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Transgenic Corn Oil for Biodiesel Production Via Enzymatic Catalysis with Ethanol

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This work aims to study the production of fatty acid ethyl esters (FAEE) from a transgenic corn oil, through transesterification using supported enzymes as catalyst (Lipozyme TL IM from Novozymes) and ethanol as reagent. First, the corn oil was characterized for its acid value (0.26 mg KOH/g oil), iodine number (127 g iodine/100 g oil), kinematic viscosity at 40 ºC (36.27 mm^2/s), density at 20 ºC (919 kg/m^3) and water content (749 mg/kg). Second, the best operating conditions to perform the transesterification reaction were determined, by testing the following experimental conditions: oil/ alcohol molar ratio (1:3, 1:6 and 1:9), catalyst/ oil mass ratio (2.3, 2.8 and 3.3 wt%), reaction time (8 and 12 h) and reaction temperature (35 and 45 ºC). Results showed that although some of the quality parameters analyzed were out of the EN 14214:2009 standard limits (namely the kinematic viscosity, water content, acid value and group I metals) the best operating conditions for a good biodiesel quality (with 69.2 wt% of FAEE content) and the highest reaction yield (98.95 wt%) are an oil/ alcohol molar ratio of 1:6, a catalyst/ oil mass ratio of 2.8 wt%, a reaction time of 12 h and a reaction temperature of 35 ºC.

1. Introduction

The need to reduce dependence on fossil fuels and the greenhouse gas emissions, led to the search of different types of feedstocks that can be used for the industrial production of biodiesel (Mata et al., 2010a,b, 2011a,b). Among the several biomass sources available and suitable for biodiesel production, vegetable oils have been used not only due to their properties, but also because they represent an important alternative, helping to decentralize biofuel generation by acting as a strong support to family farms, creating better living conditions (infrastructure) in impoverished regions, enhancing regional capabilities and providing alternatives to economic problems and difficult socio-environmental solutions (EC Directive, 2008).
The most common industrial process for biodiesel production is essentially chemically catalyzed, although the enzymatic pathway has aroused great interest in the scientific community. A common feature of the current technological developments in this field is the attempt to improve the reaction conditions, making them viable and available for industrial applications (Nielsen and Rancke-Madsen, 2011). Despite some drawbacks, mainly economic, the enzymatic process, once optimized, can present very interesting advantages over the chemical process, namely the ease of the catalyst separation, the possibility of obtaining products with higher purity and of using hydrated ethanol in the

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reaction. As main disadvantages one can refer to the relatively long reaction times and the still high cost of enzymes (Gog et al., 2012).
On the other hand, at industrial level biodiesel production is performed using methanol as reagent as it is cheaper and promotes faster reaction rates (Morais et al., 2010a,b). However, methanol has some disadvantages for handling as it is hazardous to health and to the environment (Stamenković et al., 2011). Also, it is derived from fossil fuels what makes the resulting biodiesel not completely obtained from renewable sources. Alternatively, ethanol can be used since it is not so dangerous for human health and the environment and it can be obtained from renewable sources (e.g. bioethanol from sugarcane or lignocellulosic materials).
Hence, the reasons highlighted above justify the study of the FAEE's production by enzymatic catalysis with ethanol as reagent. To this purpose, this work evaluates the effect of different operating conditions (oil/alcohol molar ratio, catalyst/oil mass ratio, reaction time, and reaction temperature) in order to find the best ones. Also, for the best conditions, the resulting biodiesel is characterized according to the EN14214: 2009 standard that regulates the quality of FAME (fatty acid methyl esters) since currently it doesn't exist in Europe a FAEE specific standard.

2. Materials and methods

2.1 Corn oil characterization

The corn oil was characterized for the following properties: acid value (titrimetric method, according to EN 14104:2011standard), iodine number (titrimetric method using Wijs reagent, according to EN 14111:2009 standard), kinematic viscosity (determined at 40 ºC, using a glass capillary viscometer, Cannon-Fenske routine viscometer, series 200, according to EN ISO 3104 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 Biodiesel production through enzyme-catalyzed transesterification

Around 200 g of oil was weighed to a screw cap Pyrex bottle, with 500 mL of capacity and the bottle was placed in a thermostatic bath to heat the oil to the reaction temperature. Then, a certain mass of enzyme was weighted and the ethanol needed was measured in a hood and added to the oil. The required volume of alcohol was measured and a certain mass of enzyme was weighed, in accordance with the respective trial, and added to the oil together with a magnetic stir bar. Then, the transesterification reaction took place for a certain time period in a stirring bath (at 60 rpm) at the desired temperature depending on the trial conditions. 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 75 g of glycerin (72% purity) was added and left to stand for 15 minutes. The denser phase (glycerol) was removed from the bottom of the separating funnel to a previously weighed beaker in order to determine the mass of the recovered crude glycerol.
The less dense phase (biodiesel) was purified with a cation-exchange (acidic) resin, where biodiesel was passed through a column (5 cm in diameter and 30 cm in length) packed with a 15 cm length of ion-exchange R (Lewatit GF 202) that retained the impurities (water, ions of K, and glycerol), at a mean flow rate of 2 bed volumes/h (or about 236 cm^3/h). Lewatif GF 202 is a macroporous cation-exchange (acidic) resin (R). The R beads are uniform, 0.65 mm in diameter, with a density of 1.24 g/mL and a bulk density of 0.740 g/mL. Biodiesel was then subjected to distillation at 80 °C to recover the excess alcohol still existing.
Finally, the purified and neutralized biodiesel was placed in a beaker and about 2 g of diatomaceous earth was added to remove any water remaining in biodiesel, the mixture was stirred for about 15 min, after which biodiesel was left to stand and then filtrated through cellulose membranes (47 mm diameter, 1.2 µm pore) to remove the diatomaceous earth using a vacuum pump. The purified biodiesel was stored in glass flasks for its subsequent characterization.

2.3 Biodiesel characterization

The most important quality parameters of biodiesel were evaluated according to the EN 14214:2009 standard that defines the quality requirements of FAMEs. The acid value was determined using a

titrimetric method, according to the standard ISO 14104:2011. The kinematic viscosity was determined at 40 ºC, using glass capillary viscometers, according to the standard ISO 3104:1994. The density was determined at 15 ºC, using a hydrometer method, according to the EN 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 EN 14103:2010 standard. This analysis was performed using a gas chromatograph (DANI GC 1000 DPC) equipped with an AT-WAX (Heliflex Capillary, Alltech) column (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 255 ºC and the oven temperature to 195 ºC. The carrier gas used was Nitrogen, at a flow rate of 2 mL/min. Injection was made in a split mode, using a split flow rate of 50 mL/min (split ratio of 1:25), and the volume injected was 0.1 µL.

3. Results and discussion

3.1 Corn oil characterization

In order to characterize the corn oil used in this work for biodiesel production some of the most important parameters were determined experimentally as shown in Table 1.

Table 1: Experimental characterization of corn oil

Parameter	Unit	Value*
Density at 20 ºC	kg/m^3	919±0
Kinematic viscosity at 40 ºC	mm^2/s	36.27±0.01
Water content	mg/kg	749±14
Acid value	mg KOH/g oil	0.26±0.02
Iodine number	g iodine/100 g oil	127±1
Higher heating value	MJ/kg oil	39.8±0.2
Average molecular mass	g/mol	865.4±8

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

The transgenic corn oil used in this experimental work has a vegetable origin and was bought from a local commercial retailer. Regarding the organoleptic parameters, the oil showed a yellowish color, clear, with no deposit, smell like vegetable oil and liquid texture at room temperature (of about 20 ºC).
The values obtained experimentally for the corn oil density and iodine number are within the expected ranges of the NP-946:1981 standard for corn oil (915-924 kg/m^3 and 103-128 g/100 g oil, respectively). The iodine number indicates the degree of the oil unsaturation. According to Moretto and Fett (1998), the density of triacylglycerides is lower as lower is its molecular mass and as higher is the degree of unsaturation. Also the acid value obtained experimentally for corn oil is below the maximum limit of this standard (0.60 mg KOH/g). The acid value is one main indicatives of the vegetable oil quality. Also, high oil acidity can neutralize an alkali-catalyst during transesterification being necessary a much higher amount of catalyst to perform reaction efficiently. According to Dantas et al. (2006), the high free fatty acids content influences the hydrolysis and oxidation of biodiesel.
For kinematic viscosity the value obtained experimentally is close to the one indicated by Balat (2008) for corn oil that is 34.9 mm^2 / s (measured at 38 °C). The water content of this oil is considered low and thus, it is suitable for the transesterification reactions. High water content can result in a lower solubility of oil in the alcohol, with a consequent drop in the ethyl esters yield (Silva, 2005). The higher heating value of this oil is according to the one (39.5 MJ / kg) reported by Balat (2008) for corn oil.

3.2 Biodiesel obtained by enzyme-catalyzed transesterification

In this work FAEE were produced by enzymatic transesterification using absolute ethanol (containing no water) as reagent and Lipozyme TL IM as catalyst. It was varied the oil/ alcohol molar ratio (1:3, 1:6 and 1:9), the catalyst/ oil mass ratio (2.3, 2.8 and 3.3 wt%), the reaction time (8 and 12 h) and the reaction temperature (35 and 45 ºC). Results are shown in Table 2, including some of the most important quality parameters for the obtained biodiesel characterization.

Table 2: Characterization of biodiesel obtained by enzyme-catalyzed transesterification with absolute ethanol as reagent and Lipozyme TL IM as catalyst

Parameters	Trials							EN14214 limits
	1a	2a	3a	4a	5a	6a	7a	
Oil/ alcohol molar ratio	1:3	1:3	1:6	1:9	1:6	1:6	1:3	
Reaction time (h)	8	12	12	12	12	12	12	
Reaction temperature (ºC)	35	35	35	35	35	35	45	
Enzyme catalyst/ oil mass ratio (wt%)	2.8	2.8	2.8	2.8	2.3	3.3	2.8	
Reaction yield (wt%)	73.46	95.30	98.95	94.60	84.00	87.50	83.55	
Density at 15 ºC (kg/m^3)	889	890	890	890	892	890	892	860-900
Kinematic viscosity at 40 ºC (mm^2/s)	12.57	8.95	9.15	13.18	10.45	8.09	9.03	3.50-5.00
Water content (mg/kg)	558	962	1966	1637	388	8297	6844	≤ 500
Iodine number (g/100 g)	102	104	92	99	96	102	102	≤ 120
Acid value (mg of KOH/g of biodiesel)	0.98	0.90	0.60	0.52	0.54	0.76	1.09	≤ 0.50
Group I metals ($Na^+ + K^+$) (mg/kg)	11.8	11.8	11.8	11.8	11.8	11.8	11.8	≤ 5.0
Copper strip corrosion (3 h at 50 ºC)	1A	1A	1A	1A	1A	1A	1A	class 1
Average flash point (ºC)	>150	>150	>150	>150	>150	>150	>150	101
CFPP (ºC)	+4	+4	+4	+4	+4	+4	+4	≤ +5*

* Limit for temperate climates.

As shown in Table 2, higher reaction yields are obtained for 12 h than for 8 h, revealing that 12 h should be sufficient to perform the reaction and to favor conversion of oil into biodiesel.

Comparing the oil/ alcohol molar ratios (1:3, 1:6 and 1:9), keeping constant the other operating conditions, the obtained reaction yields were respectively 95.30, 98.95 and 94.60 wt% (trials 2a, 3a and 4a). Therefore one may conclude that an even higher ratio doesn't improve the oil conversion into biodiesel although the transesterification of vegetable oils is kinetically favored when excess of alcohol is used in relation to triglycerides (Demirbas, 2008). A possible explanation is that a too high oil/ alcohol molar ratio can interfere with the separation of glycerol due to its increased solubility in ethanol and the presence of glycerol in the reaction medium favors the formation of triglycerides. A similar conclusion was reached in the Encinar et al. (2002) study, showing that an oil/ alcohol molar ratio of 1:9 was quite appropriate to perform transesterification as below this ratio the reaction could be incomplete, and above this ratio there would be a greater accumulation of glycerol which would impair performance in ethyl esters. In this work however oil/ alcohol molar ratio of 1:6 was enough to obtain the best reaction yield.

By comparing the catalyst/ oil mass ratios of 2.3, 2.8 and 3.3 wt% (Ferreira et al., 2008), keeping constant the other operating conditions, the obtained reaction yields were 84.00, 98.95, and 87.59 wt% respectively (trials 5a, 3a and 6a). Therefore one may conclude that an excess of enzyme (3.3 wt%) does not promote an increase in the conversion efficiency of oil into biodiesel, but on the other hand the use of less amount of enzyme (2.3 wt%) may not be sufficient.

Comparing the reaction temperatures (35 and 45 ºC), keeping constant the other operating conditions, (trials 7a and 2a) results show that at 35 ºC higher reaction yield (95.30 wt%) was obtained than at 45 ºC (83.55 wt%). The reason could be a protein denaturation at the higher temperature with consequent loss of the enzyme activity, since for enzyme Lipozyme TL IM the optimal temperature range of operation of this protein is between 30 and 40 °C, according to producer instructions.

Therefore, for the highest reaction yield (trial 3a) the best operating conditions to perform the transesterification reaction are an oil/ alcohol molar ratio of 1:6, a catalyst/ oil mass ratio 2.8 wt%), a reaction time of 12 h and a reaction temperature of 35 ºC, for which the obtained reaction yield was 98.95 wt%.

Concerning the quality of the produced biodiesel, Table 4 shows that regardless the operating conditions density is within the standard limits. All the kinematic viscosity values are out of the standard

limits and increase with the oil/ alcohol molar ratio from 1:3 to 1:6 and 1:9, being 8.95, 9.15 and 13.18 mm^2/s, respectively (trials 2a, 3a and 4a). Also, the viscosity decreases as the catalyst/ oil mass ratio increases from 2.3, 2.8 and 3.3 wt% being respectively 10.45, 9.15 and 8.09 mm^2/s (trials 5a, 3a and 6a). The water content is much above the maximum standard limit except for trial 5a. The iodine number of biodiesel is below the maximum standard limit in all samples. The acid value is out of the standard limit in all samples, but it is just slightly above the maximum limit in trials 4a and 5a (0.52 and 0.54 mg KOH/g respectively). Also, the acid value decreased with the oil/ alcohol molar ratio from 1:3 to 1:6 and 1:9, being 0.90, 0.60 and 0.52 respectively (trials 2a, 3a and 4a) and increased with the catalyst/ oil mass ratio from 2.3, 2.8 and 3.3 wt%, being respectively 0.54, 0.60 and 0.76 mm^2/s (trials 5a, 3a and 6a). The value of alkaline metals (Na + K) is above the maximum standard limit (5.0 mg/kg) with an average value of 11.8 mg/kg. All the biodiesel samples had a flash point higher than 150 °C which is above the minimum standard limit of 101 °C and all the biodiesel samples are non-corrosive as the copper strip corrosion tests showed that they are within class 1 according to the standard. Concerning the CFPP (cold filter plugging point) the value of +4 °C falls within class A (+ 5 °C) meaning that it can be used in temperate countries, such as Portugal.
In summary, one can conclude that the best biodiesel quality was obtained by enzyme-catalyzed transesterification at oil/ alcohol molar ratio of 1:6, a catalyst/ oil mass ratio of 2.8 wt%, a reaction time of 12 h and a reaction temperature of 35 ºC, as concluded previously for the best reaction yield (trial 3a). For these operating conditions the resulting biodiesel samples were characterized by gas chromatography to determine the esters content and the relative percentage of each ester and the molecular mass of biodiesel (Table 3).

Table 3: FAEE content, esters characterization and molecular mass of biodiesel obtained by enzyme-catalyzed transesterification with absolute ethanol

Parameter	Value
FAEE content (wt%) (EN14214 limit: ≥96.5%)	69.2
Relative percentage of FAEE (wt %):	
myristate (C14:0)	7.64
palmitate (C16:0)	15.60
stearate (C18:0)	4.61
oleate (C18:1)	22.54
linoleate (C18:2)	42.97
linolenate (C18:3)	4.65
arachidate (C20:0)	1.06
behenate (C22:0)	0.95
Total (wt%)	100.0
Average molecular mass of FAEE (g/mol)	301.7

As shown in Table 3 the FAEE obtained from the corn oil are mainly composed of unsaturated esters (70.15%) and just 29.85% are saturated esters. Among the unsaturated esters special attention should be given to linolenate (C18:3) as the 14214:2009 standard establishes the maximum limit of 12% (wt / wt) for this ester, which is verified in this case (4.65 wt%). From the FAEE identified linoleate (C18:2) followed by oleate (C18:1) are the most significant (with 42.97% and 22.54% respectively), which is according to Balat (2008). The average molecular mass of FAEE was also determined that is 301.7 g/mol.

4. Conclusions

This work studied the production of FAEE from corn oil by enzymatic catalysis with Lipozyme TL IM from Novozymes, using absolute ethanol as reagent. Results show that the best operating conditions are an oil/ alcohol molar ratio of 1:6, a catalyst/ oil mass ratio of 2.8 wt%, a reaction time of 12 h and a reaction temperature of 35 ºC. For these conditions it is possible to obtain a reaction yield of 98.95 wt% with a FAEE content of 69.2 wt%, being linoleate (C18:2) and oleate (C18:1) the most significant esters (with relative percentages of 42.97 wt% and 22.54 wt% respectively). The quality of biodiesel was also assessed, according to the most important parameters defined in the EN14214:2009 standard.

Although some 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 a better quality biodiesel concerning the several parameters analyzed.

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