1 Introduction

1.1 Petroleum economy related problems

1.1.1 Energy demand growing and fossil fuels dependency

Most of modern societies energy demands are currently covered by means of energy sources relying on petroleum, as well as natural gas fields, coal and charcoal. Petroleum economy has always suffered from a series of problems related to heterogeneous distribution of big oil fields and the need of transport of oil and oil derivatives across the world (Fortman *et al.*, 2008), (Jegannathan *et al.*, 2009). Therefore, energy dependence of fossil fuels consumer countries and the logistical problems associated with transport and distribution of petroleum and oil derivatives through sea freight transport, oil pipelines or gas pipelines have always generted an upward trend in the cost of petroleum or fossil fuels related energy sources.

The above mentioned issues will go worse in the medium term, taking into account the forecasted depletion of main oil fields by XXI century and the unstoppable growing of both world population and energy demand. According to United States Department of Energy, global energy consumption will practically double in the next two decades (Conti & USDE, 2009), (Fortman *et al.*, 2008).

1.1.2 Impact of fossil fuels burning over environment and human health. CO_2 and other greenhouse gases role. SO_2, aromatic compunds and particles related problems.

CO_2 and other greenhouse gases accumulation as a consequence of fossil fuels burning in industry and transport is having a deep impact worldwide, both over ecosystems degradation because of the presence of contaminants derived from

hydrocarbons and over global climate alterations by means of greenhouse effect enhancement. European Union is close to reach the Kyoto Protocol objectives (to reduce, by 2020, greenhouse gases emissions (GHG) by 20% compared with 1990 levels), but if the target stated by European Commission for 2030 is considered (reaching a GHG emissions reduction of 40% compared wth 1990 level), there is the need for achieving a more intense decarbonisation of both the economy and power, heating and transport systems of the EU. Although energy efficiency and renewable energies share in EU-28 power systems has grown both in the short-term from 2008 to 2012 and in the long-term in the 1990-2015 period, EU-28 progression in the achievement of 2020 Kyoto Protocol target seems to rely, according to the different Eurostat indicators evolution, not only in the efforts on renewable energies efficiency but rather in the reduction of energy demand because of economic crisis affecting the Eurozone since 2010. In the medium-term, it is hard to decouple economic activity growth from energy demand and fuels demand for freight and passengers transports across Europe. In order to avoid a bounce up effect in energy demand and fossil fuels share in transport with the end of the economic crisis, renewable biofuels like biodiesel must be more competitive to balance the high dependency of road transport that characterices the Europe freight and passengers transport, whith over 80% of passengers transport and over 75% of freight transport are undertaken by road. Besides, EU transport model must be modified to change the freight and passengers transport modes, reducing the share of road transport and increasing the ise of railway and inland european waterways, due to the much lower carbon footprint of later transport modes compared to former ones. Biodiesel production from agriculture, industry and urban wastes and residues could palliate diesel powered trucks and cars carbon footprint in

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european roads and reduce the harmful effects over environment, ecosystems and human health of fossil origin diesel burning during the transitional period needed to modernise the EU-28 transport systems, providing leeway for action to recently incorporated member states and member states with lower transport infrastructure budget or gross domestic product (GDP), where a slower improvement of road and railways system and an increase in the transport needs due to borders with other member states elimination generates a relatively higher increase in GHG emissions compared to more wealthy member states or those with a more efficient railway focused transport system. In this context, the quest for more sustainable energy sources is becoming a pressing need rather than idealistic or utopic solutions to environmental problems (Kurkowiak, 2015).

1.2 Renewable alternatives to petroleum

1.2.1 Hydrogen

Hydrogen is, at least in theory, a clean fuel, due to the fact that its combustion only yields water vapour. However, its production at industrial scale is based in the catlyisis by Fischer-Tropsch reaction from natural gas, through a classical petrochemical industry process that requires huge amounts of energy and is based in the intensive use of fossil fuels. Nevertheless, several biologycal processes can be used for hydrogen production from renewable sources, from urban wastewater tratment plants sludges fermentation by *Clostridium bifermentans* (Wang *et al.*, 2003) or *Enterobacter aerogenes* (Ito *et al.*, 2004) to use of genetically modified phototrophic microorganisms carrying clostridial hydrogenases (Miyake & Asada, 1997), or subjected to engineering

of the photosynthetic antena complex to yield H_2 from electron transport chains that occur in photosynthesis proteic machinery (Kondo *et al.*, 2002), (Kondo *et al.*, 2006).

Production methods based on photobioreactors have been object of intensive study in terms of photobioreactors design and operation conditions optimization. It is remarkable the research developed in Japan by Miyake *et al* at the research centre RITE (Research Institute of Technology for Earth), or the studies developed by Wakayama *et al* (Miyake *et al.*, 1999), (Wakayama & Miyake, 2002). A good understanding of bioreactors design details and design general process can be found in (Fedorov *et al.*, 1998) and (Wolfrum & Watt, 2002).

Even though research in biohydrogen production begins to give promising results, it still has several handicaps, like energy losses by heating of compressors needed to liquate the gaseous Hydrogen (van Groenestijn *et al.*, 2002) or the need of developing and building a new infrastructure able of safely distribute the hydrogen. In this context, the irruption of carbon nanotubes technology could provide the materials needed to store industrial amounts of hydrogen in a safe and economic way, but it is still in an incipient state (Chen *et al.*, 2008), (Bento, 2008), (Ball & Wietschel, 2009).

1.2.2 Bioethanol

Bioethanol is obtained from sugar rich feedstock or easy to transform in simple sugars feedstock which are fermented to produce ethyl alcohol. World ethanol production in 2007 was 12500 galon billions, and the main producers were Brasil and U.S.A., which used as feedstock sugarcane and corn grain respectively and produced jointly 62% of bioethanol global market.

Bioethanol production accounts for three steps: Firstly, compound sugars are hydrolised to glucose. Them, a second step of glucose fermentation proceeds, yielding ethanol and carbon anhydride. The third step is a thermochemical process, involving the diluted alcohol distilation to yield absolute ethanol.

The intensive use of crops needed for human nourishment and livestock feeding, alongside with the shortening of fertile farm land and the increase in primary products, turns the use of such crops for bioethanol production a new problem rather than, as initially was believed, a solution to climate change and sustainable fuels related problems (Fortman *et al.*, 2008). However, there is another potential source of feedstock for bioethanol production: lignocellulosic biomass, which includes agriculture wastes (corn and wheat straw, and sugarcane bagasses), grass crops (forage plants such as alfalfa in Europe or the poaceous *Panicum virgatum* in USA and Mexico (named switchgrass or *pasto varilla*) (van Dam *et al.*, 2009), forestry residues, recycled paper and other residues (Fukuda *et al.*, 2006). When lignocellulosic materials are used as feedstock, a pretreatment step is required, which consists in a chemical or enzymatic hydrolisis in order to remove the lignin (Jegannathan *et al.*, 2009), and results in a lower efficiency of the bioethanol production process.

Even though the solution to the above mentioned efficiency loss is still under development and the bioethanol production industry mainly operated with easy to pretreat vegetal residues or even easy to directly ferment by *Saccharomyces cerevisiae* sugars rich vegetal materials, some advances are being achieved, due to the interest of several big corporations of biotechnology, finances and other sectors in the final stages of Bush' s administration (Schubert, 2006) but also because of improvements achieved with cellulase enzymes typical from fungi belonging to *Trichoderma* genus (Fukuda *et*

al., 2006), (Lo, 2008), (Rahman *et al.*, 2009), (Jegannathan et al., 2009). New strategies have been developed, changing the focus to microorganisms engineered to carry simultaneously both cellulase and enzymes pyruvate decarboxylase and alcohol dehydrogenase, needed to ferment sugars to ethanol (van Zyl *et al.*, 2007), (Lu, 2006), (Jegannathan *et al.*, 2009).

1.2.3 Biodiesel

Biodiesel is a mixture of mono-alkyl fatty acid esters (FAAE) obtained by transesterification of fatty acids and alcohols (ethanol or methanol), using mainly vegetable oils as feedstock. When methanol is used to produce biodiesel, esters are named fatty acid methyl-esters or FAME. If, on the contrary, ethanol is used, esters are named fatty acid ethyl esters or FAEE. The properties of both methanol or ethanol derived biodiesel are similar, being the low cost and wide availability of methanol the main reason of its predominant use in biodiesel industry, in spite of its high toxicity, flamability and volatility (Ranganathan *et al.*, 2008), (Fjerbaek *et al.*, 2009).

1.2.4 Biodiesel advantages as biofuel

The main advantage of biodiesel as biofuel is that it keeps the CO_2 balance due to its production from renewable biologycal sources. It also presents a series of environmental benefits such as low carbon monoxide, sulfur, aromatic hydrocarbons and ash particules emissions. partículas de hollín. Besides, biodiesel is not toxic and is completely biodegradable (Kalscheuer *et al.*, 2006).

Thanks to its high flash point, biodiesel has a low flamability and is safe to handle. Besides, it provides a good lubrication to the engine, reducing its wearing. Blendings of any ratio of biodiesel and conventional diesel can be used in conventional diesel engines directly without problems in combustion chamber or injection lines (Utlu

& Kocak, 2008), or only with minor modifications (Fang *et al.*, 2008), (Kegl, 2008), (Zheng *et al.*, 2008) and the biofuel can be distributed using the currently available infrastructure (Kalscheuer *et al.*, 2006), (Shieh *et al.*, 2003), (Sahoo & Das, 2009).

1.2.5   Alcohols and fatty acids sources

Biodiesel is produced mainly from vegetable oils like soybean oil, jatropha oil, rapeseed oil or palm oil, as well as from sunflower seed oil, corn or peanut oil, canola oil or cotton seed oil (Ranganathan *et al.*, 2008), (Peterson, 1986). Biodiesel can also be produced from animal fats (beef tallow, pork fat), waste cooking oils, butter, fat from sewage collectors and septic tanks, waste lubricanting oils and algae oil (Peterson, 1986).

1.2.6   Biodiesel production by chemical catalysis

Commercial biodiesel is currently produced by means of the transesterification reaction of rapeseed, soybean or sunflower seeds oil by chemical catalysis. Usually, methanol is the alcohol used for the transesterification (also named alcohlysis) of the triglycerides from which the vegetable oil is composed because methanol is cheaper than etahnol. The alcoholysis process is generally divided into three reversible steps. Along the first reaction, triglycerides are transformed in diglycerides. Diglycerides are subsequently transformed into monoglycerides during the second reaction. The monoglycerides obtained in the second reaction are transformed in glycerol during the third reaction. Along the three mentioned reactions, esters are produced, and the mixture of those esters, once purified, constitute the biodiesel (Schwab *et al.*, 1988). The catalysts used can be acid ones, like sulfuric acid, or basic ones, usually sodium hydroxide; although alkaline catalysis uses to be the preferred method because it presents a series of advantages over the acid one.

1.2.6.1 Alkaline catalysis

One of the main advantages of alkaline catalysis (Figure 1) is its outstanding versatility, as it allows to obtain biodiesel virtually from every kind of vegetable oil or fat. The alcohol: oil molar ratio ranges from 1:1 to 6:1. Nevertheless, a 6:1 ratio gives a higher yield in the alkali conversion without expending a great amount of alcohol (Trent, 1945). The amount of catalyst added to the reactor ranges from 0.5 % to 1% (w/w) (Srivastava & Prasad, 2000), but some authors recommend much lower values, between 0.005% and 0.35% (w/w) (Aksoy *et al.*, 1988, Wimmer, 1995). Besides, alkali driven catalysis yields are higher than acid driven catalysis and the biodiesel obtained with basic catalysts is less corrosive than the produced with acid ones.

However, alkali caalysis has some drawbacks, especially the partial saponification of the reaction mix in presence of water. Soaps increase the catalyst consumption and reduce its activity, driving to increased viscosity and gels formation, and making difficult the glycerol separation. Other problems are the huge amount of energy required to keep the reaction rate, as the temperature of operation is, at least, 60°C (Fukuda *et al.*, 2001a); and the risky and hard glycerol and methanol separation by means of methanol evaporation, neutralization and concentration; alongside with the need of separating both the catalyst and the saponified byproducts from the biodiesel. On the other hand, big amounts of alkaline waste water are generated, requiring treatment prior to its dumping.
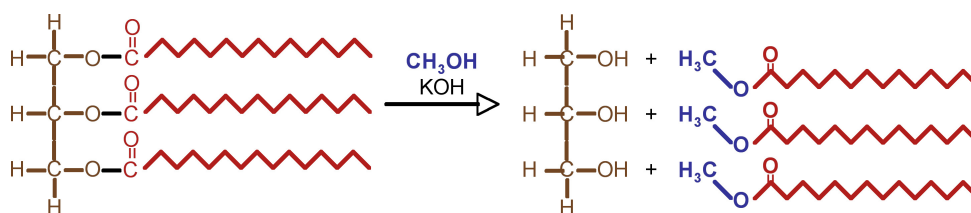
Figure 1. Alkali catalysed transesterification reaction

1.2.6.2 Acid catalysis

Acid catalysis is the second more used biodiesel production process. An acidic homogeneous or heterogeneous catalyst is used. Within the former ones, sulfuric acid is the most used, although clorhydric acid, sulfonic acid *p*-toluene and sulfonic acid methane are also used. Within the later ones,the most frequently used are exchange resins with strong sulfonate and acidic zeolites.

Similarly to alkaline catalysis, when an acid catalyst is used the triglycerides conversion is more efficient when the alcohol steichiometric excess, but in such situation the recovery of glycerol is more difficult, so the optimal proportion between alcohol and oil must be established in each case, although the molar ratio 30:1 can serve as a refference value. The kind of alcohol, as well as the oil, are similar to those described for alkaline catalysis. The estimated amount of acidic catalyst added to the reactorranges from 0.5 to 1 mol %, with a typical value of 1 %, although some authors suggest a 3.5 mol % (Zhang *et al.*, 2003), (Aksoy *et al.*, 1988). The temperature range whithin the reaction is feasible ranges from 55 to 80 ºC. A high yield in esters production is obtained, but with the drawback of a very extense reaction time, over 24 hours. Several research groups proposed the esterification of triglycerides contained in animal fats and oils by means of a two steps process, using an acidic catalyst in the first step and an alkali catalyst in the second one. These methods can have some specifications like a high temperature of reaction, from 60 to 120ºC, in order to reduce fat interesterification (Aksoy *et al.*, 1988), (Kawahara & Ono, 1979), the use of acidic cationic exchange resin as solid catalyst (Jeromin *et al.*, 1987) or the removal of

glycerol prior to the second step of the process. However, the slow diffusion of triglycerides across the catalyst pores in heterogeneous systems like supported metals, basic oxides or zeolites lowers the transesterification reaction rate, involving a very slow progression of the reaction and forcing to rise the alcohol: glyceride ratio in order to obtain yields over 70%.

1.2.7 Biodiesel production by enzymatic catalysis

Figure 2 shows the fatty acids and ethanol transesterification process by enzymatic catalysis. Generally, chemical transesterification processes present two drawbacks. First, the process is relatively slow. On the other hand, chemical methods require the removal of catalyst and byproducts generated by saponification of free fatty acids.
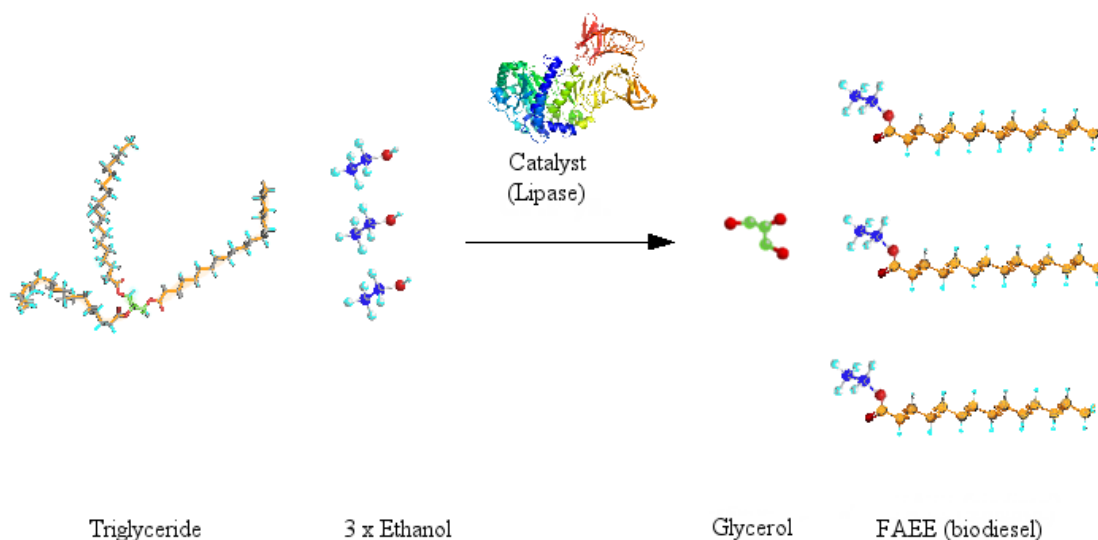

Triglyceride   3 x Ethanol   Glycerol   FAEE (biodiesel)

Figure 2. FAEE synthesis catalysed by lipase. Lipases (EC 3.1.1.3.) are able to catalyse the transesterification of acyl groups present in triglycerides to the alcoholic electrons aceptor (ethanol). Thus, a mixture of FAEE is obtained, alongside with glycerol as byproduct.

Saponification problems are more severe when the water richness in the feedstock rises, driving to a lower process reliability, given the important quality oscillations that can occur in the vegetable oil used as feedstock. By means of

enzymatic catalysis, part of the chemical catalysts related problems can be solved. Besides, enzymes can operate in solutions containing a certain amount of water and catalyse transesterification reactions at lower temperatures than traditional chemical processes required operation temperatures. Therefore, the use of enzymes to produce biodiesel can improve substantially the final product quality, simplifying and even avoiding the *downstream* refining process (Fukuda *et al.*, 2001a) and significantly reducing the energy costs of transesterification reaction maintenance (Jaeger & Eggert, 2002) thanks to the soft operation of enzymes at relatively low temperatures, within the 20 to 70ºC range, being even able to work at lower temperatures, like lipases obtained from psicrophilic microorganisms (Joseph & Ramteke, 2008), which is the strongest aspect of lipases compared to traditional chemical catalysts (Fjerbaek *et al.*, 2009).

Lipases are enzymes able to catalyse reactions such as glycerol hydrolysis, alcoholysis, acidolysis, and recently it has been found that lipases can also be used for catalyse transesterification and esterification reactions. Their biocompatibility, biodegradability and environmental safety make lipases a cornerstone for the developing of green and sustainable technologies such as biodiesel production, although they also have some drawbacks, especially the low stability of these enzymes, their susceptibility to methanol driven deactivation or the low reaction rate exhibited; and the last but not the least, lipases are expensive enzymes because of the difficulties for obtaining and purifying them. Nevertheless, protein engineering, lipase enzymes inmovilization on cheap and ease to manipulate carriers alongside with the reutilization of enzymes and optimization of operation conditions during reaction development could bring key advances in terms of lipases efficiency improvement and production and handling costs reduction (Iso *et al.*, 2001), (Hsu *et al.*, 2002).

1.2.8   Microdiesel

Conventional biodiesel is not a fully renewable energy source, because of the fossil origin of the majority of methanol used for its synthesis (It is obtained by natural gas cracking). The use of bioethanol would make biodiesel to become a more sustainable product, but it still would keep not solving the big issue of high energy consumption associated to the classical chemical catalysis, needed to keep the transesterification reaction within acceptable yield thresolds. By means of the introduction of microbiological origin lipases, biodiesel production at low temperatures close to room temperatute would be possible, but a sharp costs rise would be associated with the high price of lipases and their rapid deactivation or inhibition in presence of methanol or ethanol, as well as by the action of diffeerent substrates present in the reactor (Ranganathan et al., 2008), (Fukuda *et al.*, 2001a). Microbiological production of fatty acids ethyl esters (FAEE) yielding biodiesel can be developed not only using lipases obtained from microorganisms, but also with microorganismos subject of metabolic engineering.

Anyway, thus obtained FAEE of microbiological origin are named microdiesel (Kalscheuer et al., 2006) (Fjerbaek et al., 2009). Besides, optimization of temperature, aireation and or shaking condirtions of reactor are needed, in order to determine the best operation condirions for every case to obtain the highest biodiesel yield with a technically feasible and economically profitable process (Shieh et al., 2003), (Ebrahimpour et al., 2008).

1.3 Wastes use for (bio)diesel production

Direct use of vegetable oils in biodiesel production is expensive and has a low feasibility and sustainability. However, the use of oil processing wastes or the use of waste oils brings to a significant reduction in this biodiesel production feedstock costs.

1.3.1 Oils refining wastes

Wang *et al* proposed the use of soybean oil deodorization distillate (SODD). It is a byproduct of soybean oil refining process that accounts for approximately 0.3-0.5% of the processed soybean oil volume. SODD contains 25-35% of free fatty acids (FFA) and 45-55% of triglycerides, so around 80% of SODD can be transformed in biodiesel (Wang *et al.*, 2006). The production system proposed by Wang *et al* uses SODD and methanol, which, by the action of lipases from *Thermomyces lanuginosa* and *Candida antarctica*, are transformed in FAME in presence of tert-butanol and silica gel or 3Å molecular sieve (added to the reactor after two hours of incubation in order to keep under control the amount of water present in the reacrion mix) (Wang *et al.*, 2006).

Park *et al* used waste activated bleaching earth, a waste from the palm or rapeseed oil refining industry, as substrate for the production of biodiesel with *Candida cylindracea* lipase. Waste activated bleaching earth (ABE) accounts for 35-40% of oalm or rapeseed oil and can be used for synthesising a wide range of bulk chemicals, including biodiesel. Park *et al* used fuel oil or kerosene as solvent for the reaction mix, and methanol was the alcohol used for transesterification itself. After a 12-24 h incubation period at a temperature of 15-55ºC with an stirring rate of 30 rpm, obtained FAEE were extracted from ABE by a french press filter with a vacuum pressure of 0.5 MPa. A 98% (w/w) FAME yield was found after 24 h in the temperature range 15-20ºC, a 97% FAME yield (w/w) was obtained after 12 h incubation within the range 25-45ºC,

and only 79% FAME yield was obtained when operation temperature was set at 55ºC, indicating thermal inactivation of the enzyme.

1.3.2 Waste cooking oils

Al-Zuhair *et al* studied biodiesel production by lipases from *Candida antarctica* and *Pseudomonas cepacia* immobilised on ceramic beads or in free form from simulated waste cooking oil (SWCO). They compared the efficiency of four experimental systems, namely: free *C. antarctica* lipase with and without organic solvent, *C. antarctica* lipase immobilised on ceramic beads with and without organic solvent, free lipase from *Burkholderia cepacia* with and without organic solvent; and finallye *B. cepacia* lipase immobilised in ceramic beads with and without organic solvent. In every cases methanol was ised as alcohol. The best yields were obtained with the bacterial lipase. Al-Zuhair *et* al reported a higher biodiesel production when they worked with immobilised enzymes than when free enzymes were used, probably due to the reduction of enzymes inactivation driven by methanol denaturalisation thanks to the shielding effect of the clay structure associated microenvironment surrounding immobilised lipases (Chen *et al.*, 2009) and (Dizge *et al.*, 2009).

1.3.3 Waste waters

Urban waste waters and food and agriculture industries waste waters are highly contaminant and hard to treat residues. Nevertheless, given the high amounts of organic matter present in these effluents, their sludges can be used for the production of several biofuels. There is an extense scientific literature focused on biogas and hydrogen production from these residues and, in spite of the lack of the development at industrial scale of biohydrogen production, the majority of waste water treatment plants currently are equipped with anaerobic digesters where methane is obtained and burned in

generators yielding more than enough energy for the plant installations function (Wang *et al.*, 2003), (van Groenestijn et al., 2002). Similarly, several works focused on *in situ* transesterification of waste water treatment plants (WWTP) sludges by chemical catalysis (Mondala *et al.*, 2009). A new conception of biodiesel production from cited sludges relies in the microdiesel production from fats and the carbon sources therein contained, especially in WWTP placed close to farms and food industries. This new technology could represent a significative improvement in waste waters management, simultaneously treating them and producing a renewable, ready to use in transport and industry biofuel, given its similar to conventional diesel physicochemical characteristics (Zappi *et al.*, 2005).

This study aims, within other objectives, the study of lipase activity of several bacterial strains isolated from fat rich WWTP sludges, and their application, both in free state and immobilised on several carriers, to the production of biodiesel of microbiologycal origin (also named microdiesel) by means of sludge fats fatty acids transesterification with ethanol.

1.3.4 Bacterial strains survival in WWTP sludge

As will be explained below (section 1.4), there is plenty of information about the parameters that intervene in the process of microdiesel production. Thus, it is convenient taking intoaccount the quality variations of the sludge used as feedstock for the production of microdiesel and its influence in the viability of the bacterial strains used for catalyse the transesterification reaction. The cited documentation describes in deep detail the influence of rain water in the efficiency of WWTP, although usually this kind of literature focuses mainly in the solutions to purely engineering related problems that occur both in treatment plants and in the sewage and rain drainage networks, as

well as in the modelling of the behaviour exhibited by these systems, by means of deterministic models or using more flexible and interactive solutions, such as artificial neural networks. These modelling works pay less atention to the alterations of the microbiota usually present in treatment plants, responsible for the different processes that enable the reduction of the amount of suspended solids, organic matter and pathogens in the treated water. The studies based in field sampling are scarce, mainly because of the inherent difficulties of this sampling campaigns, that in addition are carried out in draught periods (Lessard & Beck, 1993), (Rouleau et al., 1997), (El-Din & Smith, 2002), (Gernaey et al., 2005), (Cho *et al.*, 2010). On the other hand, tha majority of studies rely on classical parameters like Chemical Oxygen Demand (COD), Total Suspended Solids (TSS), Volatile Suspended Solids (VSS) and ammonia concentratation. According to Sakrabani and colleaguess, the afore mentioned parameters can not explain in deep by themselves the degree of biodegradability of the organic matter present in waste water. Their works point, besides, to the necessity of dividing/splitting the COD in several fractions with different microbiologycal properties, using as organization criteron the value obtained for the Oxygen Uptake Rate (OUR) from bottle samples. According to these authors, the higher biodegradability is observed in waste waters dragged to the trearment plants in the onset of rains (Sakrabani *et al.*, 2009), (Vollertsen & Jacobsen, 2009).

1.4 Physico-chemical parameters in the enzymatic production of biodiesel

Antczak *et al* make a distinction within basic parameters and subparameters to consider the factors intervening in lipase driven biodiesel production (Antczak *et al.*, 2009). Within the basic parameters we can find, in first place, the election of lipases, which can be intracellular or extracellular -being the later ones the most commercialy

extended- and can be subject of modification or stabilisationa processes prior to their use to catalyse the transesterification reaction. Another crucial point is the election of the precursors (fats and alcohols) and the system configuration, making a distinction within organic solvent free systems and systems based on the use of bigger or smaller amounts of such solvents. Finally, another determinant factor of the process is temperature, which, according to Antczak *et al*, has influence in the subparameters. These subparameters are hydric activity, amount of added water, use of additives such as silica or zeolites (Antczak *et al.*, 2009), pH of microenvironment surrounding lipase enzymes (Shah *et al.*, 2004), (Aloulou *et al.*, 2007) or glycerol concentration (Belafi-Bako et al., 2002), (Dossat et al., 1999),(Watanabe *et al.*, 2007).

1.4.1 pH and temperature

In a study about influence of pH and detergents presence over the activity of lipases from *Thermomyces lanuginosus* and *Yarrowia lipolytica*, Aloulou *et al* reported that the combined effect of pH and adequate amounts of different detergents allowed for modulating the lipolytic activity of cited enzymes. Although the cited work was focused in the modification of lipases extracted from microorganisms to use them in substitution therapy for patients affected by pancreas exocrine activity defficiency, their conclussions are useful for understanding the role of pH and emulssion state of reaction mix in lipases dependent processes. In this context, interfaces formation comprising oil-enzyme, oil-water or water-enzyme can conduct both to an excessive adsorption of enzyme over substrate and to a defficient adsorption rate or, eventually, to the desorption of enzyme and substrate. Keeping control of the proportion of emulsifiers in the reactor and adjusting reaction mix pH can be useful to optimise enzyme operation conditions (Aloulou *et al.*, 2007).

On the other hand, both pH value during the transesterification reaction and pH value when pretreatments of lipase are undertaken prior to its use have strong influence over lipase activity, as well as over emzyme deactivation driven by distortions of its tertiary structure (Shah *et al.*, 2004). The election of an apropiate pH value can result, therefore, in the optimisation of temperature conditions and the enhancement of enzyme thermostability. Gutarra *et al* reported a high stability for *Penicillium simplicissimum* lipase within the pH range 4.0-6.0. Lipase activity was maximal at 50ºC within cited pH value range, and enzyme kept stable and active (although with reduced activity levels) even at high temperature, namely at 70ºC (Gutarra *et al.*, 2009b), (Srinivas *et al*., 2009).

1.4.2 Water, free fatty acids, solvents and carriers

Lipases require a certain amount of water around them to keep their structure and stay active, but, as the transesterification reactions catalysed by these enzymes occur over water-insoluble substrates, an excessive amount of water can lead to drastic decreases in transesterification processes yield (Gilham & Lehner, 2005), (Shah *et al.*, 2004). To face water presence in the fats used as feedstock for biodiesel production, several strategies can be adopted. One of such strategies is the use of organic solvents (Wang *et al.*, 2006), (Shah *et al.*, 2004), (Rosa *et al.*, 2008), detergents or ionic liquids (Ha *et al.*, 2007) that emulsify fats and facilitate the appearance of interfaces where union of lipase and substrates can perform efficiently. Fukuda *et al* adopted a different perspective to afford the problem, developing and patenting a method for the enzymatic production of biodiesel in presence of water. However, lipase recovery and biodiesel purification steps represent a problem to make the system viable (Fukuda *et al.*, 2001b). A third approach to the water problem involves the use of molecular sieves with different pore diameter (Shah *et al.*, 2004), lipase immobilisation over carriers like

kaolinites, zeolites or microporous matrixes based in light and easy to manipulate materials, such as polyurethane or polystyrene (Iso *et al.*, 2001), (Hsu *et al.*, 2002), (Dizge & Keskinler, 2008) and the use of cellular *whole cell* biocatalysts, based on the adsorption of chemically pretreated microorganisms cells to carriers in order to provide more protection to the enzyme (Fukuda *et al.*, 2008).

1.5   Microorganisms with biotechnologycal interest for biofuels synthesis

1.5.1   Lipolytic microorganisms

Isolation of lipases producing microorganisms is always a target, because of the versatility and utility of these enzymes. Given that the different lipases applications, enzymes with different characteristics can be needed, namely lipases with a higher or lower selectivity for certain substrates, or higher or lower stability and activity at different temperatures and under different pH, osmotic strength and organic solvents concentration conditions, the isolation of microorganisms with new lipases is linked to the development of new methods of biofuels production, additives used in food industry or different fatty acids derivatives used in cosmetics or pharmaceutical industries (Joseph *et al.*, 2008), (Takac & Unlu, 2009), (Kandasamy еt аl., 2010), (Uttatree еt аl., 2010), (Stergiou еt аl., 2013), (Whangsuk еt аl., 2013).

One of the most efficient strategies for isolation and cultivation of lipolytic microorganisms is the use as selective media of several industrial wastes and other fatty acids rich materials. Thus, citing some examples, lipolytic strains belonging to *Bacillus* genus using olive oil mill wastewater (OMW) as selective medium; lipolytic strains of the genera *Acinetobacter*, *Microbacterium*, *Proteus*, *Pseudomonas*, *Sphingobacterium*,

Lactococcus, *Enterococcus*, *Delftia* or *Brevundimonas* can be isolated from refrigerated milk (Hantsis-Zacharov & Halpern, 2007); or lipolytic strains belonging to *Penicillium* genus can be isolated from waste cooking oils (WCO) or oils processing wastes (Gutarra *et al.*, 2009a), (Li *et al.*, 2009), (Kumar *et al.*, 2012).

1.5.2 Bioethanol production

Although several ethaol producing microorganisms can be cited, especially genetically modifiedt ones, such as *Zymomonas mobilis*, *Pichia stipitis* or *Eschericia coli*, the most used microorganism in bioethanol production are several species of yeast from *Saccharomyces* genus. Both traditional strains used in brewering and bread production industries, like *Saccharomyces cerevisiae*, *S. carlsbergensis*, *S. ellipsoideus*, *S. fragilis* y *S. pombe*; and new strains subjected to genetical engineering, obtained by different selection methods from *wild* strains of the cited yeast genera and species, can b*e* used *for* bioethanol production. For brine fermentation, *Torula cremoris* or *Candida pseudotropicalis* are used, although different species of *Saccharomyces* could also be applied (Joshi *et al.*, 2012). Anyway, yeasts are carefully selected for their high growing and fermentation rates, high ethanol production yield, ethanol and glucose tolerance, osmotolerance, low optimal fermentation pH, high fermentation temperature and physico-chemical stress resistance. Glucose tolerance facilitates the conversion of concentated substrates in concentrated products, lowering energy needs for distillation and splitting. Osmotolerance facilitates the use of bulk feedstocks like blacks*trap molasses*, with a high salts content. Besides, osmotolerance allows the recycling of distillates with high protein concentration, reducing additionally distillation costs of final splitting steps. Acidic pH fermentation minimises fermenter contamination by competing microorganisms risk. Physico-chemical stress resistance allows the yeast to

support both the ordinary stress associated to the normal fermenter handle and the damages originated from alterations of the fermentation process. Years of selection of different industrial strains have resulted in yeasts with specific characteristics for different applications. Many of the best yeast strains are proprietary, but others are available at different microbiolgycal cultures collections (Davis & Jung, 1974).

1.6   Fatty acids enzymatic trasnesterification

1.6.1   Lipases and esterases

The terms esterase and lipase usually are used without distinction in the literature focused on these enzymes and their applications. However, they are two clearly different groups of enzymes. Esterases and lipases can be diferentiated according to the phsico-chemical nature of the preferred substrates of both kinds of enzymes and the chain length of the acyl residues of the considered substrates.

1.6.1.1 Lipases

Lipases belong to hydrolases enzymes class (E.C.3.). They act over ester bonds (E.C.3.1) of carboxylic esters (E.C.3.1.1.). Lipases hydrolise trliglycerodes to fatty acids, diacylglycerol, monoacylglycerol and glycerol; and therefore are named triacylglycerol acyl hydrolases (Carriere *et al.*, 1994). Besides, lipases or triacylglycerol hydrolases (EC 3.1.1.3) are able to catalyse carboxyl ester bonds hydrolysis into triglycerides, yielding free fatty acids (FFAs) and glycerol at lipid-water interface of emulsions; acting preferably over water insoluble substrates such as long chain fatty acids containing triglycerides (Jaeger & Reetz, 1998). These enzymes usually have a good chemoselectivity, regioselectivity and enantioselectivity, although they also present high substrate unspecificity and can present their activity optimum within a wide temperatures range (Joseph *et al.*, 2008). Fats hydrolysis is the primary

reaction catalysed by these enzymes, although they can catalyse also transesterification, interesterification, acidolysis, alcoholysis and aminolysis. Therefore, lipases have multiple industrial applications, from food industry to pharmaceutical industry, as well as agrochemicals and cosmetics production (Pandey *et al.*, 1999), (Jaeger *et al.*, 1999).

1.6.1.1.1 Lipases are able to work under extreme temperatures

Although one of their main attractives as alternative catalysts in biodiesel industry is their soft working temperatures, lipases family accounts for members able to work under thermophilic regime, such as lipase from *Burkholderia cepacia*, which reaches its activity maximum at 60ºC (Dabkowska & Szewczyk, 2009) , and hyperthermophilic regime, such as *Thermoanaerobacter thermohydrosulfuricus* SOL1 and *Caldanaerobacter subterraneus subsp. tengcongensis* lipases (Royter *et al.*, 2009), which have their activity maximum at 75ºC and can tolerate high temperatures until 95ºC. On the other side of the operation temperature spectrum, lipases obtained from psicrophilic and psicrotrophic microorganisms are found, having their activity optimum at 20ºC and keeping stable at a wide temperature range, provided that operation temperature is below 65ºC. For instance, *Bacillus sphaericus* MTCC 7526 lipase presents its optimum at 15ºC and keeps stable up to 30ºC, always under pH = 8 (Joseph *et al.*, 2006), whereas *Microbacterium phyllosphaerae* lipase presents its optimum at 20ºC and becomes unstable over 35ºC, always, as the former lipase, under pH = 8 (Joseph *et al.*, 2006).

1.6.1.2 Esterases

Esterases or carboxyl ester hydrolases (EC 3.1.1.1 and EC 3.1.1.2) are a group of enzymes able to catalyse the breaking and formation of ester bonds (Bornscheuer,

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2002). EC 3.1.1.1 and EC 3.1.1.2 esterases can hydrolyse carboxyl ester bonds only in aqueous solutions and preferably actuate over water-soluble substrates. Besides, they can actuate only over triglycerides containing long chain fatty acids.

1.6.2 Lipase and esterase activities measuring

Lipases and esterases activities can be determined quantitatively and qualitatively by several methods. Gilham *et al* make a robust review of the dofferent techniques used to study the action of lipases and esterases over different substrates (Gilham & Lehner, 2005), classifying them in three big categories: chromogenic assays, fatty acids release quantification and fluorescence assays.

1.6.2.1 Chromogenic assays

One of the most extended methods consist in the use of *p*-nitrophenyl compounds with different aliphatic acyl chains length. Release of *p*-nitrophenol by the action of tested lipases is spectrophotometrically detected as an increase in the absorbance at a 410 nm wavelength (Figure 3).
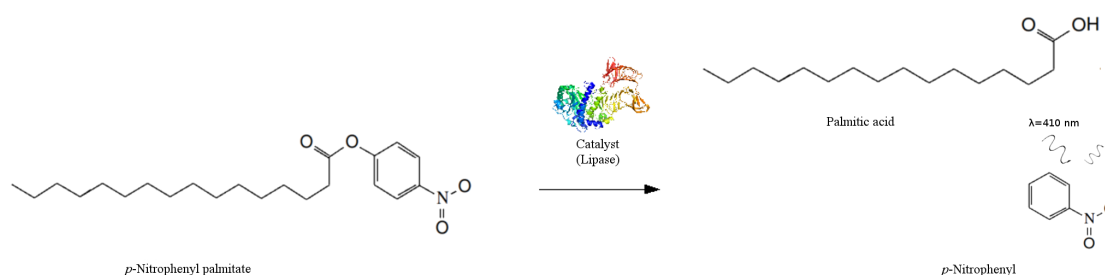

Figure 3. Breaking of *p*-nitro phenyl palmytate by lipase enzyme

p-nitrophenyl esters with different acyl chain length are commercially available . Short chain ones are used for esterase activity determination, whereas long chain ones are used for lipase activity study. The wide diffusion of this protocol resides in the fact that the only a required instrument is an ultraviolet-vissible spectrophotometer, usually

avaiable at a research facility. However, there are some drawbacks for this method, especially the need for emulsifying agents such as Triton X100 or arabic gum, the absorbance changes experienced by *p*-nitrophenyl under acidic pH or spectrophotometry limitations such as the turbidity of samples obtained from cellular lysates. Another limitation of this protocol is the emulsifying agents precipitation and interface disruption at substrate-enzyme interface when the assay is carried out at temperatures lower than 20ºC.In spite of its limitations, this protocol has been, with some modifications detailed in the Materials and Methods section 3.7, the choice protocol to characterise extracellular lipases produced by the studied strains (Gilham & Lehner, 2005).

Other colorimetric methods rely on naftyl esters hydrolysis. Naftol, complexed with a diazonium salt, brings a colored product which presence can be determined at 560 nm wavelength. Similarly to *p*-nitrophenol, different naftol esterd are commercially available (For more details, consult (Gilham & Lehner, 2005) and (Gandolfi *et al.*, 2000)).

1.6.2.2 Released fatty acids quantifying

Spectroscopical assays can be used for measuring turbidity increase generated when fatty acids released by lipase enzyme actuation are precipitated using Calcium. Turbidity increase is meassured at 500 nm wavelength.

Chromatography, either thin layer chromatography, gases chromatography or high performance liquid chromatography (HPLC) is used for directly determinying fatty acids release with a high sensitivity, and allows for the use of physiologically relevant substrates, but it only yields final point results and does not allow for kinetical meassures.