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Enzyme-catalyzed production of biodiesel as alternative to chemical-catalyzed processes:
advantages and constraints

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Running title: Enzymatic biodiesel towards industrial implementation

Abstract

Biodiesel represents an interesting alternative to fossil fuels. Traditionally the standard method for biodiesel production from oils is alkaline–catalyzed transesterification. Chemical catalysis can be replaced by enzymatic catalysis using lipases (EC 3.1.1.3, triacylglycerol acyl hydrolases), obtained from plants, animals or microorganisms. These biocatalysts act at milder temperature and normal pressure conditions, resulting in lower energy consumption. Also, undesirable side-reactions do not occur, originating pure products.

Refined vegetable oils are the most common feedstocks for biodiesel production, accounting for 70-80% of the overall biodiesel production costs. The search for low-cost feedstocks, i.e. non-edible oils and high acidic waste oils/greases, is an alternative to make biodiesel competitive. Non-regioselective and *sn*-1,3-regioselective lipases can catalyze esterification of free fatty acids and transesterification of triacylglycerols with good yields. The lipases used as catalysts for biodiesel production must present alcohol resistance, thermo-tolerance, high stability and activity.

Recently, enzymatic processes for biodiesel production have been implemented at industrial scale. Despite this trend, the conventional chemical process still remains the most popular, mainly due to the high cost of commercial lipases.

This review consists of an update of the state of the art of enzymatic biodiesel production, including legislation, feedstocks, lipases used for biodiesel synthesis, the role of acyl acceptors and strategies to avoid lipase inactivation, the mechanisms proposed for biocatalysis and the enzymatic bioreactors used. In addition, the economics of the bioprocess is also presented.

Keywords: *Biodiesel, lipases, immobilization, enzymatic reactors, legislation, oils feedstocks, economics*

102

103 **1. Introduction**

104 Biodiesel production is widely implemented at industrial level, representing an increased alternative to

105 fossil fuel by the depletion of fuel reserves, with environmental benefits, because their use reduces CO

106 and polycyclic aromatic hydrocarbon emissions [1]. The use of biodiesel is also favored by the legislation

107 and mandates by the countries in the last years.

108 The standard method in industry to transform oil into biodiesel (fatty acid ethyl esters, FAEEs; or fatty

109 acid methyl esters, FAMEs) is alkaline–catalyzed transesterification. However, some problems have been

110 described chemical biodiesel industries, namely (i) the low quality of glycerol produced due to the

111 presence of impurities formed by undesirable side-reactions, catalyzed by the non-selective chemical

112 catalysts, (ii) the need for high cost operations for biodiesel recovery and purification and (iii) the

113 generation of large amounts of pollutant alkaline effluents during catalyst inactivation and biodiesel

114 washing and recovery steps.

115 To overcome these problems, chemical catalysts can be replaced by enzymatic catalysts (lipases, EC

116 3.1.1.3, triacylglycerol acyl hydrolases) which are highly selective and can act at lower temperatures and

117 normal pressure conditions. These properties allow for lower energy consumption than in chemical-

118 catalyzed processes, and the formation of pure products in higher yields, since undesirable side-reactions

119 do not occur. Also, in lipase-catalyzed processes, raw materials of different origins and low-quality (e.g.

120 high free fatty acid and water contents) can be used. In the presence of high acidic oils, lipases can

121 convert free fatty acid (FFA) to FAMEs (or other alkyl esters) by esterification and TAG to FAMEs by

122 transesterification.

123 When immobilized lipases are used, the end of the reaction is easily controlled by removing the

124 biocatalyst from the reaction medium by filtration or centrifugation and easier phase separation is

125 observed because no emulsions are formed [2-4]. Fewer unit operations are needed, since biodiesel

126 washing to remove the catalyst and purification steps are no longer necessary, with a subsequent

127 reduction in the volumes of generated effluents.

128 The main limitations to substitute chemical catalysis by enzymatic catalysis of biodiesel in industrial

129 large-scale processes are the cost of the lipases [5] and the cost of raw materials to ensure the economic

130 feasibility of the bioprocess. In fact, the cost of feedstocks represents 70- 85 % of production cost of

131 biodiesel [6-10]. Nowadays the use of feedstock from nonedible, waste, wood, and microbial oils is the

132 cheapest alternative [11]. Thus, the response of the scientific community has been since 2007 a deep

133 research in this field.

134 When the key words enzym* biodiesel production are enter in ISI Web of Knowledge (IWK) data base,

135 3541 articles are selected from 2007, with significant increasing every year up to 2015 when the scientific

136 production seems to attain a stationary phase of around 550 articles per year. This data show the

137 importance in the scientific community of enzymatic biodiesel production.

138 In the following sections, an update of the current progress on the enzymatic-catalyzed biodiesel

139 production including an overview of world legislation, potential new feedstock of oils, economics

140 evaluation and industrial scale production are summarized.

141

142 **2. Biofuels and Biodiesel**

143 Biofuels are fuels produced from biomass feedstocks, used primarily for transportation, as substitutes or

144 blended with fossil fuels. They are renewable and help to reduce pollution. Although net greenhouse

145 gases (GHG) emissions depends on specific feedstock and production process. It is now well known that

146 advanced biofuels (from non-food, waste or lignocellulosic feedstocks) are better in terms of reduction of

147 GHG emissions [12, 13].

148 Biofuels market is estimated at 115 millions of tons for 2018 with an annual increase of 3% [14]. The US

149 Environmental Protection Agency (EPA) identified ethanol and biodiesel as the most viable biofuels by

150 2022 [15], whereas in Europe biodiesel has been used for more than 20 years and it is now a well-

151 established industry that provides 220000 jobs [16].

152 In this review, we refer to biodiesel as mono-alkyl esters of fatty acids, not to renewable diesel or green

153 diesel, which is a mixture of diesel-like hydrocarbons, obtained by hydrogenation of vegetable oils

154 (HVO), and is often also called biodiesel. Indeed, diesel motor was originally designed to work with

155 vegetable oils and later redesigned to work with fossil diesel. For this reason, diesel motors do not need

156 for major modifications to work with biodiesel. However, biodiesel must comply with standards and

157 maximum blends recommended by motor fabricants (Table 1). For instance, a maximum of 7 % (v/v) of

158 biodiesel in allowed in commercialized diesel for road transportation in Europe (EN 590:2014).

159   Table 2 presents the major advantages and drawbacks of biodiesel as biofuel. As it can be seen,

160   environmental advantages are considerable. Biodiesel can also be used as efficient heating oil, and in the

161   USA it is commercialized under the name of "bioheat" [17].

162

163   Table **1**. Biodiesel quality legislation and available blends in selected countries/regions.

Country/Region	Blend* – Normative
European Union	B5-10, B25-30, B100 – EN 14214
United States	B1-B5 – ASTM D975, B6-20 – ASTM D7457, B100 – ASTM D6751
Brazil	B2-7, B100 – ANP 44/2014
Argentina	B5, B20, B100 – IRAM 6515-1
Mexico	B5, B33, B100 – To be published in 2017
Indonesia	B20, B100 – SNI 7182:2012
Japan	B2-5 – JIS K 2390

164   *Volume percentage of blend is indicated as the number following the B (v.g. B5 is 5% biodiesel).

165

166   Table **2**. Advantages and drawbacks of biodiesel as biofuel [6, 7, 10, 18].

Issue	Advantages	Drawbacks
Environmental	-Reduction of carbon monoxide (-50%) and dioxide (-78%) -Reduction of PAH (-75-85%) -Reduction of carbon particles (-69 %) -Elimination of sulfur -Reduction of nitrogen oxides (-10 %)* -Non-toxic -Biodegradable	-Increased aldehydes emissions -Increased NOx emissions*
Motor performances	-Better lubricity -Better ignition (as it contains oxygen) -Less noise -Safer (non-inflammable)	-Corrosion, obstruction and oxidation problems when using biodiesel that does not complies with quality standards
Economics	-Energetic security -Fossil diesel imports reduction	-High cost of feedstocks (vegetable oils)

167   *Some tests report reduction and others increased emissions of NOx; NOx: nitrogen oxides; PAH-
168   polycyclic aromatic hydrocarbons.
169

170 Regarding biodiesel production in the world, in 2015 the US leaded the production followed by Brazil,

171 Indonesia, Germany, France and Argentina. World production reached 7952 million of gallons (26.8

172 millions of tons) in 2015 (Figure 1).

173

Top Countries (2015) Biodiesel Production (millions of gallons/year)
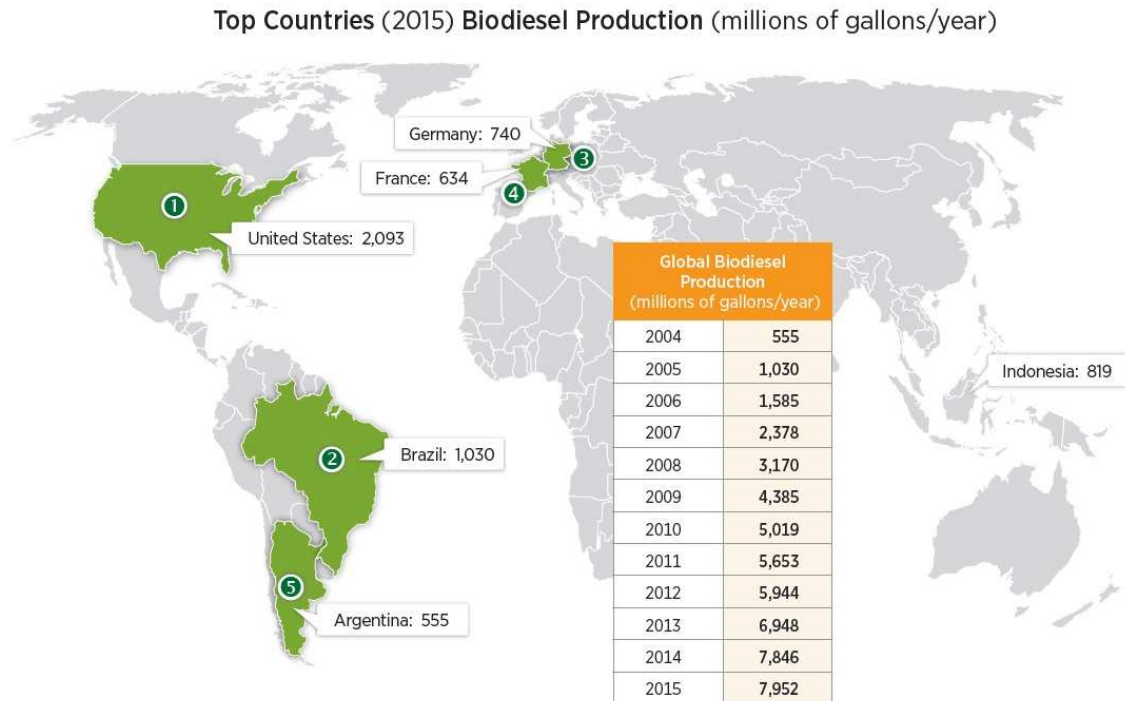
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175 Figure **1**. Production of biodiesel in the world in 2015 [19]. 1 liter equals 0,26 US gallons.

176

177 **2.1 Bioenergy legislation in the European Union**

178 The European Union establishes that until 2020, in each Member-State, the share of energy from

179 renewable sources in all forms of transport is at least 10 % of the final energy consumption in transports

180 (Directive 2009/28/CE). This goal can be attained by blending different biofuels. Also, the increase in

181 energy efficiency is an absolute need to attain fixed targets of bioenergy consumption, in a sustainable

182 way, if the global trend of energy consumption for transports continuous to increase. Energy efficiency is

183 also important for the reduction of GHG emissions.

184 By 31 December 2020, a reduction by at least 6 % of GHG along the life cycle, per unit of energy of fuels

185 used in transports in EU (e.g., road vehicles, non-road mobile machinery, agricultural and forestry

186 tractors, recreational craft when not at sea) is mandatory for fuel and energy suppliers. Blending of

187 biofuels has been one of the methods used to reduce the intensity of GHE of the fossil fuels supplied.

188 Sustainability criteria for biofuels are also established by the EU (Directive 2009/28/CE).

189 Nowadays, biofuels are mainly produced from crops installed in agricultural lands and pastures. The

190 increase in raw-materials for biofuels is only possible either by the intensification of current production

191 and/or by using non-agricultural lands. In addition, research on the development of novel advanced

192 biofuels not competing with food crops must be encouraged. Further studies on the impact of different

193 crop groups (e.g. oil crops, sugar crops, cereals and other starch-rich crops) on both direct and indirect

194 land use change should be promoted.

195 The use of advanced biofuels, obtained from wastes and algae, must be implemented, since it represents a

196 high decrease in greenhouse gas emissions, has a low risk of indirect modifications of land use. Advanced

197 biofuels do not compete with food and feed markets, for the use of arable land. However, this type of

198 biofuels is not yet commercially available in high amounts. Thus, research focused on advanced biofuels

199 is needed.

200 The consumption of advanced biofuels is promoted in the EU. It would be desirable to reach by 2020 a

201 significantly higher level of consumption of advanced biofuels compared to the current situation.

202 However, the minimum consumption level in each Member State will be a non-legally binding national

203 target to achieve within the obligation of ensuring that the share of energy from renewable sources in all

204 forms of transport in 2020 is at least 10 % of the final consumption of energy in transport in that Member

205 State. To prepare for the transition towards the use of advanced biofuels, the amount of biofuels and

206 bioliquids produced from oil crops, sugar crops, cereals and other starch-rich crops grown as main crops

207 for energy purposes on agricultural land must be restrained. The targets are set out in Directive

208 2009/28/EC.

209 The EU is moving towards a "Recycling Society", where waste generation must be avoided and, instead,

210 its use as resource is desirable following a waste hierarchy (Directive 2008/98/CE; Directive

211 2009/28/EC). The waste hierarchy is based on a priority order established according to the best overall

212 environmental option in relation to waste legislation and policy. Member States should support the use of

213 recyclates together with the waste hierarchy to become a recycling society. Whenever possible, the

214 landfilling or incineration of such recyclates must be avoided.

215

216 **2.2 Bioenergy legislation in other countries**

217 Under the Energy Policy Act of 2005, the US Congress created the renewable fuel standard (RFS)

218 program, which was and later expanded under the Energy Independence and Security Act of 2007, with

219   the aim to reduce GHG emissions and expand the nation's renewable fuels sector. The Environmental

220   Protection Agency (EPA) authorizes annual quotas dictating the percentage of the total amount of motor

221   fuels consumed in the USA that must be blended with biofuels. The target of renewable fuels by 2022 is

222   36 000 million of gallons (136 274 million liters), with an increasing percentage of cellulosic and

223   advanced biofuels (Figure 2). Regarding biodiesel, the target for 2017 is set to 2 000 million of gallons

224   (7.6 million liters) [20].

225   In Brazil, in 2013 the share of renewable energy in the total primary energy supply was around 40 % and

226   around 28 % for bioenergy. It has 75 % of renewables in its electricity supply, which qualifies Brazil as a

227   low carbon economy [21]. The official document that is driving the national policy framework of

228   renewable energy in Brazil was announced in December, 2015, in the Paris Conference (COP 21). Brazil

229   set targets of GHG reductions of 37 % below 2005 levels by 2025 and 47 % below 2005 levels by 2030.

230   Brazil also intends to increase the share of sustainable biofuels to 18 % by 2013. This includes increasing

231   levels of advanced biofuels and increasing 78% the share of biodiesel in diesel blends to 4 602 million of

232   tons of oil equivalent (Mtoe) by 2023 [21]. A concern in Brazil is Amazonia deforestation. However,

233   Brazil has reduced the deforestation rate in the Brazilian Amazonia by 82 % between 2004 and 2014 and

234   it is also strengthening policies and measures to achieve zero illegal deforestation and also reforesting 12

235   million hectares by 2030 [21].

236   China currently produces about 3 000 million liters of ethanol and about 1140 million liters of biodiesel

237   per year. The Chinese government has set targets to increase annual biofuels production to 12 700

238   million liters of ethanol and 2 300 million liters of biodiesel by 2020. However, it is highly unlikely that

239   these targets will be met, because biofuels received little attention in the recently released 13th five-year

240   plan for China and no exact output targets were given for biofuels [22].

241   In some countries, mandates are not still set. However, in spite of this, biofuels have been incipiently in

242   the market and they are becoming an attractive alternative to increasing fossil fuel prices. This is the case

243   in Mexico, where an expected increment of 30% in fossil fuel prices for 2017 is triggering markets for

244   ethanol [23] and biodiesel [24]. Whit the aim of developing research and markets for renewable energy,

245   the Mexican Ministry of Energy (SENER) and the National Council of Sciences, (CONACYT) are

246   financing National Innovation Centers on Renewable Energies (CEMIEs) through the Sustainable Energy

247   Fund (FSE). In 2016, the Mexican Innovation Center for Bioenergy (CEMIE-BIO) started its activities.

248   The CEMIE-BIO has been divided into five national clusters, each one focusing on a specific biofuel:

249   biodiesel, bioalcohols, biogas, bioturbosine and solid biofuels [25].

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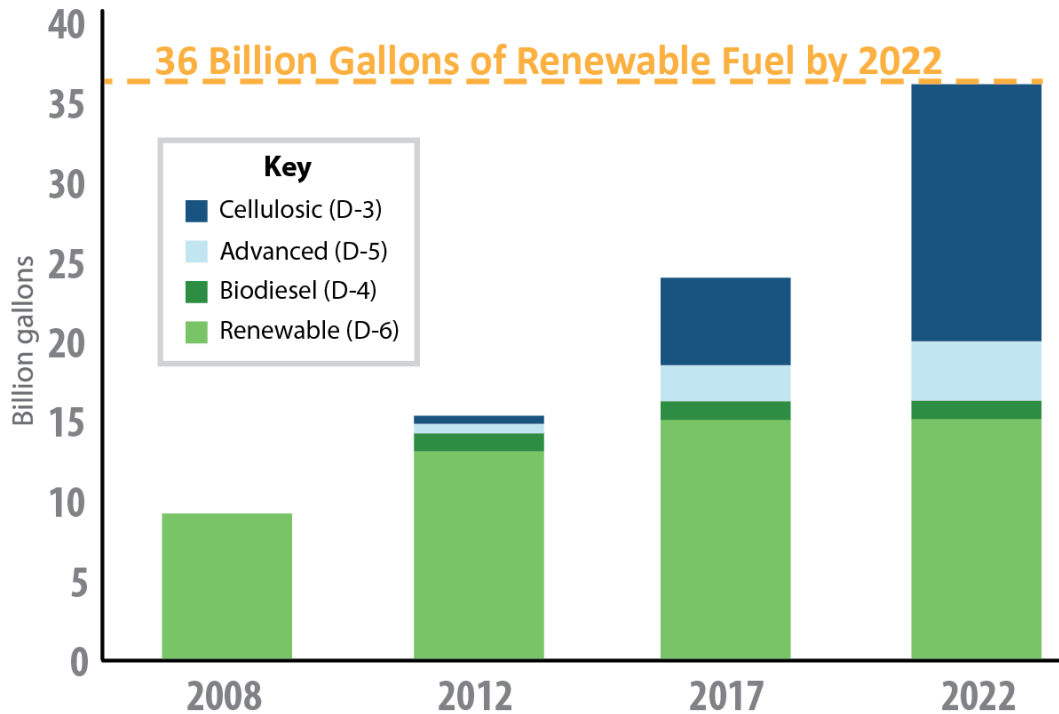
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252   Figure **2**. Targets by 2022 for the Renewable Fuels Standard (RFS) program in the USA [26].

253

254   **3. Biodiesel production process**

255   Biodiesel is derived from fatty acids or from its acylglycerols through esterification or transesterification

256   reactions with an alcohol. Water is coproduced from esterification reaction, whereas glycerol is obtained

257   in transesterification starting with triacylglycerols (TAG) (Figure 3). Reaction proceeds in the presence of

258   a catalyst, being basic catalysts the most common used (generally, sodium or potassium hydroxide or

259   methoxide), acid (usually, hydrochloric acid or sulfuric acid), or enzymatic (lipases). Reaction could also

260   be uncatalyzed when supercritical alcohol is used. Methanol is the most commonly used alcohol, that is

261   way biodiesel is currently referred as FAMEs (Fatty Acid Methyl Esters), but ethanol has shown to be a

262   real alternative to methanol, in some biodiesel standards (e.g. Brazilian biodiesel). When ethanol is used,

263   biodiesel is referred as FAEEs (Fatty Acid Ethyl Esters). Other larger-chain alcohols have been tested to

264   minimize the inactivation problems of lipases when methanol is used as alcohol.

265

A

$$R_1-OOCH \quad + \quad R'OH \quad \xleftrightarrow{\text{Catalyst}} \quad R_1-OOC-R' \quad + \quad H_2O$$

Fatty acid   Alcohol   Fatty acid alkyl   Water
                                             ester

B

$$
\begin{array}{l}
CH_2-OOC-R_1 \\
CH-OOC-R_2 \quad + \ 3\,R'OH \quad \xleftrightarrow{\text{Catalyst}} \\
CH_2-OOC-R_3
\end{array}
\qquad
\begin{array}{l}
R_1-OOC-R' \\
R_2 \ \ OOC-R' \quad + \\
R_3-OOC-R'
\end{array}
\qquad
\begin{array}{l}
CH_2-OH \\
CH-OH \\
CH_2-OH
\end{array}
$$

Triacylglycerol   Alcohol   Fatty acid alkyl   Glycerol
                                             ester

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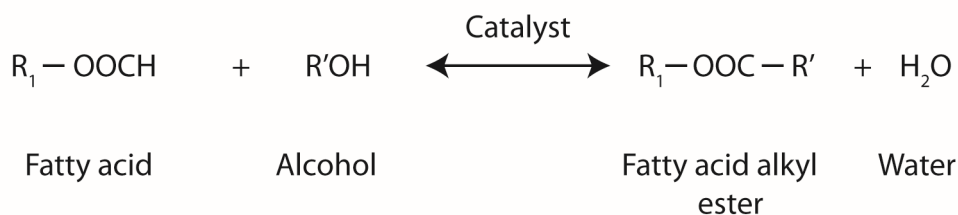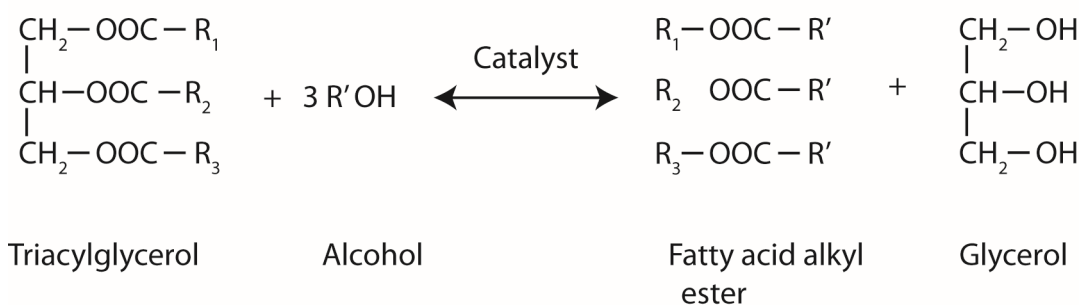
Figure **3**. Esterification (A) and transesterification (B) reactions to produce biodiesel (fatty acid alkyl esters).

3.1 Chemically catalyzed reactions

Table 3 presents illustrative examples of conditions and conversion of biodiesel produced with chemical catalysts, using either edible or non-edible oils as feedstock. Chemical catalysis with alkalis has been traditionally used to produce biodiesel and it is a well-established process with the advantage of the low cost of the catalyst. The high rate of conversion of TAG into FAMEs, in relatively short periods of time (less than 2 hours), makes this one of the most efficient and cost effective transesterification processes. Alkaline catalysis is also less corrosive than acid catalysis. However, it presents some problems related to downstream operations. For instance, glycerol recovery is difficult and time consuming. Biodiesel washing and purification steps require a high consumption of water and produce large volumes of alkaline effluents that need to be treated [2]. Furthermore, conversion of low cost raw materials such as used frying oils into biodiesel is a complicated task, if the oil presents a high free fatty acid content (>1%). Soap formation occurs, reducing the yield in methyl esters [3]. The use of solid catalysts (generally metallic oxides) have the advantage to be recyclable. The use of guanidine carbonate (organic basic catalyst) has also been reported: during boiling at reflux, guanidine carbonate disintegrates into guanidine and carbon dioxide in presence of methanol, but not with other alcohols [27]. When the oil contains a high percentage of FFA (e.g. crude or waste frying oils), acid catalysis or a previous step of acid esterification becomes necessary to avoid soap formation.

287 Table **3**. Examples of biodiesel production catalyzed by chemical catalysts.

Oil		Alcohol:Oil Molar ratio	Catalyst type	[Catalyst] (%, w/w oil)	Reaction conditions	FAMEs Yield (%, w/w)	Reference
Edible	Crude Corn	7.6:1	Sodium methoxide	1.7	50 ℃, 0.5 h	96	[28]
	Refined Corn	9.0:1	Sodium methoxide	2.0	50 ℃, 0.5 h	94	[28]
	Neutralized Olive Residue	9:1	Sodium methoxide	1.9	68 ℃, 0.5 h	95	[29]
	Refined Olive Residue	7:1	Sodium methoxide	1.6	55 ℃ 0.5 h	95	[30]
	Crude Rapeseed	6.1	Sodium methoxide	1.4	58 ℃ 0.25 h	99	[31]
	Sunflower	9:1	Potassium hydroxide	0.28	70 ℃	96	[32]
	Rapeseed Soybean	2.8:1 (methanol:FFA)	Guanidine carbonate	0.5-1.3	64.7 ℃ (Boiling Temperature) , 1 h	99	[27]
	Palm	12:1	KF/hydrocalcite (solid base catalyst)	3	65 ℃, 3 h-5 h	85 92	[33]
	Safflower	6 :1	Potassium hydroxide Sodium hydroxide Sodium methoxide Potassium methoxide	1	60 ℃, 1.5 h	98 (Sodium methoxide)	[34]
	Soybean	9:1	Mg–Al hydrocalcites	1.3	40 ℃, 1.3 h	95	[35]
	Cottonseed	7:1	Magnetic solid base	5	60 ℃, 1.7 h	99.6	[36]
	Peanut	6 :1	Potassium hydroxide	0.5	60 ℃, 1.5 h	95	[37]
	Refined rapeseed		Sodium hydroxide	1	60 ℃, 1.5 h	97	
	Palm	6:1	Sodium methoxide	0.75	45 ℃, 1.5 h	95.4	[38]

|	Feedstock	Ratio	Catalyst	Amount	Conditions	Yield	Ref
	Sunflower	6:1	Calcium oxide	7	65 ℃, 1 h	100 (Ecodiesel)	[39]
Non-edible	Waste frying oils	7.4:1	Sodium hydroxide & ultrasonication (24 kHz, 200 W)	1.5	60 ℃, 0.7 h	98	[40]
	Datura stramonium L.	6:1	Sodium methoxide	1.8	50 ℃, 0.5 h	72	[41]
	Crude cardoon	6.4:1	Sodium methoxide	1.4	52 ℃, 0.5 h	97	[42]
	Jatropha	7.28:1	Potassium hydroxide	2.06	61 ℃, 1.5 h	81.9	[43]
	Sterculia foetida seed	8:1 (2 stepwise addition)	Sodium hydroxide (2 stepwise addition)	2	60 ℃, 2 h	98.2	[44]
	Yeast *Rhodosporidium tortuloids* Y4	20:1 (v/w dried biomass)	Sodium hydroxide (*in-situ* transesterification)	4 g/L biomass	50 ℃, 10 h	97.7	[45]
	Waste agro-residues (banana peel, copra meal, corn cob, grape stalks, sugarcane bagasse)	10:1 (v/w biomass)	Sulfuric acid (*in situ* acid transesterification)	1:1 (0.2 M alcoholic sulfuric acid solution v/w biomass)	65 ℃, 8 h	Not reported	[46]
	Karabi seed (*Cascabela thevetia*)	6:1	Esterification: (Sulfuric acid) + Transesterification: (Sodium hydroxide)	1 % (w/w) (sulfuric acid) + 0.5 % (w/w) (Sodium hydroxide)	60 ℃, 1 h + 2 h	Not reported	[47]

288

289

290   **3.2 Non-catalyzed reactions**

291   Non-catalyzed production of biodiesel under supercritical conditions has also been reported. At high

292   pressure and temperature, alcohol reaches the supercritical fluid (SCF) region (Figure 4), but pressure

293   must be below the pressure required to condense it into a solid. At SCF region, liquid and gas phase have

294   the same density and the state is an intermediate between such phases and small changes in pressure and

295   temperature permits to manipulate SCF physical properties. This tunability near and above critical

296   conditions, turns SCF into remarkable and reusable solvents for extraction, but in biodiesel case it allows

297   the uncatalyzed reaction. Critical temperature, critical pressure and density and supercritical conditions

298   for methanol and ethanol are 239.6 °C, 8.09 MPa, 272 kg.m^{-3} and 240.9 °C, 6.14 MPa, 276 kg.m^{-}

299   ^{3}respectively [48].

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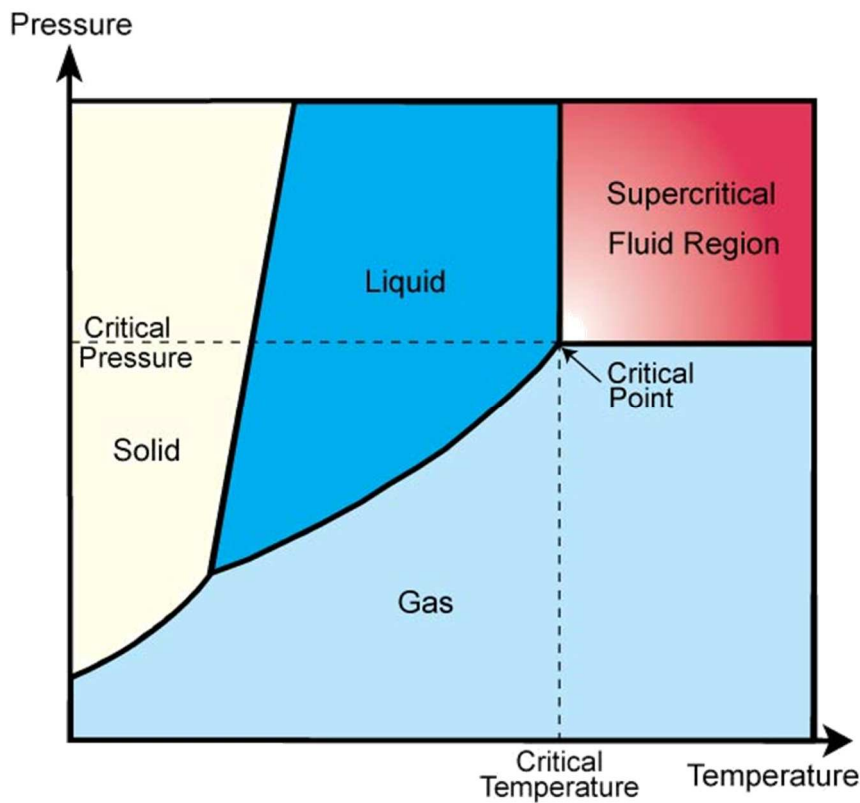
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302   Figure **4**. Pressure-temperature phase diagram showing supercritical fluid region.

303

304   In addition of molar ratio alcohol to oil, temperature and pressure are key parameters in supercritical

305   production of biodiesel. An increase in temperature leads to better reaction conversions, but at elevated

306   temperatures (> 350 °C) a decrease in reaction yield is observed because of oil decomposition, while

307   pressure influences the properties of the SCF such as density and viscosity. The best temperature and

308   pressure depends on the length and degree of saturation of the fatty acid chains of the oil as well as the

309   configuration of reactor (batch or continuous) [49]. Table 4 shows conditions and performances in

310   supercritical processes for various feedstocks. In tubular reactor lower conversions are observed and the

311   highest conversion was obtained in a spiral reactor (Figure 5). In supercritical alcohol processes, high

312   molar excess of ethanol is used. Supercritical CO_2 could be used as solvent to decrease alcohol needed.

313   The observation of the phase behavior of the system CO_2+biodiesel+methanol or ethanol showed that

314   alcohol improves biodiesel solubility in the system [50, 51]. A cosolvent could increase reaction yield:

315   CO_2 and alkanes have been added in small amounts to the alcohol in supercritical processes to improve

316   the yield [52-54]. Supercritical dimethyl carbonate (scDMC, Figure 6) has also been used instead of an

317   alcohol to produce biodiesel in an uncatalyzed process. An uncatalyzed process using DMC at

318   atmospheric pressure has also been reported to reach high conversion (98%), but still using high

319   temperatures (up to 450 °C) [55]. Uncatalyzed two-step process is carried out under more moderate

320   temperature and pressure compared to the one-step process [56, 57]. High energy and equipment cost of

321   supercritical process could be counterbalanced by integrative process combining simultaneous extraction,

322   reaction and purification, achieving high quality biodiesel and coproducts in a single step.

323

324   Table **4**. Examples of supercritical biodiesel production.

Feedstock/alcohol/cosolvent	T (°C)	P (MPa)	Alcohol : oil molar ratio	Reaction time (min)	Esters (% wt)	Reference
BATCH REACTOR						
Waste canola/methanol	270	10	≈28*	45	96.4	[58]
Soybean/methanol/propane	320	NR	33	10	95	[52]
Rapeseed/methanol	350	45	42	4	95	[59]
Cottonseed/metanol	350	NR	41	5	95	[60]
TUBULAR REACTOR						
10 % FFA soybean/ethanol	300	20	40	49	90	[61]
Palm olein/methanol	350	35	40	15	85	[62]
Palm/methanol	350	NR	40	20	80	[54]
Soybean oil/ethanol	320	15	40	45	80	[63]
SPIRAL REACTOR						
Canola/methanol	350	20	40	10	100	[64]

325   NR: Not reported. *Mass ratio of 1

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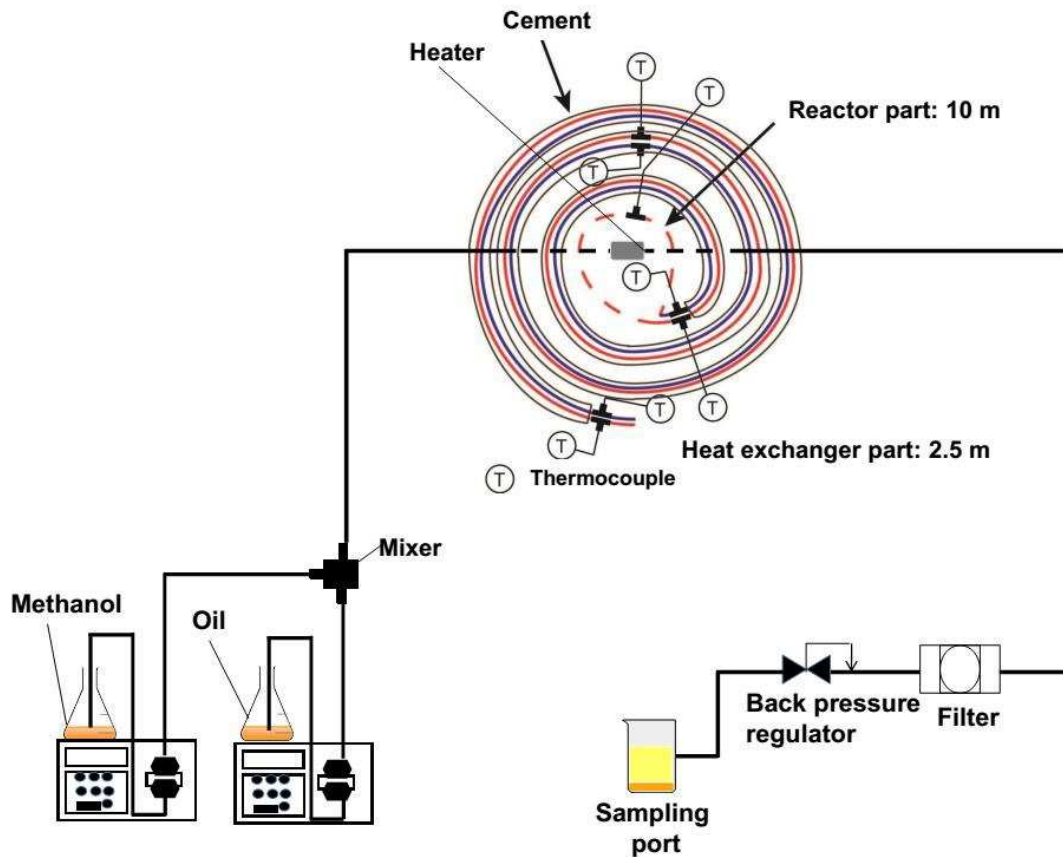
Figure **5**. Spiral reactor for supercritical biodiesel production [64].

328
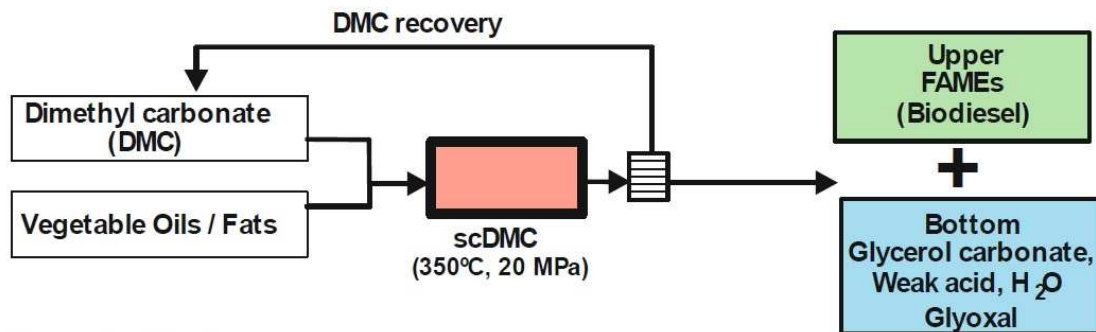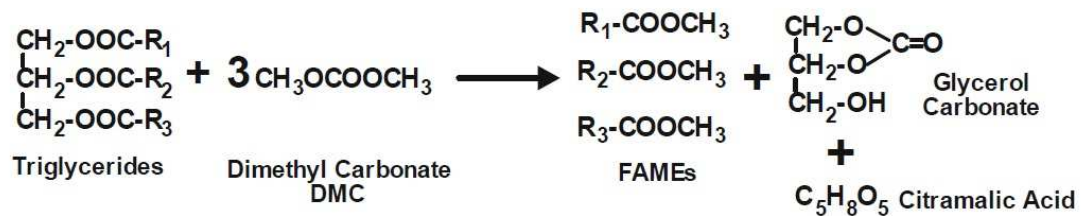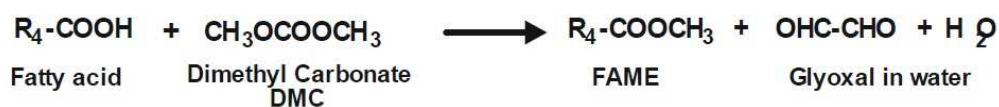
Figure **6**. Uncatalyzed process for biodiesel synthesis using supercritical dimethyl carbonate [65].

330

3.3 Enzyme-catalyzed reactions

332   Currently most common techniques used for biodiesel production are chemical catalysis using liquid

333   alkali or acid. The previously described processes present some technical and environmental problems

334   that make enzyme production of biodiesel an alternative to the non-catalyzed and chemical catalyzed

335   reactions. The enzymes capable of catalyzing biodiesel synthesis are esterases, lipases and

336   acyltransferases (see section 5).

337   Lipases (EC 3.1.1.3.) catalyze the hydrolysis of the ester bond of mono-, di- and triacylglycerols (TAG)

338   producing free fatty acids and glycerol. However, under favorable conditions such as organic solvent

339   systems and low water content, lipases catalyze synthesis reactions on different types of substrates [66].

340   The enzymatic process shows some advantages over the traditional processes, for instance lipase

341   biodiesel production can be carried out under mild reaction conditions (<70°C) reducing operational costs

342   [67], it allows an easy product and glycerol recovery and products are of high quality [7, 18, 68]. This

343   process avoids soaps formation and FFA and TAG can be esterified in one single step. One of the most

344   interesting advantages of enzyme-catalyzed biodiesel production is the high substrate specificity and

345   selectivity of lipases [5]. The differences observed for lipases in substrate specificity have been used to

346   design a *combi-lipase* for the hydrolysis of heterogeneous substrates [69]. However, for industrial

347   production, the use of lipases present some disadvantages including lipase cost, alcohol inhibition, lipase

348   inactivation and slow reaction rates [3, 18, 67]. Some factors that can affect enzymatic synthesis of

349   biodiesel are discussed in section 5.4.

350
351   **4. Feedstock (oils)**

352   Refined vegetable oils have been the most used feedstocks for biodiesel production using alkaline

353   catalysts. Animal fats (e.g. tallow, lard, chicken fats) are highly viscous and due to their high content in

354   saturated fatty acids (SFA), they are solid at ambient temperature. Biodiesel from animal fats presents

355   poor cold temperature performance in internal combustion engines [70]. Usually animal fats are blended

356   with vegetable oils to be used as raw-material for biodiesel production.

357   The use of refined oils accounts for 60-88 % of the overall biodiesel production costs [6-10]. A

358   substantial cost reduction may be attained if completely refined oils are replaced by neutralized oils, in

359   alkali-catalyzed transesterification, or degummed oils in lipase-catalyzed process [9]. In fact, 95 %

360   FAMEs were obtained after 30 min of alkali transesterification when refined [28] or only neutralized

361   olive residue oils were used [29].

362   The search for low-cost feedstocks, namely (i) oils from crops with high oil productivity/ha such as palm

363   oil or jatropha oil [9, 71], and (ii) non-edible oils, is a way to reduce biodiesel costs and make it

364   competitive with petrol-diesel [6, 9].

365   In the last years, the debate concerning the use of edible oils for energy purposes has also promoted the

366   search for non-edible oils and fats. Tables 3, 6, 7 and 8 show some examples of using edible or non-edible

367   oils as raw-material for biodiesel production either by chemical (Table 3) or enzyme catalysis (Tables 6, 7

368   and 8).

369   In addition to the non-edible oils referred in Tables 3 and 7, it is worth noticing also the following

370   potential feedstocks: castor (*Ricinus communis* L.) [72, 73], *Brassica carinata* A. Braun [74], stillingia oil

371   [75], siberian apricot (*Prunus sibirica* L.) [76], rendered chicken oil [77], waste frying oils and high

372   acidic greases from restaurants [40, 78], and oils from food waste [79].

373   Some of these non-edible crops may have great potential as bioenergy crops. That is the case of *Cynara*

374   *cardunculus* L. and *Brassica carinata* for the Mediterranean region. *Cynara cardunculus* L. (a thistle

375   currently known as cardoon) is a perennial and spontaneous plant that grows in harsh soils and dry

376   climate conditions, with a long productivity period (15–20 years). In 2005, *Cynara* was recognized by the

377   European Commission as an energy crop for biomass and oil production (EC Regulation n71701/2005,

378   October 18).

379   *Brassica carinata* A. Braun is a plant from Ethiopian highlands well adapted to semiarid climates with

380   mild to hot temperature. Its seeds are rich in non-edible oil due to the high content of erucic acid. This

381   crop presents higher oil yields than edible oilseed crops (3000 kg oil/ha *vs* 850 kg sunflower oil/ha).

382   Thus, *B. carinata* may be a promising oleaginous crop for biodiesel production even for the

383   Mediterranean region [74].

384   For tropical regions, *Jatropha curcas* L. has been considered as a miracle crop for biofuel that could

385   become the solution to energy independence and poverty eradication in these regions. Jatropha is a small

386   tree or shrub from the North-eastern part of South America that was disseminated by Portuguese

387   navigators to other countries in Africa and Asia [80]. Nowadays, jatropha is found in almost all tropical

388   and subtropical regions in the World. Some important claims have been ascribed to this crop, namely (i)

389   high oil yield production, in sub-humid tropical and subtropical environments, (ii) a long productive

390   period of 30-50 years, (iii) jatropha grows and is potentially productive in semi-arid areas, on poor,

391   degraded and saline soils, and (iv) jatropha is drought resistant and therefore can be used to combat

392   desertification and soil erosion. Jatropha seeds are rich in non-edible oil (up to 45 %, w/w), with more

393   than 75 % unsaturated fatty acids. This oil is rich in oleic (mean 40%) and linoleic (mean 40%) acids and

394   poor in linolenic acid (mean 0.22%), with high content of beta-sitosterol (71% of total sterols) making it

395   adequate for biodiesel production [81]. Jatropha oil may be easily converted by alkaline-catalysed

396   transesterification into biodiesel that meets American and European standards. Due to these properties,

397   several governments, international organizations and NGOs promoted the planting of *Jatropha curca*s L.

398   in the African continent. However, the expected results in terms of oil productivity of this crop under

399   adverse growing conditions (marginal lands and droughts) were not observed.

400   Other non-edible crops have also been studied in order to find low-cost alternative oils for biodiesel

401   production. *Datura stramonium* L. is a plant from America that is spread worldwide. It is a weed of

402   irrigated crops, toxic for animals and humans due to the presence of alkaloids (daturin and atropine). The

403   oil in its seeds (about 23 % w/w) is rich in linoleic (56.4 %), oleic (26.4 %) and palmitic (13 %) acids and

404   adequate as feedstock for biodiesel [41].

405   *Pongamia pinnata* tree is native from humid and subtropical environments and it is usually planted to

406   control soil erosion. *P. pinnata* seeds contain about 25 % oil that can be extracted by mechanical

407   expellers.

408   Siberian apricot (*Prunus sibirica* L.) is a greening tree in China with seed kernels containing 44-58 % oil

409   which showed to be an adequate raw-material for biodiesel [76].

410   *Sterculia foetida* is a soft wooded tree natural from East Africa to North Australia, but also growing in

411   Myanmar, Sri Lanka, India, Ghana and Puerto Rico. Seed kernels contain 30–35% oil (w/w) rich in

412   cyclopropene fatty acids namely sterculic and malvalic acids to an extent of 50–55%. The properties of

413   the biodiesel obtained with *S. foetida* oil are similar to that of sunflower, soybean and rapeseed oil-based

414   biodiesels except for the pour point. Thus, the presence of cyclopropene fatty acids did not limit the use

415   of these esters as biodiesel [44].

416   Karabi (*Cascabela thevetia*) is an ornamental tree that grows naturally in the north-east part of India. Its

417   seeds contain about 60-65 % (w/w) of non-edible oil in their kernel, with a high oil productivity per ha.

418   The oil is rich in oleic (44 %), palmitic (20.7 %) linoleic (20.8 %) and stearic (12.4 %) acids, which

419   makes it adequate for biodiesel production [47].

420   Besides non-conventional vegetable oils, animal fats, waste frying oils and oils from waste and agro-

421   residues, microbial [45, 82] and marine or heterotrophic algal lipids [83, 84] were considering promising

422   feedstock for biodiesel production due to their potential high productivity. Microalgae containing 70 or

423   30 % oil (w/w) in biomass could provide, in theory, 136.900 L and 58.700 L of oil per ha (Christi, 2007).

424   These values would represent about 23-fold the production of palm fat and 72-fold the production of

425   jatropha oil for the most productive algae (Schorken and Kempers, 2009). However, these algae

426   productivity values are rather optimistic. The high investment costs together with high energy demand for

427   harvesting algae biomass at low concentration have been some constraints on the industrial scale-up of a

428   cost-effective biodiesel production from algal oils [85, 86]. The costs of the salts used to obtain 1 kg of

429   algal biodiesel are similar to the price of 1 kg of petrol-diesel. Also, the energy demand for algal biodiesel

430   production is several-fold higher than the energy income from its combustion [85] According to [9],

431   production costs of oil obtained from algae grown in closed or open photobioreactors are never below 5

432   €/kg. Upstream and downstream processes should be optimized and intelligent technologies such as *in-*

433   *situ* wet biomass processing should be developed, in parallel with life cycle assessment evaluation [87].

434   Despite these problems, the production of these advanced biofuels obtained from wastes and algae has

435   been encouraged by the EU since it represents a high decrease in greenhouse gas emissions (Directive

436   2009/28/CE).

437   Concerning oil type, polyunsaturated oils are highly prone to oxidation which is reflected in a low

438   stability of the obtained biodiesel. Regarding the European (DIN EN 14214) and US legislations (ASTM

439   D 6751), a minimum Oxidative Stability Index of 6 h/110 ºC or 3 h/110ºC is required, respectively. In

440   addition, the limit was set at 12% for linolenic acid methyl ester and 1% for fatty acids with four or more

441   double bonds in European biodiesel, to avoid the use of polyunsaturated oils as biodiesel raw-material.

442   To ensure oxidative stability for the biodiesel, a maximum iodine value (IV) of 120, a parameter related

443   with the unsaturation of fatty acids, is permitted by the European legislation (DIN EN 14214). It means

444   that highly unsaturated oils (e.g. fish and marine oils) cannot be used for biodiesel production. Also,

445   soybean oil (IV: 117-143), normal sunflower oil (IV: 110-143) and safflower oil (IV: 126-152) hardly

446   meet the requirements to be used as potential biodiesel feedstock in Europe [88]. Partial hydrogenation of

447   these oils may be an option to lower iodine value, increase biodiesel oxidative stability and meet both

448   European and American standards. However, the production of partially hydrogenated methyl esters from

449   refined oil would increase biodiesel production costs. An increase by around 0.04 €/L was estimated for

450 partially hydrogenated methyl esters of soybean oil [89]. Blending high iodine value biodiesel with

451 biodiesel of lower IV to achieve the desired properties of the fuel is a current industrial practice.

452 The addition of antioxidants such as BHT and Bis-BHT may be an option to increase biodiesel oxidative

453 stability. These antioxidants are highly soluble in biodiesel, they are commercially available at acceptable

454 prices, they are not corrosive and they do not contain either acid or sulfur and nitrogen [90].

455 Oil quality parameters, including water, free fatty acids (FFA) and oxidation products contents, greatly

456 influence the yield and properties of the final product (biodiesel) and may determine the feasibility of the

457 transesterification process. High moisture content (> 0.3 %) interferes negatively with the activity of the

458 alkaline catalyst, inactivating it. When alkaline catalysts are used, the oil should have FFA content below

459 2%. The FFA will react with the catalyst to form soaps, the catalyst is inactivated and a considerable

460 decrease in the yield in methyl esters is observed [3]. In addition, an increase in viscosity and gel

461 formation, which makes it difficult to separate glycerol, are observed [91, 92]. Also, oxidation products in

462 the oil interfere negatively with the activity of alkaline catalysts [91].

463 In general, most of the low-cost raw-materials (e.g. used frying oils or crude oils) have high acidity and

464 high amounts of oxidation products. In these cases, the traditional approach consists of the esterification

465 of FFA with methanol in excess, catalyzed by an acid catalyst (usually sulfuric acid), and followed by

466 alkaline transesterification (see section 3.1). [93] proposed the replacement of acid-catalyzed step by the

467 enzymatic esterification to remove FFA of high acidic rapeseed oil prior to the alkaline transesterification

468 process.

469

470 **5. Lipases**

471 **5.1 Structural characteristics of lipases**

472 Lipases structure is characterized by a common α/β hydrolase fold [66], a conserved catalytic triad

473 formed by a nucleophile (serine, cysteine or aspartate), an acidic residue (aspartate or glutamate) and a

474 histidine [94] and an oxyanion hole that stabilizes the tetrahedral intermediate formed during the reaction

475 [66]. In addition, most of the lipases have an important structural feature, a "lid" [95], which is a flexible

476 structure formed by one or more α-helixes that cover the active site of lipases. The lid is responsible for

477 the conformational changes of lipases: in their closed conformation, the lipase is inactive since the lid

478 covers the active site; in the presence of a lipid water interface the lid uncovers the active site, allowing

479   the access of the substrate [96-98]. The movement of the lid in the presence of lipid water interface is

480   known as interfacial activation [96] and it is also implied in the selectivity of lipases [99, 100].

481

482   **5.2 Sources and selectivity of lipases**

483   Lipases can be obtained from different organisms including plants, animals and microorganisms [66].

484   Plant lipases do not have commercial applications while those from animal and microbial origins are

485   widely used [101]. The most interesting lipases used in industrial processes are those obtained from fungi,

486   bacteria and yeast [102]. Lipases from microorganisms are easier to produce and more abundant,

487   therefore represent the most studied lipases in biodiesel production. Lipase producing microorganisms

488   include *Bacillus* sp., *Pseudomonas* sp., *Staphycococcus* sp., *Aspergillus* sp., *Candida* sp., *Rhizopus* sp.,

489   *Thermomyces* sp., - and *Yarrowia* sp. among others [102].

490   Screening of lipase producing microorganisms is usually carried out in oily environments. Screening

491   techniques generally use agar plates where oils are used as carbon source allowing easy identification of

492   lipase producing microorganism [103, 104]. These traditional techniques do not identify the lipase gene

493   encoding nor can be applied for uncultivable organisms [66].

494   New techniques have emerged for the identification of new lipases. Metagenomics represents a technique

495   capable of isolating genes from unknown or uncharacterized species [105]. For example, novel lipases

496   have been successfully identified from metagenomic libraries obtained from soil samples [106], tidal flat

497   sediments [107], and oil contaminated soil [108]. PCR with degenerated oligonucleotides has also been

498   used for identification of lipases in DNA libraries [109]. After identification, novel lipase genes can be

499   expressed in yeast or bacteria [108].

500   An important characteristic of lipases is their selectivity, which is the property related to their preference

501   for specific substrates [66, 102]. Selectivity is classified in type-selectivity, regioselectivity and

502   enantioselectivity. Type-selectivity relates to lipase preference for mono-, di-, or triacylglycerols, fatty

503   acid chain length, degree of unsaturation and potential substrate substitutions. Lipase selectivity for

504   specific chemical groups is part of type-selectivity and is also known as chemo-selectivity. Lipases

505   regioselectivity refers to their preference versus a specific ester bond in the glycerol backbone of TAG

506   and can be *sn*-1 (3) or *sn*-2. Finally, enantioselectivity refers to lipases preference for one enantiomer of a

507   chiral molecule.

508 When regioselectivity lipase are used only two moles of FAMEs are formed per mole of triacylglycerol

509 (TAG), instead of three moles of FAMEs per mole of TAG, the theoretical maximum biodiesel yield is 66

510 mol-%. The formation of glycerol is thus replaced by 2-MAG, a product with interesting applications as

511 emulsifier in food, pharmaceutical and cosmetics industries, with a higher value added than glycerol

512 [110].

513 Several microbial lipases show *sn*-1 (3)regioselectivity including lipases from *Yarrowia lipolytica*,

514 *Rhizomucor miehei*, *Rhizopus oryzae* and *Thermomyces lanuginosus*. Few lipases like those from

515 *Staphylococcus* show *sn*-2 specificity [111]. Lipases can also be non-selective and act randomly on the

516 ester bonds of triacylglycerols. Non-selective and *sn*-1,3-regioselective lipases are capable of carrying out

517 esterification of FFA and transesterification of TAG with good yields. Therefore, they are of interest for

518 enzymatic biodiesel production. Despite of the *sn*-1,3-selectivity of certain lipases good yield can be

519 obtained due to the behavior known as acyl migration. Hydrolysis of TAG with this type of lipases

520 produces 1,2-DAG or 2,3-DAG and 2-MAG, unstable molecules that after acyl migration change to 1,3-

521 DAG and 1-MAG or 3-MAG and can be used by the lipase [102]. Studies about the *sn*-1,3-selective

522 lipase of *R. oryzae* showed that acyl migration is independent of enzymatic catalysis [112] being the

523 temperature and the water activity, important factors for acyl migration [113]. It has been reported that

524 immobilization of lipases can promote acyl migration [2]. Acyl migration is important for enzymatic

525 production of biodiesel and should be considered in kinetic studies [114].

526

527 Table **5**. Lipases specificity. (L) long-chain fatty acids, (S) short-chain fatty acids and (M) medium-chain
528   fatty acids. From [115].

Lipase source	Fatty acid specificity	Regio specificity (*sn*)
A. niger	S, M, L	1, 3 >> 2
Y. lipolytica	S, M, L	1, 3 > 2
T. lanuginosus	S, M, L	1, 3 >> 2
M. javanicus	M, L >> S	1, 3 > 2
R. miehei	S > M, L	1 > 3 >> 2
Pancreatic	S > M, L	1, 3
Pre-gastric	S, M >> L	1, 3
P. roquefortii	S, M >> L	1, 3

R. delemar	M, L >> S	1, 3 >> 2
R. javanicus	M, L > S	1, 3 > 2
R. japonicus	S, M, L	1, 3 > 2
R. niveus	M, L > S	1, 3 > 2
R. oryzae	M, L > S	1, 3 >>> 2
P. fluofescens	M, L > S	1, 3 > 2
P. sp.	S, M, L	1, 3 > 2
R. arrhizus	S, M > L	1, 3

529

530

5.3 Heterologous expression of lipases

531

532 Lipases have limitations such as methanol inhibition, temperature tolerance, stability and activity. In

533 addition, lipase-producing organisms can present low production yields [102] which may be an

534 inconvenient when these lipases are to be used as catalysts for the industrial production of biodiesel.

535 Furthermore, some lipases from natural sources do not have the selectivity, activity and stability required

536 for the industry. Lipases can be improved using protein engineering and cloned for their heterologous

537 expression on different organisms. Also, they can be modified to improve desired characteristics using

538 rational design [116] or directed evolution [117]. Rational design requires information about the 3D

539 structures of the lipase and the structure-function relation, while directed evolution is based on random

540 mutagenesis [118]. Lipases evolution using both approaches generates smaller libraries easier to test

541 [119]. The characteristics that are usually improved include activity, selectivity, stability in organic

542 solvents, thermostability and pH stability [102].

543 Heterologous expression is a technology of great interest for their reproducibility and high yield

544 production of recombinant lipases [102, 120]. Bacterial, yeast and filamentous fungi are the most

545 common host systems used for the heterologous production of lipases [121]. Choosing the best cell

546 factory requires a deep study of several parameters such as on inserting multiple copies of the gene of

547 interest, the type of glycosylation, the adequate folding, and on the economics of scale-up. Unfortunately,

548 there are no rules and the choice depends on the target lipase [122]. The most common prokaryotic

549 systems used for heterologous expression of lipases are *E. coli, B. subtillis, A. eutrophus* and the

550 *Pseudomonas*-based systems developed by DOW Chemical Company [121]. In prokaryotic systems

551 *Saccaromyces cerevisiae, Y. lipolytica, Aspergillus* sp and *P. pastoris* are the most common cell factories

552   to produce heterologous lipases [102].

553   Among them, the methylotrophic yeast *P. pastoris* is the most popular expressing many lipases from

554   bacteria to mammalians species, for instance *Candida* sp., *Rhizopus* ap., *Yarrowi*a sp., *Aspergillus* sp.,

555   *Rhizomucor* sp, *Thermomyces* sp, *Penicillium sp, Serratia sp, Galactomyces sp, Malessezia Pseudomonas*

556   *sp, Bacillus sp,* [102, 121].

557   Some examples of recombinant lipases for biodiesel have been produced in *E. coli, S. cerevisiae, P.*

558   *pastoris* and *A. oryzae* [102]. Lipases from *P. aeruginosa* [123]*, P. fluorescens* [124]*, B.*

559   *amyloliquefaciens* [125]*, B. subtilis* [126]*, B. thermocatenulatus* [127]*, Proteus mirabilis* [128]*, P.*

560   *vulgaris* [129] and *Staphylococcus haemolyticus* [130] were expressed in *E. coli.* Lipases from *C.*

561   *antarctica* [131]*, Y. lipolytica* [132] and *R. oryzae* [133] have been expressed in *S. cerevisiae. P. pastoris*

562   has been used for expression of lipases from *R. miehei* [134]*, R. oryzae* [135 , 136, 137]*,* a chimera from

563   *R. oryzae* and *R. chinesis* [120] and *P. cyclopium* [138]. For fungi heterologous expression, the most used

564   genera are *Aspergillus* sp. and *Trichoderma* sp. [134]. The commercial immobilized biocatalyst Novozym

565   435 is a recombinant *C. antarctica* lipase B expressed in *A. niger*, immobilized onto acrylic macroporus

566   resin and is one of the most used lipases in biodiesel production [139].

567   The heterologous production of two of the most used lipases, *C. rugosa* and *R. oryzae* produced in

568   different cell factories has been reviewed [121].

569

570   **5.4 Factors affecting lipase performance**

571   The enzymatic production of biodiesel is mainly affected by the following factors: lipase specificity and

572   selectivity, feedstock, type of immobilization, acyl acceptor type and concentration, reactor configuration,

573   reaction temperature, water content, presence or absence of solvent and reaction time among others [7,

574   18, 114].

575   Water content is essential to maintain structure and function of lipases [140]. In addition, the presence of

576   water increases interfacial activation between the aqueous-organic phase [141]. In transesterification

577   reactions with lipases, optimal water content is important, since the enzyme is unable of catalyzing the

578   reaction in absence of water because no water-oil interphase is formed [101]. However, high water

579   activity will increase hydrolysis and reduce transesterification yields [141]. High amounts of water can

580   also affect the access of hydrophobic substrates to the enzymes in porous hydrophilic supports because

581   the pores will be filled with water [140]. In solvent and solvent-free systems the optimum water content

582   depends on the lipase, immobilization support and organic solvent; ranges from trace amounts to up to

583   20% wt have been reported [3, 18, 114, 141-143].

584   Lipase-catalyzed production of biodiesel at moderate temperatures is an advantage of the enzymatic

585   process since it reduces energy consumption. Lipases have different optimal temperatures ranging from

586   20-70°C depending on their sources [141]. In the transesterification reaction, the reaction rate increases

587   with temperature until the optimal temperature is reached [144]. Temperatures above the optimal

588   temperature cause enzyme denaturation, reducing conversion [141, 144]. In general immobilization is a

589   good strategy to improve enzyme temperature stability [145]. Several factors affecting optimal

590   temperature reaction include immobilization method and support used, lipase stability, type of alcohol,

591   alcohol to oil molar ratio and type of solvent [141].

592   Although, productivities are higher in solvent-free systems, lipase-catalyzed esterification and

593   transesterification proceed faster in solvent than in solvent-free systems where alcohol in excess acts as

594   solvent. This is due to both mass-transfer issues and alcohol inhibition [146] which decreases with

595   alcohol chain length. Strategies to avoid alcohol inhibition are discussed in section 7.

596   Optimal solvent depends on reactor and reaction type: while for esterification hydrophobic solvent is a

597   good choice [147], for transesterification hydrophilic solvent is a better choice [148]; especially in

598   continuous systems [149], because a hydrophilic solvent decreases alcohol inhibition by decreasing its

599   thermodynamic coefficient [147]. Indeed, thermodynamical modeling could be a useful tool to choose

600   solvent and substrate concentrations in lipase-catalyzed reactions [150, 151]. Response surface

601   methodology could also be a useful tool to optimize reaction conditions [152].

602   In most cases, the optimum methanol/oil molar ratio (MR) corresponds to the stoichiometric value of 3:1,

603   for non-regioselective lipases, or slightly higher values. However, higher molar ratios were used in batch

604   methanolysis catalyzed by *Burkholderia cepacia* lipase immobilized on modified attapulgite (MR =

605   6.6:1) [153] or by *B. cepacia* lipase immobilized on silica-PVA matrix and used in continuous ethanolysis

606   (MR Ethanol/oil = 7) [154]. This can be explained by the high tolerance of *B. cepacia* lipase to alcohols,

607   namely to methanol [155].

608   It is worth to notice that lipases can efficiently use crude oils, most of them with high free fatty acid

609   contents, in transesterification reactions, which is not an option with alkaline catalysts [78, 84, 156-159].

610   The presence of high FFA in crude olive residue oil (19 % FFA; [137]) or in jatropha oil (18.3 %; [157])

611   improved reaction efficiency.

612

6. Lipases used as catalyst for biodiesel production

6.1 Free lipases

In biodiesel production, the most widely used lipases are from *C. antarctica, C. cylindracea, C. rugosa, P. cepacia, P. fluorescens, R. oryzae, Rhizomucor miehei, T. lanuginosus, A. niger* and *R. delemar* [141], being generally used in their immobilized form. The use of some commercially available lipases, either in their free form such as lipases from *P. fluorescens* (Lipase AK, Amano), *B. cepacia* (Lipase PS, Amano), and *T. lanuginosus* (Lipase LA201 and Lipopan 50BG, Novozymes), or immobilized lipases from *T. lanuginosus* (Lipozyme TL, Novozymes), *R. miehei* (Lipozyme RM, Novozymes) and *C. antarctica* (CALB) have been reported [102].

Few studies have been carried out about biodiesel production using free lipases. However, in recent years it has been drawn attention due to its lower preparation costs, when crude extracts with lipase activity are used [160]. Kaieda *et al.* [161] studied methanolysis of soybean oil using free lipases from *C. rugosa, P. cepacia* and *P. fluorescens*. Reaction rates with *C. rugosa* and *P. fluorescens* lipases decreased at low water content, while for *P. cepacia* lipase, the reaction rate was higher at low water content. This lipase also showed good methanol resistance. The lipase from *P. fluorescens* was also tested in its free and immobilized form in the reaction between triolein and 1-propanol or 1-butanol [162]. Free and immobilized lipase from *P. fluorescens* catalyzed the reaction. However, the reaction was faster (10 h) and with better yields with the immobilized lipase than with the free lipase (25h). The use of combination of free lipases was studied by Guan *et al.* [138], who expressed lipases from *R. miehei* and *P. cyclopium* in the cell factory *P. pastoris*. Using *R. miehei* lipase as catalyst, the methanolysis of soybean oil reached 68.5% yield while no reaction was detected with *P. cyclopium* lipase. A combination of these two lipases gave a reaction yield higher than 95% due to the difference in the specificities. *R. miehei* lipase was also expressed in *P. pastoris* by Huan *et al.* [134] and used in the methanolysis of microalgae oil. The lipase was stable over six months at 4°C and gave a reaction yield in methanolysis of 91%.

The commercial free lipase NS81006 from the genetically modified *Aspergillus niger* was studied for biodiesel production [160, 163]. Fatty acid methyl (FAMEs) and ethyl (FAEEs) esters were produced from soybean oil obtaining yields of 95.1% [160] and 90% [163] respectively. Lipase NS81006 was stable for five batches in the production of FAEEs after simple separation of the water phase [163]. Another commercial lipase studied is Callera Trans L. Ethanolysis of rapeseed oil using Callera Trans L

642   formed a biphasic system with conversion of 97.8% [164]. Callera was also tested for methanolysis of

643   corn, rapeseed and crude soybean oils obtaining reaction yields higher than 95 % [165].

644   Table 6 presents examples of biodiesel produced by free lipases.

645 Table **6**. Examples of biodiesel production catalyzed by free lipases.

Lipases	Oil	Alcohol	System	Reaction conditions	Yield (%)	Reference
Cryptococcus spp. S-2 yeast	Rice bran Olive Rapeseed Soybean	Methanol	Solvent-free High water content medium: 80 % (w/w of oil)	120 h, 30 °C,	80.2 (rice bran oil)	[166]
C. rugosa *B. cepacia* *P. fluorescens*	Soybean oil	Methanol	Solvent-free Water 0-20% wt	90 h, 35 °C, 150 rpm	90 80 90	[161]
P. fluorescens	Triolein	Propanol Butanol	Solvent-free	25h, 35 °C	90 80	[162]
R. oryzae	Crude vegetable oils (soybean, palm and rapeseed) from waste bleaching earths	Methanol	Solvent-free High water content medium: 75 % (w/w of oil) = 36.9 MR water/oil	35 °C, 96 h	55 (palm oil)	[167]
R. miehei expressed in *Pichia pastoris* *P. cyclopium* expressed in *Pichia pastoris*. *R. miehei* + *P. cyclopium*	Soybean oil	Methanol	Solvent-free Water 28.6% wt	12h, 30 °C, 180 rpm	68.5 ND >95	[138]
Lipase NS81006 from *Aspergillus niger*	Soybean oil	Ethanol	Solvent-free Water 20% wt	8h, 45 °C, 1200 rpm	90	[163]
R. miehei expressed in *Pichia pastoris*	Microalgae oil	Methanol	Hexane	24h, 30 °C, 150 rpm	91	[134]
Lipase NS81006 from *Aspergillus niger*	Soybean oil	Methanol	Solvent-free Water 10% wt	8h, 55 °C	95.1	[160]
Callera Trans L	Crude soybean oil	Methanol	Solvent-free Water 3.5% wt	24h, 35 °C, 250 rpm	>95	[165]
Callera Trans L	Rapeseed oil	Ethanol	Solvent-free Water 10% wt	35 °C, 1200 rpm	97.8	[164]
T. lanuginosus	Rapeseed and soybean oil	Methanol	Solvent-free Water 2-20% wt	24h, 35 °C	92-97	[168]

646 (ND- not detected)
647

6.2 Whole cells

Another alternative for enzymatic biodiesel production is using whole cell displaying lipase activity as biocatalyst. Bacteria, yeast and fungal species can be used as whole cell biocatalyst [68]. Whole cell refers to intracellular lipases or lipases that are attached to the cell wall of microorganisms [169]. Whole cells do not require enzyme extraction or purification steps, reducing operational costs [141]. In addition, whole cell biocatalysts may show high operational stability, high enzyme activity and immobilization can be done simultaneously during fermentation [140]. Some disadvantages of this method include mass transfer limitation and the need for aseptic handling to avoid contamination [140]. An interesting approach of whole cell is the production of recombinant proteins on the cell surface of the host by fusion with its cell surface proteins [102]. Three types of whole cells can be used for biodiesel production: wild-type lipase producing cells, yeast-based surface display technology, and genetically engineered cells [140]. Microorganisms that naturally produce cell bound lipases are classified as wild type lipase producing cells [140], such as *Rhizopus oryzae* whole cell [170]. Cell surface is a technique that exploits the functional element of microbes to place the enzymes on the extracellular cell surface of the microorganisms [140].

Whole cells from *R. oryzae* have been used for biodiesel production [11, 171-173]. Lipase-producing *R. oryzae* cells were immobilized during batch cultivation in polyurethane foam biomass and used for the methanolysis of soybean oil in a packed bead reactor [174]. Using a flow rate of 25 L/h resulted in methyl ester yields over 90% with conserved conversion of 80% after 10 repeated-batch reaction cycles of 72 h in the PBR. Immobilized *R. oryzae* whole cells inside biomass support particles (BSPs) were treated with cross-linking glutaraldehyde to improve stability [175]. Tests showed that treated cells were more stable in the presence of methyl esters after six batches of 72 hours each. *R. oryzae* IFO4697 whole cell was used for methyl ester production by direct esterification of oleic acid, reaching a biodiesel yield of 90% after 48 h[172]. *R. oryzae* IFO4697 whole cell was also tested on different vegetable oils; under optimal conditions, and three stepwise methanol addition an ethyl ester yield of 86% was obtained after 72h [173] and of 90 % in the presence of 15 % water were reported, also after 72 h reaction [171]. This biocatalyst was also tested in a tert-butanol system that reduced negatives effects caused by methanol [170]. *R. oryzae* whole cell biocatalyst were immobilized in BSPs and tested in oil from *Jatropha curcas* [176]. Methanolysis of jatropha oil gave better yields with the whole cell biocatalyst (80% wt, 90 h) than with Novozym 435 (76% wt, 90 h).

678   *R. miehei* lipase has been displayed in *P. pastoris* cell surface and used for biodiesel production from

679   soybean oil [177]. The whole cells showed good stability in isooctane system where methanol was

680   provided in a three-stepwise addition procedure to reduce lipase inactivation. Under these conditions a

681   methyl ester reaction yield of 83.1% was obtained after 72 h. Lipase 2 from *Y. lipolytica* is an

682   extracellular lipase that was displayed on the surface of the yeast to test its applications as a whole cell

683   catalyst [178]. Results showed that the cell-bound lipase was more thermostable than the free lipase and

684   was capable of producing biodiesel with a yield of 84.1% after 33 h. Yan *et al.* [179] co-displayed the

685   lipase B from *C. antarctica* and the lipase from *T. lanuginosus* on the surface of *P. pastoris* cell to

686   produce a combined whole cell. The use of co-displayed whole-cells showed high biodiesel conversion

687   (95.4%, reaction time 12.6 g) and only 16% loss in activity was detected after 15 cycles.

688   The lipase B from *C. antarctica* was produced as whole-cell biocatalyst in *A. oryzae* with high

689   esterification activity [180]. This biocatalyst was stable for 20 cycles of 24 hours with a conversion over

690   80%. *A. oryzae* was also used for the expression of the thermostable lipase from *Geobacillus*

691   *thermocatenulatus* [181]. This lipase was highly tolerant to organic solvents and gave nearly 100%

692   methanolysis of palm oil (96 h). *Fusarium heterosporum* lipase was expressed in *A. oryzae* cells,

693   immobilized in BSP and tested in six packed bed reactors [182]. Under optimal conditions, a product with

694   96.1% of methyl esters was obtained after the sixth column. Combination of whole-cell biocatalyst using

695   lipases from *R. oryzae* and *A. oryzae* was also tested as an alternative of biodiesel production in ionic

696   liquids [183]. This combination gave conversion over 95% after 72h. However, the use of ionic liquids as

697   solvent in biodiesel production is not yet industrially viable due to economic and toxicity issues. Other

698   lipases producing whole cell biocatalyst include *A. niger*, *R. mucilagenosa*, *Pseudomosa* sp., *P.*

699   *fluorescens* and *Candida* sp. [101]. *E. coli* can also be used for the production of whole-cell biocatalyst.

700   The lipase from *Serratia marcescens* YXJ-1002 was cloned and expressed as an intracellular lipase in *E.*

701   *coli* [184]. Using this biocatalyst, biodiesel was produced from waste grease in a non-solvent system with

702   yields of 97% after 72h.

703

704   **6.3 Immobilized lipases**

705   The main reasons why lipases are not yet widely used in the industry are their cost, longer reaction time

706   and consequently lower productivity, compared with alkaline catalysts. An essential strategy to lower the

707   cost of the enzymatic process is the multiple reuse of the biocatalyst or its use in continuous bioreactors,

708   which can be achieved by using immobilized enzymes.

709   Immobilization by adsorption, cross-linking, covalent binding, entrapment and encapsulation are the most

710   commonly used methods for improving lipase operational stability and to make lipases more appealing

711   for industrial use [6, 185].

712   Table 7 shows some interesting examples of the production of biodiesel from edible or non-edible oils, in

713   solvent-free or in presence of an organic solvent, catalyzed either by commercial or by non-commercial

714   immobilized lipases. The replacement of high-cost commercial immobilized lipases (e.g. Lipozyme RM

715   IM, Lipozyme TL IM and Novozym 435, from Novozymes A/S, Bagsvaerd, Denmark) by non-

716   commercial lipases and the use of novel carriers have been attempted during the last years. Recombinant

717   *Rhizopus oryzae* lipases [82, 137, 156, 158, 186], *Carica papaya* lipase [158], *Cryptococcus spp.* S-2

718   yeast lipase [166], the lipase/acyltransferase from *Candida parapsilosis* [159] and intracellular Cal A and

719   Cal B lipases [83] are examples of non-commercial enzymes tested for biodiesel production. Synthetic

720   resins [82, 137, 156, 158, 159, 186, 187], silica derivatives [154, 157, 188], modified attapulgite [153]

721   and calcium alginate [83] are examples of carriers used for enzyme immobilization.

722   The *sn*-1,3 regioselective *Thermomyces lanuginosa* lipase was immobilized on iron oxide nanoparticles,

723   to facilitate biocatalyst recovery with a magnet, and used in the presence of silica to facilitate acyl

724   migration and promote FAMEs synthesis [189]. Also, the use of Amberlite IRA-93 resin, to immobilize

725   the *sn*-1,3 regioselective recombinant *R. oryz*ae lipase, showed to accelerate acyl migration allowing the

726   conversion of TAG into FAMEs and glycerol [186].

727   From the examples presented in Table 7, the time needed to attain reaction equilibrium is between 0.5 to

728   120 h. longer reaction times reflect on a lower biodiesel productivity, which can be easily calculated from

729   these examples. The highest FAEEs productivity (33 % FAEEs/h) was obtained by ethanolysis of

730   soybean oil catalyzed by *Thermomyces lanuginosa* lipase immobilized on iron oxide nanoparticles [189]

731   followed by 19 % FAEEs/h when Lipozyme TL IM was used [190]. In methanolysis, in hexane medium,

732   the highest productivity value (154 % FAMEs/h) [191] followed by 19 % FAMEs/h [78] were attained

733   with Lipozyme RM IM as catalyst. In solvent-free media, the highest productivity values were 16 %

734   FAMEs/h in the methanolysis of crude jatropha oil catalyzed by a recombinant lipase from *Rhizopus*

735   *oryzae* immobilized in synthetic resins [158]. FAMEs or FAEEs productivities of enzymatic processes

736   are lower than those attained by chemical catalysis (Table 3). However, the overall time of enzyme-

737 catalyzed process is similar to that of chemical-catalyzed biodiesel processes because the time-consuming

738 and high cost downstream operations needed when chemical catalysts are used (e.g. catalyst inactivation,

739 biodiesel washing, glycerin purification) are not required in enzyme-catalyzed processes.

740 Table **7**. Examples of biodiesel production catalyzed by immobilized lipases.

Biocatalyst	Biocatalyst load	Oil	System	Reaction conditions	Yield (%)	Stability	Reference
Lipozyme IM 60 (M. miehei lipase) SP435 (*C. antarctica* lipase B) Other powdered lipases (*G. candidum, B. cepacia, R. delemar*)	10 % (oil weight)	Tallow High acidic greases from restaurants Rapeseed Soybean Olive	Hexane MR alcohol/ oil = 3	45 ℃, 5 h	Lipozyme IM 60: 77-94.8 (methanol); 68-98 (ethanol); 98-100 (butanol); SP435: 83.8 (2-butanol)	Not evaluated	[78]
			Solvent-free MR alcohol/ oil = 3	45 ℃, 5 h	SP435: 90.3 (isopropanol); 96.4 (2-butanol)	Not evaluated	
B. cepacia lipase (PS30, Amano) immobilized on diatomite	10 % (oil weight)	Palm kernel	Solvent-free MR alcohol/ oil = 4	40 ℃, 8 h	Ethanol: 72 t-butanol: 62 1-butanol: 42 n-propanol: 42 iso-propanol: 24 methanol: 15	Not evaluated	[192]
		Coconut	Solvent-free MR alcohol/oil = 4	40 ℃, 8 h	Ethanol: 35 1-butanol: 40 Iso-butanol: 40 1-propanol: 16 methanol: traces	Not evaluated	
Lipozyme TL IM (*T. lanuginosa* lipase) Immobilized *P. fluorescens* (AK) Lipozyme RM IM	10 % (oil weight)	Cotton seed Peanut Sunflower Palm olein Coconut Palm kernel	*n*-hexane MR alcohol/oil = 3	40 ℃, 24 h	97	Not evaluated	[187]
			solvent-free with methanol (3-step methanol addition)	40 ℃, 24 h	>90 (Lipozyme RM IM; AK) >60 (Lipozyme TL IM)	Lipozyme RM IM: 70 % activity after 168 h	

						Lipozyme TL IM: 75% activity after 120 h; 35% residual activity after 192 h (repeated 24 h-batches)	
			solvent-free with 2-propanol (3-step methanol addition)	40 ℃, 24 h	50-65	Not evaluated	
Lipozyme TL IM (*T. lanuginosa* lipase) Lipozyme RM IM Immobilized *P. fluorescens* (AK) in polypropylene (EP 100) Immobilized *C. antarctica* A, *C. antarctica* B & *R. miehei* lipases in celite or EP 100	10 % (oil weight)	sunflower	*n*-hexane or petroleum ether	40 ℃, 24 h	80 (RM IM, TL IM and *P .fluorescens* lipase)	Not evaluated	[193]
			solvent-free MR alcohol/oil = 4.5 (3-step methanol addition)	40 ℃, 24 h	>90 (*P.fluorescens* lipase in EP 100)	Lipozyme RM IM stable over 120 h (repeated 24 h-batches) Lipozyme TL IM: 50 % activity after the second reuse	
Lipozyme TL IM (*T. lanuginosa* lipase)	10 % (oil weight)	soybean	Solvent-free MR alcohol/ oil = 3 (3-step methanol addition)	40 ℃, 12 h	98	94 % activity after 15 batches	[194]

Lipozyme RM IM	9 % (oil weight)	soybean	*n*-hexane MR methanol/oil = 2.37	50 ℃, 0.5 h	77	Not evaluated	[191]
Novozym 435 (*C. antarctica* lipase B)	10 % (oil weight)	jatropha *Pongamia pinnata* (karanji) sunflower	MR Ethyl acetate/oil = 11	50 ℃, 12 h	91.3 90 92.7	Stable after 12 repeated batches (144 h) Activity = 0, after 6th batch when ethanol is used	[195]
Novozym 435 (*C. antarctica* lipase B)		Soybean	MR methanol/oil = 4.3	52 ℃, Flow rate= 0.1 mL/min	75.2	Continuous reactor	[196]
Lipozyme TL IM (*T. lanuginosa* lipase)	15 % (oil weight)	Soybean	Solvent-free MR ethanol/oil = 7.5 4 % water (w/w oil)	32 ℃, 5 h	96	Not evaluated	[190]
Novozym 435 (*C. antarctica* lipase B) *R. oryzae* lipase immobilized on biomass support particles	4 % (oil weight)	Jatropha	MR methanol/oil =3	30 ℃, 90 h 30 ℃, 60 h	76 80	93.8 % of initial activity after 5 batches (90 h each) 91.1 % of initial activity after 5 batches (60 h each)	[176]

E. aerogenes lipase immobilized on activated silica	50 U/g oil	Jatropha	MR methanol/oil =4	55 ℃, 48 h	68	Negligible activity loss after 7 batches (48 h each).	[188]
Novozym 435 (*C. antarctica* lipase B	3% (oil weight)	Sunflower	Solvent-free MR methanol/oil =3 (3 stepwise addition)	45 ℃, 50 h	95.65	$t_{1/2}$= 82.4 h after 5 batches (50 h each).	[197]
			Solvent-free MR methyl acetate/oil =3 (3 stepwise addition)	45 ℃, 50 h	99.83	$t_{1/2}$= 1728 h after 5 batches (50 h each).	
			Solvent-free MR methyl acetate/oil =12 (one step addition)	45 ℃, 8 (fed-batch P BR: flow rate 16.6 mL/min)	96.2	No loss of activity after 72 h (8 batches)	
Recombinant *R. oryzae* lipase immobilized on Amberlite IRA-93	24 U/g oil	Soybean	Solvent-free MR methanol/oil = 4.8 60 % water/oil	37 ℃, 48 h	90.5	No deactivation after 7 batches (48 h each)	[186]
B. cepacia immobilized on polyacrylonititrile membrane	0.35 % (oil weight)	Soybean	Solvent-free 51 % methanol Methanol/water = 4:3.84 (w/w) = 1.83 (mol/mol)	30 ℃, 24 h	70 (pure soybean) 90 (soybean with 50 % FFA)	91 % activity after 10 batches (240 h)	[135]

B. cepacia lipase immobilized on hydrophobic silica	14.8 % (oil weight)	Jatropha	Solvent-free MR methanol/oil = 3; 0.6% water (w/w) based on the total mass	40 ℃, 12 h	90 (batch)	Not evaluated	[157]
				40 ℃, flow rate 0.6 mL/h)	95, continuous reactor,	80% activity after 49 d continuous operation	
B. cepacia lipase immobilized on modified attapulgite	10 % (oil weight)	Jatropha	Solvent-free MR methanol/oil = 6.6	35 ℃,	24 h	94		[153]
B. cepacia lipase immobilized on silica-PVA matrix	7.8 g biocatalyst	Babassu Macaw palm	Solvent-free MR Ethanol/oil= 7	50 ℃, flow rate 0.78 mL/h)	87.6 , continuous PBR	$t_{1/2}$= 453 h (babassu) $t_{1/2}$= 478 h (macaw palm oil)	[154]
Recombinant *R. oryzae* lipase immobilized on octadecyl-Sepabeads	40 mg of biocatalyst	Olive	Solvent-free MR alcohol/ oil = 3 7 stepwise methanol addition	30 ℃, 2 h	40,96	2 reuses	[156]
Recombinant *R. oryzae* lipase immobilized on HFA-Relizyme	4000 U/g oil	alperujo oil* with 19 % FFA	Solvent-free MR alcohol/oil = 1 3-stepwise methanol addition	30 ℃, 6 h	28,6	9 reuses	[136]
Recombinant *R. oryzae* lipase immobilized on Relizyme OD403	4000 U	*Candida* sp. yeast oil	*n*-hexane (oil/solvent = 1:5) MR alcohol/oil = 1 6 stepwise methanol additions	30 ℃, 4 h	40.6	70% activity after 6 reuses of 4 h each	[82]
Recombinant *R. oryzae* lipase immobilized on different resins	10 % (oil weight)	Crude jatropha	Solvent free	30 ℃, 4 h		$t_{1/2}$= 16-579 h	[158]

(Lifetech ECR8285M, AP1090M, ECR1030M; Amberlite IRA-96; Lewatit VP OC 1600) *Carica papaya* lipase immobilized on Lewatit VP OC 1600			MR methanol/oil = 3 (7 stepwise methanol additions)		51-65	$t_{1/2}$= 27 h (repeated batches)	
Lipase/acyltransferase from *C. parapsilosis* immobilized on Lewatit VP OC 1600 and Accurel MP1000	10 % (oil weight)	Crude jatropha	Biphasic oil/water medium MR methanol/oil =6	30 ℃, 8 h	80.5 (Accurel preparation) 93.8 (Lewatit preparation)	No deactivation after 5 reuses of 8 h each	[159]
T. lanuginosa lipase Immobilized on iron oxide nanoparticles	0.8 % (14 U) weight oil	Soybean	solvent-free MR ethanol/oil=4 2 % (w/w) water 20 % silica (to facilitate acyl migration)	40 ℃, 3 h	99	Not evaluated	[189]
Novozym 435 (*C. antarctica* lipase B)	30 % (w/w biomass)	Lipids from *Aurantiochytrium* sp. (heterotrophic microalgae) rich in FFA	In-situ esterification in dimethyl carbonate	50 ℃, 12 h	89.5	Not evaluated	[84]

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6.4 Combination of lipases

Combination of lipases is an alternative to increase reaction yield in biodiesel production. Due to lipase differences in selectivity, when a blend of different lipases is used, each one will attack preferential targets, and a total conversion is reached [18]. Several lipases combinations have been studied; examples of these studies are presented in Table 8. Lard and oils from rapeseed, soybean, palm, stillingia and olive have been used with lipase combinations to produce biodiesel. An interesting combination of lipases is the lipases from *C. antarctica* and *T. lanuginosus*, since the first limiting step is conversion of diacylglycerols (DAG) into monoacylglycerols (MAG), while the second rate limiting step is the conversion of TAG into DAG [141]. Li *et al.* [198] studied methanolysis of rapeseed oil with the commercial lipases Lipozyme TL IM and Novozym 435. Reaction using Lipozyme TL IM had a yield of 85%, while 90% was obtained with Novozym 435. Combination of both lipases gave 95% conversion under the following optimal conditions: tert-butanol/oil (1:1 v/v); methanol/oil (4:1, molar), 3% Lipozyme TL IM and 1% Novozym 435 based on oil weight. A combination of Lipozyme TL IM and Novozym 435 was also used for biodiesel production from lard, obtaining better results with the mixture (yield of 97.2 %, 72 h) than with the single lipases (N435 90.5%, 72h, TL IM 72.8%, 72 h) [199]. This enzyme combination was also used in a co-solvent system of tert-butanol/acetronitrile obtaining a conversion of 96.4%, where the compound-lipase was recycled 30 times in 12h batch reactions [75].

Immobilized lipases from *R. oryzae* and *C. rugosa* were used for biodiesel production from soybean oil [200]. Reactions with only one lipase produce yields lower than 70 % (30 h). However, a combination of both lipases increased conversion up to 99% (30 h). This process was optimized using supercritical carbon dioxide with a mixture of immobilized *R. oryzae* and *C. rugosa* lipases (1:1), reaching 99% conversion in only 2h [201]. Other lipase combinations studied include immobilized *R. oryzae* with *C. rugosa* lipases [202], immobilized *P. fluorescens* with *C. rugosa* lipases [202], immobilized *T. lanuginosus* lipase combined with Lipozyme RM IM [203, 204] and Lipozyme TL IM, Lipozyme RM IM and Novozyme 435 [203].

In summary, enzyme-catalyzed biodiesel production requires a free or immobilized lipase (or lipase combination) capable of accepting all types of acylglycerols (mono-, di- and triacylglycerols) and free

771   fatty acids. In addition, other properties like alcohol tolerance, thermo tolerance, stability, good activity

772   and reaction yields on non-aqueous media and reusability are desired [2, 140].

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775 Table **8**. Examples of biodiesel production catalyzed by combination of lipases (wt% is based on oil mass).

Lipases	Oil	Alcohol	System	Reaction conditions	Yield (%)	Reference
Lipozyme TL IM (20% wt) Novozym 435 (2% wt) Lipozyme TL IM (3% wt) + Novozym 435 (1% wt)	Rapeseed oil	Methanol	tert-butanol	12h, 35 °C, 130 rpm	85 90 95	[198]
R. oryzae immobilized (30% wt) *C. rugosa* immobilized (30% wt) *R.oryzae* (15% wt) + *C. Rugosa* (15% wt)	Soybean oil	Methanol	Solvent free Water 10% wt	30h, 45 °C, 200 rpm	70 20 99	[200]
P. fluorescens immobilized (10% wt) *P. fluorescens* (5% wt) + *Candida rugosa* immobilized (5% wt) *C. rugosa* immobilized (5% wt) + Novozym 435 (5% wt)	Palm oil	Ethanol	Solvent free Water 2% wt	12h, 45 °C, 500 rpm	85 85 45	[202]
Novozym 435 (3% wt) Lipozyme TL IM (8% wt) Novozym 435 (1.96% wt) + Lipozyme TL IM (2.04% wt)	Lard	Methanol	tert-butanol	20h, 50 °C	90.5 72.8 97.2	[199]
R. miehei expressed in *Pichia pastoris* *P.cyclopium* expressed in *P. pastoris*. *R. miehei* + *P. cyclopium*	Soybean oil	Methanol	Solvent free Water 28.6% wt	12h, 30 °C, 180 rpm	68.5 ND >95	[138]
Novozym 435 (1.96% wt) + Lipozyme TL IM (2.04% wt)	Stillingia oil	Methanol	tert-butanol /acetonitrile	20h, 40 °C, 200 rpm	96.4	[75]
T. lanuginosus immobilized (25% wt) Lipozyme RM IM (25% wt) *T. lanuginosus* immobilized (20% wt) + RM IM (5% wt)	Soybean oil	Ethanol	Solvent free Water 4% wt	10h, 30 °C, 200 rpm	<80 <40 90	[204]
R. oryzae (10% wt) + *C. Rugosa* (10% wt)	Soybean oil	Methanol	Solvent free	2h, 45 °C, 250	99.9	[201]

			Water 10% wt	rpm, 130 bar		
Lipozyme TL IM (13.7% wt) Novozym 435 (13.7% wt) Lipozyme RM IM (13.7% wt) TL IM (4% wt) + Novozym 435 (8% wt) + RM IM (1.7% wt)	Olive oil	Ethanol	Solvent free Water 4% wt	18h, 35,9 °C, 180 rpm	<50 <50 <50 95	[203]
Lipozyme TL IM (15% wt) Lipozyme RM IM (15% wt) TL IM (7.9% wt) + RM IM (7.1% wt)	Palm oil	Ethanol	Solvent free Water 4% wt	18h, 37.7 °C, 180 rpm	<50 <50 81	[203]

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779   **7. Acyl acceptor and strategies to avoid lipases inactivation by methanol and glycerol**

780   **7.1 Importance of the selection of acyl acceptor**

781   One of the important decisions in biodiesel production is the choice of the alcohol (acyl aceptor) which

782   has a significant impact on the associated costs of the bioprocess, on the properties of the biodiesel, and

783   on the catalyst [205]. The most common alcohols used are short-chain alcohols, namely methanol an

784   ethanol. The choice depends on the availability of the alcohol and price in the biodiesel producer country.

785   Nevertheless, methanol is the most common used acyl acceptor due to economic reasons [110]. However,

786   the use of methanol in enzymatic biodiesel production is one of the bottlenecks of the process because of

787   the inactivation caused in the majority of free and immobilized lipases, reducing the number of

788   reutilization of the biocatalyst and subsequently increasing the cost of the process due to the frequent

789   substitution of the biocatalyst.

790   A deep state of the art of the molecular and kinetic effect of methanol has been made by [110]. There is

791   not a single mechanism to describe this negative interaction and it can be considered as the sum of the

792   effect of different factors such as solubility and miscibility of substrates and denaturation and inhibition

793   of the biocatalyst. Two main different mechanisms has been proposed for this phenomena: (i) a high

794   alcohol concentration causes a not correct folding of the lipase with subsequent irreversible deactivation

795   [206], and (ii) the role of organic solvents acting as a competitive lipase inhibitors [207]. Blocking the

796   access of the TAG to the biocatalyst and/or adsorption of alcohol onto polar immobilized support have

797   also been proposed [18].

798   The tolerance of lipases to methanol depends on the source of lipase producer microorganism. *Candida*

799   *antactica* lipase B (CALB) is an example of a lipase with low performance in enzymatic biodiesel

800   production [3], conversely to *Burkholderia sp.* lipase which has a high stability in the presence of

801   methanol [155, 208].

802   The approaches to overcome this problem are the immobilization of the biocatalyst (section 6.3) or the

803   design of process strategies (section 7.2). The use of protein engineering methods to improve the enzyme

804   resistance to methanol, has not been fully explored and the reported results are rather scarce [110, 139].

805   The use of ethanol has several advantages compared with methanol as acyl acceptor: in terms of green

806   product, ethanol is better when it is obtained from renewable sources and not from fossil fuel, it causes

807   lower lipase inactivation and, in terms of biodiesel performance, FAEEs present some characteristics

808   better than FAMEs although FAMEs have a higher maximum engine performance [18].

809   Recently, the use of a mixture at different ratios of both acyl acceptors has been tested in lipase-catalyzed

810   biodiesel production. The different reactions rates for the acyl acceptors improved the solubility of them

811   in the organic phase. In addition, the characteristics of the final biodiesel (a blend of FAMEs and FAEEs)

812   have been also improved [209, 210].

813   It is known that the inactivation effect of acyl acceptor decreases and the miscibility in the oil increases

814   with the increase in the number of carbon atoms of the alcohol [211, 212]. Not only long chain alcohols,

815   but also secondary and branched alcohols have been used in biodiesel synthesis, being the preference for

816   the alcohol specific for each lipase [3, 10, 18, 213]. However, although the obtained biodiesel properties

817   are better when long-chain alcohols are used, the cost of them is the main drawback to substitute

818   methanol and ethanol.

819   Esters has also been proposed as acyl acceptor as an alternative to the alcohols. Methyl and ethyl acetate

820   are the most common used esters [195, 197, 214]. High yields has been reported [195, 197], and

821   triacetylglycerol is obtained as final product instead of glycerol [18] with no negative effect on lipases

822   and with applications in many fields [195].

823   Solvents as dimethyl carbonate (DMC), have demonstrated to be potential candidates to acyl acceptors

824   [39, 84].

825   Norjannah *et al.* (2016) [18] present a summary with the advantages and disadvantages of acyl acceptors

826   in enzymatic reaction. Reviews on biodiesel synthesis using different types of acyl acceptor can be found

827   in the literature [3, 9, 18, 213].

828

829   **7.2 Strategies to overcome the problem of lipase inactivation by methanol and glycerol.**

830   Different strategies have been implemented to overcome the problem of alcohol inactivation:

831   The use of organic solvents is a common strategy to minimize the inactivation effect of acyls acceptors on

832   biocatalyst and to improve the solubility between triacylglycerols and alcohols [213]. It also reduces mass

833   transfer problems by decreasing the viscosity of the reaction media and favouring acyl migration

834   phenomena. In some cases, the use of solvents also avoids the need for the stepwise alcohol addition. The

835   selection of the solvent is correlated with its log P value. Hydrophobic solvents such as petroleum ether,

836   isooctane, n-hexane, n-heptane are the most common used with a lop P value around or higher than 4.

837   However, Li *et al.* (2010) [75], working with commercial lipases in the methanolysis of vegetable oil,

838   reported that the best results were obtained with solvents with a log P value in a range between 1.4-1.52.

839   is The most suitable solvent was tert-butanol which minimized also lipase denaturation caused by

840   glycerol [215]. Lower yields were obtained with more hydrophobic solvents (log P > 2). Nevertheless,

841   with the objective to get a greener bioprocess, the use of solvent should be avoided for the negative

842   effects that solvents provoke on the environment.

843   The quality of the glycerol produced by lipase-catalyzed transesterification is higher than that obtained

844   from alkaline catalysis. However, if glycerol adsorbs to the enzyme carrier it may cause lipase

845   deactivation decreasing process efficiency [194, 216]. In the transesterification of soybean oil with

846   methanol catalyzed by Lipozyme TL IM, iso-propanol was used for glycerol removal from the support

847   between reuses, to improve the operational stability of the biocatalyst ([194]; Table 7).

848   Another approach to overcome glycerol problem is the use of *sn*-1,3-regioselective lipases, instead of non

849   regioselective lipases, to synthesize biodiesel (fatty acid alkyl esters). The final products are FAMEs and

850   2-monoacylglycerols (2-MAG) [39, 110, 156, 158, 217, 218].

851   This strategy was first developed and patented by Luna *et al.* (2007) [217]: "Ecodiesel-100" is defined as

852   a mixture of two parts of fatty acid ethyl esters (FAEEs) or FAMEs, and one part of MAG, with minor

853   quantities of diacylglycerol (DAG) obtained by partial ethanolysis (or methanolysis) catalyzed by *sn*-1,3-

854   selective porcine pancreatic lipase. "Ecodiesel" is a glycerol-free biodiesel. This novel biofuel containing

855   FAMEs/MAG or FAEEs/MAG blends presented similar physical properties to those of conventional

856   biodiesel [219].

857   However, when *sn*-1,3 regioselective lipases are used and a maximum conversion of oil into FAMEs is

858   desired, acyl migration can be promoted by the presence of silica in the reaction medium ([189]; Table 7)

859   or by using some synthetic resins ([186]; Table 7).

860   Since short chain alcohols have a negative impact on lipase stability reducing the transesterification

861   yields, stepwise addition of methanol or ethanol, with the objective to minimize the contact between high

862   concentrations of methanol and lipase, is frequently carried out to protect the biocatalyst against alcohol

863   inactivation [18, 82, 156, 158, 187, 193, 194, 197]. Generally in stepwise addition the concentration of

864   methanol is lower than 1/3 molar equivalent. It is important to notice that the problem of solubility in

865   solvent-free systems of acyl acceptor is higher at the beginning of the biocatalysis when TAGs are the

866   major compounds. However, this solubility increases along the reaction because it is higher in FAMEs

867   than in TAGs [110].

868   Other novel approaches are the use of salt-solution based reaction systems [220], the use of supercritical

869   carbon dioxide or dimethyl carbonate, or ion liquids, which are presented in section 3.2

870   As previously commented, other approach to avoid biocatalyst inactivation by short-chain alcohols is the

871   use of esters as alkyl donors, namely ethyl acetate, methyl acetate or dimethyl carbonate. In the

872   production of fatty acid ethyl esters from jatropha, karanji or sunflower oils, catalyzed by Novozym 435,

873   ethyl acetate was used as solvent and alkyl donor instead of ethanol. A high operational stability of

874   Novozym 435 was observed in presence of ethyl acetate while a complete loss of activity was observed

875   after the sixth batch when ethanol was used ([195]; Table 7). Using the same biocatalyst for FAMEs

876   synthesis, carried out in batch stirred tank reactor or in batch packed bed reactor with recirculation, the

877   best results in terms of productivity and operational stability were obtained when methyl acetate was used

878   as alkyl donor ([197]; Table 7). Dimethyl carbonate (DMC) was successfully used as lipid extraction

879   reagent, alkyl acceptor and reaction medium for biodiesel synthesis by *in-situ* esterification of the lipids

880   rich in FFA from the heterotrophic microalgae *Aurantiochytrium* sp. [84] (Table 7).

881   In the presence of high water contents in reaction media, methanolysis showed to be efficiently catalyzed

882   by free *Rhizopus oryzae* lipase [167] or free *Cryptococcus spp.* S-2 yeast lipase [166] (Table 6). These

883   results suggest that water prevents the inactivation of the enzyme caused by single addition of methanol

884   [166, 167].

885   Also, acyltransferases are interesting biocatalysts with great potential for industrial applications in green

886   oleochemistry industry, as a novel alternative to the use of lipases. In this field, the lipase/acyltransferase

887   CpLIP2, secreted by the yeast *Candida parapsilosis*, stands out for its great potential. This biocatalyst is

888   one of the few enzymes that preferentially catalyzes alcoholysis over hydrolysis, when in aqueous or in

889   biphasic aqueous/organic media [159, 221-228]. Undesirable hydrolysis reaction is limited by the

890   competition between the alkyl acceptor (methanol) and water, favoring the short chain alcohol, even in

891   systems with a high molar excess of water [228]. This acyltransferase is active in an acidic to neutral pH

892   range (3-7), with an optimum for the alcoholysis reaction at pH of 6-6.5 [221] and temperature of 30 ºC.

893   CpLIP2 is highly active towards long-chain unsaturated triacylglycerols [223]. CpLIP2 was immobilized

894   on two synthetic resins (Accurel MP 1000 and Lewatit VP OC 1600) and successfully used as catalyst for

895   the transesterification of crude jatropha oil with methanol, in a lipid/aqueous system. Both enzyme

896   preparations presented high activity (80.5 % and 93.8 %, with CpLIP2 on Accurel MP 1000 or on Lewatit

897   VP OC 1600, respectively) and batch operational stability along 5 consecutive 8 h batches ([159]; Table

898   7).

899

900   **8. Mechanism of enzymatic esterification and transesterification.**

901   For biodiesel production the synthesis reactions of interest are esterification and transesterification

902   (Figure 7) [7, 66]. Esterification is the reaction between a free fatty acid and an alcohol to produce an

903   ester and release a molecule of water and transesterification refers to the reaction between a TAG and an

904   alcohol where the ester group of the TAG is removed to form three molecules of fatty acid alkyl ester and

905   one molecule of glycerol.

906   The transesterification reaction follows several steps and three different kinetic models have been

907   described for enzymatic transesterification [3, 7, 140, 229, 230]. The first mechanism is the direct

908   alcoholysis of acylglycerols into fatty acid alkyl ester (Figure 7). In this mechanism, the first step is the

909   action of the lipase on the ester bond of the TAG to produce a fatty acid alkyl ester and a DAG, which are

910   then converted to a MAG, and a second fatty acid alkyl ester. In the final step, the lipase acts on the

911   monoacylglycerol to produce glycerol and a third molecule of fatty acid alkyl ester [7].
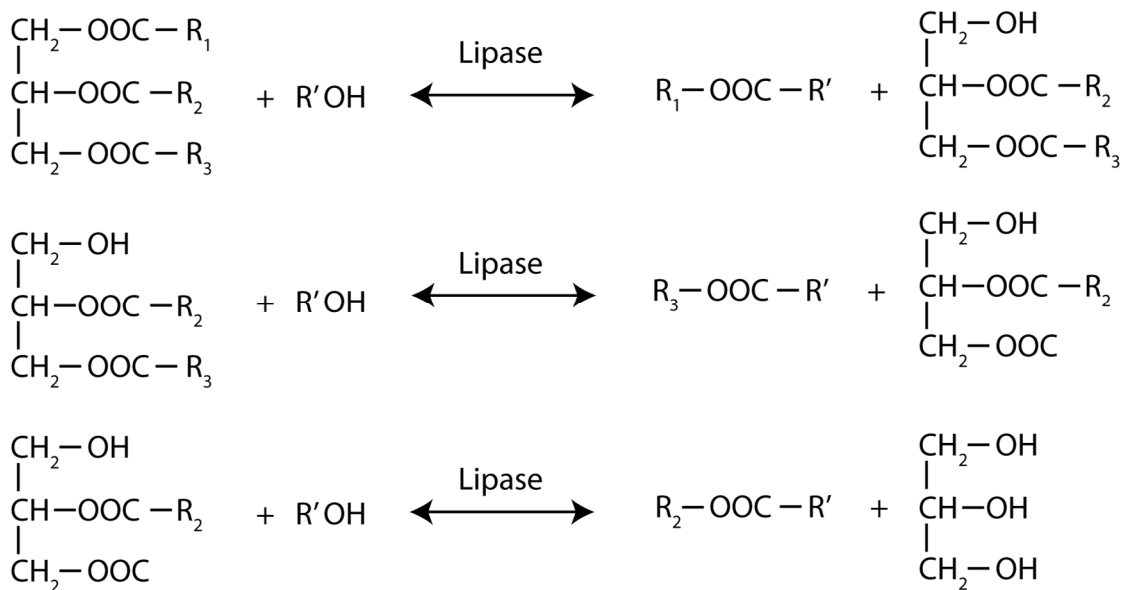
913   Figure **7**. Lipase-catalyzed transesterification steps.

914

915   The second mechanism is a two-steps process in each ester bond, starting with the hydrolysis to produce

916   acylglycerols and free fatty acids and a subsequent esterification of the free fatty acids [3]. The third

917   kinetic model presents a combination of direct alcoholysis and the two-step reaction of hydrolysis of

918   acylglycerols followed by esterification of the free fatty acids [137, 231].

919   Lipase-catalyzed transesterification follows a Ping-Pong Bi-Bi mechanism [140, 229, 230] and has four

920   steps [7, 229]. The mechanism (Figure 8) initiates with a nucleophilic addition to form the enzyme-

921   substrate complex, where the nucleophile is the oxygen in the O–H group on the enzyme. Then there is a

922   proton transfer from the conjugate acid of the amine to the alkyl oxygen atom of the substrate, forming a

923   glycerol moiety. When a triacylglycerol is the initial substrate, a diacylglycerol would be formed and

924   when a diacylglycerol is the substrate, then a monoacylglycerol would be formed. In the third step, the

925   oxygen atom from an alcohol molecule, usually methanol or ethanol, is added to the carbon atom of the

926   C=O of the acyl enzyme intermediate to form the acylated enzyme–alcohol complex. In the final step,

927   the enzyme oxygen atom of the complex is eliminated and a proton is transferred from the conjugate acid

928   of the amine, resulting in fatty acid methyl or ethyl ester depending on the alcohol used.
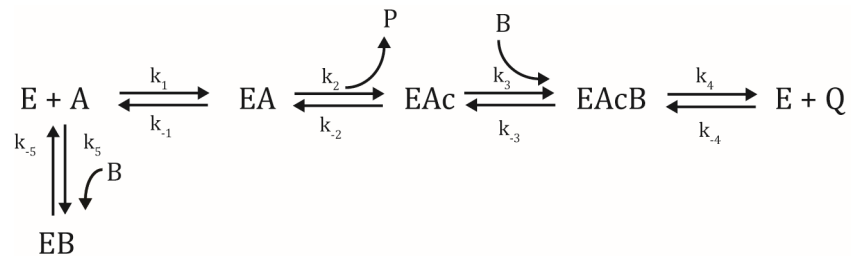
929
930   Figure **8**. Pong Bi-Bi mechanism with competitive inhibition from the nucleophile B. E: enzyme. A and
931   B: substrates. P and Q: products. EAc and EacB: intermediate complex of enzyme and substrate. EB:
932   inactive complex enzyme-substrate B.
933

934   The previously described mechanism is based on the assumption that the first product of the reaction is

935   the fatty acid ester followed by the glycerol moiety and that no alcohol or substrate inhibition is present.

936   Al-Zuhair *et al.* [229] have shown that the first product of the reaction is the glycerol moiety and that the

937   fatty acid ester is the final product. Since inhibition is a rather frequent problem in lipase-catalyzed

938   biodiesel production, Al-Zuhair *et al.* [229] also proposed a kinetic model that considered both alcohol

939   and substrate inhibition. This adapted model was capable of predicting the behavior of the

940   transesterification of palm oil with methanol in n-hexane medium with immobilized *Rhizomuchor miehei*

941   lipase as catalyst. Chersilp *et al.* [232] proposed three kinetic models for biodiesel production from palm

942   oil and ethanol with immobilized *Pseudomonas sp.* lipase. These models considered the effect of

943   substrates and products during the entire reaction. The kinetic study showed that the hydrolysis of the

944   TAG ester bond and esterification of the free fatty acid occurred simultaneously instead of hydrolysis

945   followed by esterification. In addition, they reported that the constant rates of palm oil esterification were

946   higher than the rates of the hydrolysis reaction. Li *et al.* [231] used free lipases as catalysts for the

947 reaction between soybean oil and methanol. As for lipase-catalyzed esterification [147], the system of Li

948 *et al.* followed a Ping-Pong Bi-Bi mechanism with methanol inhibition, showing that the methanolysis

949 reaction and the hydrolysis followed by esterification occurred simultaneously. Furthermore, the

950 enzymatic rate constants showed that direct transesterification is the preferred pathway for this system.

951 Similar results were found by Canet *et al.* [137] for the transesterification using immobilized lipases from

952 *Rhizopus oryzae* where the reaction was also a combinations of direct alcoholysis with the two-step

953 reaction of hydrolysis and esterification.

954
955 **9. Bioreactors types and operational strategies used in Biodiesel production**

956 The selection of the most suitable bioreactor for enzymatic transesterification has to take into account the

957 considerations made in previous sections. Mainly these bioreactors work with immobilized lipases to

958 increase the productivity and reduce the cost of the biocatalyst per ton of biodiesel and minimizing the

959 problems associated to the scale-up of the process.

960 Four different configurations of enzymatic bioreactors are described in the literature, working either in

961 batch or in continuous mode: stirred tank reactors (STRs), packed bed reactors (PBRs), fluidized-bed

962 reactors (FBRs) and membrane reactors (MBRs) [3, 5, 110]. To select the best design and operation mode

963 of the enzymatic bioreactor, the knowledge about kinetics, hydrodynamics and mass transfer of the

964 bioprocess is necessary. Poppe *et al.*, (2015) [5] present the main variables involved in the selection of the

965 enzymatic bioreactor with immobilized enzymes.

966 Most of the studies are batchwise but the implementation in continuous bioreactors is also described with

967 very high operational stability results [154, 157, 196].

968 STRs working in batch mode are the simplest configuration but, in most of the situations, it requires long

969 reaction times [101]. The break of the immobilized particles with enzyme lost to the reaction medium,

970 due to mechanical agitation, is an associated problem. However, it can be avoided with the use of porous

971 baskets containing the immobilized particles [233] or also replacing STRs by PBRs with recirculation.

972 PBRs are the most common bioreactors used in continuous enzymatic biodiesel production. Generally,

973 they offer better performance than STRs, mechanical shear stress is reduced, the technology is cheaper,

974 the reutilization of the enzyme does not need a prior separation and it can work at lower enzyme-substrate

975 ratio [110]. However, the formation of channelling due to the immiscibility of the substrates, lower yields

976 compared with batch operational mode, flow rates limited to a compromise between pressure drop values,

977 minimal diffusion layer, and higher mass transfer limitation problems are the main drawbacks of this

978　configuration [5, 234]. A comparison of biodiesel yield in batch and continuous flow packed bed reactor

979　is presented by Tran *et al.* (2014) [235].

980　FBRs are an alternative of PBRs to avoid some of the drawbacks. Substrate is flown in the FBRs with up

981　flow at an optimized flow rate let the fluidization of the biocatalyst, providing a free movement of the

982　biocatalyst around the FBRs. With this configuration, mass transfer is improved, although lower yields

983　are generally obtained. The size of the particles of the biocatalyst can be lower than in PBRs but other

984　factors as the viscosity and the density of the substrates and biocatalyst have to be taking into account

985　[236]. The scale up of this configuration is more complex than with PBRs [5].

986　An ultrafiltration membrane bioreactor is an adequate reactor to operate with reverse micellar medium.

987　Although being more expensive than conventional reactors, it can integrate the biocatalysis, the

988　downstream and the reuse of biocatalyst [237]. This type of reactor has been used in the production of

989　alkyl esters by transesterification reaction using methanol, ethanol or butanol as acyl acceptors, using

990　cutinases as biocatalyst. It worked continuously for more than 28 days with a productivity value of 500

991　kg product·kg enzyme^{-1}·day^{-1} [238].

992　A summary of an update of different bioreactors configurations, the source of lipase, the conversion or

993　productivity reached, the acyl acceptor used and the stability of the biocatalyst shown in Lotti *et al.*

994　(2015) [110] is presented in Table 9.

995　In the last year, non-conventional reactors have been tested in biodiesel production. Ultrasound systems

996　are one of them. Basically, ultrasounds modify the temperature and pressure of the microenvironment,

997　enhancing substrate dissolution, improving mass transfer, inducing conformational modifications in the

998　protein and perturbed weak interactions. A save in energy compared with mechanical agitation is also

999　reached. Thus, a reduction of reaction time, yield increasing, and the possibility to make chemo, regio and

1000　stereoselective reactions that in standard conditions are not possible, are the advantages of the ultrasound

1001　technique [239]. This technology has been applied to enzymatic biodiesel production with higher

1002　enzymatic activity and negligible loss of enzyme activity [120]. The studies has been mainly performed

1003　with commercial lipases (Novozym 435, Lipozyme RM IM) using methanol or ethanol as acyl acceptor,

1004　with conversions higher than 75 %, in solvent-free or in solvent media [239]. The production of FAMEs

1005　from waste grease using *C. antarctica* B lipase as biocatalyst in an ultrasound system, reached a yield of

1006　98.2 % after 20 min reaction time [240]. The synthesis of biodiesel from sunflower using Lipozyme TL-

1007   IM under an ultrasound field demonstrated that no excess of methanol is necessary in the reaction. In

1008   addition, the reaction was favoured when the stoichiometric relation oil:methanol of 1:3 was used [241].

1009   *Candida rugosa* lipase immobilized onto functionalized magnetic nanoparticles (MNPs) was used in the

1010   biolubricant production from castor oil in a magnetically stabilized fluidized bed reactor: 96.9 % methyl

1011   ester yield was obtained after 24 hours of reaction. Also, after eight cycles of 24 h each, no significant

1012   loss of activity was observed [242]. This reactor presents advantages comparing to conventional FBR

1013   including lower pressure drop and better mass transfer [243].

1014   *Candida rugosa* lipase in a solvent-free system was also tested in the methanolysis of canola oil in

1015   capillary channel reactors: the yield of methanolysis was improved up to 4-fold, compared with

1016   conventional approaches [244].

1017 Table **9**. Examples of processes for the lipase-catalyzed production of biodiesel in bioreactor
1018

Lipase	Conversion/Productivity	Reutilization	Reactor	Strategy	Reference
R. miehei Lipozyme IM	79%	15	STR	n- Hexane as solvent Alcoholysis	[245]
Candida cylindracea	98%	24 h	STR	Diesel or kerosene as solvent Methanol	[246]
C. antarctica Novozyme 435	95.6%	1	STR	Methanol stepwise	[197]
C. antarctica Novozyme 435	99.8%	5	STR	Methyl acetate	[197]
C. antarctica Novozyme 435	96,2%	72 h	PBR	Methyl acetate	[197]
C. antarctica Novozyme 435	99 %	20 cycles	PBR	Methanol, 10 passes per column	[216]
Fusarium solani Cutinase	500 g of product g biocatalyst^{-1} day^{-1}	28 days using a cutinase mutant	MBR	Reverse micelles	[238]
C. rugosa	87%	50 h	PBR	Methyl acetate	[247]
NS88001 and C. antarctica Novozyme 435	1.556 kg FAEEs kg catalyst^{-1} h^{-1}	---	PBR	Two-stage processes Ethanol	[248]
Burkholderia	67%	---	PBR	Methanol,	[235]
C. antarctica Novozyme 435	>80%	5 cycles	STR	Presence of tert-butanol. methanol	[215]
B. cepacea	67%	---	PBR	Methanol	[235]
Callera^{TM}	98%	24	STR	ethanol	[164]
B. cepacea	87.6 %	$t_{1/2}$ = 478 h	PBR	ethanol	[154]
C. antarctica Novozyme 435	96.2%	Increased extracting glycerol	PBR	Methanol, extracting glycerol	[249]
B. cepacea	84%	5 days	PBR	Methanol	[250]
B. cepacea	97.3	$t_{1/2}$ = 1540 h	Two-stage PBR	Ethanol, extracting glycerol	[154]
NS-40116 T. lanuginosus lipase	8.5 g of product g biocatalyst^{-1} h^{-1}	5 cycles	CSTR 40 m^3	Methanol added in continuos.	[251]

|					
C. antarctica *Novozyme 435*	98.1 9.9 mol of ester g biocatalyst^{-1} min^{-1}	15 days without loss of activity	FBR	Ethanol, extracting glycerol	[252]
C. rugosa and *R. oryzae cells*	85-81%	---	PBR	n-hexane as solvent, methanol	[253]
B. cepacia	85%	Very stable	PBR	methanol	[235]
Lipozyme TM IL	92%	$t_{1/2}$ = 45 cycles	PBR	Ethanol, extracting glicerol	[254]
Candida sp. 99-125	96%	---	Rotating PBR	Methanol	[255]
Recombinant R. oryzae	50.4	1 cycle	STR	7 Methanol stepwise	[256]
Recombinant R. oryzae	73.6	More than 2 cycles	PBR	7 Methanol stepwise	[256]

1019
1020

10. Economic evaluation and industrial scale production

Historically biodiesel price has been related to vegetable oil prices and to fossil diesel prices (Fig 9), but

also to tax incentives. However, as previously referred, the cost of the feedstock oil still represents 60-

88% of the production cost of biodiesel [6-10, 257] (see also Fig. 10). The use of waste fats could reduce

biodiesel production costs and decrease GHG emissions [12, 258, 259].

Figure **9**. Historical biodiesel price and production in the US [19]. 1 US gal = 3.7854 L.
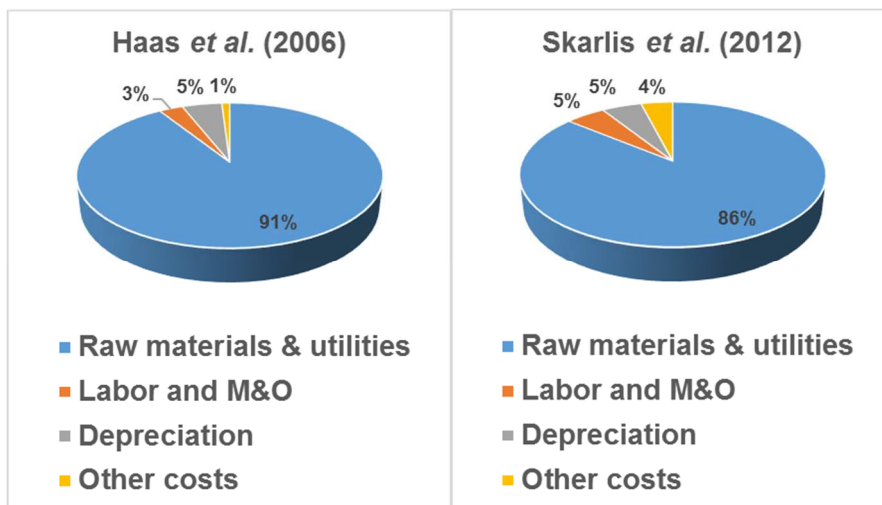
Figure **10**. Production cost of biodiesel. Based on data from Haas *et al.* (2006) [8] (left) and Skarlis *et al.*
(2012) [257] (right).

1033   Sales of coproduced glycerol also improve biodiesel profits, but this implies an additional step of glycerol

1034   purification. Glycerol purification is more difficult when alkali catalyst is used, but enzymatic catalysis

1035   produces a cleaner glycerol. Glycerol has a wide range of applications including personal care,

1036   pharmaceuticals, foods and beverages [260]. The global demand for glycerol was around 2 million tons in

1037   2011 and is expected to reach 3 million tons by 2018, worthing an estimated USD $ 2 100 million [260].

1038   Removal of alcohol excess and water is also necessary to obtain biodiesel that complains with quality

1039   standards. Figure 11 shows the scheme of a biodiesel production process with homogeneous catalysis and

1040   basic purification steps. Models of biodiesel cost and local producer experiences indicate that capital

1041   investment is pay back in about two years for alkali-catalyzed process. For feedstocks with high FFA

1042   content, acid neutralization process is reported to be affordable [259]. However, lipase-catalyzed

1043   processing or neutralization is becoming attractive for the industry as well because it allows for the use of

1044   cheaper feedstocks. The influence of the enzymatic support on biodiesel economics has also been

1045   evaluated. The ideal support should not retain glycerol, which is responsible for enzyme deactivation, and

1046   because it is also a valuable byproduct [261].

1047

1048   Tax incentives were a key factor in the development of biofuels industry in many countries. For instance

1049   in USA, to ensure compliance, companies that refine, import or blend fossil fuels are periodically

1050   required to demonstrate they have met their RFS quota (see section 2.2). Renewable identification

1051   numbers (RINs) are credits used for compliance, and are the "currency" of the US RFS program. A RIN

1052   is a 38-digit number that serves as a "proof-of-purchase" for companies to submit to the EPA as proof

1053   that they have complied with terms of the RFS [262]. The ability for independent fuel marketers to sell

1054   renewable fuels at lower prices while improving profit margins by selling RINs, has given biofuel

1055   marketers independence of the fossil fuel market.

1056   Argentina has rapidly become an important producer and exporter of biodiesel, in part due to preferential

1057   tax regimes and exemption of taxes for biodiesel used in electricity generation. These tax incentives have

1058   been renewed for 2017 under the 1326/2016 edict.

1059
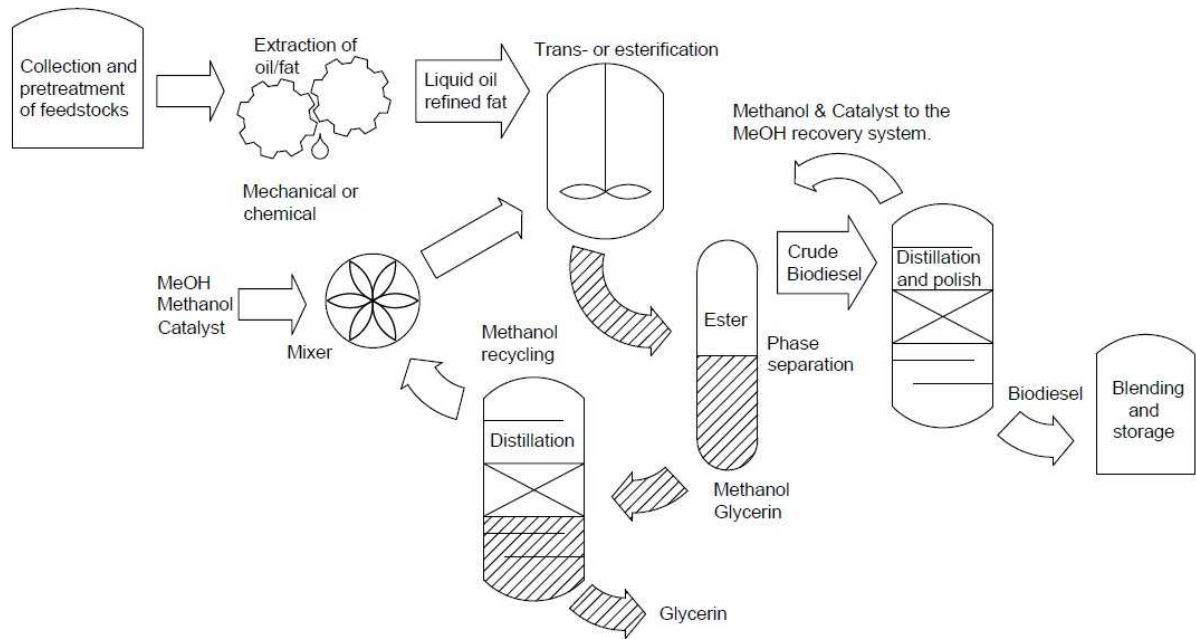
Figure **11**. Sheme of biodiesel production process with homogeneous catalysis.

11. Conclusions

Nowadays, there are already some examples of enzymatic processes for biodiesel production implemented at industrial scale and the number of pilot and industrial scale plants greatly increased in recent years. In 2006 and 2007, two Chinese companies, Lvming Co Ltd. and Hainabaichuan Co. Ltd., launched two enzymatic biodiesel production units, with a capacity of 10,000 and 20,000 t/year (doubled to 40,000 t/year in 2008), respectively. The first biodiesel production unit uses waste cooking oil as feedstock, and immobilized lipase of *Candida* sp. as biocatalyst. The second unit produces biodiesel based on waste palm oil and uses the immobilized *Candida antarctica* lipase (Novozym 435) commercialized by Novozymes A/S, Bagsvaerd, Denmark, as catalyst. In 2012, the American company Piedmont Biofuels (North Carolina) established a new technology (FAeSTER) for a continuous biodiesel production using immobilized or liquid enzyme [101]. Purolite (Bala Cynwyd, PA) and Transbiodiesel (Shfar-Am Israel) and Sunho Biodiesel Corporation (Taipei, Taiwan) are also industrial producers of enzymatic biodiesel [214].

In spite of this trend, the chemical catalysis process still remains the most popular on an industrial scale mainly due to the high cost of commercial lipases. Thus, it is necessary to improve the enzymatic

1081 technology, increasing the productivity of the bioprocess and reducing the cost of the bioprocess. To
1082 attain this goal, it is necessary to act in a multidisciplinary approach of Genetic engineering, Bioprocess
1083 engineering, including the production of recombinant lipase in the most adequate cell factory, Enzyme
1084 engineering and applied Biocatalysis. It is a fact that the approach to "create" by genetic engineering, a
1085 lipase with a high tolerance to methanol, high biocatalyic performance and high resistance to work at
1086 higher temperatures and under harsh conditions is not corresponding with the important advances get in
1087 the other aspects.

1088 Also, the use of low cost non-commercial biocatalysts, presenting both high transesterification activity
1089 and operational stability, as an alternative to commercial biocatalysts, is a solution to reduce enzymatic
1090 biodiesel production costs and making it competitive with chemical processes.

1091 The price of biodiesel is highly affected by the market price fluctuation of oil feedstock. Thus the
1092 commercial efficiency and competitiveness of biodiesel market needs the development of high-valued
1093 product from the FAMEs as raw-material, under the concept of a biodiesel refinery [10].

1094 Other approach to minimize the cost of the global process is the production of heterologous lipases, using
1095 the crude glycerol obtained in the same biodiesel industry, without high purification, as carbon source.
1096 The presence of low methanol concentration and other possible contaminants jointly in the matrix of
1097 crude glycerol is not a problem for *P. pastoris*, one of the most popular cell factories to produce
1098 recombinant lipases.

1099 In conclusion, enzymatic biodiesel, as a green alternative to chemical biodiesel, has a potential economic
1100 growth in the near future.

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