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Bioresources for Third-Generation Biofuels

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1. Introduction

Modern societies' welfare relies greatly on fossil fuels. The current energy model, based on the extensive utilization of fossil fuels, is affected by economic and environmental problems. The United States Department of Energy 2009 report estimates that, within the next two decades, global energy consumption will double (Conti, 2009). On the other hand, the European Commission 2009 report indicates that the management of climate change problems in Europe, since 2000, has been globally unfavourable. Nevertheless, there are some positive signs, such as the 1.4% reduction in 2007 of CO_2 emissions with respect to the figures obtained from 2000 to 2004 in the European Union of Fifteen (E-15). However, considering the 27 European states (E-27), and paying attention to the consumption and production of renewable energy and biofuels, the reduction in emissions has not fulfilled the European Union objectives. Among the motives of this negative evaluation, the fall in the companies' productivity, increased transport and industry emissions and the reduction in research and development areas can be cited (Radermacher, 2009). First- and second-generation biofuels could ameliorate or solve the associated fossil fuel depletion problems, although their recent implantation has raised some doubts. The main problems associated with biofuels are the food vs. fuel controversy; the agricultural and forestry land usage and the actual sustainability of biofuels' production. Third-generation biofuels, based on the microbiological processing of agricultural, urban and industrial residues, could be a possible solution. However, several technical problems must be solved to make them economically viable and easily affordable for the industry (Robles-Medina et al., 2009).

2. First-generation biofuels

The parallel progression in energy demands over depleting oil reserves and rising greenhouse gas emissions entails a high risk of severe impacts on biodiversity, humankind food security and welfare. Thus, a new energy model is needed, based on greener and renewable energy sources, and cleaner as well as more sustainable fuel technology (Fortman et al., 2008; Jegannathan et al., 2009).

2.1 Biogas, syngas, vegetable oils blends and Fischer Tropsch liquids

The first response of heavy industry to the current energy and environmental problems includes some old systems, such as syngas and Fischer Tropsch liquids. Current advances in technology and engineering could bring new opportunities to these classical chemistry and biochemistry solutions, associated with fuel shortage situations such as the Arab oil embargo of the 1970s, or the Second World War. Some of these will be detailed below.

2.1.1 Biogas

Biogas is an attractive source of energy primarily because it is renewable and enables the recycling of organic waste. The production of biogas from manure can help to manage the problems associated with this residue, contributing to the reduction of the greenhouse gas methane. Besides, biomethanation is not only useful for energy production, but also for cleaning up solid waste in urban areas. Compared with bioethanol from wheat and biodiesel from rapeseed, biogas production based on energy crops could generate about twice the net energy yield per hectare per year. Furthermore, biogas could be produced from the by-products generated by the current bioethanol and biodiesel industries (Jegannathan et al., 2009).

Biogas production is based on bacterial methanogenesis in the absence of air of organic matter in a water solution. The process occurs in three steps. The first, hydrolysis, is carried out by strict anaerobes such as *Bacteroides* or *Clostridia*, and facultative anaerobes such as *Streptococci*. It involves the enzymatic transformation of insoluble organic material and higher molecular mass compounds such as lipids, polysaccharides, proteins, nucleic acids etc. into soluble organic materials – energy and cell carbon sources such as monosaccharides and amino acids, among others. In the second step, acidogenesis, other types of microorganisms ferment the mentioned products to acetic acid, hydrogen, carbon dioxide and other lower weight simple volatile organic acids, such as propionic and butyric acid, which are converted to acetic acid. Finally, organic acids, hydrogen and carbon dioxide are converted into a mixture of methane and carbon dioxide by the methanogenic bacteria such as *Methanosarcina spp*. or *Methanothrix spp*. (consuming acetate), as well as microorganisms such as *Methanobacterium sp*. and *Methanococcus sp*., or others that consume hydrogen and formate to yield methane (Jegannathan et al., 2009).

In spite of its attractions, biogas has only been used in rural areas of developing countries and has received investment from governmental and non-profit organizations. The absence of private investment is due to some technical limitations that hamper its economic viability. The process is relatively slow and unstable, and requires large volumes of digester. The decrease in gas generation during the winter season is a serious problem, and can lead to the clogging of the reactor. Other causes for the reduction in gas production are pH and temperature variations, so the loading rate and solid concentration have to be continuously maintained (Jegannathan et al., 2009).

2.1.2 Syngas, biosyngas and Fischer-Tropsch derivatives

Synthetic gas, known as syngas, is a mixture of H_2, CO and CO_2 in different proportions. Traditionally, syngas was produced through gasification of coal at high temperatures, but it can also be produced by methane reformation (submitting the methane to a high temperature water steam stream, or hydrocracking) or by gasification of biomass. In the latter case, the obtained gas is called biosyngas. Syngas and biosyngas can be used directly as fuel, but they also can serve as precursors for other fuels, such as hydrogen, obtained by the compression of carbon monoxide and dioxide. Also, by Fischer-Tropsch synthesis (FTS), short and long chain hydrocarbons can be obtained from the aforementioned H_2, CO and CO_2 mixture (Srinivas et al., 2007). Fischer-Tropsch synthesis was discovered in the first half of the twentieth century and developed for large-scale production during the Second World War. It is based on the polymerization, through successive stages, of H_2 with CO and CO_2, yielding linear hydrocarbons. Iron, cobalt or ruthenium can be used as catalysts (Huber et al., 2006). FTS can be developed at high or low temperature. The high temperature FTS is

performed at 330–350ºC yielding mostly short-chain hydrocarbons (gasolines) and light olefins in a fluidized-bed reactor. On the other hand, low temperature FTS develops at 220–250ºC in a slurry bubble column reactor, and waxes and long-chain hydrocarbons are obtained (Bludowsky & Agar, 2009). As FTS is an extremely exothermic reaction, it can be coupled with biomass gasification. However, FTS has some drawbacks, such as the fact that complex mixtures of different chain lengths are always obtained. Thus, FTS products have to be separated prior to subsequent processes (Huber et al., 2006).

2.1.3 Vegetable oil blends

The direct usage of crude or filtered vegetable oils for diesel engine fuel is possible by blending them with conventional diesel fuels in a suitable ratio. These blends are easy to obtain and keep stable for short-term use. But vegetable oils present high viscosity, acid contamination and free fatty acids that lead to gum formation by oxidation, polymerization and carbon deposition (Ranganathan, 2008). Thus, the long-term utilization of vegetable oils for fuel leads to filter clogging, nozzle blockage and deposits in the combustion chamber (Sidibé et al., 2010). Alongside the long-term problems in injection systems, filters and combustion chamber, doubts about the sustainability of using crude vegetable oil for fuels have to be considered. Vegetable oils are expensive, and their direct use in engines or as feedstock to produce petro-diesel substitutes would encounter the same economic and environmental problems that affect the conventional biodiesel and bioethanol industries (UNCTAD, 2010).

A more interesting solution is the usage of waste cooking oil (WCO; also called waste frying oil, WFO). Waste cooking oil is widely produced, inedible, and could serve as a low-cost and almost ready-to-use substitute for fossil origin diesel. As crude vegetable oil, waste cooking oil has a high viscosity. Besides, it is enriched with free fatty acids and, hence, can generate clogging problems in unmodified diesel vehicles, especially in temperate climates and during the ignition of the engine. Viscosity problems are usually bypassed by blending WCO with petrol diesel or by using transesterification to produce biodiesel (Pugazhvadivu et al., 2005; Al-Zuhair et al., 2009; Chen et al., 2009). Al-Zuhair et al. studied the production of biodiesel with lipases from *Candida antarctica* and *Burkholderia cepacia*, both free and immobilized in ceramic beads, with or without solvents. They found that clay micro-environments protected immobilized *B. cepacia* lipase from methanol damage (Al-Zuhair et al., 2009). Also, Pugazhvadivu et al. proposed solving the injection and filter-clogging problems by preheating the waste cooking oil (Pugazhvadivu et al., 2005), by comparing the performance of a diesel engine when using conventional diesel and waste frying oil, preheated at different temperatures, as fuel. They found that preheating the waste frying oil to 135ºC improved the overall yield of the engine. In particular, the brake specific energy consumption and brake thermal efficiency were improved, and the engine exhaust CO and smoke density were reduced considerably compared to WFO preheated at 75ºC. They concluded that WFO could be used as a diesel fuel by preheating it to 135ºC.

2.2 Bioethanol and biodiesel

Bioethanol and biodiesel are frequently claimed as the most realistic alternatives to fossil fuels. These renewable fuels can be extensively produced, and both the fossil fuel distribution and engines can be easily adapted to work with blends of ethanol and gasoline, diesel and biodiesel, or even pure ethanol and pure biodiesel (Da Costa et al., 2010). But, in order to play a significant role in fossil fuel substitution, these renewable fuel industries

should overcome technical limitations in production process efficiency and feedstock-related issues (UNCTAD, 2010). Decisions about feedstock election, catalysis technology or energy gain along the production process are of paramount importance for proper biodiesel and bioethanol production.

2.2.1 Bioethanol and biodiesel production

Bioethanol is produced from simple sugar-rich raw materials or from starchy feedstock, from which simple sugars can be easily processed and released, which are fermented to produce ethanol. Bioethanol production comprises three steps. Firstly, the complex sugars are hydrolysed to release glucose. Subsequently, the glucose is subjected to a second fermentation step carried out by yeasts such as *Saccharomyces cerevisiae;* for example, yielding ethanol and carbon dioxide. The third step consists of a thermochemical process and is based on the distillation of the diluted ethanol to obtain highly concentrated ethanol. When using lignocellulosic raw materials such as agricultural residues (corn stover, straw, sugar cane bagasse), forestry waste, wastepaper and other cellulosic residues, a chemical or enzymatic hydrolysis pretreatment to degrade the lignin is needed. This additional step reduces the efficiency of the process. Some improvements have been achieved by the engineering of cellulases from the *Trichoderma* genus fungi (Fukuda et al., 2006) and the utilization of microorganisms able to simultaneously express the cellulase and enzymes needed for the ethanol fermentation pathway, such as piruvate descarboxilases and alcohol dehydrogenases (Lu et al., 2006; van Zyl et al., 2007; Jegannathan et al., 2009; Rahman et al., 2009; van Dam et al., 2009). However, these improvements have still not generated an efficient and economically affordable process.

With regard to biodiesel, it consists of a mixture of fatty acid alkyl esters (FAAE) obtained by the transesterification of fatty acids and straight chain alcohols (generally ethanol or methanol), mainly from vegetable oils. When methanol is the alcohol of choice, the term used to refer to the biodiesel is fatty acid methyl esters (FAME), while the ethanol-derived biodiesel is known as fatty acid ethyl esters (FAEE). The properties of the biodiesel obtained from ethanol or methanol are very similar, but methanol is the preferred alcohol in spite of its toxicity and fossil fuel origin because of its low cost and wide availability (Ranganathan et al., 2008; Fjerbaek et al., 2009).

The commercially delivered biodiesel is mainly obtained by the chemical transesterification of the triglycerides contained in sunflower, rapeseed or palm oil. This process can be carried out by acid and alkaline liquid catalysts (Kawahara & Ono, 1979; Jeromin et al., 1987; Aksoy et al., 1988; Fukuda et al., 2001), or heterogeneous solid catalysts such as supported metals, basic oxides or zeolites (Cao et al., 2008). The preferred catalysts are the liquid ones, particularly the alkaline ones, because these catalysts are cheap, very versatile and yield less corrosive fuel than the acid catalysts. Also, liquid catalysts are preferred because the reusable solid catalysts are still withdrawn with mass transfer and reactant diffusion problems. However, the alkaline catalysis has several limitations, especially the futile consumption of the catalyst, problems of viscosity, mass transfer and recovery of biodiesel and by-products owing to the saponification of the catalyst and free fatty acids in the presence of water (Freedman et al., 1984; Zhang et al., 2003; Jaruwat et al., 2010). These problems are bypassed by high temperature reaction conditions, addition of organic solvents to manage the water presence or enhance the interface formation, or increase of the alcohol:catalysts ratio (Kawahara & Ono, 1979; Fukuda et al., 2001). Thus, the process requires high energy inputs to maintain high temperatures conducive to viable

transesterification rates, and to separate methanol. Besides, the process generates alkaline waste water that requires treatment prior to its disposal (Jaruwat et al., 2010). Jointly, all these negative factors raise doubts about the sustainability and environmental benefits of the biodiesel industry.

2.2.2 Bioethanol and biodiesel advantages and drawbacks

Extensive bioethanol and biodiesel implantation has been followed by a panoply of economic, sociopolitical and environmental issues (Guerrero-Compeán, 2008). It is worth noting the strong dependency of these biofuels industries on crops used for human nourishment and the feeding of livestock (UNCTAD, 2010). Although a large number of patents have been proposed to solve many technical problems, the sudden peak in demand for biofuels has uncovered serious technical limitations of the currently used production systems. As a consequence, a growing controversy about the real sustainability and environmental friendliness of the actual biofuels industry has been generated (Fortman et al., 2008; Abdullah et al., 2009; Demirbaş, 2009; Yee et al., 2009; Jaruwat et al., 2010).

In addition, the consequences of biofuel production for farming practices or food markets, as well as real greenhouse gases (GHG) emission reduction along the biofuel life cycle, represent an important issue that, frequently, is not clearly treated. Parameters such as the kind of biofuel under study, feedstock, and energy inputs needed to maintain the process of transformation need to be taken into account. Also, the possibility of cogeneration of electricity or the exchange of energy between the biofuel synthesis and the feedstock transformation processes must be added to the model. Thus, wide variations in the net energy gain and consumption of resources can occur owing to the different assumptions made to calculate the overall benefits and drawbacks. Timilsina and collaborators draw a general picture of this issue over the OECD estimations. According to these authors, the most efficient biofuel production scheme is represented by sugarcane-based bioethanol in Brazil, with a 90% GHG reduction with respect to the gasoline equivalent. This high efficiency relies mainly on the high yield of this crop and the usage of sugarcane as an energy source for production plants and the cogeneration of electricity. Second-generation biofuels based on cellulosic feedstocks present a 70–90% GHG reduction relative to gasoline or diesel. Combined with electricity cogeneration, this kind of biofuel could have an even greater effect on GHG reduction, but they are still under development. Ethanol from sugar beet GHG reduction ranges from 40 to 60%, while wheat-based ethanol presents a 30–50% GHG reduction. The corn-based production of bioethanol is the least GHG-reducing biofuel and presents a low efficiency at GHG reductions varying from 0 (even negative in some cases) to 50% compared to gasoline (OECD, 2008; Timilsina & Shrestha, 2010).

3. Second-generation biofuels

Theoretically, biofuel implantation in transport and industry should solve, or at least improve, the ecological and economic problems derived from the unsustainability of the fossil fuel-based energy model.

However, recent field experiences indicate a much more complex scenario. The market economy and unbalanced relations between different sectors of the economy and national markets generate unpredictable dynamics of fuels' raw material prices. In this context, the development of subsequent new commercial and industrial opportunities has altered the already unstable behaviour of the agricultural international markets. The sudden peak in demand for grain, owing to its usage as a raw material for the production of ethanol, has

abruptly increased the prices of corn (Fischer et al., 2009). The demand pressure has operated similarly in the palm oil market, generating a palm oil tree and soy culture surface expansion in several regions, with spectacular dimensions in South-East Asia (Abdullah et al., 2009; Jaruwat et al., 2010), where the biofuels fever threatens biodiversity and has a deep social impact because of the proliferation of unregulated, intensive, agricultural practices and the switching of oil usage for traditional human nutrition, housekeeping and livestock feed (Fortman et al., 2008; Guerrero-Compeán, 2008; Demirbaş, 2009; UNCTAD, 2010; Yee et al., 2009).

3.1 Feedstock costs and biofuel competition

Biodiesel usually costs over 0.5 US$/l, compared to 0.35 US$/l for petroleum-based diesel (Demirbaş et al., 2009). It is reported that the high cost of biodiesel is mainly due to the cost of virgin vegetable oil (Krawczyk, 1996; Connemann & Fischer, 1998). For example, the soybean oil price is currently 1.27 $/l while the palm oil price is 1.18 $/l (World-Bank, 2011). Biodiesel from animal fat is currently the cheapest option (0.4–0.5 US$/l), while the traditional transesterification of vegetable oil is, at present, around 0.6–0.8 US$/l (Bender, 1999). Zhang et al. (2007) stated that there is no global market for ethanol. Within the reasons for this, crop types, agricultural practices, land labour costs, production plant sizes, processing technologies and government policies can be cited. The cost of ethanol production in a dry mill plant currently totals 0.44 US$/l. Corn represents 66% of operating costs while energy (electricity and natural gas) to fuel the production plant represents nearly 20% of operating costs. Nevertheless, ethanol from sugar cane, produced mainly in developing countries with warm climates, is generally much cheaper to produce than ethanol from grain or sugar beet (Bender, 1999). For this reason, in countries like Brazil and India, sugar cane-based ethanol is becoming an increasingly cost-effective alternative to petroleum fuels. On the other hand, ethanol derived from cellulosic feedstock using enzymatic hydrolysis requires much greater processing than from starch or sugar-based feedstock, but feedstock costs for grasses and trees are generally lower than for grain and sugar crops. If targeted reductions in conversion costs are achieved, the total cost of producing cellulosic ethanol in EOCD countries could fall below that of grain ethanol.

Estimates show that ethanol in the EU becomes competitive when the oil price reaches 70 US$/barrel, while in the USA it becomes competitive at 50–60 US$/barrel. For Brazil and other efficient sugar producing countries such as Pakistan, Swaziland and Zimbabwe, the competitive ethanol price is much cheaper, between 25–30 US$/barrel. However, anhidrous ethanol, blendable with gasoline, is still more expensive, although prices in India have declined and are approaching the price of gasoline. Although the feedstock costs represent the majority of biofuels' cost, the production plant size can reduce the final cost of the fuel. Thus, the generally larger USA conversion plants produce biofuels, particularly ethanol, at lower cost than plants in Europe. Production costs are much lower in countries with a warm climate such as Brazil, with less than half the costs of Europe. But, in spite of the reduced costs of production, ethanol from Brazil is competitive with gasoline owing to the huge sugar cane production and the cogeneration of electricity (Demirbaş et al., 2009).

3.2 Brazilian and USA models of implementation for the bioethanol industry

Since the Arab oil embargo of the 1970s, Brazil has made an incomparable effort in the reduction of its energy dependency by intensifying and extending sugar cane-based bioethanol production. Although the alternative periods of scarcity and abundance of oil

have marked fluctuations in the strength of the Brazilian Alcohol National Programme (Proalcool), the global trend has been an ascending progression in the total production of alcohol, as well as in the yield per hectare of sugar cane, and the implantation of this alcohol as transportation fuel. Today, Brazil is the second largest worldwide ethanol producer. In this way, Brazil has reduced its energy dependency, and has become the first ethanol exporter. According to Brazilian Government data, this milestone has been achieved on the basis of rural employment and welfare improvement. The key aspects of the Proalcool programme are a combination of technological advances, social planification and projection of the bioethanol industry. According to the Brazilian Government (Da Costa et al., 2010), owing to the high productivity of sugar cane, Brazil has expanded ethanol production and use without a significant increment in the fertile land surface used to cultivate sugar cane, or a food vs. fuel competition. However, there are several authors who are not so enthusiastic with the success of the Brazilian model, and point to the sugar cane industry as one of the reasons for the losses in biodiversity and the expansion of agricultural land over doubtfully catalogued marginal land, which is more relevant and dangerous than the Brazilian Government data indicates (Coelho et al., 2008; Gauder et al., 2011).

On the other hand, the American bioethanol industry choice of corn grain as its raw material has been followed by a dramatic rise in the prices of corn derivatives. Although the USA production of bioethanol supersedes the Brazilian one, the production:consumption ratio of the former (1:3) is much smaller than the latter (8:3). Despite its commercial orientation, the global efficiency of the USA model is low compared with the Brazil system and relies on the high importation taxes that protect the American industry from foreign ethanol inputs (Da Costa et al., 2010). Finally, the narrow margin of the USA production:consumption ratio suggests that the model has reached a production glass ceiling that blockades the medium-term implantation of biofuels in American society and hampers their exportation (UNCTAD, 2010; Da Costa et al., 2010).

3.3 Europe and Asia: Chemically catalyzed biodiesel

The European and Asian strategy to improve climate change and fossil fuel depletion problems is based mainly on the chemically catalyzed biodiesel obtained from vegetable oils. There is a variety of feedstocks for the production of this biofuel, from inedible oils, (mainly rapeseed oil in Europe or jatropha oil in Asia), to edible oils (principally sunflower oil in Europe and palm oil or soybean oil in Asia, although corn, peanut, cotton seed or canola oil can also be cited) (Ranganathan et al., 2008; Abdullah et al., 2009). As the elected method for industrial biodiesel production is chemical catalysis, these vegetable oils are preferred to other heterogeneous lipids sources. These other lipids need pretreatment prior to their use (Peterson, 1986; Fortman et al., 2008), and include waste frying oils, waste-activated bleaching earth from the oil refinery industry, and even animal origin lipids such as beef tallow, lard, yellow grease and poultry grease or fat from fat traps, septic tanks, or waste water sludges. The need for economically viable vegetable oils for biodiesel production implies the cultivation of greater areas with oil-producing crops such as sunflowers or palm oil trees. Thus, the previously mentioned rising corn prices, owing to the derivation of huge amounts of grain for the industrial production of bioethanol, is neither an isolated case in developing biofuel industries nor the only aspect of the biofuel industry issue. Like the bioethanol industry, the European and Asian biodiesel industries have the energy and chemical problems associated with the current biofuels model. These limitations can be summarized according to nearly obsolete technology, being strongly

dependent on chemical catalysis, non-renewable materials and promotion of non-sustainable market and farming practices (Guerrero-Compeán, 2008; Demirbaş, 2009; UNCTAD, 2010).

3.4 Technical aspects of biodiesel production

The industrial production of biodiesel needs to solve several technical problems in order to obtain this kind of biofuel in an efficient and sustainable way. The physical factors to consider can be summarized by pH, temperature, hydric activity, solvents and supports. Depending on the catalyst used to drive the transesterification reaction, some of the cited factors have different impacts on the global efficiency and feasibility of the process. A non-optimal configuration of the system can reduce significantly the biodiesel yield and compromise the viability of the production plant, especially if the upstream by-products, excess catalyst or auxiliary devices for solvent recovery hinder an easy, clean and rapid downstream processing of the biofuel.

3.4.1 pH, temperature and hydric activity

As mentioned above, the chemical catalysis of the transesterification reaction requires high temperatures to achieve an acceptable reaction rate. In the case of the alkaline catalysis, the minimal temperature to produce conventional biodiesel is 60ºC, while in the acid catalysis the temperature ranges from 50 to 80ºC (Robles-Medina et al., 2009). Acid catalysis is slower than the alkaline one and generates a more corrosive fuel, so alkaline catalysts are preferred by the industry. It incurs a great energy cost in order to initiate and maintain the reaction (Kawahara & Ono, 1979; Aksoy et al., 1988; Cao et al., 2008). However, the utilization of sodium hydroxide as a catalyst has a serious limitation in the form of saponification of free fatty acids if water is present. This drives the increased consumption of the catalyst and downstream processing problems, such as the separation of glycerol and unreacted precursors. The solutions to manage this problem include using only virgin oils, often edible vegetable oils, instead of oils with high free fatty acids and water content, such as waste cooking oils or animal origin fats, as well as other residual fats. Higher temperatures, up to 120ºC, and the addition of organic solvents, or additional steps for free fatty acids esterification with sulphuric acid before performing the alkali-catalyzed transesterification are quite common as well (Jeromin et al., 1987).

When lipases are used as catalysts, it is possible to get over the saponification problems owing to their ability to transesterificate alcohols with both triacylglycerols and free fatty acids. Besides, lipases work as well in the presence of water. In fact, they need a certain hydric activity to maintain their tridimensional structure, so the presence of water is not a problem with this kind of catalyst — although excessive hydric activity affects the transesterification reaction because the substrates are water insoluble (Jaeger & Eggert, 2002; Shah et al., 2004; Gilham & Lehner, 2005; Fjerbaek et al., 2009). Lipases can operate at low or relatively low temperatures in the range of 20 to 70ºC, and at even lower temperatures if the enzyme has been obtained from psycrophilic microorganisms (Dabkowska & Szewczyk, 2009). Depending on the chosen lipase and preparation (free, immobilized or *whole cell catalyst*), lower temperatures (below 65ºC) can be applied to avoid the thermal denaturation of the enzyme, thus saving in production costs (Fukuda et al., 2008). Within the thermostable lipases, we can cite *Burkholderia cepacia* lipase (Amano PS lipase, from Amano Pharmaceutical Co., Japan), that reaches its highest activity at 60ºC (Dabkowska & Szewczyk, 2009), and the lipases obtained from *Thermoanaerobacter thermohydrosulfuricus*

SOL1 and *Caldanaerobacter subterraneous* subsp. tengcongensis, which show their activity maximum at 75ºC and tolerate temperatures as high as 95ºC (Royter et al., 2009).
On the other side of the spectrum, the lipase from *Bacillus sphaericus* MTCC 7526 presents its optimal temperature at 15ºC, keeping stable until 30ºC, and the *Microbacterium phyllosphaerae* lipase presents the optimal temperature at 20ºC and deactivates when the temperature exceeds 35ºC, with the pH value fixed at 8 for both psycrophilic enzymes (Joseph et al., 2006; Srinivas et al., 2009). Therefore, pH plays an important role in the enzymatic production of biodiesel because it influences both the reaction rate and the thermal stability or solvents' susceptibility of the lipases. An adequate pH can facilitate the optimization of the operation temperature and improve the activity of the enzyme. Gutarra and collaborators reported a high stability of the *Penicillium simplicissimum* lipase in the pH range 4.0–6.0, that showed the maximal activity at 50ºC and remained stable and active (although with a lower activity) even at 70ºC (Gutarra et al., 2009).

3.4.2 Heterogeneous catalysts and immobilized enzymes

An alternative to the chemical transesterification of low quality oils with a relatively high concentration of water or free fatty acids consists of heterogeneous catalysis using acidic cation-exchange resins, supported metals (Zabeti et al., 2009), basic oxides or zeolites (Knezevic et al., 1998; Suppes et al., 2004). Even low cost alternatives such as waste eggshell have been proposed as well (Wei et al., 2009). These kind of catalysts are considered as an intermediate and relatively low-cost solution between the traditional homogeneous catalysts and the lipases. However, the cited heterogeneous catalysts are affected by the slow diffusion of the triglycerydes through their pores and require a higher alcohol:oil ratio to accelerate the reaction, in order to increase the production (Zabeti et al., 2009). Nevertheless, the heterogeneous catalysts can serve to improve the reusability and efficiency of immobilized enzymes and *whole cell catalysts*. Immobilization of enzymes on inert materials such as porous ceramic beads (Iso et al., 2001) or polymeric resins (Dizge et al., 2008; Dizge et al., 2009) can improve their performance. This improvement is owing to the protection that the pores' microenvironment brings to the enzyme, avoiding the inhibition or damage of the enzyme caused by methanol or solvents. Another attractive approach to the immobilization is the so-called protein-coated microcrystals technology (PCMC). PCMC is based on the use of crystalized proteins as a support to the lipases, or in the direct use of crystalized lipases as solid catalysts (Raita et al., 2010). However, the real increase in reaction rate and enzyme stability with these immobilization techniques is usually lower than the theoretically expected. One of the reasons for these lower rate issues responds to the blockage of the pores of the used material as support because of the precipitation of glycerol or the insufficient circulation of substrates around the enzyme (Zabeti et al., 2009). The knowledge generated by the intense research on production and use of supports, resins and porous metallic alloys can be useful for enzymatic production of biodiesel. With this comparative approach, the optimization of the immobilized enzyme technology could be a reality in the short rather than medium term.

3.4.3 Alcohol to oil ratio and solvents

Depending on the kind of catalyst used and the selected operation conditions in the biodiesel production plant, the alcohol to oil molar ratio will present a wide variation. Adding excess alcohol is a common practice, and could serve as reference. However, excess alcohol use implies higher reactant associated costs, especially when the alcohol of choice is

ethanol, which is more expensive than methanol. Thus, a more detailed approach to the system optimization in terms of minimal alcohol consumption is needed. Besides, a fine adjustment of the alcohol to oil ratio allows the maximal biodiesel production in the shortest possible time span and with the lowest energy input (Shieh et al., 2003).

The optimization is a relatively simple task when homogeneous catalysts such as sulphuric acid or sodium hydroxide are used to perform the conventional transesterification of vegetable oils with methanol. High yields are achieved with a methanol to oil ratio of 1:1 with an alkaline catalyst (although to improve the yield this proportion rises to 6:1) and a 30:1 ratio when an acid catalyst is used (Zhang et al., 2003).

However, in the case of lipase-catalyzed biodiesel, the situation is more complex and the molar ratio of alcohol to oil varies depending on the type of lipase, the use of an immobilized or free enzyme, and the alcohol used. Similar to the chemical catalysts, an increase of the molar alcohol:oil ratio elevates the efficiency of the reaction, but an excessive alcohol content inhibits and even damages the enzyme, especially when using methanol and free enzymes. Although the lipase-based solvent-free systems are under intensive research, owing to advantages such as the direct saving in solvents and the indirect cost reductions in downstream processes, the utilization of lipases does not necessary mean abandoning the use of a certain amount of solvents. The addition of solvents like *t*-butanol, diesel oil, hexane or dioxane to the precursors of biodiesel usually allows a better mixing of the reactants. Thus, solvents relieve the problems associated with the different water solubility of lipids and alcohols. In addition, solvents provide a more durable interaction between the enzyme and its substrates, and can favour the circulation of reactants through resins and support pores in immobilized enzyme systems. This improved circulation confers some protection to the lipases against inhibition by substrates and damages by excessive alcohols. However, solvents' addition has to be carefully studied, since an excess of solvent or an inadequate amount of solvent can affect the enzyme activity and stability. For example, Shieh et al. studied the optimal operation conditions to transesterificate soybean oil with methanol by *Rhizomucor miehei* lipase immobilized on macroporous weak anionic resin beads. They found that the best transesterification rate was obtained when the methanol:oil molar proportion was 3.4:1 at 36.5ºC (Shieh et al., 2003). Raita et al. studied the transesterification of palm oil with ethanol by *Thermomyces lanuginosa* lipase-coated microcrystals in the presence of *t*-butanol. In this case, the optimal conditions were ethanol to fatty acids 4:1 molar ratio and *t*-butanol:tryacylclycerides 1:1 molar ratio, at 45ºC (Raita et al., 2010. However, Tongboriboon et al. worked on the solvent-free transesterification of used palm oil with *Thermomyces lanuginosa* and *Candida antarctica* lipases immobilized in porous polypropylene powder, reporting that the best yield was achieved at an ethanol to oil ratio of 3:1, and the yield decreased when the molar ratio was increased to 4:1 at 45ºC (Tongboriboon et al., 2010). These authors pointed to the inhibition of the enzymes by an excessive amount of ethanol, although it is worth emphasizing that they worked on a solvent-free system, so the enzyme was relatively vulnerable to alcohol-driven damage. On the other hand, Shah et al used 4:1 ethanol to oil molar ratio as standard reaction settings in their study about the transesterification of jatropha oil with ethanol at 40ºC. The experimental design consisted of a solvent-free system and three different lipases (free and immobilized on Celite), namely *Chromobacterium viscosum*, *Candida rugosa* and *Porcine pancreas* lipases, although they did not try different alcohol to oil molar ratios (Shah et al., 2004).

4. Third-generation biofuels

As a response to the problems associated with the recent worldwide implantation of second-generation biofuels, some authors propose focusing on the processes involved in the production of such biofuels. This new approach consists of the utilization of microbial enzymes to achieve the current chemical pretreatment steps of cellulosic or starchy raw materials (Carere et al., 2008). Microorganisms deal with the degradation of lignocellulose, hemicellulose or lipid-rich materials by means of enzyme catalyzed processes at near to room temperature. Therefore, microbial enzymes could be used to make the current biofuels industry cleaner and greener. Furthermore, the production of biofuels would be coupled with the management of woody and oily wastes, converting these residues into suitable and cheap raw materials (Steen et al., 2010).

4.1 Microalgae-based biodiesel production

Another promising lipids source, still not implemented but currently being studied worldwide, is represented by microalgae. Microalgae have a high potential as biodiesel precursors because many of them are very rich in oils, sometimes with oil contents over 80% of their dry weight, although not all species are suitable as biodiesel production oils (Chisti, 2008; Manzanera, 2011). Besides, these microorganisms are able to double their biomass in less than 24 hours, achieving a reduction between 49 and 132 fold in the medium culture time required by a rapeseed or soybean field. Furthermore, microalgae cultures require low maintenance and can grow in wastewaters, non-potable water or water unsuitable for agriculture, as well as in seawater (Mata et al., 2010). The production of microalgae biodiesel could be combined with the CO_2 removal from power generation facilities (Benemann, 1997), the treatment of waste water from which microalgae would remove NH_4^+, NO_3^- and PO_4^{3-} (Aslan & Kapdan, 2006), or the synthesis of several valuable products, from bioethanol or biohydrogen to organic chemicals and food supplements (Banerjee et al., 2002; Chisti, 2007; Rupprecht, 2009; Harun et al., 2010). However, microalgae biomass-based biofuels have several problems ranging from the optimization of high density and large surface units of production to the location of the microalgae production unit. Anyway, the main decisions to take are the adoption of open or closed systems, and the election of batch or continuous operation mode. As will be discussed below, depending on the system and mode of operation choice, there will be different advantages and drawbacks.

4.1.1 Open vs. closed systems

Microalgae can be cultivated in open-culture systems such as lakes or (raceway) ponds, and in closed-culture systems called photobioreactors (PBRs). Open-culture systems are normally cheaper to build and operate, more durable and have a higher production capacity than PBRs. However, open systems are more energy expensive in terms of nutrient distribution owing to mass transfer problems, and have their depth limited to 15 cm, to ensure that the microalgae receive enough light to grow. Moreover, ponds are more sensitive to weather changes, and temperature, evaporation and light intensity controls are not feasible. Furthermore, these open systems require more land area than PBRs, and are more susceptible to contamination, both from bacteria and from microalgae present in the surroundings of culture installations (Manzanera, 2011).

In contrast, PBRs are more flexible and are intensive land-usage systems that can be configured according to the specific physical-chemical requirements of the algae of choice,

allowing the cultivation of species unsuited to open ponds. Nutrient homogenization, light distribution, pH, temperature, CO_2 and O_2 control can be achieved in photobioreactors. Thus, closed systems provide more stable and appropriate growing conditions, allowing higher cell densities and minimizing contamination. Nevertheless, PBRs have several technical problems that make them non-competitive in applications that can be achieved in raceway ponds. Such problems are overheating, bio-fouling, shearing stress, oxygen accumulation, scaling-up difficulties and the high costs of building, operation and maintenance (Chen et al., 2011).

Within these problems, it is worth highlighting capital building investment and high operation costs. PBRs biomass production costs may be one order of magnitude higher than in open systems. If the biomass added value is high, PBRs can be competitive. Otherwise, open ponds will be the preferred option. However, the evaluation of performance of open and closed systems is complex and depends on several factors, such as algal species or productivity computation method. Three parameters are commonly used to evaluate productivity in microalgae cultivation installations. Firstly, volumetric productivity (VP), that is, productivity per unit of reactor volume ($g/l \cdot d$). The second parameter is area productivity (AP), defined as productivity per unit of ground area occupied by the reactor ($g/m^2 \cdot d$). The third one is illuminated surface productivity (ISP), namely the productivity per unit of reactor illuminated surface area ($g/m^2 \cdot d$). Nevertheless, the election of closed or open systems relies on more aspects apart from productivity, as will be discussed below (Richmond, 2010).

4.1.2 Continuous vs. batch operation mode

PBRs can be operated in batch or continuous mode. There are several advantages when using them in continuous mode. Firstly, continuous culture provides a higher control than batch mode. Secondly, growth rates can be regulated and keep in a steady state for long periods, and the biomass concentration can be modulated by dilution rate control. In addition, results are more reliable and reproducible owing to the steady state of continuous reactors, and the system yields better quality production (Molina et al., 2001).

However, there are limitations that can make the continuous process unsuitable for some cases. One of these limitations is the difficulty in controlling the production of some non-growth-related products. For instance, the system often requires feed-batch culturing and continuous nutrient supply that can lead to wash-out. Filamentous organisms can be difficult to grow in continuous PBRs because of the viscosity and heterogeneity of the culture medium. Another problem is that the original strain can be lost if it is displaced by a faster-growing contaminant. The contamination risk and loss of reliability of the bioreactor becomes more relevant when long incubation periods are needed, so the potential initial investment in necessary better quality equipment could rise and hamper the economic viability of the production unit (Mata et al., 2010).

The possible coproduction of high value chemicals could lead to the solution of the above problems, but it implies taking multiple parameters and options into consideration. The microalgae production units will suffer drastic changes, both in the operational aspect (temperature, insolation, wind, microalgal and bacterial or fungical contaminations etc.) and in the commercial one (oscillations in value of by-products, improvements in centrifugation or extraction strategies or development of non-algal biofuels, etc). Taking into consideration all the above mentioned parameters, it can be ascertained that any microalgae-based biodiesel project is unique. Hence, such projects must be designed by thinking in terms of a flexible or even multipurpose and adaptable installation (Richmond, 2010).

4.2 Biodiesel production from oily biomass

Microalgae are not the only option to produce biofuels from oily biomass. Multiple prokaryotes and eukaryotes can accumulate high amounts of lipids. But, as occurred with microalgae, not all species are suitable for biodiesel production owing to differences in the kind of storage lipids. Thus, as stated by Waltermann & Steinbüchel (2010), many prokaryotes synthesize polymeric compounds such as poly(3-hydroxybutyrate) (PHB) or other polyhydroxyalkanoates (PHAs), whereas only a few genera show accumulation of triacylglycerols (TAGs) and wax esters (WEs) in the form of intracellular lipid bodies. On the other hand, storage TAGs are often found in eukaryotes, while PHAs are absent, and WE accumulation has only been reported in jojoba (*Simmondsia chinensis*). All these lipids are energy and carbon storage compounds that ensure the metabolism viability during starvation periods. Similar to the formation of PHAs, TAGs and WE, synthesis is promoted by cellular stress and during imbalanced growth; for instance, by nitrogen scarcity alongside the abundance of a carbon source (Kalscheuer et al., 2004).

The most interesting prokaryote genera in terms of accumulation of TAGs are nocardioforms such as *Mycobacterium sp*., *Nocardia sp*., *Rhodococcus sp*., *Micromonospora sp*., *Dietzia sp*., and *Gordonia sp*, alongside streptomycetes, which accumulate TAGs in the cells and the mycelia. TAGs storage is also frequently shown by members of the gram-negative genus *Acinetobacter* (although, in this case, WE are the dominant inclusion bodies components) (Waltermann & Steinbüchel, 2010). Within eukaryotes, with the exception of algae, yeasts of the genera *Candida* (non *albicans*) (Amaretti et al., 2010), *Saccharomyces* (Kalscheuer et al., 2004; Waltermann & Steinbüchel, 2010) and *Rhodotorula* (Cheirsilp et al., 2011) are the most interesting ones to produce biodiesel feedstocks.

Steinbüchel and collaborators have worked on the heterologous expression of the non specific acyl transferase WS/DGAT from *Acinetobacter calcoaceticus* ADP1 in *Saccharomyces cerevisiae* H1246 (a mutant strain unable of accumulating TAGs) (Kalscheuer et al., 2004). These authors found that the yeast recovered the ability to accumulate TAGs, as well as fatty acid ethyl esters and fatty isoamyl esters. This finding showed that the *Acinetobacter calcoaceticus* transferase had a high potential for biotechnological production of a large variety of lipids, either in prokaryotic and eukaryotic hosts. From this basis, as will be discussed in detail in Section 4.3, they worked on *Escherichia coli* TOP 10 (Invitrogen) and obtained an engineered strain able to produce fatty acid ethyl esters (biodiesel) directly from oleic acid and glucose (Kalscheuer et al., 2006).

Another possibility is combining the biomass obtained from microalgae and yeast, as recently proposed by Cheirsilp et al. (2011). These authors studied a mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* in industrial wastes. The used effluents, including both a seafood processing wastewater and molasses from a sugar cane plant. They found a synergistic effect in the mixed culture. *R. glutinis* grew faster and accumulated more lipids in the presence of *C. vulgaris*, that acted as an oxygen generator for yeast, while the microalgae obtained surplus CO_2 from yeast. The optimal conditions for lipid production were 1:1 microalga to yeast ratio initial pH of 5.0, molasses concentration at 1%, 200 rpm shaking, and light intensity at 5.0 klux under 16:8 hours light and dark cycles (Cheirsilp et al., 2011).

4.3 Whole cell catalysts

Pure or immobilized enzymes obtained from microorganisms could reduce the energy costs of industrial ethanol and biodiesel production. Nevertheless, the cellulases used to treat

(ligno)cellulosic materials such as forestry residues, waste paper or straw are difficult to purify, like the lipases used for the transesterification of lipids yielding biodiesel. Hence, their price is still too high to make their usage economically viable (Shieh et al., 2003; Ranganathan et al., 2008). Another limiting factor for the use of enzymes is the inactivation and inhibition by reactants and substrates. These drawbacks are the object of an intensive effort to make possible the reutilization of enzymes through protein engineering (Ebrahimpour et al., 2008), in order to increase their stability and activity. Research interest is also targeted on immobilization in different supports or the usage of genetically engineered microorganisms, called whole cell catalysts, which carry the necessary enzymes, avoiding their exposure to inhibiting substrates and operating as microrefineries (Kalscheuer et al., 2006). In the case of biodiesel microbiological production that will be revealed in detail below, the authors proposing and developing this technology refer to this third-generation biofuel as 'Microdiesel'. The microbial production of biodiesel requires the construction of genetically modified microorganisms, able to transesterificate ethanol with lipids and, if possible, able to produce it by themselves to optimize the whole process. Since their 2006 work on microdiesel production on the laboratory scale using an engineered *Escherichia coli* strain, Steinbüchel and collaborators have established the guidelines of microdiesel industry development. Their approach consisted of expressing heterologously in *E. coli* the genes from *Zymomonas mobilis,* encoding for piruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*), as well as the *Acinetobacter baylyi* non specific acyl transferase ADP1 (*atfA*). The obtained strain was able to carry out the aerobic ethanol fermentation from sugars, as well as the enzymatic transesterification of this alcohol with the fatty acids derived from the lipidic metabolism, yielding FAEE, referred to as 'microdiesel' by the authors (Kalscheuer et al., 2006). Recently, Elbahloul and Steinbüchel have used the aforementioned microdiesel producing *E. coli* at a pilot plant scale, using glycerol and sodium oleate as carbon and fatty acids sources respectively, with promising results (Elbahloul & Steinbüchel, 2010). Nevertheless, their conclusions for both studies indicate that there is still a long way to go to the industrial application of their findings, and that the technique needs to be modified to make the engineered strains adaptable to different lipids rich sources and to lignocellulosic raw materials. These modifications would allow the usage of forestry and agricultural wastes, making the biodiesel production process at least as versatile as chemical transesterification.

4.4 Microdiesel production from residues

Vegetable oils are expensive and require large areas of farmland for their production, so the direct usage of these oils for biodiesel production is expensive and unsustainable. However, there are multiple and as yet unexploited alternative fatty acid sources. Similarly, bioethanol production for its direct use as a biofuel or as a biodiesel precursor requires huge amounts of corn grain or sugar cane. Nevertheless, industrial residues such as the vegetable oil refinery waste, as well as farming, forestry, livestock and domestic solid and liquid waste (Chen et al., 2009; Dizge et al., 2009) are widespread and huge sources of lipids and carbon. Wang et al. proposed the soybean oil deodorizer distillate (SODD), a by-product from the soybean oil refineries that represents 0.3–0.5% of the soybean oil processed, to produce biodiesel. With 45–55% of triglycerides and 25–35% of free fatty acids, these authors estimated that around 80% of the SODD can be transformed into biodiesel in a transesterification with methanol by the *Thermomyces lanuginosa* and *Candida antarctica* lipases in the presence of tertbutanol and 3Å molecular sieve (Wang et al., 2006). Park et al.

used waste-activated bleaching earth (ABE), a residue of the rapeseed or palm oil refinery industry that stores 35–40% of oil and can be used to synthesize multiple bulk chemicals, including biodiesel. As in the Wang example, these authors chose methanol as alcohol, but their solvent choice was fuel oil and kerosene, the catalyst was *Candida cylindracea* lipase and the obtained FAME was extracted with a filter press (Park et al., 2008). Al-Zuhair and colleagues studied the production of biodiesel from simulated waste cooking oil (SWCO) with free- and immobilized- on ceramic beads *Candida antarctica* and *Burkholderia cepacia* lipases, with or without organic solvent. They obtained the best yield when they used *B. cepacia* without organic solvent, and observed that the system worked better when the enzymes were immobilized, probably because the clay structural microenvironments offered the lipases protection against the methanol derived denaturation (Al-Zuhair et al., 2009). Recently, Steen et al., among others, have proposed the direct fermentation of cellulosic biomass to produce biodiesel, fatty alcohols, waxes and other valuable chemicals (Steen et al., 2010). Their approach combines the waste management and the guidelines defined by Steinbüchel et al. with the new trends in synthetic biology and consolidated bioprocesses. This multidisciplinary approach brings a new flexible, easy-to-modify toolbox, composed of genetically modified FAEE synthetic strains, harbouring the enzymatic apparatus needed to produce ethanol from raw (hemi)cellulosic materials, to transesterificate it with fatty acids, or to synthesize both the fatty acids and the ethanol directly from the cellulose (Steen et al., 2010).

4.5 Wastewater sludges-based microdiesel

The microdiesel concept initiated by Steinbüchel et al. can be combined with the management and reutilization of waste waters by the application of microbial lipases to transesterificate the lipids present in the dairy industry or urban wastewater sludges. The lipidic fraction of sludges from urban wastewater treatment represents between 17 and 30% of the dry weight. This lipidic fraction is formed by direct absorption of fats present in the water by the sludge particles and by the phospholipids released from the cell membranes of micro-organisms, as well as from metabolites and cell lysis by-products (Boocock et al., 1992; Shen & Zhang, 2003; Jardé et al., 2005).

Lipid-rich wastewaters require pretreatment in order to reduce the amount of lipids and ease the subsequent conventional treatment. The pretreatment is usually based on physical processes, the most common of which are fat traps, tilted plate separators (TPS), and dissolved air flotation (DAF) units. In addition, centrifuges and electroflotation systems are used occasionally (Willey, 2001). Fat traps are rectangular or circular vessels through which the wastewater passes under laminar-flow conditions, at a rate that allows the lipids to rise to the surface near to the outlet end of the trap. The separation principle is based on Stoke's law, relating rising velocity of a particle to its diameter, so the theoretical separation efficiency is dependent on depth. In practice, fat traps have a depth of 1.5 m, although if the accumulation of a bottom sludge is expected, then an additional 0.5 m would be added to the total liquid depth. Gravity flow is preferred to pumping when feeding the trap, in order to minimize the wastewater emulsification. Fat traps are used in the food industry and in restaurants (Willey, 2001).

Meanwhile, tilted plate separators were developed in the petrochemical industry and are based on the fact that surface area, rather than depth, determines the oil separation. The introduction of tilted plates into a vessel provides many parallel gravity separators with a

high surface to volume ratio in a shallow tank. Typically, TPS can occupy less than 10% of the area needed to install a conventional fat trap, although they have some disadvantages. They are susceptible to fouling if solid or semi-solid fat is present in the effluent and a crane is required to remove the plate pack for cleaning. Besides, the pumping systems have to be carefully selected and controlled to avoid surging and liquid depth fluctuations (Zeevalkink & Brunsmann, 1983; Willey, 2001). Finally, dissolved air flotation units are based on the flotation of lipids by means of microbubble clouds (60-70 μm bubble diameter) created by the injection into water of 6 bar pressure air through nozzles. Microbubbles attach to the surface of the fat and oil particles, increasing their rise rate. These systems are used both in the food industry (Willey, 2001) and in the mining wastewater treatment (Tessele, 1998). Once the pre-treatment has finished, wastewater can receive further treatment prior to its disposal or biological treatment. Thus, chemical treatment may be used to reduce the total fatty matter in wastewater. Such treatment uses aluminium sulphate, ferric chloride, or more usually, lime, to break the emulsion and coagulate the fat particles. Subsequently, the fats can be separated by flotation or sedimentation. The rate of sedimentation can be improved by a second-stage flocculation, involving the addition of low levels of polyelectrolyte (0.5-5.0 mg/l) to the wastewater once coagulation has taken place (Willey, 2001).

The use of sludges to produce biofuels is not a new idea itself, but the available literature focuses mainly on the methane production by anaerobic fermentation, currently applied in the majority of waste water treatment plants (WWTP) to provide energy to these installations, or for fermentative biohydrogen production, which is still not industrially available (van Groenestijn et al., 2002; Wang et al., 2003). Several groups have studied the *in situ* transesterification of WWTP sludges, but have focused on the chemical catalysis of the transesterification with methanol (Haas & Foglia, 2003; Mondala et al., 2009). However this method still presents the same limiting factors that affect the chemical transesterification of edible vegetable oils (Freedman et al., 1984). In spite of their chemical approach, these works provide useful information about several aspects of the biodiesel production process, especially at the first stages of the process. Thus, one common problem of chemical and enzymatic biodiesel production is the need for the pretreatment of the feedstock to make its lipids easily available to the catalyst. In the case of wastewater treatment sludge, this pretreatment step usually implies the use of organic or non-polar solvents to release the lipids from the organic matter (Antczak et al., 2009; Siddiquee & Rohani, 2011).

The most extended protocols rely on chloroform:methanol mixtures, as used in the Folch's method (Folch, 1957), in which a 2:1 chloroform:methanol reactant is mixed with the sample, where water acts as ternary component to form an emulsion. After equilibration with a fourth volume in saline solution, the emulsion separates in two phases: the lower one containing chloroform:methanol:water in the proportions 86:14:1 alongside the lipids; and the upper one containing the same solvents in proportions 3:48:47 and carrying the non-lipidic components of the sample. Bligh and Dyer's method is a simplified variant of the former, but requires the re-extraction of the sample residue with chloroform (Bligh & Dyer, 1959). Nevertheless, there are some methods with near to Folch's reagent yielding which use less toxic reagents, such as pure hexane or different combinations of hexane and other solvents, such as the hexane-isopropanol (3:2) blend proposed by Hara and Radin (Hara & Radin, 1978), or the ethyl acetate-ethanol (2:1) mixture used by Lin et al. (Lin et al., 2004). For

a detailed revision of the solvents based extraction protocols, see Kuksis, 1994; Murphy, 1994; Kates, 1996.

In spite of being slightly less toxic than chloroform, the cited solvents are hazardous and present enough management risks to consider other extraction strategies. Several authors propose solvent-free methods based on ionic liquids (Ha et al., 2007), boiling the sludge or subjecting it to supercritical gases, mainly *t*-butanol (Wang et al., 2006; Royon et al., 2007), propane (Rosa et al., 2008), syngas (Tirado-Acevedo et al., 2010) and CO_2 (Helwani et al., 2009), or even to extreme pressures and temperatures (cracking) (Saka & Kusdiana, 2001). All of them are costly and not feasible with the current technology (Siddiquee et al., 2011). A more realistic and ready-to-use option is extraction using hot ethanol, which can be used to perform the lipids' extraction without using coadyuvant solvents. This approach to extraction can be illustrated with the works developed by Holser and Akin (2008) or Nielsen and Shukla (2004), among others. Although these authors have focused on the ethanol-based extraction of high value lipids from flax processing wastewater and egg yolk powder, respectively, their findings could be scaled and applied to biodiesel production from wastewater sludges. Nielsen and Shukla found that the use of ethanol at room temperature led to the extraction of nearly all the phospholipids, together with cholesterol and a minor part of the triacylglycerols, without special extraction and filtration devices. On the other hand, Holser and Akin performed a serial extraction of the lipids present in flax wastewater in three steps, under different temperature values (50, 80, 90 and 100ºC). They found that the most efficient extraction was achieved when the sample-ethanol mixture was heated to 90ºC and the reaction time was 15 minutes (Holser & Akin, 2008).

Considering the above findings, and taking into account the fact that the enzymatic production of biodiesel generally requires high alcohol to oil ratios, to improve the solubilization of the lipids and the formation of water-oil, enzyme-oil and enzyme-alcohol-oil interfaces, we propose that a suitable scheme for the production of biodiesel from WWTP sludge could be as simple as using a pressurized pretreatment tank, where the sludge is soaked in ethanol, kept at 90ºC under stirring and refluxed to subject the mixture to three extraction cycles. This is followed by incubation in a reaction tank where the extracted lipids, alongside with part of the ethanol used in the previous step, are added to a reaction mix containing the enzyme (free, immobilized or *whole cell catalyst*) and kept at the optimal temperature and pH conditions, to ensure both enzyme stability and an acceptable microdiesel production rate. Heat exchangers between the two tanks could serve to save energy, using the heat released before entering the second tank to preheat the sludge before entering the first one.

The system could even be autonomous in terms of ethanol requirements if the engineered microorganism used to produce the lipase was able to produce ethanol simultaneously, or if the cited tanks were coupled with a third reactor where bioethanol was produced from sugars present in the non-lipidic products obtained in the pretreatment tank by means of ethanol-producing yeast or bacteria strains. In the case of economic restrictions, some short-term cost reduction could be achieved by replacing the pretreatment pressurized tank with a non-pressurized unit, and keeping the temperature of the extraction mix below 79ºC, although it would imply medium-term economic losses because the lower extraction efficiency must be compensated by performing more extraction cycles at a higher reflux rate and a greater ethanol volume in the pretreatment tank, or even by the use of at least two serial pretreatment tanks.

5. Conclusion

As a short-term response to the consequences of greenhouse gas emissions and the unsustainability of the fossil fuel-based energy model, the industry has developed ready-to-use substitutes for traditional fossil fuels, delivered generally and ambiguously under the commercial 'bio' denomination. However, the first- and second-generation of so-called biofuels are neither of completely biological origin nor based on renewable and environmentally friendly feedstocks. In addition, the production techniques rely on high energy inputs, both in feedstock production (as is the case for rapeseed, soybean or palm oil) and in the biofuel synthesis (acid catalyzed biodiesel or corn bioethanol perfectly illustrate the neat energy gain problems). Alongside these problems, new and complex problems have emerged. Firstly, the increase in the prices of grain and vegetable oils used both to produce biofuels and for human nourishment and livestock feeding; and secondly, the expansion of agricultural land to increase production of sugar cane or vegetable oils to satisfy the huge demand for these sugar and lipid sources, generated by the abrupt increase in biofuels production. Thus, the development of cleaner and more sustainable biofuels is required to achieve the challenge to totally replace traditional fossil fuels by third-generation biofuels, independent of non-renewable precursors or inefficient industrial processes, that damage the environment directly and indirectly and threaten biodiversity and food security (UNCTAD, 2010).

A great variety of domestic, agricultural and industrial residues, from lignocellulosic forestry and agriculture waste to fatty acid rich waste waters, generated by the dairy, poultry or vegetable oil refinery industries, as well as the sludges from urban waste waters, can be used as precursors of biofuels. The treatment of these residues could be combined with the production of third-generation biofuels by enzymatic catalysis because the high cost of enzymes could be compensated by the low cost of the residues (or even the presence of incentives for residue reduction and management). But the massive application of these concepts requires a series of technical and biotechnological improvements, such as the optimization of lipids and sugars extraction, feedstock pretreatment processes, biofuels production plant design, heterogeneous catalysts and enzyme immobilization techniques, protein engineering of lipases, alcohol dehydrogenases or hydrolases to increase their activity and reusability, genetic engineering of microbes to facilitate both the pretreatment of precursors, and the synthesis and purification of the biofuels.

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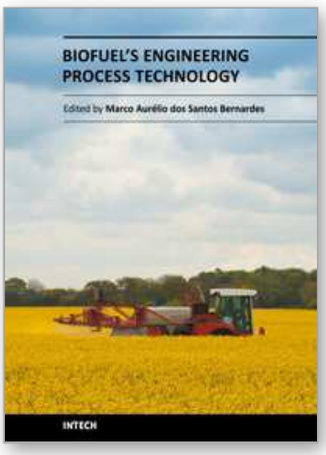

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This book aspires to be a comprehensive summary of current biofuels issues and thereby contribute to the understanding of this important topic. Readers will find themes including biofuels development efforts, their implications for the food industry, current and future biofuels crops, the successful Brazilian ethanol program, insights of the first, second, third and fourth biofuel generations, advanced biofuel production techniques, related waste treatment, emissions and environmental impacts, water consumption, produced allergens and toxins. Additionally, the biofuel policy discussion is expected to be continuing in the foreseeable future and the reading of the biofuels features dealt with in this book, are recommended for anyone interested in understanding this diverse and developing theme.

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