of specific alkyl esters with a less energy intensive process and allows transesterification of glycerides with high free fatty acid contents. On the other hand, the easy recovery of enzymes gives them reusability much higher than chemical catalysts reducing production costs [60].

The high cost of the enzymes often makes the enzymatic processes economically unattractive. However if the pollution of natural environment is also taken into consideration, these costs are questionable.

4.3. Parameters affecting enzymatic transesterification and enzyme stability under supercritical conditions

Although many enzymes are stable in SCFs, one should pay considerable attention to finding the correct reaction conditions for each substrate/enzyme/SCF system.

The advantages of using supercritical carbon dioxide as a medium for enzymatic reactions have been well documented. Frequently the temperature ranges used for employing supercritical carbon dioxide in processing are compatible with the use of enzymes as catalysts. An additional benefit of using supercritical fluids along with enzymatic catalysis is that it provides a medium for the recovery of products or reactants. However, a limitation of the process may arise from the non-polarity of carbon dioxide which preferentially dissolves hydrophobic compounds. High diffusivity of supercritical fluids and low surface tension lead to reduced internal mass transfer limitations for heterogeneous chemical or biochemical catalysis [63].

- **Effect of water activity**

Water content in reaction mixture can be expressed as water activity (a_w) or percentage concentration (%). Water activity (a_w) of reaction system is a function of water activities of individual components of this system. The value of a_w defines the availability of water in the system.

Treating the enzymes with pure CO_2 leads to a removal of the water. If the bounded water in extracted by dry CO_2 the enzyme loses its activity. On the other hand, acting with water saturated CO_2 causes again an inhibition of the enzyme and subsequent loss of activity. It was found that the optimum water activity, corresponding to the highest activity of enzyme was characteristic of both the enzyme and reaction medium composition and has to be optimized separately for each transesterification reaction [63].

❖ Type of alcohol and molar ratio alcohol:oil

Alcohols used for biodiesel production comprise methanol, ethanol, propanol, isopropanol, 2-propanol, n-butanol, and isobutanol in which methanol and ethanol are the cheapest and most often used in the industrial scale. However, these two alcohols are strong denaturing agents than longer aliphatic alcohols and inactivated enzymes [64].

Structure and concentration of alcohol affect the operational stability of the enzyme. The molar excess of alcohol over fatty acids contained in TAGs moves the reaction equilibrium in the direction of product formation but it can also inactivate the enzyme. The optimum molar ratio of substrates used for enzymatic biodiesel synthesis has to be determined separately for a given system [65].

❖ Effect of pressure:

Pressure has a direct and indirect effect on enzyme activity. The direct effect acts on the protein structure where local changes may occur. Those local changes may lead to another active site of a protein which may pose an altered activity, specificity or stability. If an enzyme is stable in a SCF, its stability is usually not influenced by pressure for pressure ranges up to 30MPa. Pressure-induced deactivation of enzymes takes place mostly at pressure exceeding 150MPa [59].

Pressure is also likely to have an indirect affect on the efficiency of the reaction by changing both the reaction rate constant and the solubility of the reactants. The possibility of applying high pressures which do not interfere in the enzyme activity is a great advantage because for running reactions this means that the solvent power of the supercritical fluid can be adjusted [63].

Pressure has not a strong impact during enzyme activity but some parameters related such as steps of pressurization and depressurization have. These problems will be discussed more deeply in section 3.4 of this Chapter.

❖ Effect of temperature:

Determination of optimal temperature for the enzymatic reactions in SCFs depends on the enzyme activity which decreases due to thermal deactivation with increasing temperature and to the physical properties of the solvent which may be favorable at higher temperatures due to lower mass transfer limitations and viscosity, surface tension and solving power.

It is important to specify that temperature influences enzyme activity much more than pressure. Most proteins denature at temperatures above 60 ◦C. Optimal temperature for enzyme catalyzed reactions in SCFs is also related to the operating pressure, since the solubility of the substrates and the products is strongly dependent on the density which can be controlled by variation of temperature and pressure.

Two effects are joined during an increase in reaction temperature: The reaction rate increases with increasing temperature, but enzyme activation/ deactivation can also occur. [59]. An optimization of temperature is need to each system taken into account the enzyme stability and the solubility of the compounds in Sc-CO2 at those conditions [63].

❖ Inhibition of enzyme

Enzyme inhibition can be caused by water, initial products or end-produts. The compounds can block the active centre of the enzyme so that no activity and selectivity is available.

Results of studies on the effect of glycerol on efficiency of transesterification indicate that it inactivates enzymes, particularly in repeated-batch processes. Dossat et al. [66] and Du et al. [67] observed the considerable drop in activity of lipases immobilized in hydrophilic matrices when exposed to glycerol. They found that glycerol molecules were adsorbed on the surface of these carriers thereby forming the hydrophilic coating which made enzyme molecules inaccessible to hydrophobic substrates. Adding a hydrophilic organic solvent can solve this

problem since glycerol is well soluble and therefore it does not form films on the carriers. Du et al. noticed that washing of the immobilized lipase with isopropyl alcohol restored its activity since glycerol was removed from the carrier [67].

4.4. Effect of pressurization-depressurization on enzyme activity

Operating with high pressure processes, the influence of pressurization – depressurization steps on the enzyme activity is very important. Bringing the enzyme under pressure does not play an important role but especially the depressurization steps have influence on the residual enzyme activity.

The supercritical fluid enters the enzyme by diffusion which normally is a relatively slow process. After a certain time, the enzyme is saturated with the fluid. In a slow depressurization process, the fluid will have enough time to diffuse out and leave the enzyme and the bulk. On the other hand, if the expansion of the fluids is too fast, the fluid cannot leave the enzyme fast enough which causes a higher fluid pressure in the enzyme in comparison to the bulk medium. Overcoming a certain ΔP, it causes an unfolding of the enzyme and destroying the structure. This effect is similar to cell cracking where the cells membranes are broken by the resulting over pressure inside the cells [63].

Giebauf et all studied the hydrolases stability in Sc-CO2 where the comparison between the effect of depressurization from liquid or supercritical phase on enzyme activity loss was made [68], [69]. By depressurization of carbon dioxide the forces formed inside the liquid phase are responsible for a significant higher protein denaturation.

CHAPTER 2: MATERIALS AND METHODS

PART I: MATERIALS

1.1 Chemicals

The edible oil used in all tests was virgin sunflower oil from FULA® . The waste cooking oil was provided by a commercial establishment building at FCT campus. The acrylic resin immobilized Lipozyme® TL IM (*Thermomyces lanuginosus* lipase) and the macroporous resin immobilized lipase Novozyme® 435 (Lipase B from *Candida Antarctica*) were acquired by Novozyms A/S, Bagsvaerd, Denmark.

All the other compounds used in this experimental work as well as all the information associated are described in the next figure.

Table 6: Compounds used in the experimental work description.

NAME	MOLECULAR FORMULA	MOLECULAR MASS (g/mol)	% PURITY	BRAND
Methanol for Chromatography	CH_4O	32,04	99,9	Sigma - Aldrich
Hexane for Chromatography	C_6H_{14}	86,18	97	Sigma - Aldrich
Carbon Dioxide	CO_2	44,01	≥99,98	Air Liquide
Ethylene Glycol	C_2H_6O	62,07	NA	Merck
Heptadecane	$C_{17}H_{36}$	240,48	≥98	Fluka
FAME mixture each 20% (m/m) (methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate)	$C_{17}H_{34}O_2$, $C_{19}H_{38}O_2$, $C_{19}H_{36}O_2$, $C_{19}H_{34}O_2$, $C_{19}H_{32}O_2$	270,46; 298,51; 286,49; 294,72	≥99	Sigma - Aldrich

1.2. Experimental set-up

The continuous biodiesel production process apparatus is shown in Figure 27. This apparatus consist of two main sections: the reaction section with a high pressure packed-bed enzymatic reactor and the separation section.

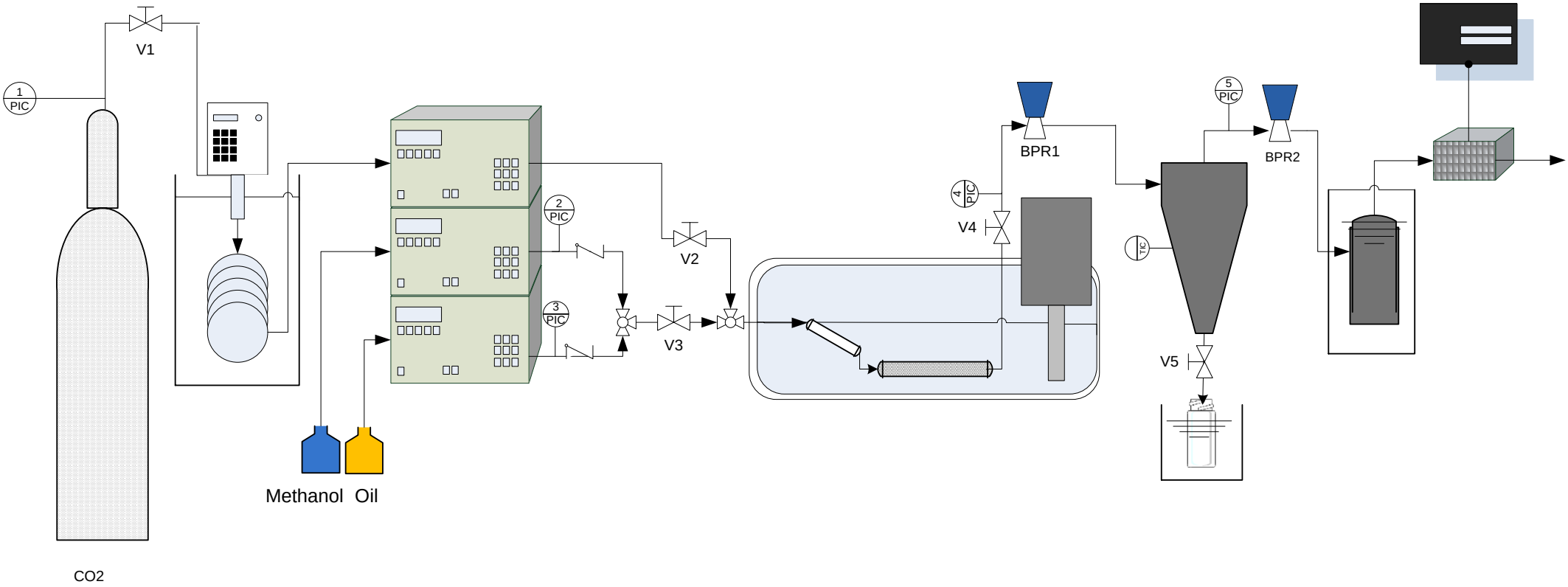

Figure 27: Experimental set-up.

This apparatus is composed by a CO_2 vessel followed by a water/ ethylene glicol refrigeration system (Julabo, model F25) to liquefy the carbon dioxide and allow it to be pumped by an HPLC pump (GILSON 305 max. flow of 25 ml/min).

REACTION SECTION

The liquid mixture (oil and methanol) is pumped through a HPLC pump system (Gilson 305/306). To ensure a homogeneous mixture, both liquid and gas are mixed in a static mixture (Kenics KBUL 3/16 with 27 mixings elements, 191 mm length and 3,4 mm inner diameter) before passing through the packed-bed enzymatic reactor already field with enzyme. The static mixer and the reactor are immersed in a water bath in which temperature is controlled by the controller JULABO P (minimum measurement interval of 1ºC). The packed bed reactor consists of a tubular stainless steel vessel .The reaction pressure is controlled with a back pressure regulator value (BPR 01) (Tescom USA) and measured by a digital pressure meter (WIKA S-10) with an interval of 1 bar.

To avoid freezing caused by the decompression of CO_2, the BPR1 is heated up with an electrical heater. The temperature is controlled by the controller (ISOPAD TD 2000) with a minimal measurement interval of 0,1ºC.

SEPARATION SECTION

The products/ CO_2 mixture obtained after the reactor which until now has always been in one phase region, is decompressed to a specific pressure to the first separator. The separator will be operated in the two phase region of the system. By separating the liquid phase from the gas phase, the reaction products can be fractionated. The separation in the first separator is controlled with a back pressure regulator (BPR 02) (Tescom USA) and is measured with an analytic pressure meter (Skalenwert 2) with a measurement interval of 1 bar.

The separation temperature is measured in-line with a digital pressure meter and a NI-CrNi thermocouple and controlled by the temperature controller HORST with an interval of 1ºC.

After the first separator, the gas phase containing the biodiesel, passes through a second separator at room pressure and cooled in ice recovering the final product. The CO_2 flow is measured at the end by passing through a mass flow meter (RHEONIK RH 007) with a capacity between 0,002 – 0,04 kg/min.

All the high pressure tubing, valves and fittings are from HIP and SWAGELOK.

PART II: CONTINOUS BIODIESEL PRODUCTION APPARATUS

In this experimental work, it was necessary to change work scale two times. The study started at a small scale (from here on referred as 1/16 *in* scale) in which was made the proof of concept. However, operational problems led to the need for a scaling apparatus and another apparatus was constructed (from here on referred as 1/8 *in* scale). Once more, in order to study some process parameters the process was also operated at a pilot scale.

2.1 Initial study: 1/16 *inch* scale

The first study occurred in the 1/16 *inch* scale apparatus. The apparatus is so called due to the use of 1/16 *inch* OD tubing. Assuming literature data for the pressure and temperature reaction, the proof of concept for the studied process was made. The experimental set-up is similar to the one presented at PART I, Section 1.2 but at a smaller scale.

Figure 28: Smaller scale apparatus (tubing of 1/16 *in*).

At this scale, the refrigeration system of CO_2 and HPLC pumps were the same to the ones presented at PART I, Section 1.2. The packed bed reactor consists of a tubular stainless steel vessel with a inner diameter of 5,4 mm and a total length of 300 mm. At the end of the reactor, a collection system was implemented. The first separation occurred at a gravitic separator. Operational problems, which will be addressed later on, had occurred caused by the small scale and another apparatus was constructed.

2.2 Parameter optimization: scale up to 1/8 *inch*

This apparatus was scaled up from 1/16 *inch* OD tubing to 1/8 *inch*. At this scale, an optimization of the reaction parameters and separation parameters was made.

Figure 29: 1/8 *in* apparatus: Scale up.

The packed bed reactor consists of a tubular stainless steel vessel with a inner diameter of 9 mm and a total length of 267 mm. The first separator is a high pressure cyclone in which is recovered the liquid phase consisted by compounds that did not react, intermediate compounds and by-products. The biodiesel is recovered in a second high pressure separator cooled in ice.

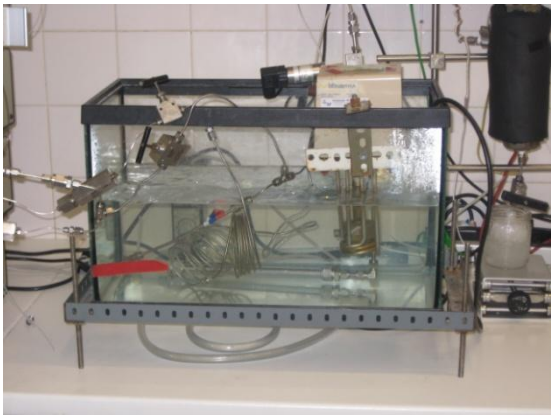

Figure 30: Reaction section.

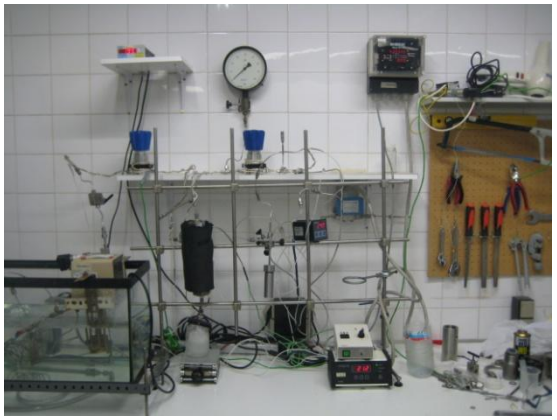

Figure 31: Separation section.

2.3 Pilot Scale

The main problem in using a lab-scale apparatus is that the very small amount of products recovered may hinder the accuracy of mass balances results. In order to solve these problems experimental work was carried out in a pilot scale.

Figure 32: Pilot scale apparatus.

This apparatus was already built and some transformations were done in order to adapt it to the experimental work.

The packed bed reactor consists of a tubular stainless steel vessel with a inner diameter of 9 mm and a total length of 400 mm. To the CO_2 refrigeration system is coupled a cryostat (Julabo immersion and Flow-through coolers, model FT400) and a circulator bath (Circulator Bath HAAKE C1-W13). The reactants and the CO_2 were previously heated by a heat exchanger. The reactor was heated up with an electrical digital heater (CAL3300, model 312-1841). A BPR is located at the reaction exit to control the pressure in the system. At the end of the installation is placed a glass trap to collecting the final reaction mixture which is immersed in an ice bath. At this scale, only a reaction study was made.

PART III: METHODS

3.1 Continuous biodiesel production

At first, both CO_2 refrigeration system and water bath are turn on until temperatures of both systems are stabilized. Then, CO_2 vessel and the valve V1 and V2 are open putting the system at the CO_2 vessel pressure of 6 MPa. CO_2 HPLC pump is turn on until the system achieved 20 MPa. After that, the CO_2 flow is changed for the operational flow rate. Methanol HPLC pump is then turn on putting some pressure into the system before pumping the oil. Oil HPLC slave pump is then turn on with the methanol HPLC pump starting to pumping the mixture of oil and methanol into the system achieving 20 MPa controlled by BPR 1.

In order to study only the reaction, samples are collected in the first separator at room pressure and immersed in an ice bath. The sample recovered in the first 30 min is neglected in order to recover the next ones with the system already stable. Samples for further analysis are recovered during 4h/5h.

To study the separation process, before starting to pump the mixture of liquids (oil and methanol), BPR 2 is progressively closed until achieved the separation pressure with the valve V5 closed. Also the electrical heat for the first separator is also turn on with the CO_2 refrigeration system and water bath at the beginning. With the CO_2 system pressure and all temperatures stable, HPLC pumps for the liquids are turn on pumping the liquids until 20 MPa controlled by BPR1. Every hour, liquid phase are recovered by opening the valve V5 recovering the mixture to a vial previously weighed. The gas phase is recovered in a steel high pressure separator also previously weighed and immersed in an ice bath or, in some cases, a dry ice bath. This separator is washed with acetone and reused for the recovery of the next sample. The same way as for the reaction study, the sample recovered in the first 30 minutes is neglected and the next samples are recovered also during 4h/5h. CO_2 mass flow is also measured by a mass flow meter at the end of the apparatus.

3.2. Sample analysis

❖ GAS CHROMATOGRAPHY

The fatty acid methyl esters (FAME), triglycerides, intermediate compounds and by-products content was determined by gas chromatography (GC) with on-Column injection.

Gas chromatography technique is based on the passage of the compounds through a capillary column by the flow of inert gas which consists in the mobile phase. These inert gases can be He, Ar, N_2, etc. The column contains a stationary liquid phase absorbed to the surface in a inert solid where the sample components are retained. The compounds can be separated according to their affinity (polarity) for the stationary phase or its boiling point. An on-Column injection means that the sample injection is made directly in the interior of the column. This kind of injector allows the placement of the syringe inside the column without depressurize it.

Figure 33: Gas chromatograph on-column TRACE GC 2000.

The chromatograph was a TRACE GC 2000 Series with a flame ionization detector (FID) with a ZB-5HT INFERNO column, 10 m x 0,32 mm , 0,10 μm film thickness and a polarity of 8 from ZEBRON. The stationary phase of the column in non-polar consisted by 5 %-Phenyl 95 %-Dimethylpolysiloxane. Thus, the FAME are separated according to the boiling points and the unsaturated compounds are eluted before the corresponding saturated acids of the same chain length. This column allows the simultaneous analysis of triglycerides and FAME content. However, an overlapping of the methyl esters peaks was observed to methyl

stearate(C18:0), methyl oleate (C18:1), methyl linoleate (C18:2) and methyl linolenate (C18:3) since the difference is only a double bond between them and they have near boiling points.

The method used was the ASTM D6584. The carrier-gas was hellium with a starting flow of 0,1 ml.min^{-1} during 1 minute and rises with the rate of 1,5 ml.min^{-1} to 1.1 ml.min^{-1} standing at this flow until the end of the program. The oven temperature was 323-453 K at 288 K min^{-1} followed by a ramp of 280 K min^{-1} until 503 K and 303 K min^{-1} until 653 K with a final holding time of 10 min. Peak identification was carried out using known standards and the software *Excalibur*.

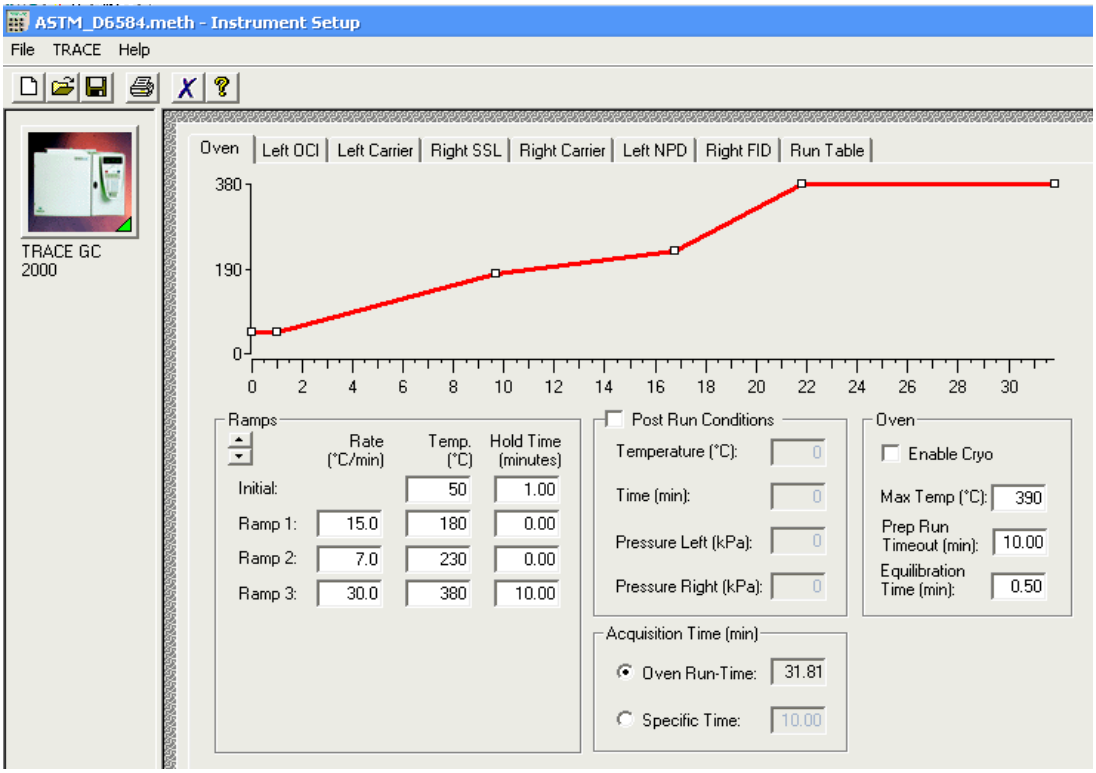

Figure 34: Method ASTM D6584 description.

The samples preparation method is described in APPENDIX C and the compounds properties in APPENDIX D.

❖ SAMPLE ANALYSIS

Through Excalibur Software, an analyze of the chromatogram is made. An example of a typical chromatogram is described in Figure 35.

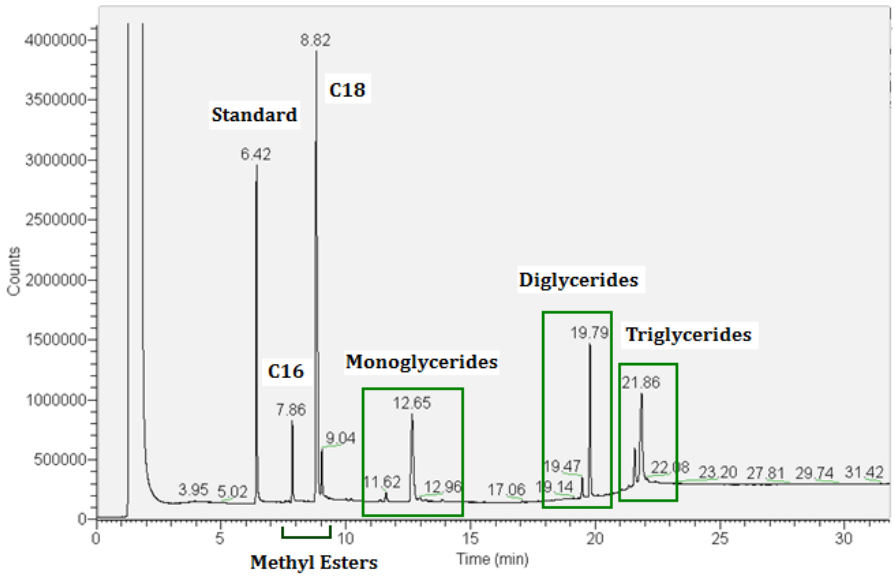

Figure 35: Typical chromatogram obtained by GC.

The first peak corresponds to the standard. Peaks with a retention time of 7 min and 8/9 min correspond to methyl palmitate (C16) and to methyl esters with 18 carbons atoms, respectively. The methyl esters stearate, oleic, linolenate and linolenic have retention times similar and a total separation is not possible with this program. Thus, a peak overlapping happens for these four methyl esters during the 8/9 min. The monoglycerides have a retention time between 11min and 15 min. After 16 min, the peaks for diglycerides appear until 19/20 min. The last peaks at 21/22 min correspond to the triglycerides that did not react.

Through the integration of all peaks and using calibration standards, it is possible to determine the concentration obtained for triglycerides and the methyl esters obtained. Calibration standards were determined for the five methyl esters (palmitate, stearate, oleic, linolenate and linonic) and for the triglycerides. For the monoglycerides and diglycerides it was not possible due to the fact that the standards were not available. The method for the determination of the calibration standards is described in Appendix E.

CHAPTER 3: RESULTS AND DISCUSSION

PART I: Initial study: 1/16 *inch* Scale

The first study occurred at a 1/16 *inch* scale. From reports in the literature, value for temperature, pressure and molar ratio were assumed for the transesterification reaction under supercritical carbon dioxide conditions using enzymatic catalysis in a continuous process. This initial study represents the proof of concept. Thus, the aim of this part was to verify if this system for biodiesel production was able to be considered as a feasible process.

The transesterification reaction was tested at p=25 MPa, T=323,15 K and molar ratio 1:29 with edible oil and methanol. These conditions were chosen according to the literature [52]. The reactor was charged with 3g of Lipozyme TL IM® corresponding to a residence time of 15 sec. At these conditions, a maximum of **86%** yield was achieved.

❖ STABILITY OF ENZIME ACTIVITY

Achieving this high yield, the process already proved its potential as an alternative process for biodiesel production. However, given that the process operates at a continuous state, it will be only an economically viable process if the enzyme maintains its activity during the operating time.

Thus, the transesterification reaction at p=25 MPa, T=323,15 K, residence time of 15 sec and molar ratio of 1:29 was tested for more than 10 hours of operation time.

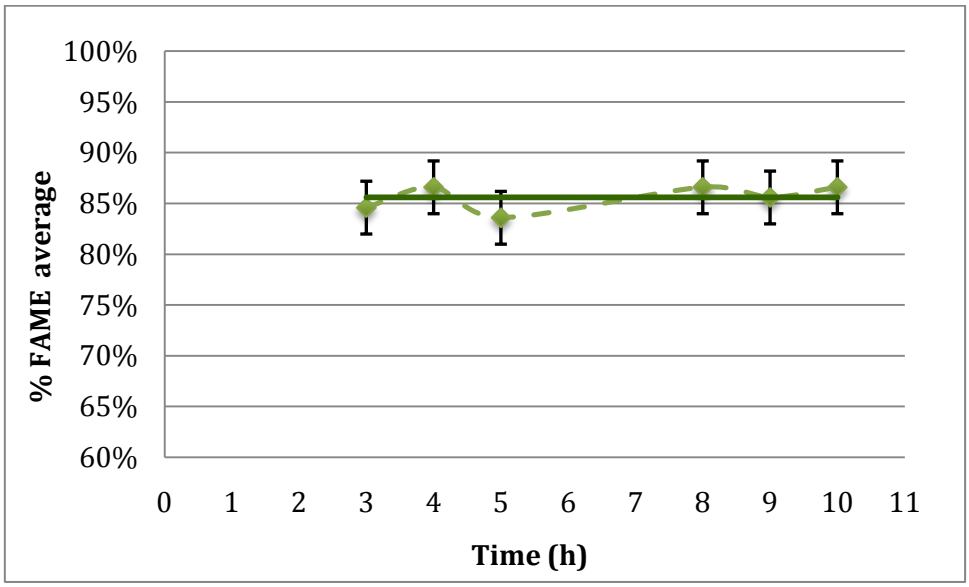

Figure 36: Study of enzyme stability.

The activity of the enzyme remained stable during the 10 hours of operation (Figure 36). Considering the experimental error, the enzyme presents an activity which results in 86% average reaction yield. Thus, not only the yield obtained was high enough to consider the process with high potential but also it was proved the stability of the enzyme activity during long periods of time. The variation between the experimental points in Figure 36 could be related to flow fluctuations in CO_2 HPLC pump and with the system stabilization.

Proved the potential capacity of the process, studies were planned in order to optimize the process. However, in several experimental works there was some discrepancy between the results and, on the other hand, operational problems had occurred. After some experiments, it was necessary to change the enzyme bed since the results suggested the idea of the enzyme to be inactive. During that operation, it was verified that the enzyme was totally compacted inside the reactor turning its removal from the reactor an extremely hard step. With this enzyme compaction degree it was possible to predict that diffusion problems could have occurred. Due to the compaction of the enzyme, some active centers could also be inaccessible. This could be an explanation for the unexpected decreasing of the

yield of some experiments. Moreover, this compaction could promote a "barrier" at the beginning of the reactor caused by an increase of pressure related to the compaction of the enzyme. Over passing that barrier, a significant pressure drop in the reactor was observed. Decreasing the pressure, oil solubility in Sc-CO_2 also decreases and the precipitation on the enzymes particles can occur resulting in a activity loss.

In order to overcome these problems, a larger scale was proposed. With higher inner diameter of the reactor, the compaction of the enzyme would not occur and that might solve the problems which caused the decreasing of the reaction yield. The optimization of the process parameters will be made at a larger scale.

PART II: Scale up to 1/8 *inch*

In PART I of the experimental work, it was proved the potential of the continuous process under supercritical conditions achieving satisfactory results. Thus, in order to study in more detail the reaction parameters and solve some operational problems described previously, it was constructed another experimental setup whose scale was two times larger than the experimental setup in PART I.

The transesterification reaction was studied for two possible alternatives depending on the type of substrate used. Methanol was applied as an alcohol for the reaction for two types of substrates: vegetable oil and waste cooking oil.

At this part of the experimental work, the goal was to study two transesterification reactions:

- Edible oil/methanol
- Waste cooking oil/methanol

In each one, the reaction parameters were optimized.

The water effect on the transesterification reaction is a parameter to take into account since it influences enzyme activity [65]. The addition of water can be done indirectly by flowing the CO_2 over water-saturated glass wool before the packed bed reactor saturating CO_2 before the contact with the catalyst. However, with the CO_2 saturated stream with water, enzymatic activity was reduced more 90% [52]. Water can also be added to the system by different water amounts directly added to the alcohol. Some authors have reported that an enzymatic activity loss becomes pronounced with a water content in the system above 2,5% resulting in a lower FAME yield [70]. Thus, an excess of water content in the system can also causes hinder enzyme activity. An optimum value must be added and it was assumed that enough water was presented in CO_2, methanol and oil to keep the enzyme hydrated.

2.1. Transesterification reaction of edible vegetable oil with methanol

2.1.1. Temperature optimization

The transesterification reaction was catalyzed by Lipozyme TL IM®. In Figure 37, it was noticed that the enzyme activity changes in a range of temperature between 303,15 – 323,15 K. All the experimental works were done at p=20 MPa, molar ratio oil: methanol of 1:29 and 20 sec. of residence time.

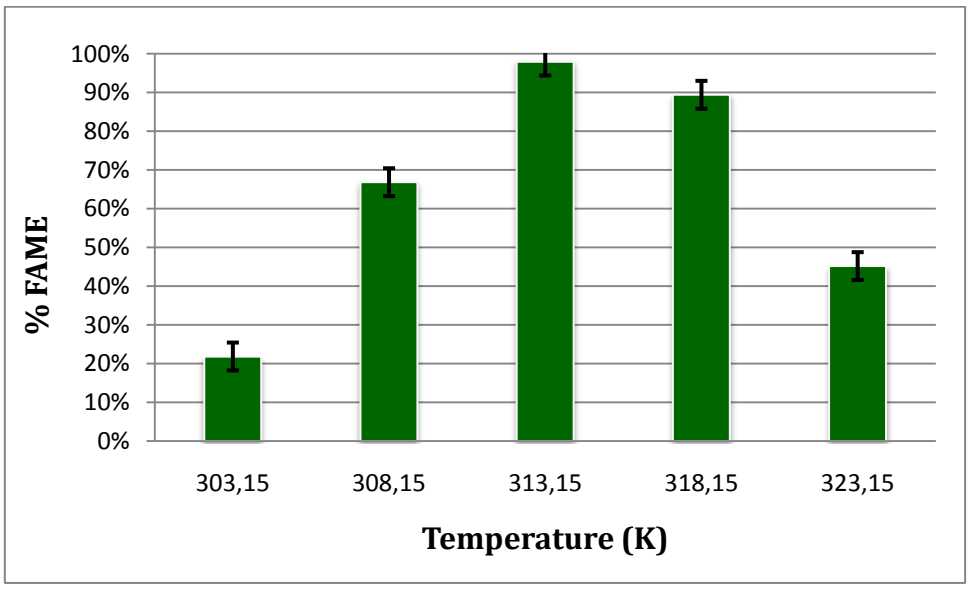

Figure 37: Temperature effect in the transesterification reaction yield.

The optimum temperature for Lipozyme TL IM® is 313,15 K achieving a **98,6%** yield (Figure 37).

In fact, several articles were published studying the enzyme activity in temperatures ranges of 303,15 – 333,15 K. For the enzymatic hydrolysis of sunflower oil in S_C-CO_2 at 20MPa, the concentration of free fatty acids increased with temperature (308,15 –318,15 K), whereas it decreased with further increase in temperature [71].

2.1.2. Residence time optimization

Each enzyme has a certain number of active centers. Once those centers are occupied, the enzyme is not able to convert more triglycerides. On the other hand, the exposure to methanol can modify the normal enzyme structure. In both cases, it can interfere in the enzyme activity resulting in a lower reaction yield [52].

The optimum residence time must be achieved not only to verify the activity of the enzyme to that time exposure but also for the design of the reactor. In a continuous process, solvent flow rate and the residence time are very important parameters to take into account on the design of the reactor since it established the optimal size (not too big representing excessive costs but also not too small in which residence time is insufficient for achieving the expected yield). Knowing the optimum residence time for the reaction, an optimization between packing size of the reactor and the solvent flow rate can be made optimizing the investment and production costs.

Experimental works at different residence times of 28, 20, 17,15, 14 seconds were made. All these experimental works were made at the following conditions: T= 313,15 K (optimum temperature already studied) and p=20 MPa. Maintaining the same enzyme quantity inside the reactor, several flow rates of CO_2 were applied representing different residence times.

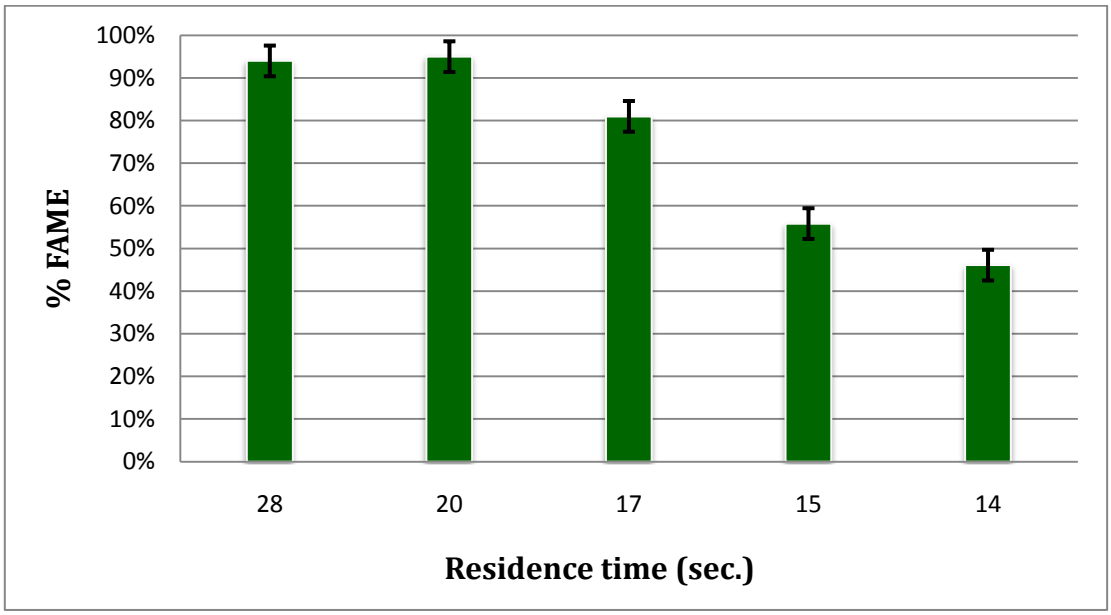

Figure 38: Optimization of the residence time.

Analyzing Figure 38, the optimum residence time was 20 seconds achieving a yield of **95,0%.**

If the mixture spent too much time contacting with the enzyme, the excessive time contact could result in a decrease on enzyme activity and, therefore, decreasing the reaction yield. This could happen at higher residence times than 20 seconds. The experimental work with a residence time of 28 seconds had already demonstrated a slightly yield decreasing since the reaction yield was 94%. Although the difference between reaction yields achieved for a residence time of 28 and 20 seconds was almost insignificant, taken into account both values, a smaller residence time is more convenient to industrial scale since it represent at the same flow rate, a smaller reactor reducing equipment costs. Thus, a residence time of 20 seconds is a better option against a residence time of 28 seconds.

2.1.3. Oil: methanol molar ratio optimization

According to the transesterification reaction stoichiometry, 1 oil mole reacts with 3 mole of methanol. However, in order to achieve a high yield the molar ratio oil: methanol must be higher than 1:3. By increasing the oil to methanol molar ratio the reaction equilibrium is shifted in the direction of product formation. Nevertheless, it is stated that methanol is very aggressive to enzyme activity [70]. At batch processes, the optimum molar ratio using methanol as an alcohol for transesterification reactor is 1:6. However, processes using higher molar ratios than 1:3 should be fed batch systems where the methanol addition can be made by stages avoiding the enzyme inhibition [72]. In a continuous process, the contact between the enzyme and the reactants is much lower than in a batch process. Thus, the molar ratio oil: methanol in a continuous process should be higher than the molar ratio established for batch processes.

The transesterification reaction was studied for the following molar ratios: 1:36; 1:29; 1:24; 1:18; 1:12. All the experiments were carried out at p=20 MPa, T= 313,15 K and a residence time of 28 seconds.

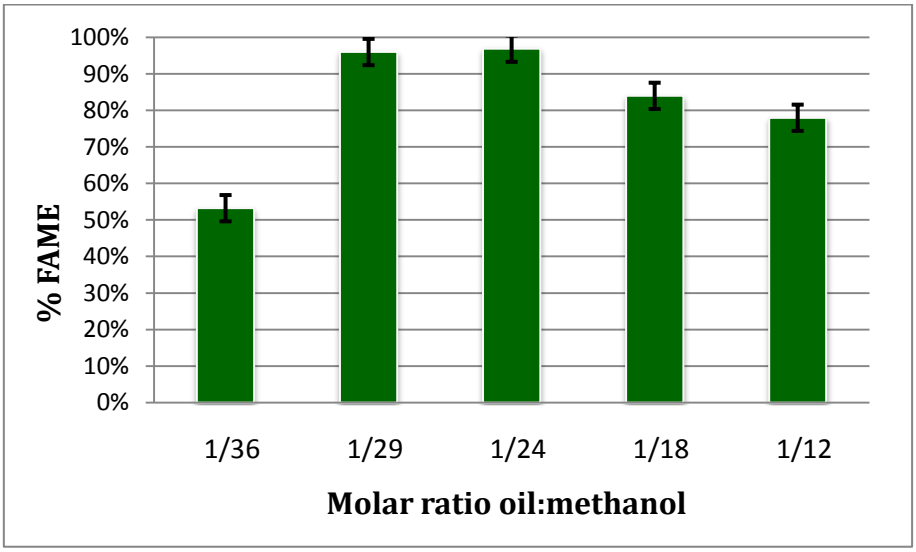

Figure 39: Experimental works results at several molar ratios oil: methanol.

As it can be seen in Figure 39, the optimum molar ratio oil: methanol is 1:24 achieving a reaction yield of **97%.**

Increasing the amount added of methanol can cause the denaturation of the enzyme resulting in a decrease of reaction yield.

According to Jackson et al. [52], the optimum molar ratio for a continuous process using methanol and enzymatic catalysis was 4:5 (volumetric ratio) which corresponds to 1:25 in molar terms. The reaction yield obtained for the molar ratio 1:24 was 96%, very similar to the yield value obtained for the molar ratio 1:25. This results its according to the study made by Jackson et al [52].

On the other hand, molar ratios lower than 1:24, the quantity of methanol added does not promote the direct reaction in order to achieve a good yield.

The three main transesterification reaction parameters using edible vegetable oil and methanol in a continuous process were optimized for 1/8 *inch* scale. The best conditions were the p=20 MPa, reaction temperature of 313,15 K, a residence time of 20 seconds and a molar ratio oil: methanol of 1:24.

2.2. Fractionation process

After transesterification reaction, the mixture obtained must be treated in order to recover the biodiesel produced. The recovery is made integrating a fractionation stage after the reaction. The separation results on the manipulation of Sc-CO_2 characteristics which allows the fractionation depending on compounds solubilities at certain conditions of temperature and pressure. The aim of this experimental part is to define the optimum fractionation conditions which allow the best product fractionation.

The mixture separation was studied for pressure range 15-12 MPa and temperature range 313,15 – 333,15 K. The reaction conditions were previously selected in order to obtain significant amount of FAME, triglycerides, mono and diglycerides to study the separation efficiency. The reaction conditions applied results in ~60% yield.

Separation was not possible at p=15 MPa and T=323,15 K. and P=14 MPa and T=323,15 K. At these conditions, all the compounds have high solubility in Sc-CO_2

and they are all recovered in the gas phase. In order to decrease the compounds solubility, separation pressure was lowered taking into account also the temperature effect.

At p=13,5MPa and T= 328,15 K, oil solubility in Sc-CO_2 is still too high recovering the gas phase with a high oil content and, consequently, biodiesel with a low purity.

Several experiments were done with constant temperatures of 323,15 and 333,15 K and pressures between 12 and 14 MPa (Figure 40).

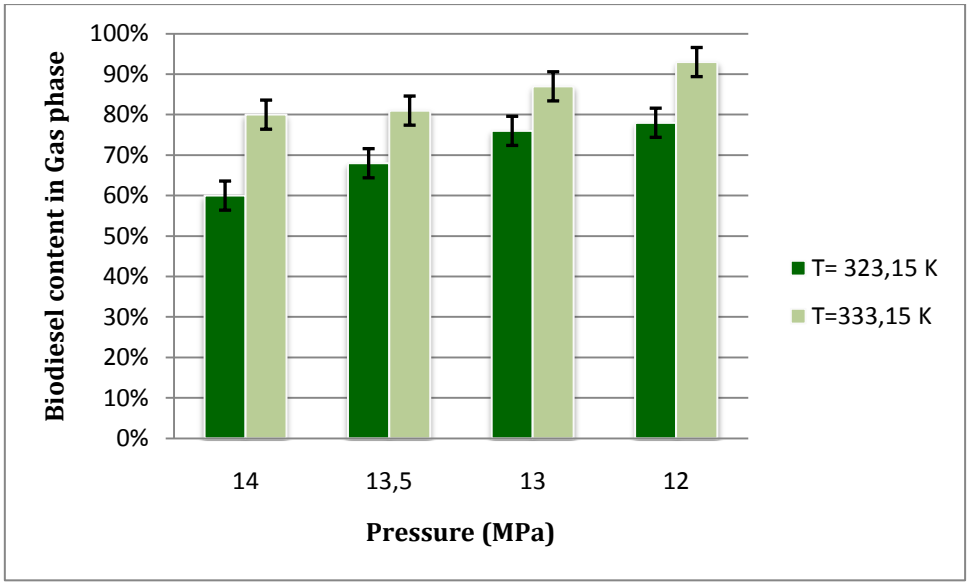

Figure 40: Separation experiments with constant temperatures of 323,15 e 333,15 K.

Through Figure 40, it is possible to analyze the temperature effect in the separation process. With a constant pressure, Sc-CO_2 density will decrease with the increase of temperature, reducing its solvent power characteristics, resulting in a decrease of oil solubility into Sc-CO_2 and consequently higher biodiesel purity in the gas phase. With a difference of only 10 K, the increase of biodiesel purity in the gas phase is significant. Considering the temperature of 323,15 K, increasing pressure results in a decrease of gas phase purity of 8% except for the increase between 13 MPa and 12 MPa which enrichment was only of 2% (76% - 78%). At lower pressures, with constant temperature, the increase effect is not so significant

since the oil solubility is already low at both pressures. At 323,15 K, the best experiment was with p=12 MPa resulting in a gas phase with a purity of 78%.

At the higher temperature of 333,15 k, the gas phase enrichment was lower with the decreasing of pressure. This could be caused by the fact that temperature effect is much higher in the separation process than the pressure effect. Normally, at higher pressure levels an increase in temperature can also result in higher solubility of substances in supercritical fluids because of increase of vapor pressure of the compounds to be solved overcomes the decrease in density [63]. Thus, at temperature of 333,15 K, decreasing the pressure results in a gas phase enrichment of 6% except for the higher pressures (14 MPa – 13,5 MPa) which difference was insignificant (only 1%).

The conditions which allow the higher Biodiesel purity were p=12 MPa and T=333,15 K. At these conditions, Sc-CO_2 was very selective achieving in the gas phase a biodiesel content of 93%.

Another aspect to take into account is the mass quantity recovered in the gas phase. High purity of biodiesel is achieved with high selectivity of Sc-CO_2 towards biodiesel but, on the other hand, with the increase of selectivity, Sc-CO_2 solvent power characteristics decrease resulting in a decrease of mass quantity recovered.

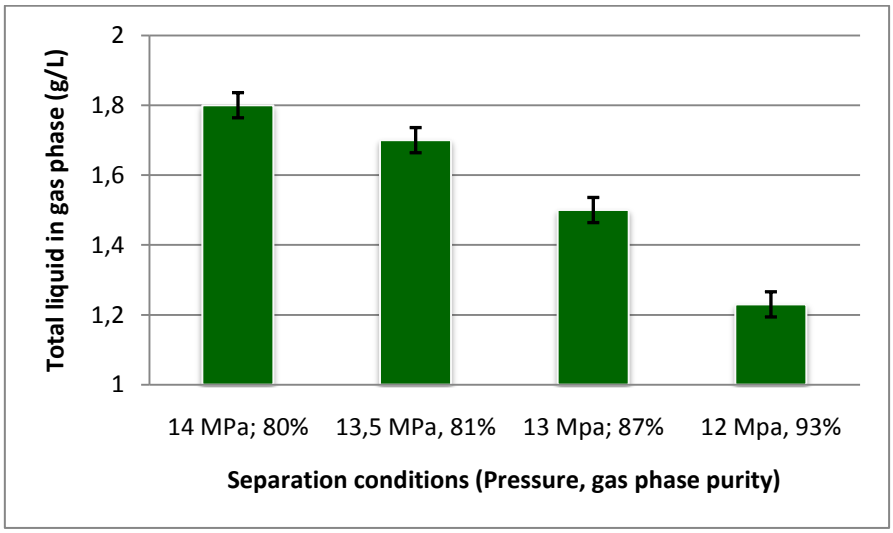

Figure 41: Total loading of gas phase recovered with the increase of pressure and T=333,15 K.

Figure 41 represents the total liquid recovered in gas phase related to the pressure applied and biodiesel purity achieved. It is important to notice that higher the biodiesel purity, lower is the total biodiesel mass recovered. Without any separation process implemented, the total loading of gas phase after the reactor is 2,6 g/L. At p=12 MPa and 333,15K, a biodiesel purity of 93% is achieved but it is only recovered a total liquid of 1,2 g/L in the gas phase. On the other hand, at 14MPa and 333,15 K, biodiesel with 80% of purity in total liquid in the gas phase of 1,8 g/L is recovered. These results were obtained with a reaction yield of 60%. This means that a 33% of biodiesel enrichment in the gas phase is possible with a fractionation process at p=12 MPa and 333,15 K. However, if the reaction yield was higher than 60%, that conditions were unnecessary. For instance, with a reaction yield of 80% and fractionation process at p=12 MPa and 333,15 K, it was possible to recover in the gas phase almost pure biodiesel but the mass recovered was too low. A fractionation process at 13,5 MPa and 333,15 K was viable since a enrichment of 20% is possible at these conditions and the mass recovered was higher.

The study of several different pressure and temperature conditions applied to the fractionation process were useful to prove the potential of Sc-CO_2 when applied to separation processes. Knowing the possible enrichment in biodiesel of gas phase at several conditions, the reaction yield obtain determines the favorable conditions that must be applied in order to obtain the biodiesel purity desirable and at the same time recovered a final significant amount.

The CO_2 recirculation was not possible at this phase. However, with further development of this process the CO_2 recirculation system must be taken into account and implemented optimizing the process.

2.3. Transesterification reaction using waste cooking oil and methanol

The substrate used for the transesterification reaction in order to obtain biodiesel must be edible oils. These oils compete with food sector resulting in an exponential increase of food prices. Waste cooking oil is considered as a viable alternative since it represents nowadays a serious environmental problem. Using this oil as raw material will not only solve an environmental problem but also reduces the production costs once waste cooking oil has no applications nowadays and is considered as a residue without commercial value.

The waste cooking oil used was collected from a commercial establishment in Faculdade de Ciências e Tecnologia campus. After frying, waste oil has several food and cooking residues. These residues must be removed before the oil could be applied as raw material for transesterification reaction since they can damage the experimental apparatus and interfere negatively in the reaction. Thus, the waste cooking oil collected was passed through a purification step before its application. That step is a simple gravity filtration using a filter where oil passes through and the residues stay retain in the filter. This step was repeated two times and after that the oil was ready to be used. At industrial scale, the oil collected must be also treated to remove the residues before its use and this stage needs to be taken into account.

Waste cooking oil has higher contain in FFA than edible vegetable oils [73]. However, Lipozyme TL IM® should be able to convert FFA and any problem should not appear related to that. Thus, the best conditions determined for the reaction between edible vegetable oil and methanol were applied using waste cooking oil in order to verify the reaction yield.

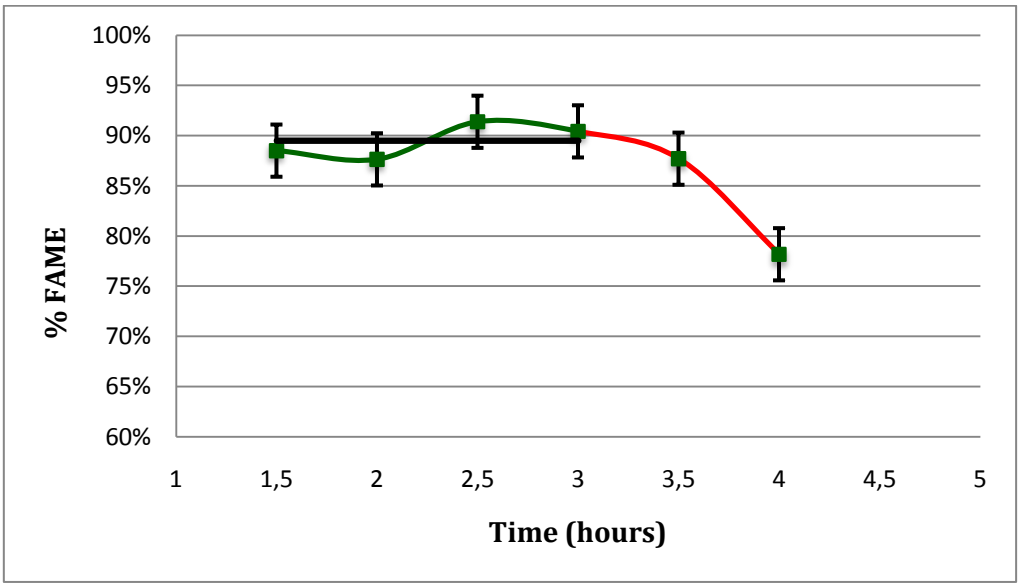

Figure 42: Reaction yield results for the transesterification reaction using the best conditions determined previously and waste cooking oil.

As can be seen in Figure 42, the average yield obtained for a reaction period of 3 hours with samples taken every 30 minutes was 89%. Comparing to experimental work made at the same conditions but using edible vegetable oil, a decrease of almost 10% was observed. Analyzing Figure 42, after 3 hours of operation a significant enzyme desactivation (represented by the red line) .can be observed. In fact, between the third and fourth hour there was a decrease in the reaction yield of more than 10%.

Other experimental work was made to verify if the enzyme looses activity during the reaction period using waste cooking oil. A reaction study was made using again the best conditions for edible vegetable oil already determined (p=20 MPa, T=313,15 K, residence time of 20 seconds and a molar ratio waste oil : methanol of 1:24) but with long reaction period (7,5 hours).

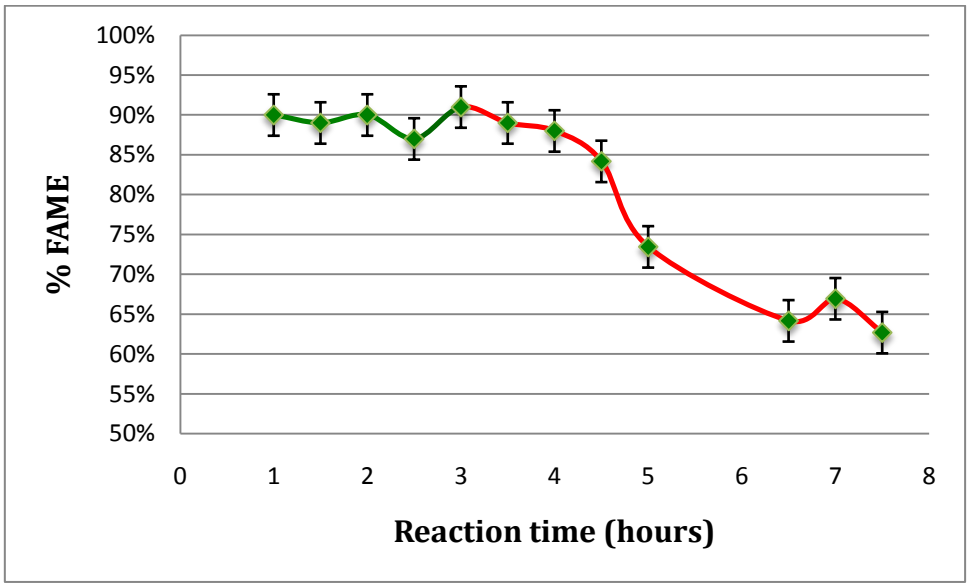

Figure 43: Reaction yield results for a long reaction period using waste cooking oil.

Analyzing Figure 43, a clear enzyme desactivation can be observed after 2,5 hours of reaction time. It was confirmed that the waste cooking oil was causing enzyme desactivation. Considering the first experiments, after 8h of reaction, enzyme looses activity resulting in a reduction of more that 30% comparing to reaction yield obtain for the transesterification reaction using edible vegetable oil.

As described in CHAPTER 1, Part II, various physical and chemical changes occur during oil frying. Resulting of this chemical changes, many undesirable compounds are formed during frying whose toxicological effects are not known fully [38]. Comparing to the results obtained with transesterification reaction using edible vegetable oil, since the only change implemented was the raw material, this desactivation might be due to the presence of some compounds in waste cooking oil which causes enzyme activity loss.

The enzyme activity loss could be also caused by two main situations; excessive methanol exposure and glicerol adsorption on the enzyme support.

Although it was a continuous process and the time of enzyme exposure to methanol is smaller compared to batch processes, over the time, enzyme could be deactivating due to the exposure to methanol. Several studies were published in

which enzyme washes were made using organic solvents such as isopropanol at the end of batch processes [73]. In order to adapt this type of system in a continuous process, two reactors would have to be operating in parallel. One was operating while the other one was washed with an organic solvent such as ethanol, isopropanol or other, recovering all the compounds that were adsorbed in enzyme packed bed. After washing, the production was sent to this reactor and the other one starts the washing stage. However, the application of an organic solvent to this called "integrated green process" is not an environmental friendly solution to this problem and other solutions must be studied and developed.

Studies about glycerol influence on the biodiesel production in a packed bed reactor were published [66]. At this study, it was demonstrated that all the glycerol produced remains adsorbed onto the enzymatic support leading to a drastic decrease in enzyme activity. It was assumed that glycerol forms a layer around the enzyme which difficult the hydrophobic substrates diffusion (mono- di, triglycerides) since glycerol is a hydrophilic compound resulting in a yield decrease over the time.

A decrease of enzyme activity was only verified using edible vegetable oil between experiments and not during the reaction time and this could be related to the system depressurization during the night which causes the oil precipitation into the enzyme structure.

The three situations: methanol exposure, glycerol adsorption and depressurization of the system between experiments occurs in both systems using waste cooking oil or edible vegetable oil but a significant decrease of enzyme activity during the reaction time was only verified for the experiments using waste cooking oil. Thus, it is possible to conclude that the loss enzyme activity is related to the waste cooking oil which composition has some compounds which might be decreasing enzyme activity during the reaction time. Further waste cooking oils analysis must be made to verify the waste oil composition and identify the compounds which might be decreasing enzyme activity.

Although a significant loss of enzyme activity was verified, a higher reaction yield could be achieved by optimizing the reaction parameters again. In order to confirm if the parameters from which was possible to achieved the higher yield with edible vegetable oil are the same for the waste cooking oil used for the transesterification reaction, higher residence time and waste oil: methanol molar ratio were tested.

Waste cooking oil transesterification reaction was carried out at 313,15 K, p=20 MPa and a molar ratio of 1:24 but the residence time was increased to 28 seconds.

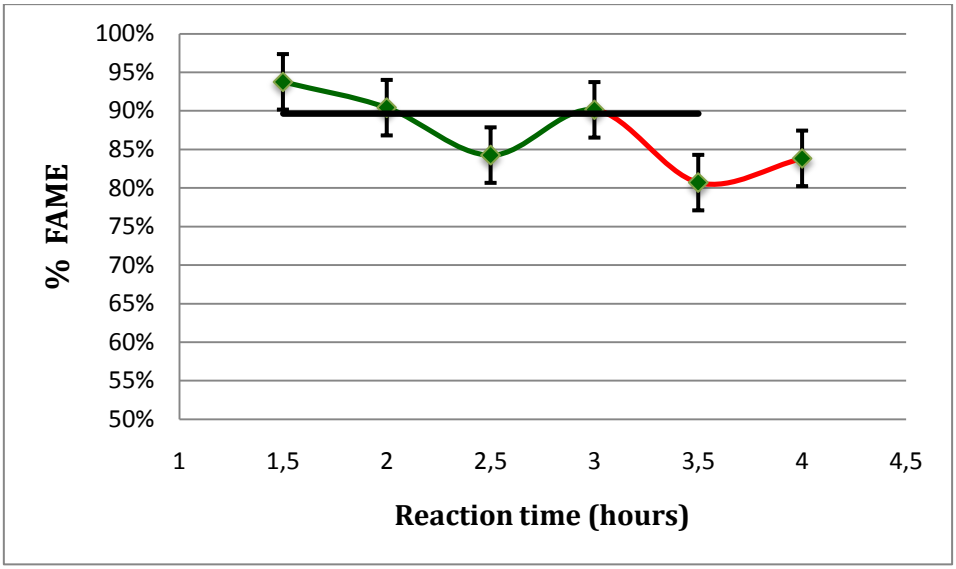

Figure 44: Transesterification reaction yield using a residence time higher of 28 seconds.

Analyzing Figure 44, using a residence time of 28 seconds the reaction yield had not increased significantly, the increase was only 1%. Once again, enzyme deactivation signals had appeared after 3 hours of reaction.

The molar ratio waste cooking oil: methanol was increased to 1:29 and 1:36 maintaining pressure, temperature and residence time values from the first three experiments (p=20 MPa, T=313,15 K, residence time of 20 seconds).

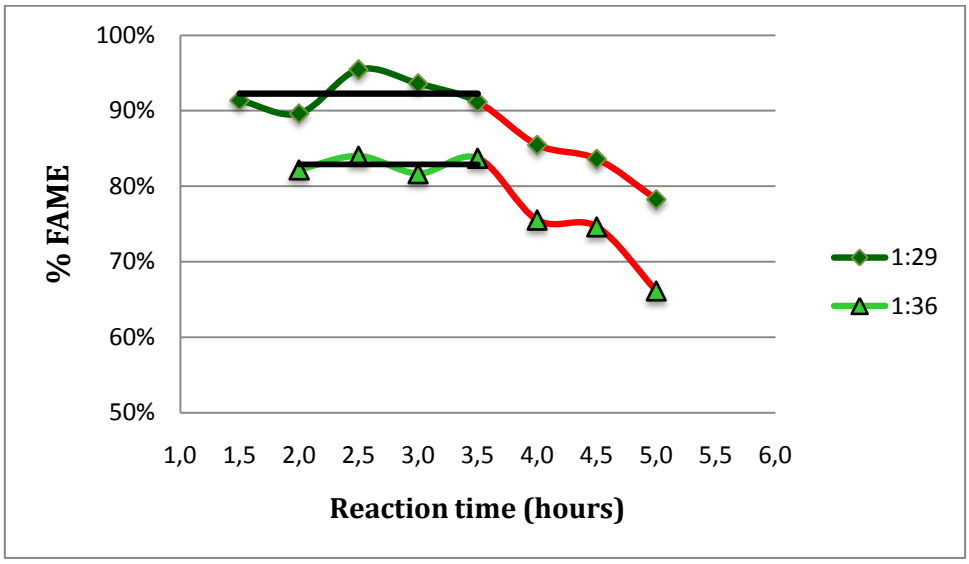

Figure 45: Transesterification reaction yield using a molar ratio of 1:29 and 1:36.

The average yield for the application of molar ratio 1:29 was 92% and for the molar ratio of 1:36 was 83% (Figure 45). Comparing to the first experiment with waste cooking oil using the best conditions determined for the edible vegetable oil (yield of 89%), a molar ratio of 1:29 allowed a reaction yield increase of only 3 % and the application of the molar ratio of 1:36 caused a decrease of 6%. The decrease with molar ratio of 1:36 could also be related to the excessive methanol addition that contributed also for the enzyme desactivation.

Comparing to the conditions applied in the first experiment using waste cooking oil, a increase of 3% was verified. The best result was achieved for the implementation of higher oil: methanol molar ratio of 1:29. The difference between the reaction yield using edible vegetable oil and waste cooking oil is not significant enough to justify the increasing of the methanol amount used when the implementation of a molar ratio of 1:29. Thus, it was concluded that the conditions p=20 MPa, T=313,15 K, residence time of 20 seconds and a molar ratio waste oil : methanol of 1:24 are also the optimized conditions that must be applied to the transesterification reaction using waste cooking oils.

Several studies of edible vegetable oil transesterification reaction were published using Novozyme 435® as biocatalyst achieving satisfactory yields [54]. It could be possible that Novozyme 435® had higher activity using waste cooking oil than Lipozyme TL IM®. Thus, experiments were made applying the same conditions as the first experiment but using now Novozyme 435® instead Lipozyme TL IM®. For the conditions p=20 MPa, T=313,15 K, residence time of 20 seconds and a molar ratio waste cooking oil: methanol of 1:24 the results are shown in Figure 46.

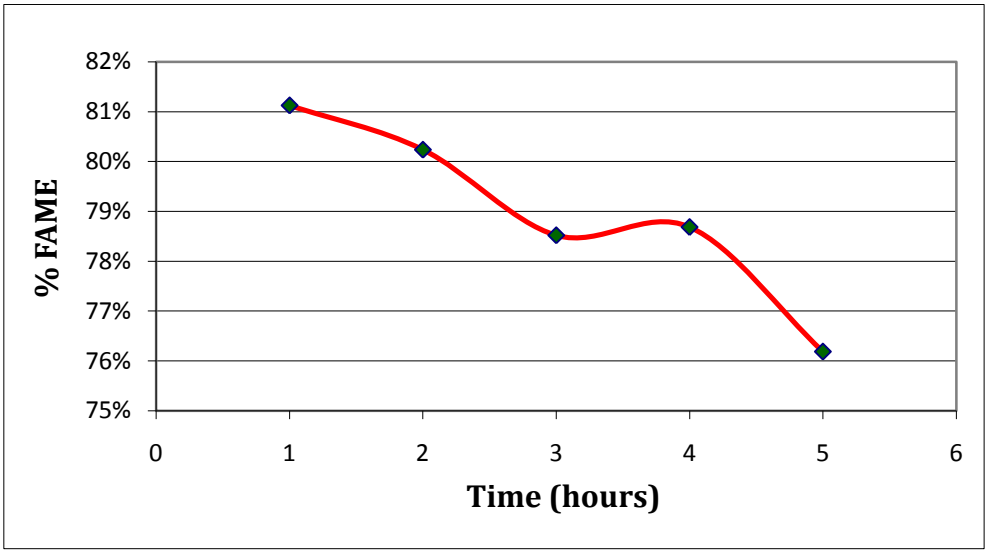

Figure 46: Transesterification reaction using waste cooking oil and Novozyme 435® as biocatalyst.

Analyzing Figure 46 it is easily confirmed that not only the reaction yield decreased sharply to 81% representing a decrease of almost 10% comparing to the transesterification reaction using Lipozyme TL IM® but also declined its activity after the first hour of reaction. Concluding, the implementation of Novozyme 435® as the only biocatalyst applied results in a lower reaction yield and lower enzyme stability compared to the application of Lipozyme TL IM®.

Samples collected in the last experiments were analyzed and they indicated the high contents of monodiglycerides and diglycerides. The Lipozyme TL IM®, theoretically, acts on the first and the third position of triglyceride molecule. Knowing that Novozyme 435® acts on the second position, joining both in the fixed bed reactor would favor the compounds conversion. The implementation of both biocatalysts was tried.

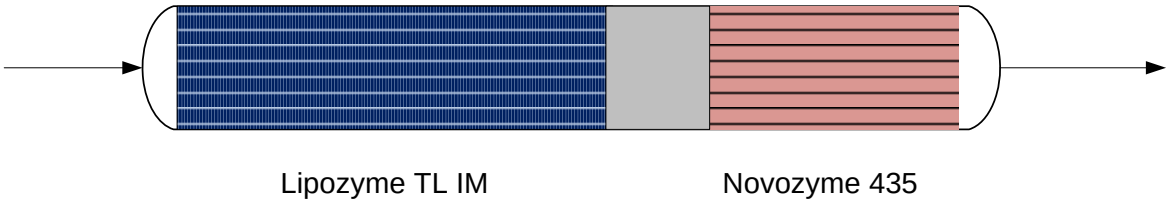

Figure 47: Schematic representation of the fixed bed reactor using two enzymatic preparations: Lipozyme TL IM® e Novozyme 435®.

Using the double mass quantity of Lipozyme TL IM® compared to Novozyme 435®, a residence time of 30 seconds was implemented. All the other parameters were maintained (p=20 MPa, T=313,15 k and molar ration waste cooking oil : methanol 1:24).

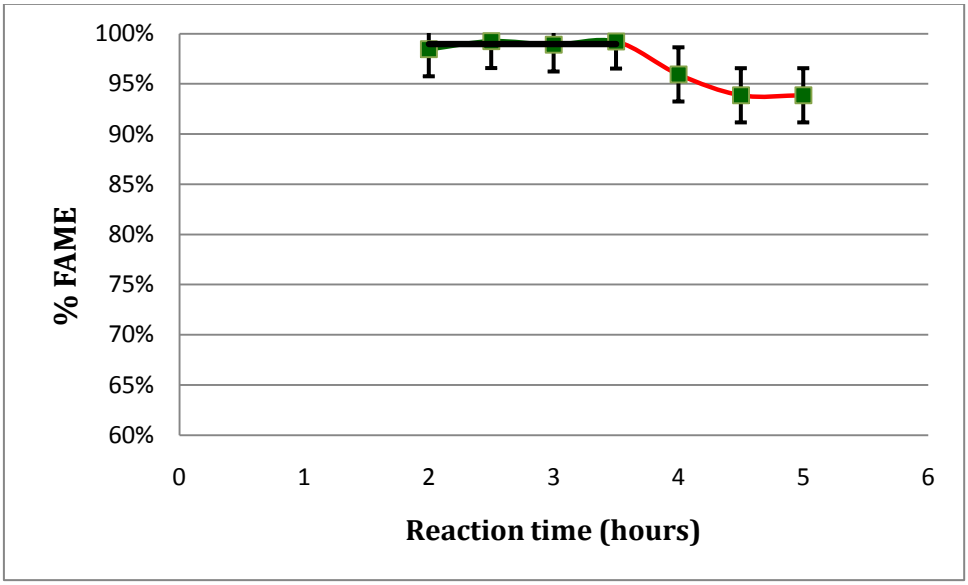

Figure 48: Transesterification reaction results using Lipozyme TL IM® and Novozyme 435®.

The average reaction yield using both biocatalysts was 98,9% (Figure 48). However, after 3,5 hours of reaction enzyme desactivation had occurred although at a lower rate compared to the other experiments. This was the best result achieved of all the experiments made with waste cooking oil. The implementation of both biocatalysts result in an increase of 10% compared to the experiments made only with Lipozyme TL IM® achieving results similar to the ones obtained for edible vegetable oil.

According to the European standard EN 14214, biodiesel in order to be commercialized needs to have a purity of 98,6%. Since the reaction yield is already high (98,9%), a fractionation process to enrich the mixture is not needed. However, glicerol and methanol must be recovered. Similar to the purification step applied to the process using edible vegetable oil, a separation step is also needed but at lower conditions since the final aim is only the glycerol and methanol recuperation. A process design was further though as a possible feasible system. Maintaining the reaction temperature of 313,15K, a separator could be applied after the reactor operating at 10 MPa achieving a gas phase enriched in methanol, glycerol and CO_2 and a liquid phase with biodiesel and a small amount of CO_2. This liquid phase could pass through another separator operating at room pressure and temperature in which biodiesel is recovered and the CO_2 is recirculated. The gas phase passes also through a third separator in which occur the fractionation between CO_2 (gas phase) and the methanol and glycerol recovered in the liquid phase. This mixture passes through a dryer in which methanol is evaporated and later condensate and glycerol is recovered almost pure. All the methanol and CO_2 recovered in several stages of the process are recirculated to the system. This process will be further studied in more detail and developed in the CHAPTER 5.

Biodiesel production using waste cooking oils and biocatalyst under supercritical CO_2 achieving high reaction yield is already a potential process. The main problem is related to enzyme stability and must be solved in order to turn this process into a viable and environmental friendly alternative which could be implemented at industrial scale. The application of edible vegetable oils should not be viewed as an alternative since the application of a residue with low commercial value and environmental problems can also lead to production of biodiesel with less harsh reaction conditions and also with high reaction yield. However, problems related to enzyme desactivation had occurred and that must be reviewed and further intensely studied in order to maintain enzyme stability during the process. This process is only viable if the enzyme would be able to maintain its activity during large periods of time and studies that confirm the reason of this instability and indicates it solution must be made.

PART III: PILOT SCALE

The glycerol recovery and identification at the 1/18 *inch* scale had become very difficult due to the formation of glycerol in low amounts which can explain why glycerol was not detected in the samples. Two options seem to be possible: adsorption by enzymatic support or the small amount of glycerol formed is dragged by methanol which is dissolved in Sc-CO_2 not precipitating in the first separator.

Operating at a higher scale, the glycerol formation could be high enough in order to be identified in the GC analysis. Thus, a pilot scale already implemented was adapted to this process. The experiment was made using a CO_2 flow rate of 47 g/min, temperature of 313,15 K, molar ratio edible vegetable oil: methanol of 1:24, p=20 MPa and a residence time of 30 seconds testing only the transesterification reaction. A glass vessel was applied after the reactor and with a back pressure regulator the mixture was recovered to the vessel. This vessel was submerged in a ice bath. The experiment ran for 4 hours. After that, it was observed in the glass vessel the formation of two distinct phases. These two phases were separated and later analyzed in the GC. Analyzing the bottom phase, a small glycerol peak was identified with GC analysis confirming that glycerol is formed but in small quantities which turn its identification a difficult step. The implementation of higher scale could solve this problem. Further studies at larger scales should be done developing separation conditions related to phase equilibrium between CO_2, methanol and glycerol in order to recover all the glycerol formed during the reaction.

Mass balance described later in CHAPTER 5 was made using values obtained in this experiment since this kind of study is made using large scales approaching the results as much as possible to the industrial scale. Processes balances will be described next.

CHAPTER 4: PROCESS BALANCES

The mass and energy balances are the basis for the design of any process. The mass balance and energy execution from a process allows the quantification of reactants and products as well as the determination of the energy associated and the design of the equipment.

The mass and energy balances were made for the process described in Figure 49.

Methanol, CO_2 and the oil are pumped by B1, B2, B3 respectively, passing through a heat exchanger HE1 in which the mixture is heated up to 313,15K and compressed to 20 MPa. Then, this mixture passes through a static mixer before entering in the packed bed reactor in which transesterification reaction occurs. After the reactor, the mixture containing oil, methanol, Sc-CO_2, FAME and glycerol passes through a heat exchanger HE2 and is heated up to 333,15 K and is decompressed to 10 MPa in the separator S1. In the liquid phase is recovered the biodiesel. Biodiesel passes through a heat exchanger HE4 to cool down to 298,15K and is decompressed to 1 MPa before entering the separator S2. The CO_2 from the gas phase is then compressed in order to be recycled and the biodiesel stored. The gas phase from the separator S1 passes through a heat exchanger HE3 cooling down to 273,15 K and decompressed to 2MPa before the separator S3 in which separation between CO_2, methanol and glycerol occurs. The CO_2 is again recovered in the gas phase and passes through a heat exchanger HE5 to be heated up to 298,15 K and is compressed to be recycled. The liquid phase containing mostly methanol and glycerol is circulated to a dryer in which methanol is evaporated. The mixture is heated up before entering in the dryer by a heat exchanger HE6 and after is cooled down in another heat exchanger HE7 to 298,15 K. Glycerol is then pumped to the glycerol recovery tank. The unreacted methanol and CO_2 lost pumped at the beginning of the process are only the amounts necessary to replace the losses assumed in the process since all the CO_2 and methanol are recirculated through their storage tanks.

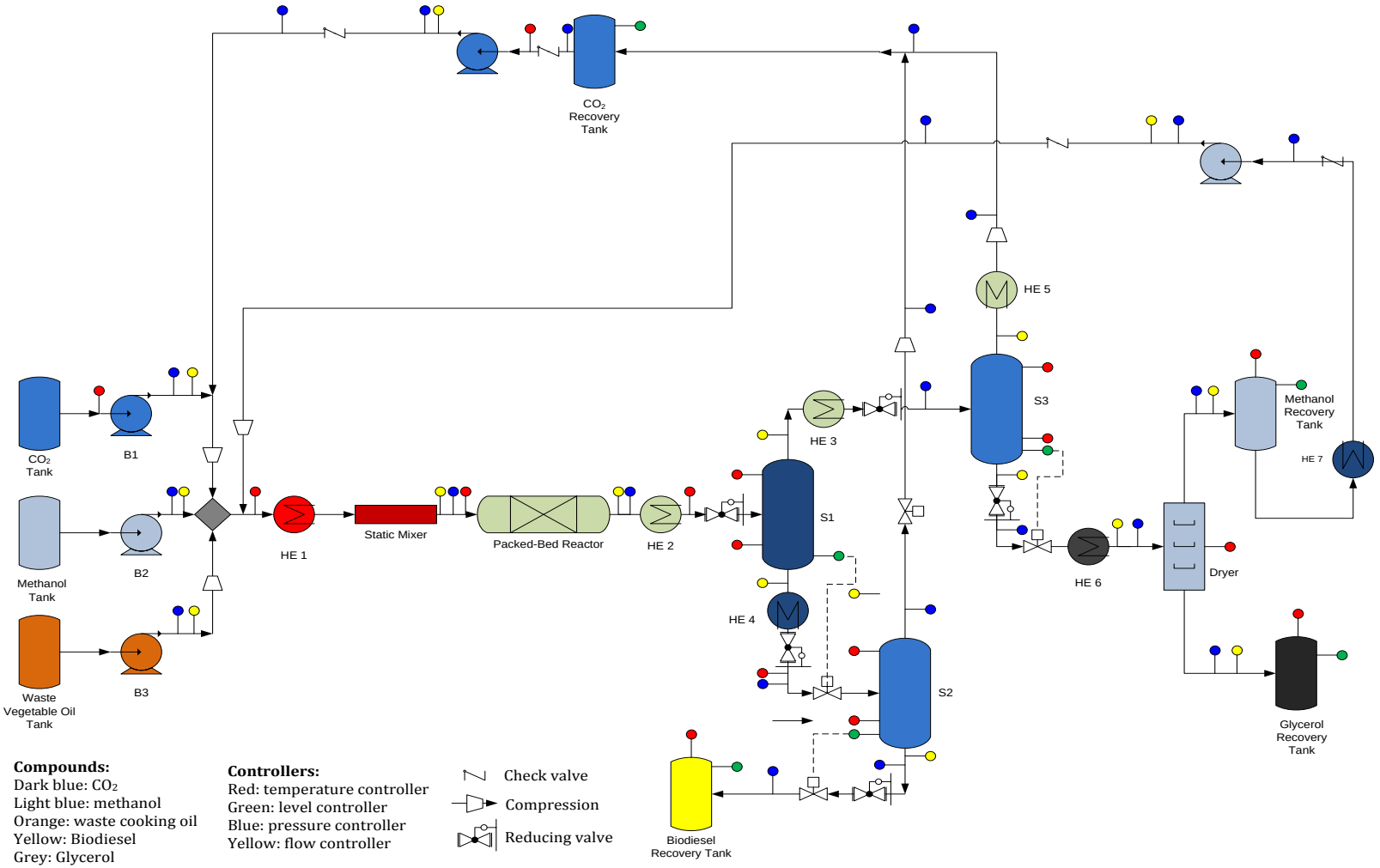

Figure 49: Process description.

PART I: Process diagram

In the process diagram (Figure 50) are represented all the unit operations that constitute the process. These are represented like blocks which are connected by lines representing streams input and output of the unit operation. These streams will then be characterized and analyzed in greater detail in the mass balance.

Table 7: Streams description from process diagram.

Stream	Composition	Stream State
1	methanol, CO_2, oil	Supercritical
2	methanol, CO_2, oil	Supercritical
3	methanol, CO_2, oil, FAME, glycerol	Supercritical
4	CO_2, methanol, glycerol	Gas/Liquid
5	Waste cooking oil, Biodiesel, CO_2	Gas/Liquid
6	Biodiesel, Waste cooking oil	Gas/Liquid
7	CO_2	Gaseous
8	CO_2	Gaseous
9	CO_2, methanol, glycerol	Gas/Liquid
10	Methanol, CO_2	Gaseous
11	Glycerol	Liquid

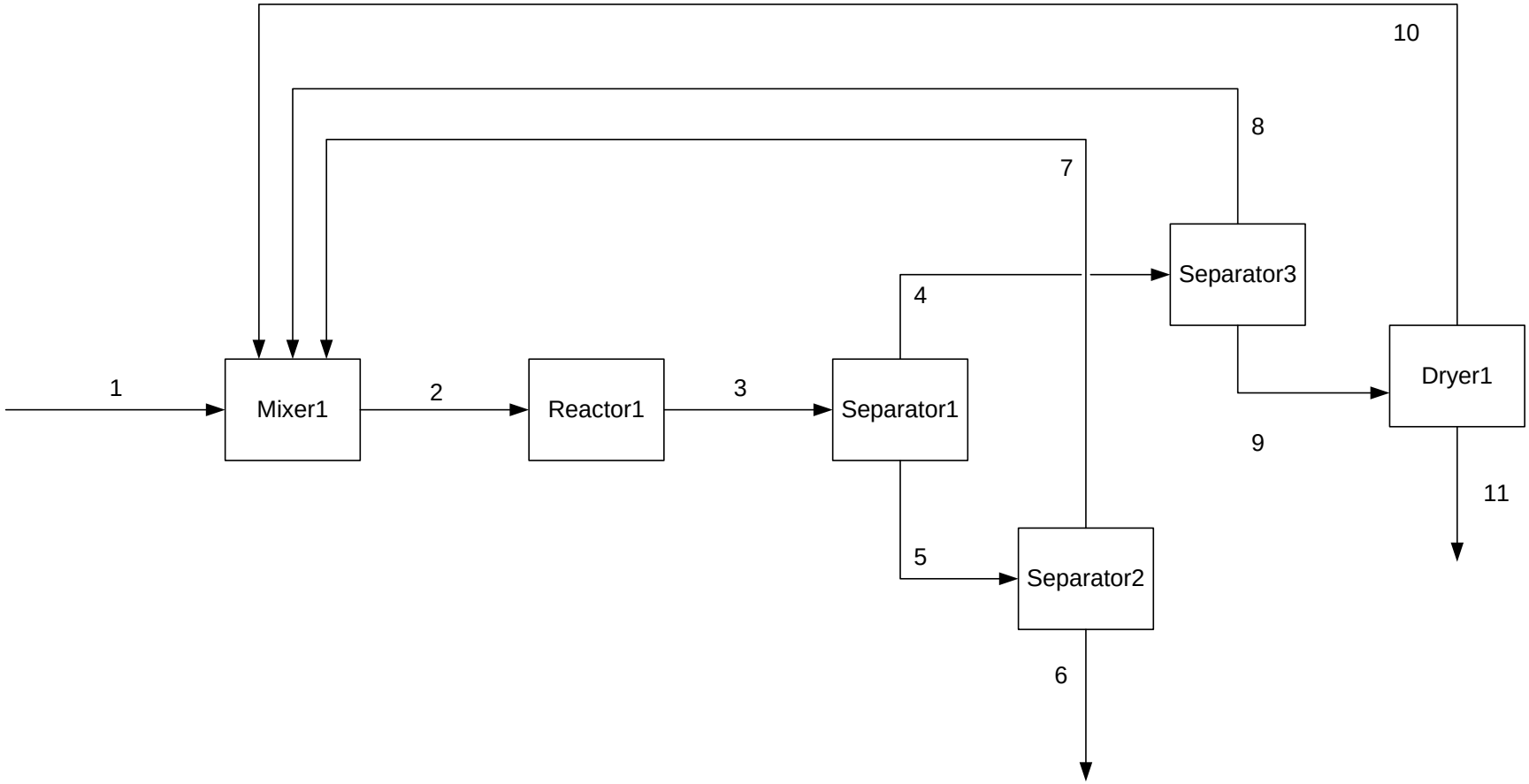

Figure 50: Process diagram.

PART II: Mass Balance

A mass balance consists in the determination of the quantities of each compound of inputs (reagents), outputs (products), accumulates (cumulative) and transformed compounds of each unit operation represented in the process diagram. The mass balance is determined by taking into account the general equation of conservation of mass given by:

$$\{\boldsymbol{Inputs}\} = \{\boldsymbol{Outputs}\} + \{\boldsymbol{Accumulates}\} + \{\boldsymbol{Consumed}\} - \{\boldsymbol{Generated}\}$$

The mass balance had been determined for a single annual operation and based on the following assumptions:

- An annual biodiesel production of 12.800 tons.
- Factory operates 330 days per year during 24 hours and taking one month to factory cleaning and maintenance.
- Stream 1 represents the reposition current whereby is added methanol, CO_2 and oil in the quantity necessary to reinsert the equivalent losses.
- The waste cooking oil used in the process is equivalent to 10% of total annual Portugal production.
- The transesterification reaction conditions are: p=20 MPa, T=313,15 K, molar ratio waste cooking oil : methanol of 1:24, residence time of 30 seconds and using as biocatalysts Lipozyme TL IM® and Novozyme 435®.
- The reaction yield is 98,9%.
- The separation in the separator 1 is at p=10 MPa and 333,15 K in which is recovered the biodiesel and the remain waste cooking oil in the liquid phase.
- The separation in the separator 2 occurs at room temperature (298,15K) and 1 MPa in which the separation between the biodiesel, the remaining waste cooking oil and the residual CO_2 is made.

- The separation in the separator 3 is at p=2MPa and 273,15K in which the majority of CO_2 is separated obtaining a mixture of methanol, glycerol and a small amount of CO_2 at the bottom.
- The mixture of methanol, glycerol and a small amount of CO_2 is dried in the dryer 1 operating at 338 K and p=0,1 MPa. Methanol is evaporated and further condensated. Glycerol is recovered almost pure.
- All the CO_2 and methanol recovered are recirculated to the system.

Table 8: Mass balance in MT/year.

MT/year	Stream 1	Stream 2	Stream 3	Stream 4	Stream 5	Stream 6	Stream 7	Stream 8	Stream 9	Stream 10	Stream 11
Waste Vegetable Oil	12,9	13,2	0,3	-	0,3	0,3	-	-	-	-	-
Methanol	1,4	11,4	10,0	10,0	-	-	-	-	10,0	10,0	-
Carbon Dioxide	9,6	2.925,0	2.925,0	2.904,4	20,6	8,7	11,9	2.875,3	29,0	28,2	0,9
Biodiesel	-	-	12,8	-	12,8	12,8	-	-	-	-	-
Glycerol	-	-	1,3	1,3	-	-	-	-	1,3	-	1,3

PART III: Energy Balance

In all industrial processes it is fundamental to take into account the energy and heat involved, i.e., the amount of energy needed to provide or to remove from the industrial process. Through the energy balances made to the system it is possible to quantify these parameters in order to verify and ensure the efficiency and economic viability of the process.

At this process, there are several operation stages that require a more precise quantification of the heat involved. These stages are related to all the heat exchangers implemented to heat or to cool down certain streams, the heat provide to the reactor as well as all the compression and decompression steps of several mixtures with CO_2.

The heat transfer involved with methanol, waste cooking oil, biodiesel and glycerol was determined by the equation given by

$$Q = m_i \times Cp_i \times \Delta T$$

with

Q : heat value to add or to remove from the system (kJ/h)

Cp_i : heat capacity of the compound (kj/(kg.K)

ΔT : T_f - T_i (K)

To determine the heat (**Q**) to add or remove to the reactor so that it remains at the desired temperature it was used the following equation:

$$Q = H_{products} - H_{reactants} - Q_{reaction}$$

in which $H_{products}$ and $H_{reactants}$ corresponds to the total enthalpy (kj/kg) from the reactants and the products calculated through the temperature of reference (298,15 K). Enthalpy from the products and the reactants is calculated through the following equation:

$$H_{products} = \int_{298,15}^{T_f} \sum m_i \times C_p(T) \times dT$$

$$H_{reactants} = \int_{298,15}^{T_f} \sum m_i \times C_p(T) \times dT$$

The total heat generated by the reaction (Q_R) is given by

$$Q_R = \sum (\Delta H_i^0 \times m_i)_{products} - \sum (\Delta H_i^0 \times m_i)_{reactants}$$

with

ΔH_i^0 : standard enthalpy formation at reference temperature (298,15K) (kj/kg)

Using these equations it was possible to determined the heat necessary to provide to the reactor.

The heat supply for the vaporization of methanol was determined by the followed equation:

$$Q = m_{MeOH} \times Cp_{MeOH} \times \Delta T + m_{MeOH} \times \Delta vap_{MeOH}$$

The heat transfer related to compression and decompression of CO_2 was determined using the software ***Aspen Plus 11.1***.

In order to optimize the process on the basis of requirements, the heat released in operations such as CO_2 compression or the heat released from cooling a mixture in a heat exchanger is further applied in operations in which heat addition is needed, decreasing the total energy amount that must be provided to the process. The heat transfer in all the process units is described in the table 10.

Table 9: Energy Balance.

Operation	Q (MJ/h)
CO_2 compression (6 MPa to 20 MPa)	-55.722
Transesterification reaction	0,31
Separation 1	56. 940
Separation 2	60,40
Separation 3	14.378
CO_2 compression (2 MPa to 6 MPa) and heating	-15.529
CO_2 compression (1 MPa to 6 MPa)	-114
CO_2 compression (0,1MPa to 6 MPa)	-126
Methanol evaporation	210
ΔE	**89,75**

Using all the heat values previously determined for all the operations, the energy balance was determined given at the a positive value of 89,75 MJ/h, i.e., taken advantage from the heat released from the CO_2 compression it is only necessary to provide to the system 89,75 MJ/h.

Considering the heat amount that must be provide to the system of 89,75 MJ/h, the amount of natural gas added to the steam boiler was determined and correspond to 36.288 m^3/year.

CHAPTER 5: ECONOMICS VIABILITY

A study of economic viability is an essential tool to support the decision to go forward with the implementation of a certain project. This will allow the analysis of the viability of the project and identify any adjustments needed to the success of the business.

This analysis is provisional, i.e., based on certain forecasts regarding the activity of the business such as number of products or services provided, the selling price, sales estimate by month and year, credit given to customers, credit given by suppliers, staff costs, overheads related to advertising, water, electricity, rent, among others. Another important parameter is the total investment value and the impact of this investment in the business usually in 3-5 years after the investment project.
A study of economic viability of this process was made but it is important to take into account that some of the assumed values are estimated and a more intensive study is needed to increase the assurance degree of these results.

To calculate the required investment is necessary to know the assets and working capital. The assets can be divided in two categories: fixed or material assets and non-material assets.

The fixed assets include all the goods, patrimonies, buildings or movables which the company needs to achieve its operational activity. These assets have a useful life exceeding one year, without the purpose of being sold or transformed (buildings, land, production equipment, vehicles, etc.). The costs associated with fixed assets are given in Table 11.

Table 10: Fixed assets costs.

FIXED ASSETS	
	Cost
Implementation	500.000 €
Land	7.500 €
Basic Equipment	6.976.969 €
Installation (50% BE)	3.488.485 €
Electric installation (8% BE)	558.158 €
Engineering (15% EB)	1.046.545 €
Laboratory	200.000 €
Vehicles	925.000 €
Others	1.200.000 €
Contingencies (10% sub-total)	1.510.266 €
Total	**16.612.923 €**

The costs associated to the installation of all the equipment or accessories needed, electric installation and engineering were determined and correspond to 50%, 8% and 15 %, respectively, of the basic equipment costs.

The implementation cost was determined assuming a price of 100€/m^2 and an area of 5.000 m^2. For the land cost, it was assumed a price of 1,5€/m^2 and an area of 5.000 m^2.

The equipment costs include the reactor, the separators, static mixer and the dryer. In the utilities, all the storage tanks and the compressor were also included in the equipment costs. In addition, all the instrumentation (indicators and flow, pressure, temperature and level controllers), valves and BPR and liquid pumps were also added (table 12).

Table 11: Detailed Equipment costs.

	Volume (m^3)	Price (€)
Reactor	3,30	650.467
Separator 1	3,32	456.366
Separator 2	3,25	452.010
Separator 3	0,02	240.096
Dryer	-	120.000
Compressor	-	400.000
Static Mixer	-	10.000
Liquid Pumps	-	425.000
Heat exchangers	-	176.000
Instrumentation	-	26.860
Valves and BPR	-	64.500
Storage Tanks	-	1.750.000
Other	-	2.325.656
Total	**-**	**6.976.969**

The non-material assets consists of elements with no physical existence but they represent significant value to the company. The costs associated to the non-material assets are summarized in the table 13.

Table 12: Non material assets costs.

NON-MATERIAL ASSETS	
Projects and engineering (15% BE)	1.046.545 €
Licenses /software/training	100.000 €
Total	**1.146.545 €**

The projects and engineering costs incorporated in the non-material assets correspond to 15% of the total basic equipment costs.

The working capital corresponds to the total amount of financial resources needed to put the factory up and running. Thus, the working capital must pay all the costs of production during the payment period plus inventories and subtracting funding from suppliers and financing short-term. The working capital can be calculated from the followed equation:

$$Working\ capital = Clients + stocks - suppliers$$

The Clients (product selling) is determined by the products income (table 14).

Table 13: Products income.

Products Income			
	Ton/ano	Price (€/ton)	Cost (€)
Biodiesel	13.085	910 €	11.907.749 €
Glycerol	1.326	- €	- €
TOTAL			**11.907.749 €**

The stocks are previously determined including the raw materials cost (table 15).

Table 14: Raw materials costs.

Raw Materials Costs			
	Ton/year	Price (€/Ton)	Cost (€)
Waste Vegetable Oil	13.200	- €	- €
Methanol	1.384	159 €	220.031 €
Carbon Dioxide	9.608	100 €	960.775 €
Enzyme	24	85.250 €	2.046.000 €
TOTAL			**3.226.806 €**

The waste cooking oil cost associated includes only its transportation by truck until the manufacturing facility. The cost was determined taken into account the number of trucks necessary, distance traveled by the trucks and the diesel consumption by the trucks.

Assuming a payment on 60 days, the working capital was determined (table 16).

Table 15: Working capital determination.

Working capital	
Clients	1.984.625 €
Stocks	2.522.426 €
Suppliers	537.801 €
Total	**3.969.250 €**

With the data calculated in the previous tables, it was possible to determine the total investment (table 17) that corresponds to the sum of total of fixed assets, non-material assets and working capital.

Table 16: Total investment determination.

Fixed assets	16.612.923 €
Non-material assets	1.146.545 €
Working Capital	3.969.250 €
Total Investment	**21.728.718 €**

In order to be competitive, the production cost must to be low enough to be possible to take profit from the product sale but at the same time must to be lower than the diesel price. The production cost was determined taking into account the costs associated to the raw material, maintenance, services, staff, electricity and heating.

Through the energy balance, the amount of natural gas added to the steam boiler is 36.228 m^3/year. With a price of 0,243 €/m^3, the annual cost for the heating provide of the system is 8.803€.

The electricity costs were determined taking into account all the equipment that requires electricity and its power. These costs are described in the table 18.

Table 17: Electricity costs.

Equipment	Power (kW)	Quantity	Consumption (7920h) MWh
Liquid pumps	34	1	266
CO_2 compressor	400	1	3168
Recycle pumps	81	1	638
Lightning	2	1	16
Controllers	0,01	74	6
Total	516	78	4.094

Thus, with a total annual consumption of 4.094 MWh/year and assuming an industrial price of 0,058 €/kWh, the total annual cost for the electricity is 236.629 €. It is possible to conclude that the total energy annual cost of the process is **245.433 €** (electricity and natural gas).

The costs associated with staff are described in the table 19.

Table 18: Staff costs.

Salaries					
Position	**Monthly Salary**	**Taxes (33%)**	**Workers**	**Monthly cost**	**Annual cost**
Financial director	4.000 €	5.320 €	1	5.320 €	74.480 €
Production director	3.000 €	3.990 €	2	7.980 €	111.720 €
Shift manager	1.500 €	1.995 €	3	5.985 €	83.790 €
Operators	1.000 €	1.330 €	15	19.950 €	279.300 €
Quality director	1.500 €	1.995 €	1	1.995 €	27.930 €

Lab manager	1.500 €	1.995 €	1	1.995 €	27.930 €
Laby technician	1.000 €	1.330 €	2	2.660 €	37.240 €
Medical assistance	750 €	998 €	1	998 €	13.965 €
Logistic / RH	1.000 €	1.330 €	1	1.330 €	18.620 €
Security	750 €	998 €	6	5.985 €	83.790 €
Maintenance	750 €	998 €	2	1.995 €	27.930 €
Sellers	1.000 €	1.330 €	3	3.990 €	55.860 €
Drivers	1.200 €	1.596 €	8	12.768 €	178.752 €
Total	**-**	**-**	**-**	**72.951 €**	**1.021.307 €**

For services costs, they were determined assuming that they correspond to 10% of the basic equipment total cost plus all the logistic for the wasted oil collection.

Table 19: Production cost estimative.

	Annual	Monthly
Raw materials	3.226.806 €	293.346 €
Maintenance	697.697 €	63.427 €
Services	1.917.377 €	174.307 €
Staff	1.021.307 €	92.846 €
Electricity	236.629 €	21.512 €
Heating	8.803 €	800 €
Total cost	**7.108.620 €**	646.238 €

From the mass balance, a total of 13.085 tons of Biodiesel is produced annually. Assuming the total production costs described in table 20, it was possible to determine a biodiesel cost production of **0,48 €/l**.

Assuming a selling price of **0,80€/l** of biodiesel lower than the diesel price of 1€/l, a profit margin of **67%** is applied. From the selling price and the annual production, it was possible to determine the revenues which correspond to **10.468.351 €/year**. The profit is then **3.359.731 €/year** corresponding to the revenues less the cost production.

These conclusions are financially attractive. However, a more detailed economic study is needed to increase the results exactitude.

CHAPTER 6: CONCLUSIONS

The continuous biodiesel production using biocatalysts under supercritical carbon dioxide conditions has proved to be a high potential process for both oil types: edible vegetable oil and waste cooking oils. However, the use of waste cooking oil as raw material achieving high yields is the major success of this study since its application has many advantages such as using a residue with low commercial value which decrease the production cost and at the same time its solves a big environmental problem related to its treatment. However, Lipozyme TL IM® used as the only biocatalyst for the transesterification reaction using edible vegetable oil has proved not to be very efficient with waste cooking oil in which FFA content is much higher. High reaction yield (98,9%) was only achieved with the addition of other enzyme, Novozyme 435® which activity proved to complement Lipozyme TL IM® activity converting almost all the mono-, di-, triglycerides and FFA. The reaction yield achieved for the transesterification reaction using waste cooking oil is high enough to simplify the purification step using intrinsic characteristics from Sc-CO_2 also reducing the production costs. Agreeing with what is written in the literature, Sc-CO_2 proved to be a good solvent and the supercritical conditions allow the implementation of a very simple and efficient process in order to recover the biodiesel with high purity level. Biocatalysts proved to be the best alternative as catalysts implementation since it was achieved also a high reaction yield without the problems seen in industrial conventional processes related to the purification step.

The major problem associated to this process is related to the enzyme stability. Enzyme desactivation was observed in several experimental works using waste cooking oil. This desactivation is related to undesirable compounds in waste cooking oil since factors such as excessive methanol amount used, glycerol adsorption on the enzymatic support and depressurization steps between experiments could also occur with the use of edible vegetable oil and the loss enzyme activity during reaction time using edible vegetable oil was not verified.

Thus, a decrease of enzyme activity must be related with certain compounds from the waste cooking oil. Further detailed analysis of waste cooking oil composition must be made in order to overcome this problem.

Studies at larger scales should be also done with the development of the optimum separation conditions determined by phase equilibrium studies between CO_2, methanol and glycerol in order to recover all the glycerol and methanol formed during the reaction.

A simplified engineering study was made for a continuous biodiesel production using enzymes as biocatalysts under Sc-CO_2. Mass balance and an energy balance assuming biodiesel annually production of 12.800 tons through 13.313 tons of waste cooking oil collected in Portugal were made. From this balances, it was possible to make a economic viability study which had proved that this process is a big business opportunity with 16.254.109€ of total investment. Selling the biodiesel with a very competitive price comparing to diesel, a annually profit of 6.317.927€ is taken from the process. Since society will continue to use diesel as the conventional transportation fuel until other technologies become more mature and efficient, this process not only is very lucrative but also would decrease our fossil fuel dependency decreasing also our expensive in fossil energy importation. In order to increase the process production, collection systems of waste cooking oil must be developed as well as incentives to promote the deposition of waste oil at the locations prepared for that. The implementation of those locations must be developed studying efficient systems and implemented them in more places.

Summarizing, this study proved that a "*green*" integrated process of biodiesel using "*green*" techniques and "*green*" catalysts could be implemented representing also a very lucrative process. Further forces must be brought together to overcome the problems of this process that make this project not yet a reality.

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APPENDIX

APPENDIX A

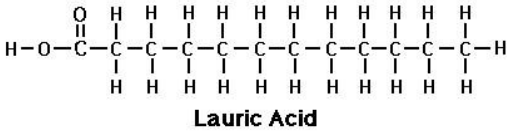
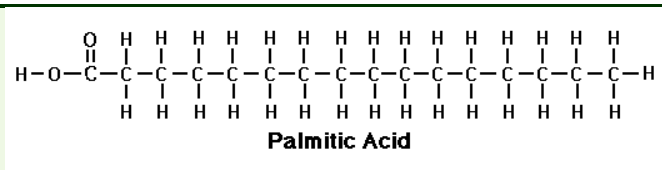
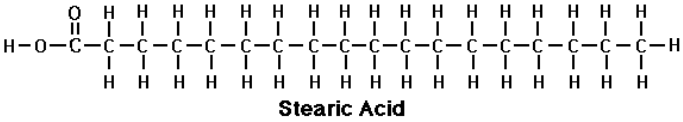
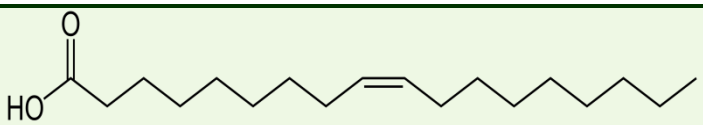
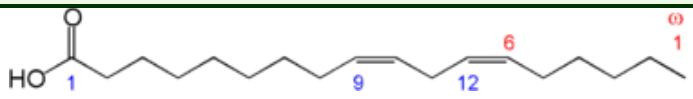
Table 20: Specifications of EN 14214 comparing to conventional Diesel [11].

PROPERTIES	DIESEL	BIODIESEL
Density 288 K	0.82-0.86	0.86-0.9
Viscosity 313 K	2.0 - 4.5	3.5 – 5.0
Flash Point (K)	> 377	> 423
Sulfur (% mass)	< 0.2	< 0.01
Sulphated ash (% mass)	< 0.01	0.02
Water (mg/kg)	< 200	< 500
Carbon residues (% weigh)	< 0.30	< 0.03
Total contamination (mg/kg)	-	< 24
Copper corrosion (3h/323K)	Class I	Class I
Cetane number	> 45	> 51
Methanol (% mass)	-	< 0.2
Ester content (% mass)	-	>96.5
Monoglycerides content (% mass)	-	< 0.8
Diglycerides content (% mass)	-	< 0.2
Triglycerides content (% mass)	-	< 0.4
Free Glycerin (% mass)	-	< 0.02
Total Glycerin (% mass)	-	< 0.25
Iodine number	-	120

Phosphorous (mg/kg)	-	< 10
Alkaline metals Na, K (mg/kg)	-	< 5
Methyl esters of linolenic acid (% mass)	-	< 12
Methyl esters polyunsaturated (≥ 4 double bonds) (% mass)	-	< 1

APPENDIX B

Table 21: Description of mainly fatty acids constituents of vegetable oils.

SATURATED			
Fatty acid	**Structural formula**	**Symbol**	**Molecule formula**
Lauric acid	 Lauric Acid	C12:0	$C_{12}H_{24}O_2$
Palmitic acid	 Palmitic Acid	C16:0	$C_{16}H_{32}O_2$
Stearic acid	 Stearic Acid	C18:0	$C_{18}H_{36}O_2$
UNSATURATED			
Fatty acid	**Structural formula**	**Symbol**	**Chemical formula**
Oleic acid		C18:1	$C_{18}H_{34}O_2$
Linoleic acid		C18:2	$C_{18}H_{32}O_2$

Linolenic acid		C18:3	$C_{18}H_{30}O_2$

APPENDIX C

❖ SAMPLE PREPARATION

For peak identification was used heptadecane as the internal standard. Thus, all samples have a concentration of standard of 12 ppm. The solvent used is hexane.

The samples obtained from the experimental work were too concentrated to be injected in the GC. Thus, dilutions were necessary before the injection. Samples must have concentrations lower than 100 ppm approximately in order to be injected in the GC. Two dilutions are made to prepare the sample for injection. A volume of 10 μL of sample is introduced in a vial previously tared (VIAL 1). 1 ml of a heptadecane in hexane solution (5 mM) is added to VIAL 1. In another vial is added 1ml of hexane (VIAL 2). 10 μL the solution in VIAL1 is added to the VIAL 2.

The VIAL 2 is already prepared to be injected with a sample concentration of about 100 ppm.

APPENDIX D

Molar mass, density and boiling points of the compounds used are described in table 23.

Table 22: Compounds properties.

	MM (g/mol)	Density (g/cm^3)	Boiling point (K)
Methanol	**32**	**0,792**	**337,85**
Glicerol	**92,1**	**1,226**	**563,15**
CO_2	**44**	**0,84**	**-**
Sunflower oil	**-**	**0,966**	**-**

The sunflower density was experimentally measured.

	Experiment 1	Experiment 2	Experiment 3
Mass (g)	1,8401	1,8654	2,3318
Volume (ml)	1,9	1,95	2,4
Density (g/ml)	0,968	0,957	0,976
Average density		**0,966**	

APPENDIX E

In order to quantify the methyl esters obtained from each experiment, the calibration standards were made for palmitate (C16), stearate (C18:0), oleic (C18:1), and linoleate (C18:2) linolenate (C18:3) methyl esters.

From the calibration standard, a relationship between the sample concentration and the peak area a can be determined.

$$\frac{[FAME]}{[Standard]} = RF \times \frac{Area_{FAME}}{Area_{\ standard}}$$

Graphically,

$$\frac{[FAME]}{[Standard]} \ vs \ \frac{Area_{FAME}}{Area_{\ Standard}}$$

it is possible to get the calibration standard with the response factor given by ***m*** value from the graphic representation.

Seven solutions were previously prepared with a known concentration and then injected. From the peak analysis similar to the one presented in the CHAPTER 2, it is possible to take the calibration standard. The method for the results analysis is further described.

Table 23: Results analysis for the calibration standards elaboration.

$[C16]_{ppm}$	$\frac{[C16]}{[Standard]}$	$\frac{Area_{C16}}{Area_{standard}}$	$[C18]_{ppm}$	$\frac{[C18]}{[Standard]}$	$\frac{Area_{C18}}{Area_{standard}}$
10	1,2475	0,518468	40	4,9901	1,85085
8	0,9980	0,4027	32	3,9921	1,2533
6	0,7485	0,3103	24	2,9941	1,0498
4	0,4990	0,2259	16	1,9961	0,673\
3	0,3742	0,1743	12	1,4971	0,5382

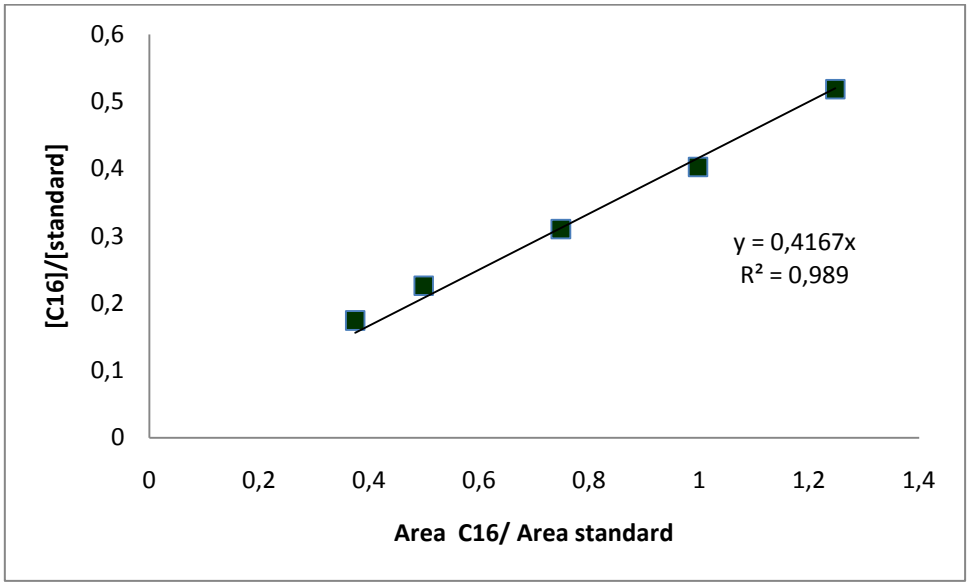


Figure 51: Calibration standard for the C16 methyl esters.

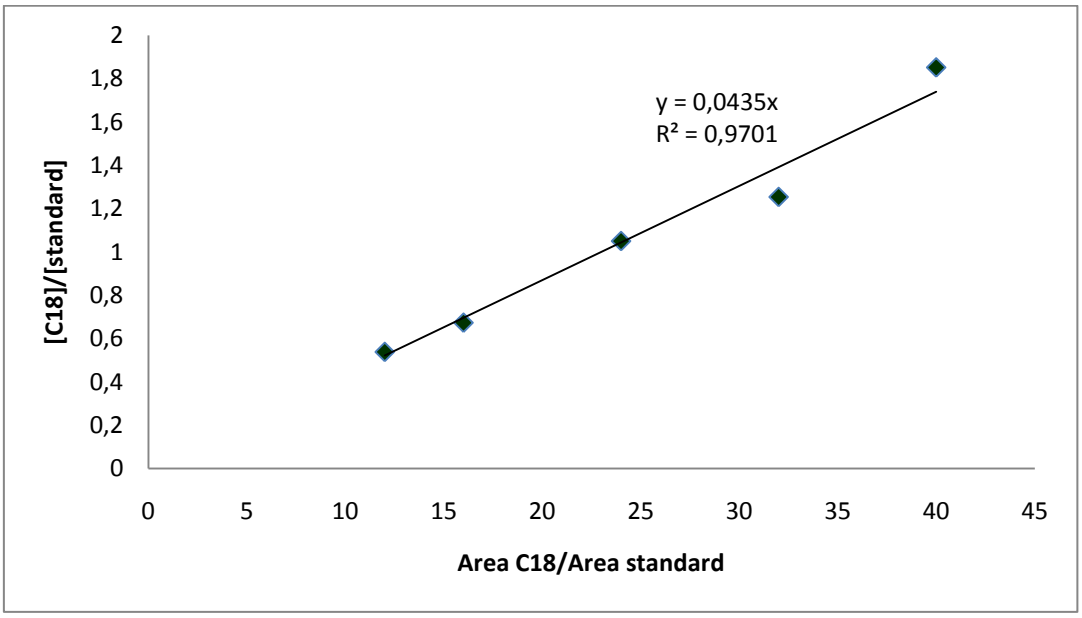


Figure 52: Calibration standard for the C18 methyl esters.

APPENDIX F: LOWRY METHOD

Lowry Method was applied in order to determine the protein concentration in both enzymes: Lipozyme TL IM and Novozyme 435. The method is based on both the Biuret reaction, in which the peptide bonds of proteins react with copper under alkaline conditions. After, the protein pre-treated with cooper solution, reacts with the Folin reagent under the Folin–Ciocalteau reaction which is poorly understood but in essence phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids. The reactions result in a strong blue color, which depends partly on the tyrosine and tryptophan content [74].

Basically, with the addition of the copper solution, reaction between peptide bonds of proteins and the Cu^{2+} ions occurs under alkaline conditions. To the complex Cu-Protein is added the Folin-Ciocalteau that contains an acid which will be reduced by the proteins into the solution with the formation of a blue colored product. The concentration of this colored product will be estimated spectrophotometrically at λ=750 nm. The stronger the color of the final product, higher is the protein content in that specific enzyme.

BSA (bovine serum albumin) was used for the preparation of a standard curve in order to get the theoretical concentration of a sample by precise weighing and dilution and the accuracy of the method was determined by comparison of the theoretical and determined values. Bovin Serum Albumin (BSA) is universally used as a standard protein because of its low cost, high purity and ready availability [75].

Since the enzyme was immobilized, it was necessary to proceed to it desorption to be possible the application of the Lowry method.

In order to obtain the solution of enzyme, an alkaline solution was first prepared in order to hydrolyze the enzyme breaking the bonds between the enzyme and the support. To 4mg of each enzyme (Lipozyme TL IM and Novozyme 435) was added 1 ml of NaOH solution 1M. Both solutions were maintained in a water bath at 100ºC during 10 min in order to accelerate the alkaline hydrolyzation. After, both solutions were transferred to an ice bath during 5 min to cool. The alkaline hydrolyzation had already occurred and it was necessary to separate the free enzyme of the remaining support. Both solutions were centrifuged at 10.000 rpm during 5 min. A first dilution 1:10 was made to each solution transferring 200 μL of the supernatant for new eppendorfs and then 100 μL for other eppendorf with 900 μL of distillated water. From this solution, another dilution was made this time 1:100 and replicated. At the end, 4 eppendorfs were obtained with free Lipozyme TL IM and Novozyme 435 (2 to each enzyme) and proceeded to the Lowry quantification method.

The solutions applied to this method were:

- **Solution A**: 3g of sodium carbonate + 0,4 g of NaOH in 100 mL of distillated water.
- **Solution 2:** Aqueous solution of sodium potassium tartarate at 4%.
- **Solution 3:** Aqueous solution of copper sulfate pentahydrate at 2%.
- **Lowry reagent solution:** 0,25 mL of Solution 2 + 0,25 mL of Solution 3 and adding to a total of 25 ml with Solution A.
- **Standard BSA solution (200 μg/mL).** The calibration standards for the spectrophotometer were prepared by diluting this solution to 20, 50 and 100 μg/mL. At the end, four solutions were obtained for the calibration standard: 20, 50, 100 e 200 μg/mL.

To the four eppendorfs obtained previously, was added 1 mL of Lowry reagent solution maintaining the solution with mixing during 10 min. After this 10 min, 200 μL of Folin-Ciocalteau was added. Between each addition the samples were shaken in vortex for 5 minutes. After 30 min, the absorbance was measured at 750

nm. The blank solution was prepared at the same way but using 200 μL distillated water instead of 200 μL of sample. The same procedure was applied to the BSA standard solutions to calibrate the spectrophotometer. All the measurements were replicated.

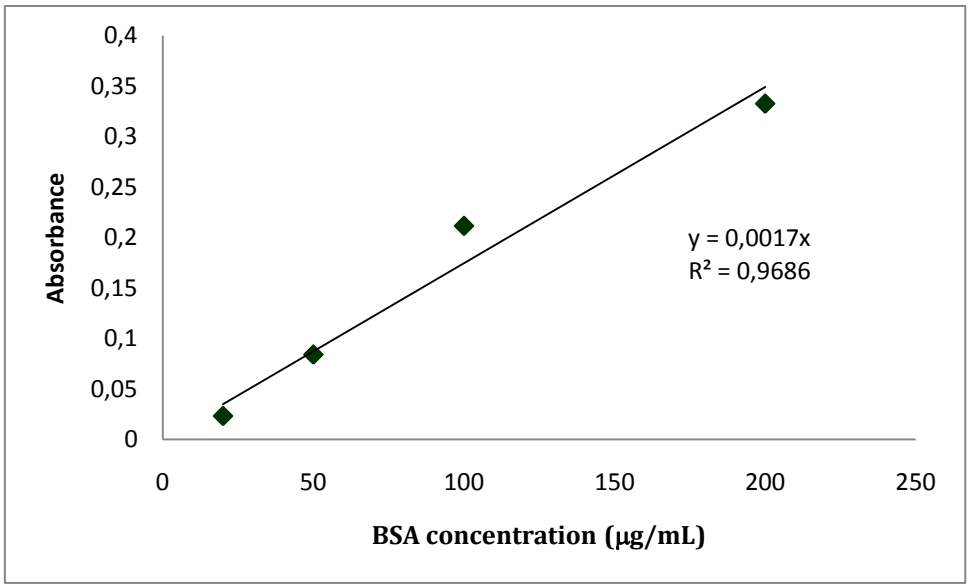


Figure 53: Calibration standard with BSA.

From this calibration, it is obtained a relationship between absorbance and protein concentration given by Absorbance= 0,0017 x [protein] (μg/mL). From the absorbance values obtained for both solutions (Lipozyme and Novozyme) using the equation, it is possible to obtain the protein content in each enzyme.

LIPOZYME TL IM	
Absorbance	0,0597
Protein content (%)	2,0
NOVOZYME	
Absorbance	0,1526
Protein content (%)	0,8