

*Review*

## **Enzymatic transesterification of lipids from microalgae into biodiesel: a review**

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**Abstract:** Biodiesel is considered as a potential interesting alternative diesel substitute due to its many advantages, such as biodegradability and non-toxicity. Biodiesel production worldwide is low and does not meet the needs of society. Therefore, biodiesel production policies have been implemented by various countries. This paper addresses the issues of plant materials containing lipids for biodiesel production and explores also the avenue of microalgae as an alternative. Various conversion technologies of lipids into biodiesel are also described and compared. Enzymatic transesterification would be most appropriate when microalgae oils are used because of their high free fatty acids content. The optimization of the enzymatic process depends on several parameters such as the catalyst, temperature, reaction time and the alcohol: oil molar ratio.

**Keywords:** Biodiesel; microalgae; enzymatic transesterification; process parameters

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### **1. Introduction**

Fuels derived from petroleum are not a long-term energy source. Indeed, the oil reserves can become depleted by 2054 [1]. Thus, biofuels such as biodiesel and bioethanol are an alternative for the replacement of petroleum fuels. More than 10% of the world energy depends currently on biofuels and wastes [2]. While the first generation of biodiesel uses edible vegetable materials for the biodiesel production, other sources of raw materials are also considered. A first alternative is the use of non-edible vegetable materials such as jatropha or castor bean with high oil yields, 1300 L<sub>oil</sub>/ha

and 380–980 L<sub>oil</sub>/ha respectively [3,4]. However, these non-edible vegetable materials are expensive. A second alternative is the use of wastes cooking oils and greases which require some pre-treatment like the removing of solid wastes [5]. A third alternative would be the use of microalgae, microscopic algae that may be grown in the presence of solar light and carbon dioxide (CO<sub>2</sub>), a powerful greenhouse gas (GHG). Furthermore, microalgae have shorter harvesting cycle, less than 1 month, compared to edible vegetable materials [6,7]. They have also the potential to produce up to 17 times more oil by hectare of culture [8].

Biodiesel is mainly produced by a transesterification process. Depending on the catalyst used, there are three types of transesterification: basic, acid and enzymatic. Enzymatic transesterification appears as the most appropriate transformation process of lipids issued from microalgae oil into biodiesel, because it allows to transform the free fatty acids (FFA) without soap formation into alkyl esters [9]. The enzymatic transesterification has several advantages compared to the basic or acid transesterification such as: easier separation of products and by-products, no soap formation, non-toxic catalyst and low reaction temperatures. However, the reaction conditions must still be optimized for industrial application. Optimization techniques, such as the pre-treatment of the enzyme (incubation with the substrates, alkyl esters or co-solvents) prior to the reaction and the control of the alcohol concentration in the medium reaction allow to obtain elevated alkyl esters yields (94–99% w/w) while reducing the reaction time (3.5–7 h) [10,11].

Finally, this paper presents an overview of the worldwide biodiesel production, raw materials and production processes most used, as well as an analysis of the parameters of the enzymatic transesterification process.

## 2. Biodiesel

### 2.1. Basic concept

Biodiesel, an environmentally friendly alternative to diesel [12,13] is produced by transformation of plant, animal or waste lipids into fatty acid alkyl esters. It helps to reduce CO<sub>2</sub> balance, since theoretically the vegetable material used to produce biodiesel, consumed CO<sub>2</sub> (through photosynthesis) during its growth [14]. Furthermore, the use of biodiesel in automobile motors can reduce emissions of pollutants (carbon monoxide (CO), hydrocarbons, polyaromatic hydrocarbons and soot) and increase slightly NO<sub>x</sub> emissions.

The quality of the biodiesel depends on the raw material and the production process (Table 1). Therefore, one of the main challenges in biodiesel production is to improve its physicochemical characteristics, to reduce its viscosity and its cloud point and to increase its cetane number. For example, the viscosity of biodiesel produced from raw materials such as rapeseed, soybean, sunflower and olive oils among others, varies between 2.83 and 5.12 cSt [15], while the viscosity of petrodiesel is about 3.0 cSt [16]. The heating value of petrodiesel between 42.5 and 45 MJ/kg is higher than that of biodiesel [17,18], which varies between 35 and 42 MJ/kg [15,19].

**Table 1. Physicochemical properties of biodiesel produced from various raw materials.**

Raw materials	Density (g/L)	Viscosity (cSt) (40 °C)	Heating value (MJ/kg)	Flash point (°C)	Cloud point (°C)	Pour point (°C)	Reference
Palm oil	880	5.7	34	164	13	-	[19]
Soybean oil	885	4.1	40	69	-2	-3	[20]
Rapeseed oil	882	4.5	37	170	-4	-12	[20]
Sunflower oil	860	4.6	34	183	1	-	[19]
Microalgae oil <i>Chlorella</i> <i>protothecoides</i>	864	5.2	41	115	-12	-11	[9]
Microalgae oil <i>Scenedesmus</i> <i>obliquus</i>	869	-	39	-	-	Cold filter plugging point: 3.5 °C	[21]

## 2.2. The world consumption and the biodiesel production policies

The world energy demand is large and growing annually [1]. The majority of the energy consumed worldwide comes from petroleum and its derivatives. In 2015, global crude oil consumption was around 92 million barrels per day while world crude oil reserves reach 1495 billion barrels [22]. If petroleum consumption remains stable, it is expected that the reserves would run out in 2054 [1]. However, global energy demand could increase by 35% between 2005 and 2030 [23]. In addition, the price of petrodiesel and the pollution generated stimulate the research and the development (R&D) of other replacement sources of fossil fuels. Therefore, the development of alternative energy sources to replace petroleum fuel is indispensable. This is the reason that led some countries (Argentina, Brazil, USA among others) to assign funds for R&D projects directed to find sustainable fuels, and to create laws promoting the use of biodiesel [24,25]. R&D has as main objective to improve the process of biodiesel production and to reduce its cost. In 2014, the global biodiesel production was of 70,792 million metric tons of oil equivalent worldwide, an increase of 7.7 fold compared to 2000 (9176 million metric tons of oil equivalent worldwide) [26]. In order to establish laws for the use of biodiesel blends, governments have implemented regulations (Table 2), the main objective being to reduce the amount of pollutant emissions caused by fossil fuels such as CO, the sulfur dioxide (SO<sub>2</sub>), etc. Thus, petrodiesel and biodiesel blends containing between 1 (B1) and 100% (v/v) (B100) of biodiesel (B5 and B20 are the most used (Table 2)) allow to reduce these emissions.

**Table 2. Producer countries and laws on the biodiesel blends.**

Countries	Organisation sponsor	Laws or legislative initiatives	Reference
European Union	Directive of the European Parliament	<i>Directive 2009/28/EC of the European Parliament and the Council of 23 April 2009 on the promotion of the use of energy from renewable sources and modifying then repealing Directives 2001/77/CE and 2003/30/E. The EU establishes the requirement of a minimum part of 10% (v/v) of biofuels in the total consumption of fuel and diesel for transport, this target to be achieved by 2020 by all States members.</i>	[24]
United States	U.S. Navy	<i>New U.S. policy. Department of the Navy. 'The marine and non-tactical diesel vehicles must run on a blend of biodiesel 20% (v/v) (B20) no later than June 2005'.</i>	[27]
Brazil	Presidency	<i>Law No. 11097, 13 January 2005. 'Expect the introduction of biodiesel into the Brazilian energy model, modifying the laws 9478, to 6 August 1997, 9847, to the October 26, 1999 and 10636 to December 30, 2002 and other measures.'</i> <i>For the combustibles sold to the consumer, the minimum value determined in the blends of biodiesel is 5% (v/v).</i>	[28]
Argentina	Senate and House of representatives	<i>Law 26.093. 'The promotion of the production and use of sustainable biofuels.'</i> <i>All combustibles diesel type must have a minimum of 5% (v/v) of biodiesel by May 2006.</i>	[25]
Canada	Federal government	<i>Canada Gazette Part 1.</i> <i>'The requirement of a content of 5% (v/v) by the presence of renewable fuel in gasoline, diesel or other liquid fuel from petroleum, from 2010 and later than in 2012'.</i>	[29]

### 2.3. Vegetable materials, raw materials for biodiesel production

The worldwide biodiesel production uses various raw materials in terms of their price and availability (Table 3). The main raw materials are the vegetable oils, the waste oils, and the animal fats. The raw materials most commonly used in the world are from vegetable origin: soybean, rapeseed, palm and sunflower (Table 4). The price of biodiesel depends on the raw material used and its oil yield ( $L_{oil}/ha$ ); the cost of the raw material can reach up to 78% of the total production cost of the biodiesel [30].

**Table 3. Worldwide biodiesel production.**

Countries	More used raw material	Production in 2009 (million tons)	Production capacity in 2010 (million tons)	Reference
European Union	Rapeseed, sunflower waste oils	9.1	23	[31]
United States	Soybean	1.8	7.2	[32]
Spain	Sunflower	0.9	4.1	[31]
Brazil	Soybean; palm; cotton; castor bean	1.4	3.4	[31, 33]
Argentina	Soybean; microalgae	1.2	2.4	[33]

**Table 4. Oil yield and price of raw materials used in the biodiesel production.**

Raw materials	Producer countries of biodiesel	Oil yields (L/ha)	Prices in 2008–2009 (\$US/ton)	Reference
Soybean	Argentina, Brazil, United States, Russia	400	617	[3,34,35]
Rapeseed	Germany, Finland, France, Italy, United Kingdom, Russia, Switzerland	1100	800	[3,34,36]
Palm	Brazil, Indonesia, Malaysia, Thailand	2400	879	[3,34,37]
Sunflower	Spain, France, Russia	690	1140	[2,34,38]
Jatropha	China, India, Indonesia, Philippines, Thailand	1300	810	[3,39]
Canola	Canada	1330	770	[34,37,40]
Castor bean	Brazil	380–978	1050	[4,41]
Coconut	Philippines, Thailand	1993–3987	780	[42,43]

The lipids extracted from the vegetable materials have a characteristic fatty acid (FA) composition (Table 5) which influences the physicochemical properties of the biodiesel such as its viscosity, density and heating value, determining thus its quality [44]. Other properties such as the acid value and iodine must also be considered in the selection of raw materials to obtain a high quality biodiesel [12,13]. Biodiesel composition also influences cold flow properties and oxidative (storage) stability. Biodiesel with high concentrations of saturated fatty acid methyl esters tends to have higher cloud point, pour point and cold filter plugging point. On the other hand, higher concentrations of saturated fatty acid methyl esters also promotes higher oxidative stability. Biodiesel producers focus also their attention on the use of other raw materials such as non-edible vegetable material or microalgae. Non-edible vegetable materials such as castor bean, jatropha and *Pongamia pinnata* [45] are currently used in several countries. The castor bean having high lipid content (53% w/w), a rapid growth and a drought resistance, is used in Brazil to produce biodiesel (Table 3) [46]. However, castor bean is not an attractive raw material because of its price (1050\$ US/ton), the highest among all the vegetable materials [4] and its extremely high viscosity even after conversion to biodiesel. Jatropha is another raw material used in Asia, India being the largest jatropha oil producer [47]. Moreover, jatropha not only has a high lipid yield (1890 L<sub>oil</sub>/ha), but also a high content of oleic (C<sub>18:1</sub>) (41% w/w) and linoleic (C<sub>18:2</sub>) (34% w/w) acids [48]. Therefore, non-edible vegetable materials are an interesting alternative to replace edible vegetable material [30].

**Table 5. Main raw materials and their fatty acid content.**

Vegetable oil	C <sub>16:0</sub> Palmitic acid	C <sub>18:0</sub> Stearic acid	C <sub>18:1</sub> Oleic acid	C <sub>18:2</sub> Linoleic acid	Reference
Palm	45.5	4.1	39	10	[49]
Soybean	12.6	6.9	35	45	[50]
Sunflower	7.7	4	29	58	[51]
Rapeseed	6.8	2.3	69	14	[50]
Microalgae ( <i>Chlorella protothecoides</i> )	8	3.2	71	15	[52,53]

#### 2.4. Microalgae, alternative raw material

Microalgae are microscopic algae that can be grown in heterotrophic, autotrophic and/or mixotrophic cultures (Table 6) [54,55]. They can use several carbon sources other than glucose, such as cassava hydrolysate, sugar cane, glycerol, etc.), during a heterotrophic culture, which allows to reduce the cost of culture [55-58].

**Table 6. Microalgae with an elevated lipid content.**

Microalgae	Type of culture	Oil content (% w/w)	Oil yield (mg <sub>oil</sub> /L <sub>culture</sub> ·d)	FA content (% w/w)						Reference
				C <sub>16:0</sub>	C <sub>16:1</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	Others	
<i>Chlorella protothecoides</i>	Heterotrophic	48	4037	8	0.3	3.2	71	15	2.6	[52,53]
<i>Botryococcus braunii</i>	Autotrophic	36	48	22	0	5	48	7.5	18	[59]
<i>Chaetoceros calcitrans</i>	Autotrophic	40	17	13	12	3.5	3.5	1	68	[60,61]
<i>Chaetoceros muelleri</i>	Autotrophic	39	22	15	25	1	3	2.5	54	[60,61]
<i>Skeletonema</i> sp.	Autotrophic	31	49	12	4.1	0.2	0.7	1.1	82	[61,62]

Microalgae are considered a more suitable raw material for biodiesel production since they present several advantages over biodiesel production from vegetable materials:

1. The culture of microalgae is flexible; microalgae can be grown in various medium such as sea water or fresh water, under several reaction conditions: temperature, pH and nutrients. Microalgae can also be grown in wastewater in the presence of CO<sub>2</sub>, and thus be used in wastewater treatments [63].
2. The oil content of microalgae can reach 77% (w/w) under certain culture conditions [6]. By comparison, soybean has an oil yield of about 18% (w/w) [64], while that of the rapeseed is about 46% (w/w) [65].
3. Microalgae proliferate rapidly. Indeed, the microalga *Chlorella protothecoides* can provide 10 g<sub>biomass</sub>/(L<sub>culture</sub>·d) [52]. In addition, microalgae culture does not use arable lands, which

makes them attractive for biodiesel production [52].

4. The oil yield from microalgae is high. The heterotrophic culture of *Chlorella protothecoides* at a pilot scale gives an oil yield of 70,000 L<sub>oil</sub>/ha [52], 17 times higher than that of palm oil in (2400 L<sub>oil</sub>/ha) [3]. All these features make microalgae particularly interesting to replace the vegetable materials in the biodiesel production. In addition, lipids extracted from *Chlorella protothecoides* produce a biodiesel having a viscosity of 5.2 cSt (40 °C), a low pour point (-11 °C), an elevated flash point (115 °C) and a heating value of 41 MJ/kg [9].

However, according to Chen et al., (2011) [66] out of 30 species of tested microalgae, the lipid yield of microalgae may vary due to multiple parameters such as the species, the culture, the bioreactor, the type, the concentration of nutrients in the culture system and the extraction method. For example, the lipid content of *Botryococcus braunii* (36% w/w) is similar to that of *Chaetoceros calcitrans* (40% w/w), but the oil productivity of the latter (17 mg<sub>oil</sub>/L<sub>culture</sub>·d) is 3 times lower than that of *Botryococcus braunii* (48 mg<sub>oil</sub>/(L<sub>culture</sub>·d)) (Table 6). In addition, there are a wide variety of species of microalgae, and their diversity in lipid composition makes some species inappropriate for the biodiesel production [60]. Indeed, unsaponifiable lipids such as sterols or pigments like β-carotene cannot be used to produce biodiesel. For example, Kim et al., (2016) [67] recently found that *Aurantiochytrium* sp. contains more than 8% (w/w) of unsaponifiable lipids. However, such compounds can be used in pharmaceutical or cosmetic industries [68-70]. For the biodiesel production, microalgae oil must contain large amounts of FA (>40% w/w), suitable for its transformation into biodiesel [30]. Microalgae rich in polyunsaturated FA produce a biodiesel with low cetane number. For example, the microalgae *Chaetoceros calcitrans* and *Chaetoceros muelleri* have respective oil contents of 39 and 31% (w/w), and an eicosapentaenoic acid content (EPA, C<sub>20:5</sub>, omega 3) elevated (>14% w/w) [60], which provides a low cetane number biodiesel. By contrast, microalgae such as *Aurantiochytrium* sp. that contain a high level of saturated FA (around 51% w/w of palmitic acid) allow the production of biodiesel with a high cetane number [67]. The culture conditions can also modify the amount of lipids, but the proportion of FA is determined by the species of microalgae (Table 6).

Recent developments in genetic engineering have also favoured the transformation of microalgae using tools such as RNA (ribonucleic acid) silencing, homologous recombination to improve their oil content and composition for subsequent biodiesel production. RNA silencing involves the introduction, in the algal cell, of a mini-gene directing the synthesis of a short RNA of a specific sequence, adopting a hairpin-like structure. Such RNA will interact with the messenger RNA of a gene of interest, resulting in its inactivation and, consequently, will decrease the expression of this gene. One of the earliest applications of this technique in the field of biodiesel production allowed redirecting the metabolic carbon into lipid biosynthesis by down regulating the expression of genes involved in starch biosynthesis [6]. However, the lipid accumulation can sometimes impair the growth of engineered microalgae [6].

A global infatuation in the development of the process for biodiesel production from microalgae oil exists at an industrial level (Sapphire Energy, San Diego, CA, USA; Seambiotic Ltd, Israel; Solazyme Inc, CA, USA). The Argentina industry Oil Fox S.A. has inaugurated in 2010 a factory for the biodiesel production from a mixture of soybean (90%) and microalgae (10%) [71]. The cost of biodiesel production from microalgae is function of the use of pond or photobioreactor [72]. It can vary between 2.4 (pond) and 10.6 (photobioreactor) US\$/L [72,73]. Gupta et al., (2016), [73] have recently developed several scenarios to optimize the use of microalgae to produce biodiesel. They

have analyzed the impact of the growth, harvesting, lipid extraction and reaction steps on the cost of biodiesel production. They found that the growth and lipid extraction steps play the most crucial role in the biodiesel production cost. However, several challenges for the large-scale microalgae biodiesel production have to be overcome and research and development beyond the bench scale that can compete with the traditional commodity oils must be pursued.

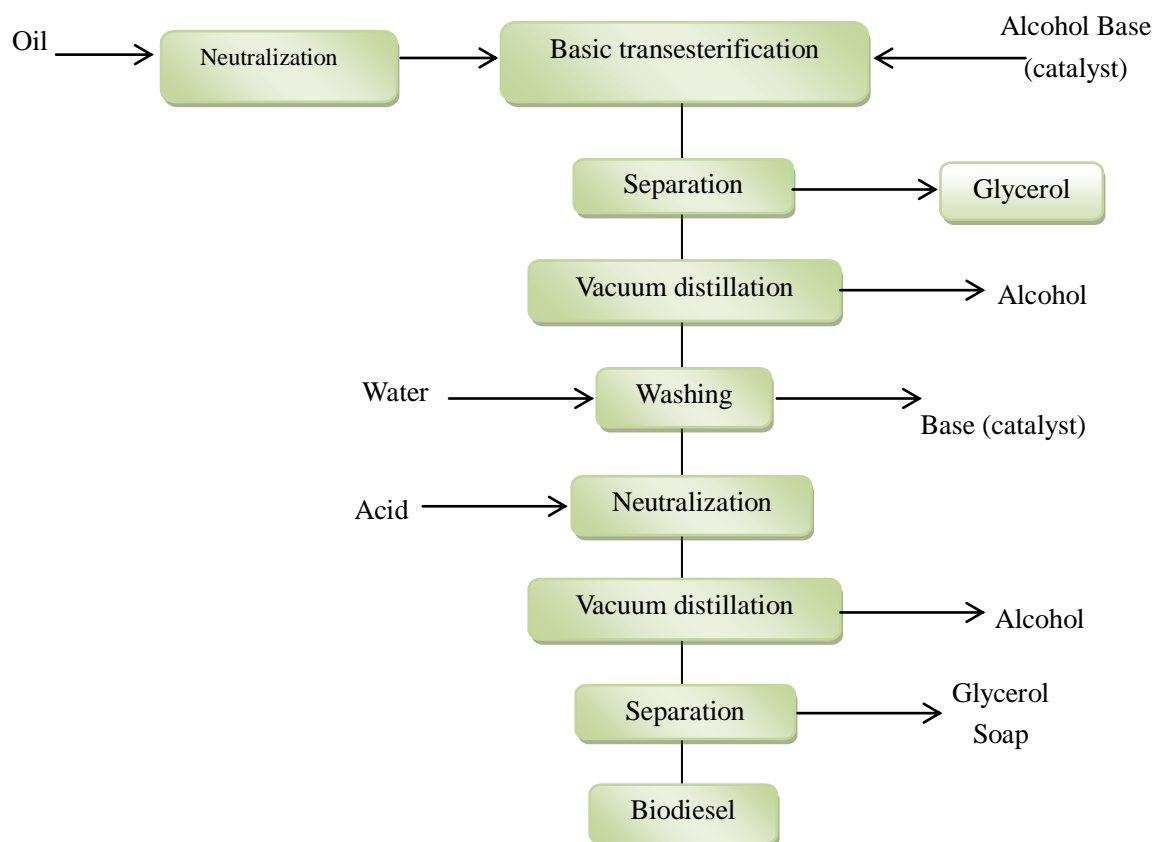
### 3. Lipids Transesterification Processes for Biodiesel Production

#### 3.1. Transesterification by basic catalysis

The basic catalysis by homogeneous or heterogeneous ways is the most used process for the biodiesel production [74]. The homogeneous catalysis is preferred to the heterogeneous catalysis, due to the cost of the basic catalyst and a short reaction time [75-77]. The main advantage of the basic transesterification compared to the acid or enzymatic transesterification is the reaction time, the basic transesterification being a fast reaction (0.5 to 9 h) (Table 7). For example, transesterification of the waste oil in the presence of MeOH and sodium hydroxide (NaOH) with a reaction time of 0.5 h reaches a fatty acid methyl esters (FAME) yield of 85% (w/w) [78]. Furthermore, NaOH and KOH are low cost catalysts (Table 7). Although the stoichiometric alcohol: triglyceride molar ratio is 3:1, the basic transesterification reaction uses an excess of alcohol and the alcohol: oil molar ratio varies between 5:1 to 30:1 [79,80]. The MeOH is the most widely used alcohol; however, other more expensive alcohols (ethanol, n-butanol) are also employed [75]. Generally, the temperature of the basic transesterification is that of the alcohol normal boiling point (65 °C for MeOH). The alkyl esters yield during the basic transesterification is elevated (generally higher than 90%) (Table 7). For example, during the transesterification of the sunflower oil in the presence of MeOH and potassium carbonate ( $K_2CO_3$ ), the FAME yield can reach 100% [81]. The main disadvantage of the basic transesterification is the soap formation: the amount of soap formed varies depending of the FFA oil content, the type of catalyst and the temperature of reaction [76]. For example, NaOH tends to induce a soap formation higher than that obtained with KOH. Thus, if the FFA oil content is higher than 0.5 mg<sub>KOH</sub>/g<sub>oil</sub>, the oil must be pre-treated before the basic transesterification, in order to remove FFA and thus avoid soap formation [82]. The basic catalysis is therefore not suitable for transformation of microalgae oil into biodiesel because of its acid value (about 9 mg<sub>KOH</sub>/g<sub>oil</sub>) [9]. The soap formed during the basic transesterification (forming an emulsion during the water wash of the biodiesel) consumes the catalyst and prevents the separation of FAME and glycerol (by-product of the oil transesterification) [82,83]. Thus, during the basic transesterification, if the oil used is rich in FFA, a deacidification step of the oil is required either by the use of alcohols [84] or by liquid-liquid extraction of the FFA [85]. However, this deacidification treatment increases the cost of the biodiesel. Nevertheless, Cai et al., (2015), [86] have recently proposed a soap-catalyzed reaction step to produce biodiesel from waste cooking oil. They have first esterified the FFA of waste cooking oil by glycerol with a basic catalysis, and then performed an alkali-catalyzed transesterification of the esterified waste cooking oil. For the soap-catalyzed step, conversion of FFA to acylglycerol was above 99% (w/w) (210 °C of temperature, reaction time of 4h, agitation 300 rpm, glycerol:FFA ratio of 1.4:1, 0.5 % (w/w) of NaOH catalyst). Then, they obtained, after the alkali-catalyzed transesterification of esterified waste cooking oil, a FAME yield of 93% (w/w) (40 °C; reaction time of 1h, agitation 300 rpm, MeOH: esterified waste cooking oil molar ratio of 6:1, NaOH: 0.33 (w/w)).



After the basic transesterification reaction, it is necessary to cool the mixture reaction in order to separate the glycerol from the biodiesel (Figure 1). The biodiesel is then purified by distillation, neutralization and washing to remove residual reagents and glycerol [99].



**Figure 1. Basic transesterification process.**

### 3.2. Transesterification by acid catalysis

The mechanism of the transesterification reaction by acid catalysis is similar to the basic transesterification, each step of the process being reversible. The acid transesterification reaction reaches a FAME yield of about 95 to 99% (w/w) (Table 8). However, the acid transesterification requires an excess of alcohol higher than that used during the basic transesterification, alcohol:oil molar ratios of the acid transesterification varying between 9:1 and 166:1 (Tables 8 and 9) [100,101]. It should be noted that the excess of alcohol does not advantage the recovery of glycerol. Another disadvantage of the acid process is the temperature (up to 120 °C) higher than that required during the basic transesterification (about 65 °C) (Table 9). In acid transesterification, large amounts of alcohol are necessary in order to use the lowest possible temperatures. For example, the transesterification temperature of jatropha oil in the presence of H<sub>2</sub>SO<sub>4</sub> is low (60 °C), but the excess of alcohol is high (alcohol:oil molar ratio of 166:1) [100]. During the acid transesterification of soybean, oil:alcohol molar ratio is low (9:1), but the temperature is high (100 °C) [101].

**Table 7. Basic transesterification of vegetable oils.**

Basic catalyst	Oil	Alcohol	Alcohol:oil molar ratio	Reaction conditions	FA conversion (%)	Alkyl esters yields (% w/w)	Reference
NaOH (Sodium hydroxide)	Sunflower	Methanol	6:1	60 °C; 2 h		97	[87]
	Frying oil	Methanol	7.5:1	70 °C; 0.5 h		85	[76]
	Frying oil	Methanol	6:1	65 °C; 1.5 h		77	[88]
	Mahua ( <i>Madhuca indica</i> )	Methanol	6:1	60 °C; 2 h	92		[89]
	Animal fats	Methanol	2-10:1	>98 °C; 0.5 h		90	[90]
KOH (Potassium hydroxide)	Frying oil	Methanol	6:1	65 °C; 2 h		94	[88]
	<i>Pongamia pinnata</i>	Methanol	10:1	60 °C; 1.5 h	92		[91]
	Rapeseed	Methanol	6:1	65 °C; 2 h		95–96	[92]
KF/Al <sub>2</sub> O <sub>3</sub> (Potassium fluoride on aluminum oxide)	Palm oil	Methanol	12:1	65 °C; 3 h		90	[93]
KF/Eu <sub>2</sub> O (Potassium fluoride on europium oxide)	Rapeseed	Methanol	12:1	65 °C; 1 h	92		[94]
KI/Al <sub>2</sub> O <sub>3</sub> (Potassium iodide on aluminum oxide)	Soybean	Methanol	15:1	65 °C; 8 h	96		[95]
Ca(OCH <sub>2</sub> CH <sub>3</sub> ) (Calcium ethoxide)	Soybean	Ethanol	12: 1	75 °C; 3 h		92	[96]
KF/ZnO (Potassium fluoride on zinc oxide)	Palm oil	Methanol	11.4: 1	65 °C; 9.7 h		89	[97]

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**Table 7. Basic transesterification of vegetable oils—continued.**

Basic catalyst	Oil	Alcohol	Alcohol:oil molar ratio	Reaction conditions	FA conversion (% w/w)	Alkyl esters yields (% w/w)	Reference
K <sub>2</sub> CO <sub>3</sub> (Potassium carbonate)	Sunflower	Methanol	12: 1	50 °C; 7.5 h	100		[98]
NaCO <sub>3</sub> (Sodium carbonate)	Sunflower	Methanol	12: 1	50 °C; 7.5 h	92		[98]
Na <sub>3</sub> PO <sub>4</sub> (Sodium phosphate)	Sunflower	Methanol	12: 1	50 °C; 9 h	90		[98]
CaO (Calcium oxide)	Sunflower	Methanol	12: 1	50 °C; 11.5 h	97		[98]
KNO <sub>3</sub> (Potassium nitrate)	Soybean	Methanol	15: 1	60 °C; 7 h	87		[95]

**Table 8. Acid transesterification of vegetable oils.**

Acid catalyst	Oil	Alcohol	Alcohol:oil molar ratio	Reaction conditions	FA conversion (%)	Alkyl esters yield (% w/w)	Reference
H <sub>2</sub> SO <sub>4</sub> (15% w/w)	Jatropha curcas L seeds	Methanol-n-hexane	166:1	60 °C; 24 h		99.8	[100]
H <sub>2</sub> SO <sub>4</sub> (0.5% w/w)	Soybean	Methanol	9:1	100 °C; 12 h	98		[101]
H <sub>2</sub> SO <sub>4</sub> (0.5 M)	<i>Chlorella pyrenoidosa</i>	Methanol-n-hexane	164:1	110 °C; 2 h		95	[102]
H <sub>2</sub> SO <sub>4</sub> (4% w/w)	Waste oil	Methanol	16:1	95 °C; 10 h	93		[103]
S-ZrO <sub>2</sub> (Sulfated zirconia, 5 % w/w)	Soybean	Methanol	20:1	120 °C; 1 h		98.6	[104]
ZnO (Zinc oxide)	<i>Pongamia pinnata</i>	Methanol	10:1	120 °C; 24 h	83		[91]

**Table 9. Comparison of various methods of transesterification.**

Characteristic of the process	Basic transesterification	Acid transesterification	Enzymatic transesterification
Alkyl esters yield	High yield (>94%)	High yield (>94%)	High yield (>90%)
Temperature	Moderate temperature reaction (50–70 °C)	Elevated temperature reaction (60–120 °C)	Low temperature reaction (30–60 °C)
Alcohol:oil molar ratio	Excess of alcohol, molar ratio = (6–12 :1)	Excess of alcohol, molar ratio = (10–166 :1)	Stoichiometric ratio, molar ratio = (3–4 :1)
Reaction time	Short (average of 4 h)	Long (average of 12h)	Long (average of 28 h)
Catalyst	Inexpensive catalyst; low catalyst concentration (average 1% w/w)	Inexpensive catalyst; low catalyst concentration (average 1% w/w)	Expensive catalyst; moderate catalyst concentration (average 8% w/w); deactivation of the catalyst because of the alcohol
Energy consumption	High, the product requires hot water washing	High, high reaction temperatures	Moderate reaction conditions
Recovery of the products	Difficult; several purification steps	Easy, the product does not require washing	Easy, the product does not require washing
Environmental impact	Using large quantities of water to wash	The process is corrosive, the acid catalyst is pollutant	Ecological; no washing step necessary
Others	Soap formation	Transformation of FFA without soap	Transformation of FFA without soap

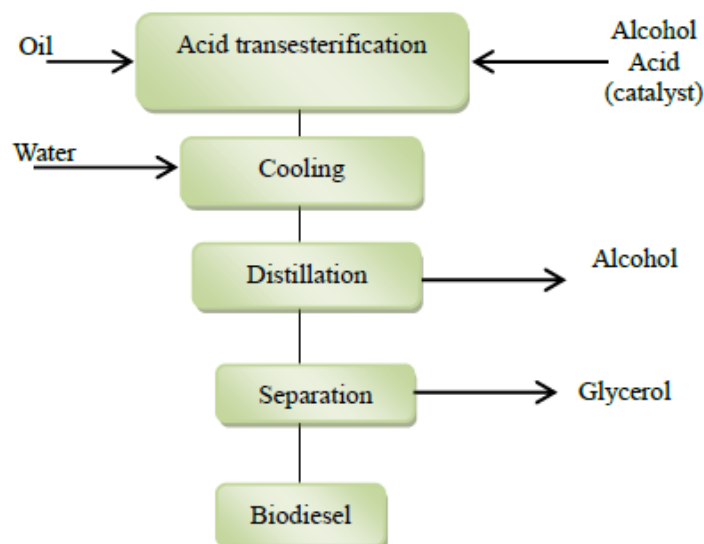
The main advantage of acid transesterification is that it allows the transesterification of oils having an elevated FFA content ( $>0.5 \text{ mg}_{\text{KOH}}/\text{g}_{\text{oil}}$ ) without soap formation. The waste and microalgae oils having an elevated content of FFA (76 and 9  $\text{mg}_{\text{KOH}}/\text{g}_{\text{oil}}$  respectively) [9,103] can thus be transformed into biodiesel by acid transesterification or by two-steps transesterification (acido-basic). The acid transesterification of waste oils in the presence of MeOH, and sulfuric acid ( $\text{H}_2\text{SO}_4$ ) gives a FAME yield of 93% (w/w) (95 °C of temperature, reaction time of 10h and alcohol:oil molar ratio of 16:1) [103]. During the acid transesterification of *Chlorella pyrenoidosa* in the presence of MeOH and  $\text{H}_2\text{SO}_4$ , 95% (w/w) of the lipids is transformed into biodiesel (90 °C of temperature, reaction time of 2 h, and alcohol:oil molar ratio of 165:1) [102].

Several recent studies have focused on the one-step in situ transesterification of wet and dried microalgae biomass into biodiesel [105,106]. In this case, the alcohol (MeOH, ethanol) and co-solvents (hexane, chloroform) participate in both lipid extraction and transesterification reaction [105,107]. For example, Choi et al., (2015), [108], have recently performed a one-step acid transesterification in situ of dried *Scenedesmus* sp. biomass. They obtained a biodiesel yield estimated at 85% (w/w) with a FAME conversion of about 96 % (70 °C of temperature, reaction time of 10 h, dried biomass-MeOH ratio of 1:22.07 (v/w), 5% (v/v)  $\text{H}_2\text{SO}_4$ ). Nevertheless, the use of wet biomass to produce biodiesel by a one-step in situ transesterification has to overcome several challenges [105], since water can limit the lipid extraction by solvents or favour the hydrolysis of the produced biodiesel [109]. Indeed, using dried *Chlorella* sp. biomass and a one-step acid transesterification method, Velasquez-Orta et al., (2013), [110] have obtained a biodiesel yield of 92% (60 °C of temperature, reaction time of 19 h, biomass: $\text{H}_2\text{SO}_4$ :methanol ratio of 1:0.35:600 (w/w/w)). However, when the *Chlorella* sp. biomass contained 10% of moisture, they got under the same operating conditions a biodiesel conversion yield of only 61% [110]. The direct transesterification of wet microalgae biomass can also be performed under microwave irradiation or supercritical conditions to improve the alkyl esters yield [111,112].

Such process avoids the lipid extraction step that is largely responsible for the cost of the microalgae biodiesel production [73].

After the acid transesterification reaction, a cooling step is required, because of the elevated reaction temperature. A subsequent distillation allows the separation of the alcohol and finally the biodiesel is separated from glycerol. The washing steps are not necessary, because there is no soap formation during the acid reaction (Figure 2).

However, the acid transesterification is rarely used at an industrial scale, because the acids catalysts like  $\text{H}_2\text{SO}_4$  (mostly used) are highly corrosive [8].



**Figure 2. Acid transesterification process.**

### 3.3. Transesterification by enzymatic catalysis

Several extracellular lipases (triacylglycerol acylhydrolases EC 3.1.1.3) such as Novozym® 435 synthesized by *Candida antarctica* or Lipozyme® TL IM synthesized by *Thermomyces lanuginosus* have been widely used as catalysts for the biodiesel production [113] (Tables 10 and 11). One of the principal advantages of the enzymatic transesterification is the ease of separation and the purification of biodiesel, because the biodiesel produced by such reaction does not require repetitive washing cycles. In addition, no soap formation is observed during the enzymatic transesterification [11]. Thus, the enzymatic catalysis is more suitable than the chemical catalysis for the transformation of microalgae oil into biodiesel because of their high FFA content (Table 9).

The lipases can be used free or immobilized (Table 12). The free enzymes show elevated activity compared with immobilized enzymes: for example, the lipase produced from free *Thermomyces lanuginosus* has an activity of about 100,000 U/g, while immobilized (on Immobead 150), shows an activity of 3000 U/g (1 U is the amount of enzyme (g) that liberates 1  $\mu\text{mol}/\text{min}$  of substrate at 25 °C). The immobilization induced changes in the enzymatic activity, according to the source of the enzyme, the type of support used and the immobilization method. Furthermore, the immobilization limits the contact between the enzyme and the substrates. The free enzymes conduct to an elevated FAME yield, generally greater than 97% (w/w), but they should be dissolved in an aqueous medium during the transesterification reaction, because they hydrolyze the ester bonds in the triglyceride-water interface [120]. However, due to the elevated cost of lipases, the heterogeneous enzymatic catalysis (immobilized enzyme) is preferable to the homogeneous catalysis (free enzyme), the immobilized enzymes being reused [103,124,125,147]. Immobilization can also improve the stability of the enzyme [148]. Figure 3 shows the heterogeneous enzymatic transesterification processes most used. The enzymatic process is environmentally friendly and a lower energy consuming compared to the chemical transesterification. For example, the temperature of the enzymatic reaction is about of 15 to 40% lower than that of the basic reaction. Furthermore, the enzymes, unlike basic and acids catalysts, are not polluting substances [149] (Table 9).

**Table 10. Homogeneous enzymatic transesterification of various oils.**

Lipase	Oil	Alcohol	Alcohol:oil molar ratio	Solvent (% v/v oil)	Catalyst concentration (% w/w oil)	Water (% w/w oil)	Reaction conditions	FA conversion (% w/w)	Alkyl esters yields (% w/w)	Reference
<i>Pseudomonas fluorescens</i>	Triolein	1-propanol	3: 1	-	10	-	50 °C; 25 h	90 (free lipase)-85 (immobilized lipase)		[114]
	Rapeseed	2-ethyl-1-hexanol	3: 1	-	3.3	3	37 °C; 48 h; 200 rpm	99		[115]
	Rapeseed	2-ethyl-1-hexanol	3: 1	-	3.3	3	37 °C; 1 h	97		[116]
<i>Pseudomonas fluorescens</i> 26-2.	Soybean	Methanol	3: 1	-	3	5	40 °C; 72 h; 200 rpm	84		[117]
<i>Candida rugosa</i>	Rapeseed	2-ethyl-1-hexanol	3: 1	-	3.3	3	37 °C; 24h; 200 rpm	98		[115]
<i>Candida rugosa</i> 1	Rapeseed	2-ethyl-1-hexanol	3: 1	-	3.3	3	37 °C; 10 h	99		[116]
<i>Candida rugosa</i> 2	Rapeseed	2-ethyl-1-hexanol	3: 1	-	3.3	3	37 °C; 24 h	99		
<i>Candida rugosa</i> 3	Rapeseed	2-ethyl-1-hexanol	3: 1	-	3.3	3	37 °C; 5 h	99		
<i>C. viscosum</i>	Rapeseed	2-ethyl-1-hexanol	3: 1	-	3.3	3	37 °C; 10 h	98		
	Rapeseed	2-ethyl-1-hexanol	3: 1	-	3.3	3	37 °C; 24 h; 200 rpm	96		[115]
<i>Rhizomucor miehei</i>	Rapeseed	2-ethyl-1-hexanol	3: 1	-	3.3	3	37 °C; 48 h; 200 rpm	87		
	Rapeseed	2-ethyl-1-hexanol	3: 1	-	3.3	3	37 °C; 24 h	94		[116]
<i>R. miehei</i> (RML) and <i>P. cyclopium</i> (MDL)	Soybean	Methanol	4: 1	-	RML (188 U) and MDL (88.2 U)/g oil	34	pH = 7; 30 °C; 180 rpm; 24 h	100		[118]

Continued on next page

**Table 10. Homogeneous enzymatic transesterification of various oils—continued.**

Lipase	Oil	Alcohol	Alcohol:oil molar ratio	Solvent (% v/v oil)	Catalyst concentration (% w/w oil)	Water (% w/w oil)	Reaction conditions	FA conversion (% )	Alkyl esters yields (% w/w)	Reference
<i>Rhizopus sp.</i>	Rapeseed	2-ethyl-1-hexanol	3: 1	-	3.3	3	37 °C; 10 h	100		[116]
<i>Rhizopus oryzae</i>	Soybean	Methanol (stepwise addition)	3: 1	-	17	10	pH = decrease 5.4 to 3.5; 35 °C; 150 rpm; 70 h		90	[119]
<i>Aspergillus niger</i>	Rapeseed	2-ethyl-1-hexanol	3: 1	-	3.3	3	37 °C; 24 h	45		[116]
<i>Pancreatic</i>	Cottonseed	Methanol	15: 1	t-butanol (75%)	0.5	5	pH = 7; 37 °C; 180 rpm; 4 h	72		[120]



**Table 11. Heterogeneous enzymatic transesterification of various oils.**

Lipase	Oil	Alcohol	Alcohol:oil molar ratio	Solvent (% v/v oil)	Catalyst concentration (% w/w oil)	Water (% w/w oil)	Reaction conditions	FA conversion (%)	Alkyl esters yields (% w/w)	Reference	
<i>Novozym 435 (C. antarctica)</i>	Waste baked duck oil	Methanol	4: 1	t-butanol (40%)	15	-	40 °C; 20 h		90	[121]	
	Sunflower	Butanol	4: 1	t-butanol (73%)	22	-	60 °C; 48 h		88	[122]	
	Cottonseed	Methanol	4: 1	-	30	-	50 °C; 700 rpm; 7 h		91.5	[123]	
	Waste oil	Methanol (3 steps)	3: 1	-	4	-	30 °C; 130 rpm; 48 h	97		[124]	
	Triolein	Butanol	3: 1	-	1.5	-	40 °C; 50h		40	[125]	
	Palm oil	Methanol	1:1	-	20	-	50 °C; 250 rpm; 8 h		95	[126]	
			Methyl acetate	12: 1							
	Cottonseed	Methanol	6: 1	t-butanol (32.5%)	1.7	-	50 °C; 24h		97	[127]	
	Olive	Methanol	8: 1	hexane	5.5	-	60 °C; 100 rpm; 24 h	94 (fresh oil)-84 (used oil)		[128]	
	Soybean	Methanol	3: 1	-	4	0.2	30°C; 150 rpm; 30 h		97	[11]	
	Soybean	Methyl acetate	12: 1	-	30	-	40°C; 150 rpm; 10 h		92	[129]	
	Sunflower	Methanol	3: 1	-	4	-	40 °C; 400 rpm; 17 h		95	[88]	
	Waste oil	Methanol (3 steps)	3: 1	-	4	-	30 °C; 130 rpm; 40 h		90	[130]	
	Sunflower	2-propanol	4: 1	-	10	-	50 °C; 150 rpm; 8 h	93		[131]	
	Rapeseed	Methanol	-	-	14	-	30 °C; 130 rpm; 24 h		91	[132]	
	Palm oil	Ethanol	4: 1	t-butanol (10%)	20	-	45 °C; 24h		87	[133]	
	Soybean	Methyl acetate	12: 1	-	30	-	40 °C; 150 rpm; 14 h		92	[134]	
Rapeseed/Soybean	Methanol (3 steps)	1:1 at each step	-	4	-	30 °C; 130 rpm; 48h	98		[135]		

*Continued on next page*

**Table 11. Heterogeneous enzymatic transesterification of various oils—continued.**

Lipase	Oil	Alcohol	Alcohol:oil molar ratio	Solvent (% v/v oil)	Catalyst concentration (% w/w oil)	Water (% w/w oil)	Reaction conditions	FA conversion (%)	Alkyl esters yields (% w/w)	Reference
<i>Novozym 435</i> ( <i>C. antarctica</i> ) <i>incubated</i>	Rice bran oil (refined)	Methanol (stepwise addition)	3.6: 1	-	5	-	50 °C; 150 rpm; 7 h	-	99	[10]
	Soybean	Methanol (stepwise addition)	3: 1	-	4	-	30 °C; 150 rpm; 3.5 h	-	98.7	[11]
	Rice bran oil (85% FFA)	Methanol (stepwise addition)	3.6: 1	-	5	-	50 °C; 150 rpm; 6 h	-	96	[10]
	Soybean (degummed)	Methanol (3 steps, 1/3 molar equivalent at each step)	3: 1	-	4	-	30 °C; 130 rpm; 48 h	-	94	[136]
<i>Novozym 435</i> <i>and Lipozyme</i> <i>TL IM</i> ( <i>T. lanuginosus</i> )	Rapeseed	Methanol	4: 1	t-butanol (1:1 t-butanol/oil ratio)	3% of TL IM and 1% of Novozym 435	-	35 °C; 130 rpm; 12 h	-	95	[137]
	Waste cooking oil	Methanol	24: 1	-	TL IM/Novo 435 = 2/1	-	20 MPa; 40 °C; 2h	-	99	[138]
<i>Novozym 435</i> <i>and Lipozyme</i> <i>TL IM</i> ( <i>T. lanuginosus</i> )	Oleic acid	Methanol	4: 1	t-butanol (1:1 t-butanol/oil ratio)	3% of TL IM and 1% of Novozym 435	-	35 °C; 130 rpm; 5 h	-	75	[137]

*Continued on next page*

**Table 11. Heterogeneous enzymatic transesterification of various oils—continued.**

Lipase	Oil	Alcohol	Alcohol:oil molar ratio	Solvent (% v/v oil)	Catalyst concentration (% w/w oil)	Water (% w/w oil)	Reaction conditions	FA conversion (%)	Alkyl esters yields (% w/w)	Reference
<i>Pseudomonas cepacia (PS-D)</i>	Triolein	1-butanol	3: 1	-	1.5	-	40 °C; 5 h	-	100	[125]
	Triolein	iso-butanol	3: 1	-	1.5	-	40 °C; 3 h	-	100	
	Triolein	Propanol	3: 1	-	1.5	-	40°C; 5h	-	100	
	Triolein	Ethanol	3: 1	-	1.5	-	40 °C; 7 h	-	92	
	Triolein	Methanol	3: 1	-	1.5	-	40 °C; 24 h	-	42	
	Triolein	2-butanol	3: 1	-	1.5	-	40 °C; 24 h	-	82	
<i>Pseudomonas cepacia (PS-C)</i>	Triolein	Butanol	3: 1	-	1.5	-	40 °C; 48 h	-	100	[125]
<i>Pseudomonas cepacia</i>	Sunflower	Ethanol	3: 1	-	2	-	40 °C; 400 rpm; 30 h	-	54	[139]
<i>Lipase AK (P. fluorescens) and Lipase AY (C. rugosa)</i>	Palm oil	Ethanol	3: 1	-	5% Lipase AK and 5% Lipase AY	2	45 °C; 6 h; 1 step	-	80	[140]

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**Table 11. Heterogeneous enzymatic transesterification of various oils—continued.**

Lipase	Oil	Alcohol	Alcohol:oil molar ratio	Solvent (% v/v oil)	Catalyst concentration (% w/w oil)	Water (% w/w oil)	Reaction conditions	FA conversion (%)	Alkyl esters yields (% w/w)	Reference
<i>PCMC</i> ( <i>T. lanuginosus</i> from <i>Aspergillus</i> <i>sp.</i> )	Palm oil	Methanol	4: 1	t-butanol (10%)	20	-	45 °C; 24 h	-	32	[133]
	Palm oil	Ethanol	4: 1	t-butanol (10%)	20	-	45 °C; 24 h	-	90	
<i>Novozym 388</i> <i>immobilized</i> ( <i>A. oryzae</i> )	Canola	Methanol (3 steps)	4: 1	-	20	-	pH = 6; 40 °C; 24 h	-	85 (adsorbed lipase)-81 (covalently bound lipase)	[141]
<i>Lipozyme</i> <i>TL-100L</i> <i>immobilized</i> ( <i>A. oryzae</i> )	Canola	Methanol (3 steps)	4: 1	-	20	-	pH = 6; 40 °C; 24 h	-	92 (adsorbed lipase)-85 (covalently bound lipase)	[141]
<i>Lipozyme TL</i> <i>IM</i> ( <i>T. lanuginosus</i> )	Soybean	Methanol (3 steps)	4: 1	-	30	-	40 °C; 150 rpm; 14 h	-	90	[142]
	Sunflower	Ethanol	3: 1	-	2	-	40 °C; 400 rpm; 30 h	-	44	[139]

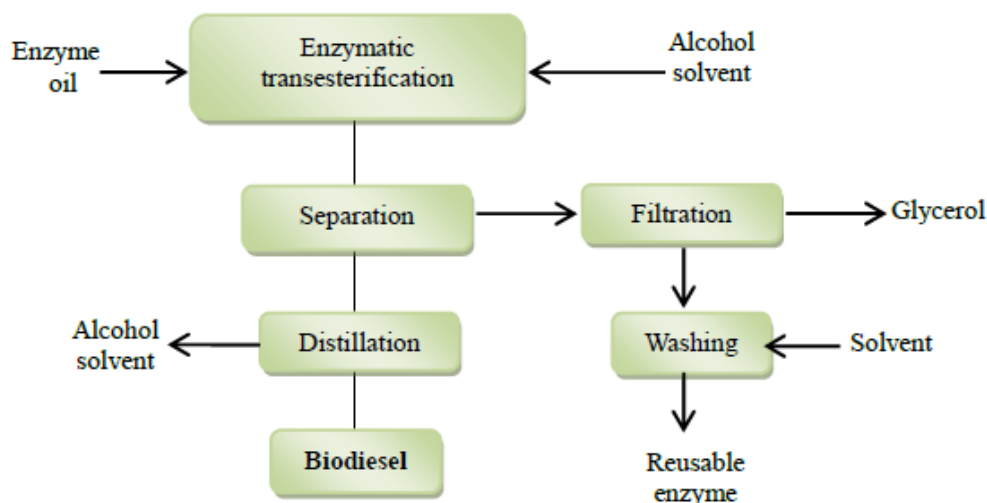
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**Table 11. Heterogeneous enzymatic transesterification of various oils—continued.**

Lipase	Oil	Alcohol	Alcohol:oil molar ratio	Solvent (% v/v oil)	Catalyst concentration (% w/w oil)	Water (% w/w oil)	Reaction conditions	FA conversion (%)	Alkyl esters yields (% w/w)	Reference
<i>Rhizopus oryzae</i> (ROL) and <i>Candida rugosa</i> (CRL)	Soybean	Methanol	1.5: 1	-	20% (ROL and CRL 1:1)	10	130 bar (supercritical) 45 °C; 250 rpm; 3 h	-	99	[143]
<i>Lipozyme IM 60</i> ( <i>R. miehei</i> ) incubated	Rice bran oil (refined)	Methanol	3.6: 1	-	5	-	50 °C; 150 rpm; 7 h		74	[10]
	Rice bran oil (85% GLA)	Methanol	3.6: 1	-	5	-	50 °C; 150 rpm; 6 h		92	[10]
<i>Lipozyme RMIM</i> ( <i>R. miehei</i> )	Triolein	Butanol	3: 1	-	1.5	-	40 °C; 25 h		100	[125]
<i>Lipozyme 62350</i>	Sunflower	Ethanol	3: 1	-	4	-	40 °C; 400 rpm; 20 h		72	[139]
<i>Candida sp.</i> 99-125	<i>Chlorella p.</i>	Methanol	3: 1	-	75	10	38 °C; 180 rpm; 12 h		98	[144]
	<i>Chlorella p.</i>	Methanol	3: 1	-	30	10	38 °C; 180 rpm; 12 h	98		[52]
	Waste oil	Methanol (3 steps)	3: 1	n-hexane	100	10	40 °C; 30 h		92	[145]
	Rice	Methanol (2 steps)	4: 1	n-hexane	20	20	40 °C; 170 rpm; 12 h		87	[146]

**Table 12. Characteristics of some immobilized lipases tested for biodiesel production.**

Characteristics	<i>C. antarctica</i>	<i>T. lanuginosus</i>	<i>P. cepacia</i>
Regioselectivity	Non specific	Position 1 and 3	-
Resistance to deactivation	Moderate	Moderate	Low
Activity (U/g)	10000	225	
Combination	<i>T. lanuginosus</i>	<i>C. antarctica</i> <i>A. oryzae</i> .	-
Tested oils	Rapeseed, animal fat, palm, rice, soybean, sunflower, triolein	Palm, rice, soybean, sunflower, triolein	Sunflower, triolein
Temperature (°C)	30–40	30–50	40
Optimal enzyme concentration (% w/w <sub>oil</sub> )	4–5	4–20	1.5
Alcohol:oil molar ratio	3–4 :1	4:1	3:1
Average alkyl esters yield (% w/w <sub>oil</sub> )	92	91	96
Average reaction time (h)	13	22	3–48
Particularities	Low alkyl esters yield; presence of butanol	High production of biodiesel from the FFA	High alkyl esters yield with long chain alcohols

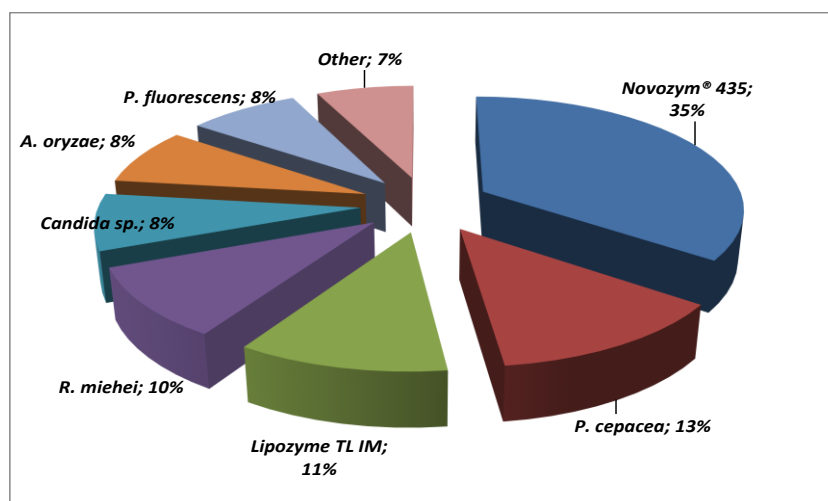
**Figure 3. Heterogeneous enzymatic transesterification process.**

However, the enzymatic transesterification reaction requires an acyl acceptor such as the MeOH, ethanol or butanol, and the enzymes can be denatured in the presence of short chain alcohols (Table 11) [113]. For example, the lipase produced from *Pseudomonas cepacia* presents an elevated sensitivity to short chain alcohols such as MeOH or ethanol. Thus, the transesterification of

sunflower oil in the presence of ethanol (reaction time of 30h, temperature of 40 °C) using this lipase provides a low FAME yield (54% w/w) [139]. Similarly, the transesterification reaction of the triolein using this lipase in the presence of MeOH reaches a FAME yield of 42% (w/w) (same operating conditions) [125]. To overcome the lipase deactivation by MeOH or ethanol, the alcohol should be added stepwise [150] or a co-solvent such as t-butanol or n-hexane can be also used [52,133]. The presence of those co-solvents increases the solubility of MeOH in the medium reaction, thereby reducing the denaturation of the enzyme; however, the use of those solvents makes the process more expensive. Another solution to avoid the use of co-solvents is the use of long chain acyl acceptors such as the 1-butanol, the 2-butanol, the isobutanol or the propanol [125]. The solubility of long chain alcohols such as the propanol and the butanol in the oils (hydrophobic substrates) is more elevated than those of short chain alcohols such as MeOH or ethanol. FAME yields around 100% (w/w) can be obtained in the presence of long chain alcohols and in the absence of co-solvents [125], but these are more expensive acyl acceptors. For example, the Novozym® 435 (*Candida antarctica*) was tested during the transesterification of soybean oil in the presence of methyl acetate (acyl acceptor). No negative effect of the methyl acetate on the enzymatic activity has been noted (FAME yield of 92% w/w) [129].

### 3.3.1. Enzyme selection for the transesterification reaction

The selection of the enzyme is crucial (Figure 4). The most used lipase in biodiesel production, the Novozym® 435 (*Candida antarctica*), is generally immobilized by adsorption on the surface of an acrylic resin. It is a lipase widely used industrially because of its non-specific regioselectivity and its activity (10,000 U/g). The concentration necessary for the transesterification of oils is moderate ( $\geq 4\%$   $w_{\text{enzyme}}/w_{\text{oil}}$ ) (Table 11), because of the enzyme activity. Novozym® 435 could also resist to the deactivation in the presence of short chain alcohols such as MeOH or ethanol. During the transesterification of rapeseed oil in the presence of MeOH and without a co-solvent, the lipase produced from the *Candida antarctica* permitted to reach a FAME yield of 91% (w/w) [132]. However, in the presence of butanol, the lipase Novozym® 435 provides a low FAME yield. For example, the lipases Lipozyme RMIM (*Rhizomucor miehei*), *Pseudomonas cepacia* (2 species PS-D and PS-C), and Novozym® 435 were tested during the biodiesel production from triolein (triglyceride of oleic acid) and butanol. The butyl ester yield was 40% (w/w) using Novozym® 435, while it was 100% (w/w) with the 3 other lipases [125]. Nevertheless, the alkyl esters yield during the transesterification of sunflower oil in the presence of butanol can reach 96% (w/w) with Novozym® 435; but the reaction requires 100% (v/v) of t-butanol (co-solvent), an elevated quantity of enzyme, 22% ( $w_{\text{enzyme}}/w_{\text{oil}}$ ) and a reaction time of 48 h [122].



**Figure 4. Lipases used in the biodiesel production.**

The lipases differ in their regioselectivities, hydrolyzing preferably the ester bondings of the triglycerides in certain positions. For example, *Rhizopus oryzae* synthesizes a sn-1,3 regioselective lipase which hydrolyzes ester bonds of triglycerides at position 1 and 3 [151]. The specific and non-specific lipases can be therefore used in combination. Li et al., (2006), [137] studied the effect of the combination of the lipases on the alkyl esters yield of the transesterification of various alcohols and oils, and have demonstrated that a combination of lipases led to an increase of the alkyl esters yield as opposed to the use of a single lipase. For example, the Lipozyme TL IM (*Thermomyces lanuginosus*) (a 1,3-specific lipase) and the Novozym® 435 (a non-specific lipase) were tested for the transesterification of rapeseed oil in the presence of MeOH. The FAME yield of the reaction reached 85% (w/w) by using Lipozyme TL IM (4%  $w_{\text{enzyme}}/w_{\text{oil}}$ ) or 90% (w/w) by using Novozym® 435 (4%  $w_{\text{enzyme}}/w_{\text{oil}}$ ) separately, while the combination of 1% ( $w_{\text{Novozym® 435}}/w_{\text{oil}}$ ) and 3% ( $w_{\text{Lipozyme TL IM}}/w_{\text{oil}}$ ) gave a FAME yield of 95% (w/w) under the same reaction conditions [137]. Lipase Novozym® 435 is costlier than Lipozyme TL IM, which means that the combination of these lipases can not only increase the FAME yield, but also reduce the operation cost. However, the activity of the Lipozyme TL IM (*Thermomyces lanuginosus*) (3000 U/g) is low compared to that of the Novozym® 435 (10,000 U/g). Furthermore, the Lipozyme TL IM is very sensitive to the presence of MeOH, thus the use of a co-solvent during the reaction is usually required [103,137].

Several studies focused on the use of lipases for the microalgae biodiesel production, (Table 11). For example, the lipase *Candida sp.* 99–125 (30%  $w_{\text{enzyme}}/w_{\text{oil}}$ ) was tested during the biodiesel production from microalgae oil (*Chlorella protothecoides*) in the presence of MeOH (alcohol:oil molar ratio of 3:1), 10% (w/w) of water and hexane as co-solvent (pH = 7.0, temperature of 38 °C and a reaction time of 12 h). The FAME yield was 98% (w/w) [52]. In the same way, the oil from the microalgae *Chlorella protothecoides* was used for the biodiesel production in the presence of an enzyme produced from the *Candida sp.* and in a co-solvent free medium reaction under the following reaction conditions: enzyme concentration of 75% ( $w_{\text{enzyme}}/w_{\text{oil}}$ ), alcohol:oil molar ratio of 3:1 and 10% (w/w) of water, the FAME yield was 98% (w/w) [144]. Interestingly, Canet et al., (2016), [151] have recently studied the impact of FFA (oleic acid) on the transesterification reaction rate of olive oil (model oil often used to mimic the oil from microalgae), [99] to FAME by immobilized *Rhizopus oryzae* lipase on Relizyme OD403/S. They found that the FAME production rate increased from  $1.8 \times 10^{-4}$  mol/min to  $3.6 \times 10^{-4}$  mol/min by adding 20% (w/w) free oleic acid in the



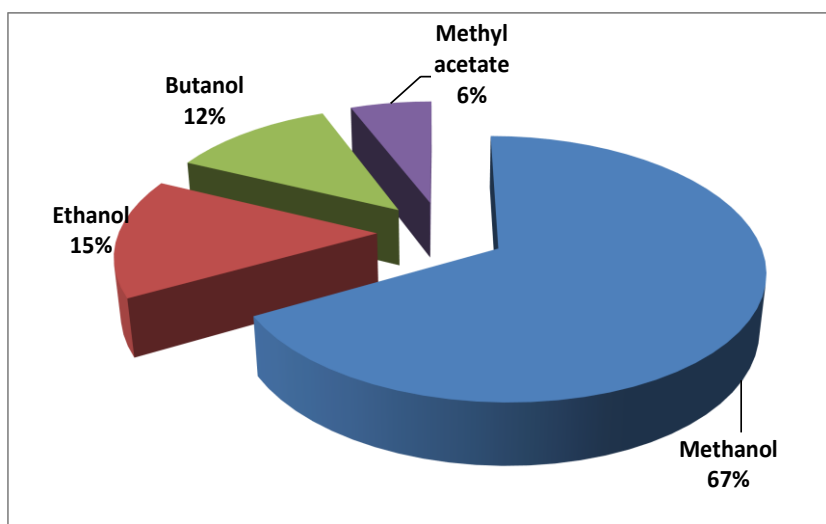
reaction medium (temperature of 30 °C, reaction time varying between 0 to 0.5 h, 32000 UA of lipase, olive oil with or without oleic acid and 160 µL of MeOH). Furthermore, the presence of oleic acid increased the stability of the immobilized lipase. Without FFA, the enzyme lost 50% of its activity after 7 cycles and became inactive after 10 cycles, while it remained almost fully active during 10 cycles by adding 10% (w/w) free oleic acid.

However, an elevated quantity of enzyme makes the process uneconomic commercially because of the enzymes cost.

### 3.3.2. The alcohol in the enzymatic transesterification reaction

The alcohol is the second important parameter to consider during the enzymatic transesterification (Figure 5). The alcohols used are MeOH, ethanol, propanol, 2-propanol, 1-butanol, 2-butanol, isobutanol and 2-ethyl-1-hexanol. MeOH is the most widely used because of its availability and its low price. However, the selection of the alcohol depends on the type of lipase, some being more sensitive to the presence of short chain alcohols such as MeOH or ethanol [113]. A stoichiometric alcohol:oil molar ratio of 3:1 is generally recommended for the enzymatic transesterification reactions. However, even if the alcohol:oil molar ratio is stoichiometric, the deactivation of the enzyme is possible. Lotti et al., (2015), [113] have recently summarized and discussed the possible mechanisms of lipase deactivation by short chain alcohols. Such alcohols can, for example, induce a partial unfolding of the protein and/or may act as competitive inhibitors of the enzyme. The deactivation caused by the short chain acyl acceptors can not only decrease the alkyl esters yield, but also increase the reaction time. For example, the transesterification of the triolein (temperature of 40 °C, catalyst PS-D (*Pseudomonas cepacia*) immobilized on diatomaceous at a concentration of 1.5% ( $w_{enzyme}/w_{oil}$ ) and alcohol:oil molar ratio of 3:1) in the presence of MeOH provides a FAME yield of 42% (w/w), with a reaction time of 24 h, whereas in the presence of alcohols such as butanol or propanol, the alkyl esters yield is about 100% (w/w), for a reaction time of 5 h [125]. Some authors prefer to control the alcohol concentration (progressive addition of alcohol) in the medium reaction to avoid the low alkyl esters yields caused by MeOH excess [141,142,146]. The lipase Lipozyme TL 100 L from *Aspergillus oryzae* (20%  $w_{enzyme}/w_{oil}$ ) was tested during the transesterification of canola oil in the presence of MeOH (MeOH:oil molar ratio of 3:1), the alcohol was supplied by three successive additions and the resulting FAME yield was high (92% w/w) [141]. However, to obtain an elevated esters yield, some authors prefer to use a high quantity of enzyme to counter the presence of an alcohol excess.

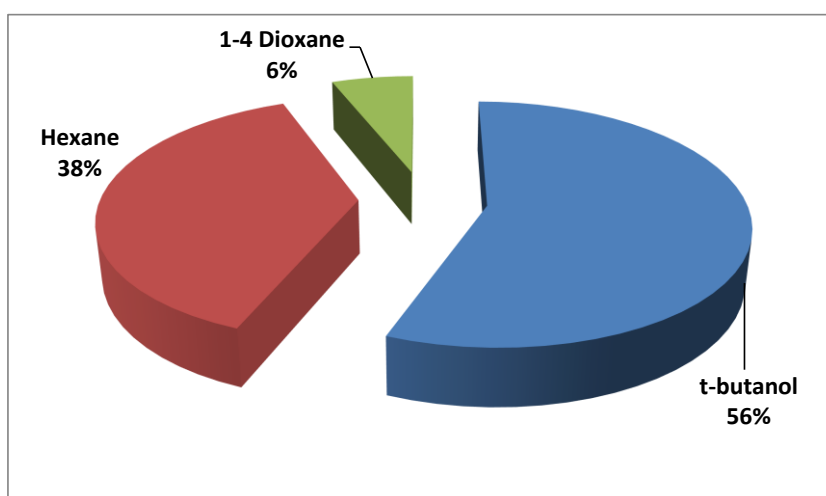
The type of alcohol used for the biodiesel production also influences the physicochemical properties of the biodiesel and the parameters of the transesterification reaction. The ethyl or butyl ester, have cloud and pour points lower than those of methyl esters [44].



**Figure 5. Alcohols used in the biodiesel production.**

### 3.3.3. The co-solvent in the enzymatic transesterification reaction

The co-solvents are used during the enzymatic transesterification in order to increase the solubility of hydrophilic substrates (alcohols) in hydrophobic substrates (vegetable oils). The vegetable oil being a non-polar substrate, the co-solvent should be a non-polar or a low polarity solvent. Hexane and t-butanol are the most used solvents during the enzymatic production of biodiesel (Figure 6). Other solvents such as toluene and acetonitrile have also been tested for the biodiesel production from cottonseed oil. For example, during the homogeneous enzymatic transesterification of cottonseed oil in the presence of MeOH (alcohol:oil molar ratio of 15:1, temperature of 37 °C and enzyme concentration of 0.5% w/w<sub>oil</sub>), the conversion of the triglycerides (72% w/w) is 2.6 times higher by using the t-butanol compared to a reaction in the absence of solvent (25% w/w). However, the conversion of the triglycerides is higher in the absence of solvent compared to the use of the acetonitrile (<1% w/w) in the medium reaction [120].



**Figure 6. Solvents used in the biodiesel production.**

It should be noted that the quantity of co-solvent depends on the sensitivity of the enzyme to the concentration of alcohol in the medium reaction. Most of the enzymes are also sensitive to the presence of glycerol. When glycerol deactivates the catalyst, the resulting alkyl esters yield is low [122]. Co-solvents can help to avoid the deactivation caused by the glycerol in the medium reaction. Raita et al., (2010), [133] studied the effect of *t*-butanol on the esters yield of the transesterification of palm oil in the presence of MeOH: alcohol:oil molar ratio of 4:1 and 20% ( $w_{\text{enzyme}}/w_{\text{oil}}$ ) of lipase from *Thermomyces lanuginosus* produced with *Aspergillus sp.* and immobilized in the form of protein-coated microcrystals (PCMC), temperature of 45 °C and reaction time of 24 h. In the presence of *t*-butanol, the FAME yield obtained was 90% (w/w), whereas in absence of co-solvent, the FAME yield was less than 70% (w/w) under the same reaction conditions [133]. Some research groups have also developed a process to remove the glycerol from the reaction medium during the enzymatic transesterification reaction [152]. However, Rodrigues et al., (2016), [150] have recently prepared immobilized sn-1,3 regioselective lipases (*Rhizopus oryzae* lipase and recombinant *Carica papaya* lipase) by adsorption or covalent binding on synthetic resins to produce biodiesel from jatropha lipids. They found that such immobilized lipases abolish the formation of glycerol, while the maximum percentage of FAME obtained in the reaction medium varies between 51.7 and 64.5% (w/w) depending on the resins and the method of immobilization used (30 °C of temperature, reaction time up to 48 h, 4% (w/w) water, methanol:jatropha lipids molar ratio of 3:1 and seven stepwise methanol additions).

#### 3.3.4. The temperature of the enzymatic transesterification reaction

The enzymes are sensitive to the reaction temperature. Consequently, this parameter plays a key role during the enzymatic transesterification reaction [114,120] (Tables 10 and 11). The optimal temperature for the enzymatic transesterification reaction is a parameter specific to each enzyme depending on whether it is derived from mesophilic (35–50 °C) or thermophilic (60–80 °C) microorganisms [153,154]. A low or elevated temperature can produce a change in the active site of the enzyme and induce a decrease of the esters yield. The enzymatic transesterification reaction is performed at a lower temperature than that of the chemical transesterification (basic or acid) (Table 9). In general, the optimal temperature range of the enzymatic transesterification is between 30 and 50 °C. During the enzymatic transesterification of cottonseed oil in the presence of MeOH using a pancreatic enzyme (free enzyme), the optimal temperature was low (37 °C) and the conversion of FA was about 75% (w/w) [120]. The temperature of the enzymatic transesterification reaction is also influenced by the form (free or immobilized) of the enzyme employed. The transesterification reactions using free enzymes exhibit lower reaction temperatures (30–50 °C) [151] (Table 11), whereas in the presence of immobilized enzymes, the transesterification temperature is generally higher (from 35 to 60 °C). During the enzymatic transesterification of olive oil in the presence of MeOH and the Novozym® 435, the optimal temperature reaction was 60 °C (an increase of the FAME yield is obtained between 30 and 60 °C, but between 60 and 70 °C the FAME yield decreases) [153]. Thus, the immobilized enzymes present improved stability at elevated temperatures compared to the free enzymes.

#### 3.3.5. The pH of the enzymatic transesterification reaction

The pH variations during the enzymatic transesterification may produce changes in the amino

acid charges of enzymes and thus affect the enzymatic activity. The enzymes hydrolyze triglycerides in a low pH interval, a neutral pH being preferred (Table 10). For example, the immobilized enzymes from *Aspergillus oryzae* catalyze the transesterification reactions of lipids in a pH interval slightly acid (pH = 6.0) [141]. Some microbial lipases can also keep their activity at a pH varying from 7.5 to 9 [148,154]. By contrast, other enzymes reach their maximum activity at low pH. For example, the lipase isolated from *Bacillus pumilus* reaches its maximum activity at a pH = 1 (50 °C of temperature, reaction time of 96 h, 5% (w/v) palm oil) [148,155]. The transesterification of rapeseed oil in the presence of MeOH catalyzed by the enzyme from *Rhizopus oryzae* reach a maximum FAME yield of 90% (w/w) at a pH = 3.5 [119].

The optimal pH value must therefore be determined experimentally and may change depending on the substrates, the temperature reaction and the type of enzymes.

### 3.3.6. The water concentration in the enzymatic transesterification reaction

During the homogeneous enzymatic transesterification, the water concentration is an important factor to consider. As the free enzymes hydrolyze the triglycerides at the substrate-water interface, the enzymatic activity depends on the water concentration in the reaction medium. The optimal value of the water concentration depends on the reaction conditions and varies between 0.3 and 34% ( $w_{\text{water}}/w_{\text{oil}}$ ) (Tables 10 and 11). For example, the transesterification of triolein in the presence of MeOH with 1,4-dioxane using the lipase extracted from *Pseudomonas fluorescens* reaches a maximum conversion of 70% (w/w) (temperature of 50 °C, reaction time of 80 h) [114], while the transesterification of rapeseed oil in the presence of MeOH and a mixture of lipases from *Rhizomucor miehei* and *Penicillium cyclopium* (pH = 7, temperature of 30 °C, reaction time of 24 h) requires 34% ( $w_{\text{water}}/w_{\text{oil}}$ ) of water [118]. The immobilized enzymes generally do not need water (Table 11). However, in some cases, the addition of a small quantity of water to the heterogeneous enzymatic reaction can increase the alkyl esters yield. During the transesterification of oils (microalgae, rice and waste oils) in the presence of MeOH and the immobilized lipase from *Candida* sp., a water concentration between 10 and 20% ( $w_{\text{water}}/w_{\text{oil}}$ ) achieves elevated FAME yields of 87 to 98% (w/w) [52,144,145,146].

### 3.3.7. Optimization of the enzymatic transesterification

In order to reduce the reaction time and avoid the catalyst deactivation caused by the presence of alcohol in the medium reaction, some optimization techniques have been recently used.

#### 3.3.7.1. Control of the alcohol concentration in the medium reaction

The addition of alcohol in a gradual manner is an often used technique in recent years, for the short chain alcohols such as the MeOH [21,156]. This technique allows to maintain a low alcohol concentration thus avoiding the deactivation of the enzyme (the use of a co-solvent is not necessary). This technique requires the determination of the optimal alcohol concentration value versus the reaction time [145,146]. Various authors state that the gradual addition of alcohol during transesterification reaction improves the alkyl ester yield. For example, during the transesterification of waste oil in the presence of MeOH, using a alcohol flow rate of 3.9 mL/h, the FAME yield is elevated (90% w/w), whereas if the alcohol is added using a flow rate of 22.3 mL/h, the FAME yield is 69% (w/w) [130]. Because this technique can also help prevent the catalyst deactivation, the

enzyme concentration used is generally low. During the transesterification reaction of vegetable oil in the presence of MeOH and of the Novozym® 435, the control of the alcohol concentration can reduce the quantity of lipase until of a minimum value of 4% ( $w_{\text{enzyme}}/w_{\text{oil}}$ ) for a FAME yield greater than 90% (w/w) [124,130,135].

### 3.3.7.2. Pre-treatment of the purified enzyme or the direct use of whole cell lipases

Another technique used to improve the esters yield and decrease the reaction time is the pre-incubation of the enzyme before the start of the reaction. The enzyme may be pre-incubated in a solution containing a solvent, an alkyl ester, the substrate or a combination. The technique consists to pre-treat the enzyme, while avoiding the deactivation caused by the acyl acceptor and the glycerol. The pre-incubation of the enzyme Novozym® 435 during 0.5 h in an alkyl ester (methyl oleate) and 12 h in the substrate (soybean oil), before the transesterification reaction, provides a FAME yield of 97% (w/w) (lipase concentration of 4% ( $w_{\text{enzyme}}/w_{\text{oil}}$ ), reaction time of 3.5 h, solvent free medium). Without prior incubation of Novozym® 435, a reaction time of 30h is required to achieve a FAME yield of 97% (w/w) under the same reaction conditions [11]. The main advantage of the enzyme pre-incubation is the increase in the initial rate of the reaction. For example, the pre-incubation of the Novozym® 435 increases the initial rate of reaction by 37% compared to the non-incubated enzyme. By combining the techniques of controlling the alcohol concentration and incubation of the enzyme, it is possible to reduce the reaction time and increase the alkyl esters yield. A short reaction time (6 h) was reached with the lipase Novozym® 435, incubated 1h in a co-solvent (t-butanol) and then in a substrate (rice oil). The MeOH was added stepwise. The FAME yield was 96% (w/w) for a catalyst concentration of 5% ( $w_{\text{enzyme}}/w_{\text{oil}}$ ) [10].

However, a new avenue in the biodiesel production from microalgae is the use of whole cells biocatalysts like lipase-synthesising bacteria, fungi or yeast [21]. These whole cell lipases can be immobilized on particles or polymeric foam, improving their stability and reusability [157, 158]. For example, whole *R. mucilaginosa* MTCC8737 yeast immobilized on sugarcane bagasse can be reused 10 times, as biocatalyst, without any lipase activity loss to convert *Chlorella salina* lipids into biodiesel with an alkyl ester yield of 89% (40 °C of temperature, reaction time of 60 h, 1.5 g biocatalyst, methyl acetate:oil ratio of 1:12, 10% (w/w) water, agitation 250 rpm) [158]. Guldhe et al., (2016), [21] have also reused, during repeated batches, immobilized *Aspergillus* sp. whole cell lipases to produce biodiesel from *Scenedesmus obliquus* lipids. They found that the FAME conversion was similar between the first and second batches (around 77% w/w), while it decreased of 15% between the first and third batches (35 °C of temperature, reaction time of 36 h, MeOH:oil ratio of 5:1, 2.5% (w/ oil weight) water, 3 stepwise MeOH additions: 2 moles at time zero and 1.5 moles at 12 h and 18 h).

The use of microbial cells synthesizing lipases as biocatalysts for microalgae biodiesel production that avoids the separation, purification and immobilized steps of the free enzymes aroused great interest for industry.

## 4. Conclusion

The price of fuel, the level of global environmental pollution and the depletion of crude oil by the middle of the 21<sup>th</sup> century requires finding other alternative energy sources. Biodiesel can substitute in part the petrodiesel. The biodiesel has similar physicochemical characteristics to the

petrodiesel, but is less polluting. Biodiesel is biodegradable and has content in sulfur compounds lower than that of petrodiesel and an elevated flash point (about 130 °C). Some countries have begun to implement laws for the production and consumption of biodiesel. Argentina, Brazil, Canada, the United States and the European Union have established that the biodiesel/petrodiesel mixture in motor vehicles must be between 5 and 20% (v/v, biodiesel/petrodiesel). In general, biodiesel is produced from extracted oils from vegetable materials such as rapeseed, soybean and palm. There are laws in biofuel-producing countries regulating the proportion of arable lands that can be used for the production of food and biofuels. These laws could limit the production of biodiesel from vegetable materials on a large scale. The microalgae could be good alternative to replace the vegetable materials. The microalgae produce generally between 8 to 24 times more oil per hectare than the vegetable materials. However, all microalgae are not suitable for the biodiesel production. The microalgae *Chaetoceros calcitrans* and *Skeletonema sp.* does have elevated oils content (39 and 31% w/w, respectively), but they have an elevated eicosapentaenoic acid content (C<sub>20:5</sub>) which can induce a low cetane number. In contrast, *Chlorella protothecoides* has an elevated content of oleic acid (about 70% w/w), making it very attractive for the biodiesel production. Another advantage of microalgae is the diversity of cultures and nutrients that can be used during their growth and reproduction. The heterotrophic cultivation of microalgae represents the best option for the large-scale production of microalgae oil. For example, 10 g<sub>oil</sub>/L·d can be obtained from the heterotrophic culture of the microalgae *Chlorella protothecoides*.

The enzymatic transesterification is the most appropriate chemical transformation process for microalgae oil, due to its elevated content in FFA (9 mg<sub>KOH</sub>/g<sub>oil</sub>). This process converts the FFA without soap formation. The most widely used enzymes in the biodiesel production are immobilized which allows to reuse them several times and facilitate separation of the biodiesel from glycerol. The enzymes being expensive, their concentration in enzymatic transesterification is an important parameter to optimize. The most commonly used enzymes in the production of biodiesel are the Novozym ® 435 (*Candida antarctica*), the Lipozyme TL IM (*Thermomyces lanuginosus*) and the *Pseudomonas cepacia*. These enzymes permit to obtain an elevated alkyl esters yield (>91% w/w). Most enzymes are sensitive to the presence of alcohols in the medium reaction. However, the lipase from *Candida antarctica* shows a resistance to the deactivation caused by the MeOH. An important parameter of the reaction is the alcohol:oil molar ratio, the value thereof varies of 3:1 to 4:1 (while that of the basic transesterification varies of 6:1 to 12:1). The MeOH is the most widely used alcohol, mainly for its cost. The use of short chain alcohols such as the MeOH or the ethanol can cause the deactivation of the enzyme. Thus the use of another solvent is sometimes necessary, such as t-butanol because of its polarity. The quantity of solvent varies between 10 and 400% (V<sub>solvent</sub>/V<sub>oil</sub>) and depends on the reaction conditions and the type of substrates used. The temperature of the enzymatic transesterification reaction is between 30 and 60 °C. The pH and the quantity of water are parameters depending on the type of enzyme. Most immobilized enzymes operate in the absence of water, while the presence of water is important to free enzymes. Novel optimization techniques such as the control of alcohol concentration and enzyme pre-incubation can reduce the time of the enzymatic transesterification reaction and increase the alkyl esters yield. Thus, these optimization techniques can be effective in reducing the production costs of biodiesel on a large scale.

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## Conflict of interest

The authors declare there is no conflict of interest.

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