Enzymatic Catalysis of Vegetable Oil with Ethanol in the Presence of Co-solvents

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This article evaluates the production of biodiesel or FAEE (fatty acid ethyl esters) from vegetable oil by enzymatic catalysis with ethanol as reagent and in the presence of hexane as co-solvent. A three-factor design of experiments was performed to evaluate the influence of the following three variables on the reaction performance (or the ethyl esters content): reaction time (24, 36, and 48 h), co-solvent percentage (10, 20 and 30 %, v/v), and oil/alcohol molar ratio (1:4, 1:6, 1:8), at fixed reaction temperature (35 ºC) and fixed mass of Lipozyme TL IM (enzyme/oil mass ratio of 1.6 wt%). Results show that the best operating conditions to perform the reaction are: oil/alcohol molar ratio of 1:4, 10 % (v/v) of hexane co-solvent, and 48 h of reaction time. Although most of the biodiesel quality parameters evaluated are out of the NP EN14214:2009 standard limits, for the above mentioned conditions it is possible to obtain a lower viscosity biodiesel, with lower density, lower *CFPP* (cold filter plugging point), and with a lower alkaline metals (Na^+ and K^+) content.

1. Introduction

Rising fossil fuel prices and the countries' environmental concerns about the harmful effects of petroleum as a fuel source are driving the mainstream awareness and interest for the development of more sustainable transportation fuel alternatives (Mata et al., 2011a). In this regard biodiesel has been pointed out as a more environmentally friendly solution to partially replace diesel fuel as well as the final destination for many fatty liquid and greasy wastes generated by human activities (Mata et al., 2010, 2011b; Morais et al., 2010a,b). For long time biodiesel has been produced industrially by transesterification using short chain alcohols and chemical catalysts, particularly alkaline, due to its high conversion rates. However, the presence of water and/or the high free fatty acid (FFA) content of many fatty wastes brought difficulties in carrying out this reaction. In an attempt to overcome these difficulties, the enzymatic transesterification has recently gained adherents, despite the enzymes cost continue to be an obstacle to its large scale implementation.

Currently, hydrolases (proteases, lipases and carbohydrolases) are the most common enzymes used in 95 % of the enzymatic processes, where 10.5 % are related to lipases. Lipases are enzymes that are able to catalyze reactions in aqueous (hydrolysis of triglycerides) or non-aqueous media (esterification activity), both under mild conditions. Depending on the lipase origin (microbial, plant or animal) it can present different catalytic activity with the substrate (Salvador et al., 2009; Franken, 2007). The

hydrolysis reaction transforms lipids into fatty acids and glycerol via a reversible reaction, so the reaction rate is directly linked to the substrate concentration, pH, enzyme concentration, temperature and the presence of inhibitors or activators, since all these factors can influence the conversion of reactants to products in a positive or negative way (Salvador et al., 2009).

The lipases catalyzed reaction present a major drawback that is the immiscibility among substrates (hydrophilic alcohol and hydrophobic triglycerides), which contributes to form an interface resistant to mass transfer. In this sense, the addition of organic solvents can enhance the solubility of the oil in the alcohol, inducing a greater catalytic activity during the transesterification reaction. Previous studies (Fu and Vasudevan, 2010) concluded that the co-solvent concentration should be low in order to prevent the lipase inactivation, which is somehow protected by the hydrophobicity of the solvent. When the limit is reached, this protection is interrupted and consequently there is a decrease in the reaction yield (Selmi and Thomas, 1998; Fu and Vasudevan, 2010). The transesterification process using co-solvents also allows the simultaneous conversion of free fatty acids and triglycerides into esters, which undoubtedly is an advantage when using low cost raw materials that will inevitably have high levels of FFA. The most common co-solvents are: iso-octane, di-metoxiethane, methyl iso-propyl ketone (MIPK), acetone, hexane, petroleum ether, tetrahydrofuran (THF) and tert-butanol (Fu and Vasudevan, 2010; Samukawa et al, 2000).

This work aims to study the production of FAEE by enzymatic transesterification from vegetable oil, using ethanol as reagent and in the presence of hexane as co-solvent. Since currently in Europe FAEE do not have a standard that defines its quality requirements to be used in vehicle engines as biodiesel, in this work FAEE were analyzed according to the (NP EN14214, 2009) standard that regulates the quality of FAME (fatty acid methyl esters).

2. Materials and Methods

2.1 Vegetable oil characterization

Vegetable oil was characterized for the following properties: acid value – titrimetric method, according to (NP EN ISO 660, 2002) standard, iodine number – titrimetric method using Wijs reagent, according to (ISO 3961, 1996) standard, kinematic viscosity – determined at 40 ºC, using a glass capillary viscometer, Cannon-Fenske routine viscometer, series 200, according to EN ISO 3104 (ISO 3104, 1994) standard, and higher heating value of the fats [determined using an oxygen bomb calorimeter, according to American Society for Testing and Materials (ASTM) method D240-87].

2.2 FAEE production through enzymatic catalysis in the presence of co-solvent

Around 300 g of oil was weighed to a screw cap Pyrex bottle, with 500 mL of capacity. Then, 5 g of enzyme was weighted and added to the oil, together with a magnetic stir bar and a certain amount of hexane and of ethanol needed, which were measured in a hood. The mixture was shaken vigorously to promote contact between oil, alcohol, catalyst and co-solvent. Then, the transesterification reaction took place for a certain time period in a stirring bath at 35 °C of temperature. After the reaction ended, the flask was removed from the bath and the mixture was filtrated to recover the enzyme.

The filtrate was placed in a separating funnel and about 100 mL of glycerin (72 % purity) was added with gentle agitation. This process was conducted in two steps, with the addition of 50 mL glycerin in each one. The denser phase (glycerol) was removed from the bottom of the separating funnel. The less dense phase (biodiesel) was subjected to distillation at 80 °C in order to recover the excess alcohol still existing and then placed again in the separating funnel, where it was washed with hot water acidified with a few drops of concentrated sulfuric acid. The washing procedure was repeated until a clear phase was obtained with a neutral pH. Finally, about 2 g of magnesium oxide (MgO) was added to remove any water remaining in biodiesel, the mixture was stirred for 1 h, after which biodiesel was filtrated through cellulose membranes (4-7 µm) to remove the MgO using a vacuum pump.

2.3 FAEE characterization

In this study, some of the most important quality parameters of biodiesel from vegetable oil were evaluated according to the (NP EN 14214, 2009) standard that defines the quality requirements of FAMEs. The acid value was determined using a titrimetric method, according to the standard (NP EN 14104, 2011). The kinematic viscosity was determined at 40 ºC, using glass capillary viscometers,

according to the (ISO 3104, 1994) standard. The density was determined at 15 ºC, using a hydrometer method, according to the (ISO 3675, 1998) standard. The flash point was determined using a rapid equilibrium closed cup method, according to the standard (ISO 3679, 2004). The copper corrosion was determined using a copper strip test, according to the standard ISO 2160:1998. The water content was determined by Karl Fischer coulometric titration, according to the standard (NP EN ISO 12937, 2003). The iodine number was determined by the titrimetric method using Wijs reagent, according to the (EN 14111, 2009) standard. The cold filter plugging point (CFPP) was determined using standardized filtration equipment, according to the EN 116:2002 standard. The FAEE content was determined by gas chromatography (GC) according to the NP EN (14103, 2010) standard. This analysis was performed using a gas chromatograph (DANI GC 1000 DPC) equipped with a TRB-WAX (Capillary Column, Teknokroma) for FAME´s/ FAEE´s (30 m, 0.32 mm internal diameter, and 0.25 µm film thickness). The injector temperature was set to 250 ºC, while the flame ionization detector (FID) temperature was set to 250 ºC and the oven temperature to 195 ºC. The carrier gas used was Helium, at a flow rate of 1 mL/min. Injection was made in a split mode, using a split ratio of 1:80, and the volume injected was 0.1 µL.

3. Results and Discussion

3.1 Vegetable oil characterization
Table 1 presents the several parameters determined experimentally for the vegetable oil characterization, including: density at 20 ºC, kinematic viscosity at 40 ºC, water content, acid value, iodine number, higher heating value, and the average molecular mass.

Table 1: Experimental characterization of vegetable oil

Parameter	Unit	Value*
density at 20 ºC	kg/m^3	920 ± 0
kinematic viscosity at 40 ºC	mm^2/s	36.35 ± 0.01
water content	mg/kg	562 ± 14
acid value	(mg KOH)/(g oil)	0.34 ± 0.02
iodine number	(g iodine)/(100 g oil)	127 ± 1
higher heating value	MJ/(kg oil)	39 ± 0.9
average molecular mass	g/mol	870.2 ± 4.5

*Experiments were performed in triplicate and data are expressed as mean ± SD (standard deviation)

According to the oil characterization results some important aspects need to be considered for this oil transesterification:
- The density value is comparable to that of sunflower oil (917 to 925 kg/m^3) according to the (NP961, 1979) standard;
- The water content exerts an inhibitory action in the alkali-catalyzed transesterification for biodiesel production. However it may be favorable for biodiesel production through enzymatic catalysis.
- The acid value is below the maximum limit of (NP961, 1979) for sunflower oil [(0.6 g KOH)/(g oil)] which is a positive factor in order to prevent the soap formation.
- The iodine value is within the range defined in the NP961:1979 standard for sunflower oil [(110-143 g iodine)/(100 g of oil)]. This parameter is directly related to the number of double bonds in the oil molecules and consequently to the larger or lower stability of the oil to oxidation.
- Regarding the organoleptic parameters, the oil sample showed a yellowish color, smell like vegetable oil and liquid texture clear without any deposit.

3.2 Experimental study for FAEE production though enzymatic catalysis
This work used a three-factor design of experiments, 2^3 centered, to study the influence of the following variables on the reaction performance: reaction time, co-solvent (hexane) percentage, and oil: alcohol molar ratio, for fixed reaction temperature (35 ºC) and fixed mass of Lipozyme TL IM (5 g Lipozyme TL IM to 300 g oil). These factors' values are shown in Table 2.

Table 2: Factors and study domain of a 2^3 centered factorial design

Factor	Low Level (-)	Center	High Level (+)
oil: alcohol molar ratio	1:4	1:6	1:8
reaction time (h)	24	36	48
co-solvent (%, v/v)	10	20	30

As shown in Table 3, the experimental matrix contained for the three factors a total of 18 trials with two replicates, sixteen of which to the factorial design and two replicates of the midpoint. The FAEE characterization and comparison with the (NP EN14214, 2011) standard limits are shown in Table 4.

Table 3: Experimental matrix of a 2^3 centered factorial design

Parameters	1a	2a	3a	4a	5a	6a	7a	8a	1b	2b	3b	4b	5b	6b	7b	8b	1c	2c
									Trials									
Oil: alcohol molar ratio	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:6	1:6
Reaction time (h)	24	24	24	24	48	48	48	48	24	24	24	24	48	48	48	48	36	36
Co-solvent (% v/v)	10	10	30	30	10	10	30	30	10	10	30	30	10	10	30	30	20	20

Table 4: Average values of the FAEE quality parameters in each two trials under the same experimental conditions

Parameters	1-2a	3-4a	5-6a	7-8a	1-2b	3-4b	5-6b	7-8b	1-2c	NP EN14214 limits
Oil: alcohol molar ratio	1:4	1:4	1:4	1:4	1:8	1:8	1:8	1:8	1:6	
Reaction time (h)	24	24	48	48	24	24	48	48	36	
Co-solvent (% v/v)	10	30	10	30	10	30	10	30	20	
Density at 15 ºC (kg/m^3)	915	907	904	905	913	911	911	910	909	860-900
Kinematic viscosity at 40 ºC (mm^2/s)	17.03	10.59	8.34	12.42	16.00	11.56	12.29	10.17	11.13	3.50-5.00
Average flash point (ºC)	158	165	163	158	153	163	155	160	160	$\geq$101
CFPP (ºC)	1/-1	-1/-6	-5	-5/-4	-2	-3/-4	-5	-4	-6	$\leq$ +5*
Water content (mg/kg)	13612	5263	9505	5377	3862	5068	4559	4997	5338	$\leq$ 500
Iodine number (g/100 g)	107	108	99	92	108	115	101	107	109	$\leq$ 120
Acid value (mg of KOH/g of biodiesel)	-	1.38	1.70	1.10	0.91	1.03	1.15	1.29	3.00	$\leq$ 0.50
Group I metals (Na$^+$+ K$^+$) (mg/kg)	3.7	5.7	2.6	8.8	4.7	5.2	5.6	1.5	2.5	$\leq$ 5.0
Copper strip corrosion (3 h at 50 ºC)	1A	1A	1A	1A	1A	1A	1A	1A	1A	class 1
Higher heating value (MJ/kg)	38.0	38.8	38.4	38.6	38.4	38.2	38.1	37.9	37.6	
FAEE content (wt %)	55.8	52.8	53.6	54.5	33.2	38.0	45.0	46.2	44.7	$\geq$96.5
palmitate (C16:0)	15.8	16.4	16.3	15.4	17.7	18.5	16.7	16.5	16.8	
stearate (C18:0)	5.0	5.2	5.1	6.8	5.7	5.6	5.6	3.3	0.8	
oleate (C18:1)	24.0	25.6	25.6	25.4	25.1	25.8	25.7	15.7	5.3	
linoleate (C18:2)	43.7	46.4	46.9	47.1	44.1	44.9	45.6	36.0	25.8	
linolenate (C18:3)	4.7	5.1	4.9	5.0	5.1	5.2	5.1	24.9	44.7	
arachidate (C20:0)	1.7	nd	nd	nd	0.8	nd	0.9	3.1	4.9	
behenate (C22:0)	5.1	1.3	1.2	0.6	1.9	nd	1.0	1.0	1.8	
Total (wt %)	879.2	870.4	870.3	869.5	871.0	866.6	864.5	869.9	871.5	
average molecular mass of FAEE (g/mol)	308.4	305.5	305.4	305.2	305.7	304.2	305.2	305.3	305.8	

nd – not detected
* CFPP (cold filter plugging point) standard limit for temperate climates

Results show that the variable that most influences the reaction efficiency (or the FAEE content) is the oil/alcohol molar ratio. The reaction time and the co-solvent percentage are also important variables

but they do not influence reaction as much as the first one. In this case the best oil/alcohol molar ratio is 1:4 since higher ethyl esters contents (from 53.5 to 57.7 wt%) were obtained in the several trials, as shown in Figure 1a. Therefore, in order to perform the enzyme catalyzed transesterification the best operating conditions are an oil/alcohol molar ratio of 1:4, 10 % (v/v) of hexane co-solvent, for 48 hours of reaction time, at fixed reaction temperature (35 ºC) and enzyme/oil mass ratio (1.6 wt%). At these conditions one obtains a lower viscosity biodiesel, with lower density, lower CFPP, and lower mass of Na^+ and K^+ metals. The second best operating conditions are an oil/alcohol molar ratio of 1:4, 30 % (v/v) of hexane co-solvent, for 24 h of reaction time, at fixed reaction temperature (35 ºC) and enzyme/oil mass ratio (1.6 wt%).

Concerning the several biodiesel quality parameters analyzed it was possible to observe some dependence among some of them. For example, Figure 1b shows that the density increases as the viscosity increases. Figure 1c shows that the acidity increases as the water content increases. Figure 1d shows that as the water content decreases the calorific value increases and this decrease is more pronounced for the oil/alcohol molar ratio of 1:4.
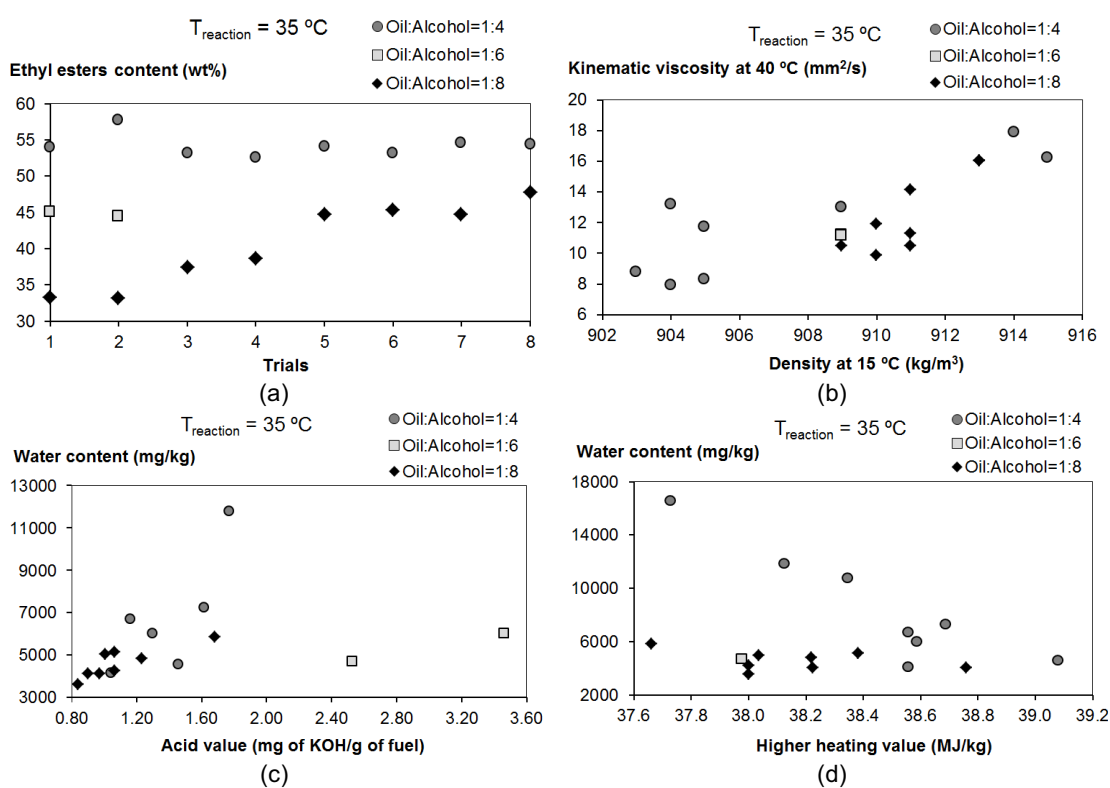

Figure 1: Experimental results of a 2^3 centered factorial design and FAEE characterization: (a) ethyl esters content per trial, (b) FAEE kinematic viscosity versus density, (c) FAEE water content versus acid value, (d) FAEE water content versus higher heating value.

This work represents an important contribution to the development of new routes for biodiesel synthesis. Despite the drawbacks still existing (mainly economic) once optimized, the enzymatic process can present very interesting advantages over the chemical process, namely the ease of the catalyst separation (if supported enzymes are used), the possibility of obtaining products with higher purity than the chemical process, and of using hydrated ethanol (with about 4-5 wt% water) in the reaction (Shah and Gupta, 2007). The reutilization of immobilized lipases is also envisaged as a way to reduce operating costs of biodiesel production (Shah and Gupta, 2007). As main disadvantages one can refer to the relatively long reaction times and the still high cost of enzymes (Gog et al., 2012).

4. Conclusions

In this work a three-factor design of experiments is done to study the influence of three variables in the production of FAEE from vegetable oil by enzymatic catalysis, using ethanol as reagent and in the presence of hexane as co-solvent. The reaction performance was evaluated by determining the ethyl esters content (wt %) by gas chromatography. Results show that the best operating conditions are: oil/alcohol molar ratio of 1:4, 10 % (v/v) of hexane co-solvent, and 48 h of reaction time. The second best operating conditions are oil/alcohol molar ratio of 1:4, 30 % (v/v) of hexane co-solvent, and 24 h of reaction time. The quality of biodiesel was also assessed, according to the most important parameters defined in the (NP EN14214, 2009) standard. Although most of the parameters determined are out of this standard limits, results have shown that for the above mentioned reaction conditions it is possible to obtain better quality biodiesel concerning the viscosity, density, CFPP, and alkaline metals (Na + K) content.

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