Research Article

Received: 14 December 2015

Biodiesel production via enzymatic transesterification with free and immobilized cutinase

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Abstract

The aim of this work was to study the *Fusarium solani pisi* cutinase behavior as biocatalyst in the biodiesel production by transesterification in reverse micelles system and immobilization of this enzyme on Zeolites and silica gel particles was made.

Free enzyme were kinetically characterized by the hydrolysis of *p*-nitrophenyl butyrate *p*-NPB. The IME3, IME11 (amine and glutaraldehyde), IME6 (amine by adsorption), IME7 (vinyl in silica) and IME5 (Zeolites) were characterized by automatic titration of the hydrolysis of tribytyrin emulsion. High enzyme activity solution (Activity 1357.2 U/ml) was used for small scale production of biodiesel by transesterification. The oil substrate (Triolein) and alcohol (Butanol), water content, free enzyme versus immobilized enzyme were tested in small scale production of biodiesel by transesterification. HPLC chromatographic profiles were used to check which type of enzyme and immobilization lead to a complete transesterification reaction of triglyceride producing glycerol and alkyl ester (biodiesel). It was observed that from the different IME the cutinase immobilization on silica gel support though amine and glutaraldehyde (Activity 748 U/ml) has higher immobilization yield (38%) than the others through biocatalysts. A fed batch strategy was efficient to increase the amount of biodiesel in reaction media when the immobilized enzyme were previously washed with isooctane reducing the amount of water.

The best immobilization of this study was silica-amine-glutaraldehyde since the increase of its quantity (300mg) decrease from 3 to 1 day necessary to total triglycerides consumption.

Keywords: Cutinase biocatalyst; Immobilization; Biodiesel; Enzymatic transesterification; Reversed micelles

1. Introduction

The increasing industrialization of the world leads to high demand of petroleum-based fuels that are the main energy source nowadays. In 1980, fuel consumption was 6630 million tons of oil equivalents (Mtoe). It almost doubled in 2012 at 12,239 Mtoe. According to the International Energy Agency estimation (IEA), global energy demand is expect to increase by 53% by 2030. Only the transport sector consume 58% of the total consume of fossil fuels [1]. Globally, we consume the equivalent of more than 11 billions tons of the oil in fossil fuel every year and crude oil reserves are vanishing at a rate of 4 billion tons a year. If this rate continues, oil deposits will be exhausted by 2052 [2].

Increases in vehicle fuel economy resulted in a decline in the petroleum and other liquids share of fuel used while consumption of liquid biofuels increases. Biofuels, including E85, biodiesel blended into diesel, and ethanol blended into motor gasoline (up to 15%), account for 4% of all petroleum and other liquids consumption by energy content in 2040 [3].

The European Directive on the promotion of the use of energy from renewable sources, Directive 2009/28/EC, established the objective that, by 2020, 20% of all the gross final consumption of energy in the Community should come from renewable sources, bearing in mind that for the transport sector, this quota

should be 10%. In order to meet this overall objective, individual objectives are laid down for the Member States and, for Portugal, the quota to be reached by 2020 will be 31% [4].

This high consumption of fuels petroleum derivate contributes to the increase of greenhouse gas (GHG) emissions which lead to many negative environmental effects like climate change, receding of glaciers, raise in sea level, and loss of biodiversity amount others. The progressive depletion of the conventional fuels due to the increased consumption led to rising of crude oil price which directly affect the global economic activity. It is necessary to study an alternative renewable, sustainable, efficient, cost-effective and with less emissions. There are many energy alternatives like biofuels, hydrogen, natural gas and syngas (synthetic gas) that are important sustainable fuels sources to consider in the near future [1].

Biofuels are an excellent choice to replace fossil fuels due to their renewability, carbon neutral, biodegradability, generating acceptable quality exhaust gases and are necessary for environmental and economic sustainability [2]. Besides that biodiesel is mainly used as an alternative fuel for diesel engines and is an environmentally friendly alternative liquid fuel that can be used in any diesel engine without modification. Since January 1st of 2010 that in Portugal the diesel is blended with biodiesel (B7) [5].

A challenge for researchers is to produce biodiesel in mass scale and with lower price in order to compete with the diesel price.

More than 95% of biodiesel production feedstock's come from edible oil, when the edible oil market increases both the cost of edible oils and biodiesel rises also [6], [7]. However there are some limitations in biodiesel production using non-edible oils such as the competition for land and water used for food and fibre production, high production and processing costs [7]. The use of cooking oil for production of biodiesel is a possible alternative due to the oil low cost of acquisition, that is 2.5–3.5 times cheaper than virgin vegetable oils, the environmental preservation, prevents water sources contamination and at the same time produces a less pollutant fuel [8], [9].

There are several methods to produce biodiesel, like pyrolysis, dilution (direct blending), micro-emulsion and transesterification. Dilution and micro-emulsion are not used because the higher viscosity and bad volatility of biodiesel feedstock, pyrolysis is a simple process and environmental friendly but transesterification is the most used for the production of biodiesel [10]. Transesterification is the process of using an alcohol like methanol or ethanol in the presence of a catalyst, such as sodium hydroxide (NaOH) or sodium methoxide (NaOMe), to chemically break the molecule of the raw renewable oil into methyl or ethyl esters of the renewable oil with glycerol as a by-product which reduces the high viscosity of oils. This method also reduces the molecular weight of the original oil to 1/3 of its former value, reduces the viscosity by a factor of ca. 8 and increases the volatility and cetane number to levels comparable to diesel fuel [11].

Enzymes are heterogeneous catalytic which are considered a good choice to produce biodiesel because they can easily treat fatty acids as well as triglycerides with higher conversions and in a presence of water however they have high production costs. [10]. The mostly biocatalyst used for the reactions of hydrolysis, esterification and transesterification

are lipases. They are characterized preferably by their catalytic action at the interface between water and an insoluble substrates or organic solvent containing the reactants. Another interesting group of hydrolytic enzymes are cutinases, which also belong to the class of serine esterase as most of the lipases do but in comparison to the lipases they are not characterized by the phenomenon of interfacial activation [12]. A way to overcome the high production cost is by enzyme immobilization. There are several techniques that can be classified into three types: carrier bonding, cross-linking and entrapment. Depending on the type of interactions between enzymes and carriers, these techniques can be further classified into irreversible and reversible immobilization. Each immobilization technique has its own merits and inevitably some disadvantages for lipase immobilization [13].

Encapsulation in the interior of the reversed micelles has the particular advantages of forming spontaneously and allowing a strict control of the water content of the reaction, which is represented by the molar ratio between water and surfactant concentration, W_o [14]. Enzymatic activity can be affected in this type of systems by W_o parameter since it determines the microenvironment of the enzyme encapsulated within the micelle [15]. In this case low values of W_o led to higher conversions on transesterification.

Among the alcohols that can be used in the transesterification reaction are methanol (CH_3OH), ethanol (C_2H_5OH), propanol (C_3H_7OH), butanol (C_4H_9OH) and amyl alcohol. Methanol and ethanol are mostly used, ethanol is a desired alcohol in the transesterification process compared to methanol because it is derived from agricultural products and is renewable and biologically less objectionable in the environment. However methanol is preferable because of its low cost and its physical and chemical advantages (polar and shortest chain alcohol) [16].

Use solvents as reaction media has advantages such as increased solubility of nonpolar substrates and products, shifting thermodynamic equilibrium to favour the ester synthesis in relation to hydrolysis, and the minimization of enzyme inhibition by substrates or products, etc [17]. There are also disadvantages of using organic media, which can lead to denaturation or inhibition of the enzyme, separation of solvent from the product, lack of “green” solvent [17].

The goal to produce biodiesel in mass scale and with lower price can be achieved by: (1) choose the cheaper feedstock and in large quantities; (2) improving the immobilization technologies; (3) optimizing the transesterification process; (4) developing novel bioreactors; and (5) intensifying the process integration to reduce the operation cost.

2. Materials and Methods

The characterization of enzyme extracts and immobilized enzyme preparations (biocatalysts), in particular and how to quantify protein, how to measure enzyme activities, the assay of substrates and products resulting from the transesterification of triolein with butanol in reversed micelles of AOT/isooctane are important parameters to quantify in this work.

2.1. Materials

2.1.1. Microorganism

The wild-type *F. solani pisi cutinase* was produced by recombinant *S. cerevisiae SU50* strain as described by Calado *et al* [18]. The

cutinase-producing *S. cerevisiae SU50* strain (Mata, leu2-3, ura3, gall:URA3, ML-S, MAL3, SUC3) contains the expression vector pUR7320 constructed and provided by the Unilever Research Laboratory, Vlaardingen, the Netherlands.

2.1.2. Chemicals

Surfactant bis(2-ethylhexyl)sodium sulfosuccinate, AOT (98 %), triolein (65 %), 1-butanol (99 %), Polyethylenimine (50%) and Glutaraldehye (25% Aq. Sol. Grade I) were obtained from Sigma. Isooctane (99.5 %) is purchased from Fluka. All eluents (acetonitrile, 2-propanol and n-hexane) of HPLC grade obtained from Lab Scan and sodium phosphate buffer (Na_2HPO_4 and NaH_2PO_4) is of analytical reagent grade from Merck. Most triglycerides are unsymmetrical, being derived from mixtures of fatty acids, but in contrast triolein is a symmetrical triglyceride which represents 4-30% of olive oil. Ammonium sulphate PA-ACS-180 was from Applichem Panreac; In the immobilization procedures was used porous silica Type LC 500A/500 (size between 2-3mm) and syloid silica Type ED2 (size between 2-5 µm), both from Grace.

3. Analytical methods

3.1. Protein Concentration assay

Protein concentration has been determined by the microplate procedure by using Pierce Bradford Protein Assay Reagent kit (BCA).

3.1.1. Cutinase Activity Assay

Enzyme activity assay is determined using *p*-NPB in a 70 mM concentrated stock solution in pure acetronitrile. 15 µl of enzyme extract, previously diluted in Tris buffer, is added to reaction mixtures composed by 1470 µl of 20 mM Tris.HCl with pH 8 and 15 µl of *p*-NPB 70 mM solution inside the glass optical cell (3 ml) mixed by a small magnetic stirrer submitted to magnetic agitation of 700 rpm (Electronic Stirrer Model 300 Rank Brothers Ltd). The cell holder is kept at temperature of 30ºC by circulating warm water from a thermostatic bath (Thermomix MM,B.BRAUN) also at 30ºC. The reaction rate is determined by monitoring spectrophotometrically the hydrolysis of *p*-NPB and formation of *p*-NP at 400 nm (Ɛ = 15,400M-1cm-1) at 30ºC for one minute. Dilutions of the enzyme samples in Tris Buffer were made in order to work in linear variation of the concentration of p-NP, i.e., intensity of its yellow colour (Hitachi U-2000 Spectrometer) using a software program (Hitachi Hyper Terminal software) to record the variation of absorption at 400 nm against time with respect to the amount of enzyme in this reaction mixture.

The solution of immobilized enzyme activities is also measured by using the Titrino method.

3.2. Karl Fisher

The content of water (in percentage) in biodiesel samples and in AOT/isooctane reaction media was measured by weighing the samples before and after injection in Karl Fisher equipment. First the Karl Fisher was equilibrated using the sample standard with 0.1% of water.

3.3. Analysis of Biodiesel using HPLC

The method that was used in HPLC is represented by figure 1.

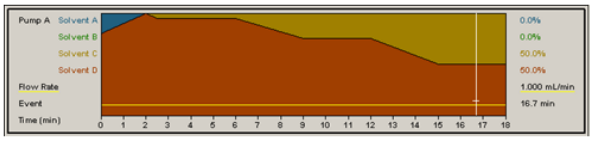

Figure 1. Method used in HPLC for biodiesel production.

The sample collected from the biodiesel reactor was first added acid to desactive the

cutinase and them diluted with hexane before to centrifugation to separate precipitates was analyzed by high performance liquid chromatography (HPLC) using a Choromolith Performance RP-18 end capped (100mmx4.6mmx2µm) column. HPLC apparatus (ELITE Hitachi La Chrom) equipped with an auto sampler (ELITE Hitachi La Chrom L-2200), a HPLC pump (ELITE La Chrom L-2130) and a UV detector (MERCK Hitachi L-2400) at 205 nm has been used. The flow rate has been maintained at 1 ml/min with injection volume of 10 µl of standards or reaction samples both previously diluted with n-hexane. In this methodology there are three mobile phases: phase D consists with acetonitrile 100%, phase A consists of pure water and phase C consists of n-hexane and 2-proponol mixture (4:5 v/v).

Triglycerides, alkyl esters, fatty esters mono and diglycerides in samples taken from the transesterification reaction media are monitored at end of reaction, and quantified by using High Performance Liquid Chromatography(HPLC).

50 µl of standards at specific concentration or reaction samples is added 1 µl of acetic acid at 58.5 mM pH 3 to correct pH value and to stop the enzymatic reaction. After that, samples have been dissolved in 949 µl of n-hexane and centrifuged (eppendorf Centrifuge 5810R) to remove any precipitate. Each vial had the supernatant of each sample with 1400 µl of volume.

3.1.1. Calibration of HPLC for analysis of alkyl glycerides and Biodiesel Production

3.1.1.1. Retention times

The figure 2 shows the chromatogram that we expect to obtain. The substrates, intermediates and products resulting from transesterification and hydrolysis can be well identify according specific time zones. The figure 2 presents a typical chromatogram for the injection of a sample of triolein in reversed micelles reaction media.

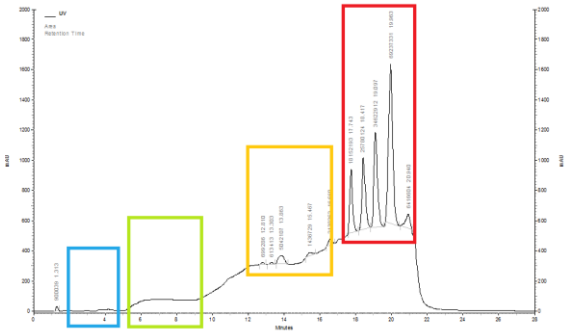

Figure 2. Resume of typical chromatogram and the time zone of substrates, intermediates and products. Blue rectangle – time zone of MG detection, Green rectangle – time zone of Alkyl Esters detection, Yellow rectangle – time zone of DG detection, Red rectangle – time zone of TG detection.

3.2. Enzymatic Activity using a Titrino

The emulsion used for measuring the immobilized enzyme preparations activity using Titrino (Metrohm) was prepared by adding to the Phosphate buffer 25mM pH 8.2 (80mL), 30mM of Tributyrin (690µL), 100Mm NaCl (0.466g) and 3.5% triton X-100 (2.8mL). Next the solution was agitated in a stirring plate during 15 minutes until make it transparent. The pH of the solution was adjusted for 7.98 value. On the reactor of Titrino (Metrohm) were put 3.950mL of Tributyrin solution, 50µL of free enzyme diluted 1:80 and automatic titration (Figure 26). . The calibration curve was made with butyric acid.

The activity of the free cutinase is about 3.5 higher by the spectrophotometric method using the p-nitrophenylbutyrate in relation to the Titrino method using emulsion of tributyrin.

4. Methods

4.1. Enzyme purification

The cutinase was precipitated from the clarified fermentation broth obtained after centrifugation of recombinant *S. cerevisiae SU50* cells solubilized with Tris buffer 20mM pH8.0 and then stored at -20ºC.

4.2. Immobilization Procedures

4.2.1. Enzyme immobilization

Porous silica gel and zeolite were used for enzyme immobilization. Silica gel was functionalized with APTS 8% (v/v) Sigma-Aldrich >98%. The cutinase was also immobilized by adsorption on Zeolite supports (see Table 1).

4.3. Biodiesel production

The transesterification of triglycerides was performed in closed small scale reactors with total volume 5.3 ml. Control experiments were performed using buffers or original supports without enzyme immobilized. All experiments, including the ones with immobilized enzyme were carried-out using optimal conditions previously defined [19].

4.3.1. Reversed micelles for biodiesel production

Cutinase is dissolved in phosphate or Tris buffer 20 mM pH 8.0. It is further microencapsulated in 150 mM AOT in isooctane with molar ratio of water: surfactant W_o as 2.7 by the injection method. This method consisted in adding drop wise the aqueous solution with cutinase to the AOT / isooctane solution while strong vortex mixing for 15 s.

For biodiesel production, the reversed micelles solution was previously dried with molecular sieve and then a total reaction volume of 5.3 ml in closed in a glass reactor (10 ml) with magnetic stirrer. The conditions buffer molarity, pH, AOT concentration and W_o were chosen taking into account the desired optimization [19]. The biodiesel production starts by adding the alcohol and triolein substrates into the reversed micellar system. By stoichiometry, for 3 moles of butanol is consumed 1 mole of triolein adding an alcohol excess of 60%. The reaction started by adding alcohol with strong vortex mixing of 15 s. (See table 3).

5. Results and Discussion

5.1. Stability of HPLC during assay of the biodiesel production

The HPLC showed instability in the areas at the same retention time of the chromatogram between consecutive days. Because of this it was impossible to perform the enzyme kinetic studies by HPLC. For a better control of this instability it was also necessary to verify individual analysis of each peak once the HPLC software does not always form the correct calculations of areas.

However, to normalize this instability of the detected areas by HPLC was decided to compare the area of each compound in relation to total area in form of percentage of each component (Fatty acid, monoglycerides, alkyl esters, diglycerides and triglycerides) of the sample.

$$Component\ percentage = \frac{Individual\ component\ area}{Total\ area}$$

5.2. Cutinase concentration and purification from the clarified fermentation broth

The cutinase activity of 179 U/ml in the clarified fermentation broth was measured. Ammonium sulfate, 33% (w/w) was added to the clarified fermentation broth, after cell separation by centrifugation, slowly and at 4ºC,

in order to precipitate the cutinase and then it was stored for 5 days at 4ºC.

After 5 days the precipitated enzyme solution was centrifuged at 9000 rpm (Sorvall RC 6 Rotor SLC-3000) and 4ºC over 20 minutes. The cutinase precipitated was re-suspended in 250 ml of Tris-Hcl 20Mm, pH 8.0, and protein concentration and its activity (Ext1) were assayed obtaining 2.52 mg/ml and Activity 1357.2 U/ml, respectively.

5.3. Cutinase immobilization and characterization

5.3.1. Amine support obtained by silanization with Aminopropyltriethoxysilane

In this method for enzyme covalent immobilization, 2g of silica supports was washed with 20ml of HCl 100mM during 2 hours. Then the particles were washed with phosphate buffer 50mM, pH 7.5 until the pH rise to 7. After, 20 ml of Aminepropyltriethoxysilane (Merk) 8% (v/v) was joined to the particles up to pH 3/4 and put it in the oven (IBALAB (Memert)) at 70ºC over 1 to 2 hours. Afterwards the particles were washed with phosphate buffer 50mM, pH 7,5 till pH rise to 7 and then put in the oven (IBALAB (Memert)) at 115ºC during 12 hours. The particles was cooled at room temperature in vacuum (Kartele®)

It was added 20 ml of glutaraldehyde 0.5% solution to the wetted silica supports contained in the vial and it was left stirring in Plate Elisa (Cod.3000356 P. Selecta ®) during 1 hour. Then the porous (Grace) silica supports were washed with phosphate buffer 50mM, pH 7.5 four times. After it was joined 100mg of porous silica to 1ml of the diluted cutinase enzyme (Ext1). The enzyme was diluted with phosphate buffer 50mM, pH 7.5 in a proportion of 1:1. (See table 1).

It was measure the volume and activity of the supernatant of IME3 (Vol 1ml; Activity 152U/ml) and IME11 (Vol 1ml; Activity 48.7U/ml. The volume and washes activity of IME3 (Vol 6ml; Activity 5.7 U/ml) and IME11 (Vol 6ml; Activity 8.2U/ml) was also measure. (See table2).

5.3.2. Enzyme immobilization by adsorption

80 mg of zeolite (IME5) and 100mg of amine support (IME6) obtained by silanization with Aminepropyltriethoxysilane were put in contact with 2 ml of cutinase enzyme (Protein 2.52 mg/ml, Activity 1357.2 U/ml) and then they were laid into an oven (Memert) at 30ºC until total drying (Table 1). After they were cooled at room temperature in vacuum (Kartele®) and stored in a refrigerator at 4ºC. The same procedure as the previous one was performed with 100mg of vinyl support (IME7) obtained by silanization with Triethoxyvinyl and instead immobilized by adsorption it was by covalent link. The values for IME5, IME6 and IME7 are represented in table 2.

The resume of biocatalysts produced are present in table 2 and the reactions that were performed with them are show in table 3.

Table 1. Biocatalyst used in all reactions.

Support	Silica gel			Zeolite
Functionalization	Amine (APTS)	Amine	Vinyl	–
1º Derivatization	Glutaraldehyde (0.5% solution)	–	–	–
2º Derivatization	–	–	–	–
Enzyme Ext1 1357.2 U/ml	IME3 (100mg) IME11 (300mg Amine Silica gel,	IME6 (100mg)	IME7 (100mg)	IME5 (100mg, Ext1 2 ml)

Table 2. Reactions of all experiments made.

Immobilized enzyme supports	Enzyme source	Supernatant activity (U/ml)	Estimated PNB (U/g)	Biocatalyst activity (U/g)	Mass of IME_{inital} (mg)	Immobilization Yield (%)	Biocatalyst activity After Biodiesel production (U/g_{wetted})
IME3	Ext1	152	5013	77.2	100	1.5	0
IME5	Ext1	0	34375	2600	80	7.6	182.8
IME6	Ext1	0	27500	3412.5	100	12.4	279.7
IME7	Ext1	0	27500	365.6	100	1.3	21.8
IME11	Ext1	48.7	1965.3	747,5	300	38.0	256.6

The content in water of the biodiesel samples and AOT/isooctane were lower than 0.1%.

Table 3. Identification of the biocatalysts (IMEs) used in the biodiesel production and respective characteristics of supernatants and biocatalyst activity.

Reactions	Tris. HCl buffer (400mM, pH8) (µl)	Triolein (g)	Extract (µl)	IME (g)	n-Butanol (µl)	AOT/Isooctane (ml)	Biodiesel from Soya oil (ml)	Biodiesel from Waste oil (ml)	Incubator
R9 (Free enzyme)	_	0.82	72 (Ext1)	_	566	5	_	_	P. Selecta with Heidolph MR Hei-Mix L stir plate
R10a), R10b) (IME3)	_	0.60	_	100(*)	283	5	_	_	
R11 (IME3)	_	0.60	_	100(**)	283	5	_	_	
R14 (IME5)	_	0.67	_	80(***)	283	5	_	_	
R15 (IME6)	_	0.65	_	100(***)	283	5	_	_	
R16 (IME7)	_	0.64	_	100(***)	283	5	_	_	
R25 (IME11)	_	0.70	_	300(*)	283	5	_	_	

(*) Washed with isooctane

(**) Wetted in phosphate buffer

(***) Dried

5.4. Biodiesel production

5.4.1. Free enzyme

It was prepared a reactor for the reaction R9 with free enzyme using Ext1 into incubator P. Selecta with Heidolph MR Hei-Mix L stir plate and the results are shown in table 4. The duration of the experiment was about 72 hours.

Table 4. Comparison of the percentage in R9 samples components.

Time (h)	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
0.5	2,55	39,40	48,84	4,31	4,89
	Fat Acid (%)	MG (%)	% Alkyl esters	DG (%)	TG (%)
72	2,82	6,83	87,52	0,00	2,82

At 0.5 hours it was observed a higher triolein consumption and alkyl esters production (48.84%), after this time until 72 hour occurred a consumption of monoglycerides around 32.6% and a biodiesel production of 39% (Total biodiesel production of 87.52%)

5.4.2. Effect of initial water content

For this it were prepared the reactions R10a) and R11 with IME3 previously washed with isooctane versus IME3 wetted in phosphate buffer, respectively. The duration of the reactions was about 72 hours into the P. Selecta incubator with Heidolph MR Hei-Mix L stir plate. The percentage of water content, measured by Karl Fisher, into reactors at time zero was about 2.5% for IME3 previously washed with isooctane and 6.7% for IME3 wetted in phosphate buffer.

Through the tables 5 and 6 is possible to observe that in R10a) the reaction was almost complete in contrast with R11 whose triglycerides consumption was low (3.5%) as well as the alkyl esters formation (20.2%).

Table 5. Comparison of the percentage in R10a) samples components.

Time (h)	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
0.5	8,21	5,55	4,26	4,79	77,19
	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
72	20,37	5,86	72,95	0,00	0,81

Table 6. Comparison of the percentage in R11 samples components.

Time (h)	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
0.5	3,80	11,16	7,76	9,63	67,65
	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
72	1,16	2,76	20,20%	11,74	64,14

The presence of water in the immobilized enzyme impaired the complete reaction to take place.

5.4.3. Effect of immobilization method

Others types of immobilization and support beyond the last one was important to test. It were used several types of supports just as zeolites and porous silica.

Cutinase adsorption on zeolite (IME6) and covalent immobilization through amine (IME5) and vinyl (IME7) functionalized silica were prepared and the tables 7, 8 and 9 indicate that the zeolite was the best immobilization method. It had the highest triglycerides consumption during the first half hour and at the end the alkyl esters percentage was around 77% which is quite good.

Table 7. Comparison of the percentages in R14 sample components (IME5).

Time (h)	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
0.5	6,64	18,33	46,05	5,75	23,23
	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
120	0,46	3,60	77,54	0,00	19,09

Table 8. Comparison of the percentages in R15 sample components (IME6).

Time (h)	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
0.5	9,08	0,00	1,18	7,87	85,47
	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
120	16,40	0,00	6,15	11,13	66,31

Table 9. Comparison of the percentages in R16 sample components (IME7).

Time (h)	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
0.5	8,78	0,44	0,00	4,44	86,35
	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
120	3,39	3,89	7,13	0,00	85,59

5.5. Addition of substrates (Butanol and Triolein)

5.5.1. Stability of free enzyme

According to table 10 showed that occurred alkyl esters production after 96 hours of about 62.43% and triglycerides consumption around 85%. The content of water was measured using the Karl Fisher and it was 0.16%. After 96 hours was added 1/3 of the initial amount of triolein and butanol and the results were observed in table 11. After the addition of the substrates, the triglycerides peak area increase around 35.8% while Alkyl esters apparently decreases 34%). The area corresponding to the triglicerides and alkyl esters remained almost the same. Past 72 hours of triolein and butanol addition (168h) the triglycerides decrease about 2.6% and the biodiesel increase only 8.6% (Table 11)

Table 10. Comparison of the percentages in R22 sample free enzyme.

Time (h)	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
0	0,00	0,00	0,00	6,25	93,75
	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
96	5,20	13,27	62,43	3,77	15,33

Table 11. Comparison of the percentages in R22 sample components (after new substrates addition).

Time (h)	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
96	2,30	6,51	27,78	12,28	51,13
	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
168	3,79	7,81	36,41	3,50	48,49

5.5.2. Stability of the immobilized enzyme preparations (IMEs).

In order to study the reuse of the enzyme it was made a reaction with fed batch strategy to immobilized enzyme with IME11, in R10b) and R25. In R10b) the amount of immobilized enzyme was 100mg and in R25 was 300mg.

The R10b) and R25 was carried out into P. Selecta with Heidolph MR Hei-Mix L with 35ºC of temperature and 900rpm of agitation.

The first experiment was with R10b) (Table 12) during 3 days and after was added Triolein and Butanol at half amount of the initial at time 0 and the final sample was taken after 72 hours of the addition (Table 13).

Table 12. Comparison of the percentage in R10b) samples components.

Time (h)	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
0.5	5,48	0,00	0,77	7,17	86,58
	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
72	10,68	10,19	71,61	2,10	5,43

Table 13. Comparison of the percentage in R10b) samples components.

Time (h)	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
72	0,93	6,19	48,83	2,99	41,05
	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
144	1,54	6,57	88,44	0,93	2,52

Through the table 12 is possible to observe that the triglycerides decrease about 81.15% and the alkyl esters increase about 70.84% after 72 hours. With the addition of Triolein and Butanol (Table 13) the triglycerides increase to 41.05% and the Alkyl esters appears to decrease but its area it is lower compared with total area. However, after 144h the reaction the alkyl esters increase to 82% while triglycerides decreases to 2.5% showing that immobilized enzyme preparation continue to be active in great contrast what has been passed in the experiment where used the free enzyme.

For the R25 (Table 14 and Table 15) the addition of substrates occurred 5 days after finished the initial incubation. For this reaction experiment (R25) using 300 mg of biocatalyst (IME11) is possible to conclude that it continue active after 5 days.

Table 14. Comparison of the percentage in R25 samples components.

Time (h)	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
0.5	9,09	0,00	3,83	3,83	87,07
	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
48	3,90	5,24	90,86	0,00	0,00

Table 15. Comparison of the percentage in R25 samples components.

Time (h)	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
48+5days	1,66	8,44	88,96	0,94	0,00
	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
48+5days	0,23	2,64	64,34	2,09	30,70
	Fat Acid (%)	MG (%)	Alkyl esters (%)	DG (%)	TG (%)
48+5days+72	0,57	3,82	82,04	0,87	12,70

During the time-out at room temperature and without agitation the enzyme reaction continued to occur but the percentage of each compound not change too much as the transesterification was near of thermodynamic equilibrium. After this, it was added 1/3 of the initial amount of Triolein and Butanol and as can be seen by table 15 the percentage of triglycerides increased and the alkyl esters decrease in comparison with the total area. At 72 hours after Triolein and Butanol addition the triglycerides decreases around 18% and de alkyl esters increase about 17.7%.This experiment proves that this immobilized enzyme preparation continue to be active after a long period of the time in the reaction media (more than 5 days).

6. Conclusion

Cutinase can produce biodiesel by transesterification of triolein and butanol in reverse micelles system based on isooctane/AOT. It is possible to immobilize cutinase in different supports using different types of immobilizations. The best immobilization was IME11 with an immobilization yield of 38%. The enzyme immobilization on silica gel with amine and glutaraldehyde by covalent binding (IME3 and IME11) was the best immobilization because 100% of triglycerides conversion was achieve for IME3 only in 3 days (or 1 day with IME11) at temperature of 35ºC and 900rpm of agitation into P. Selecta with Heidolph MR-Hei_Mix L stir plate. The feed batch strategy proved that cutinase is an unstable enzyme when used in free form but immobilized it can reach the 100%

of triglycerides conversion. The addition of 20% of biodiesel (from soya oil or waste oil) to replace the reactional medium seemed to be effective in biodiesel production although the production was low. The water content influence biodiesel production because when the immobilized enzymes were washed with isooctane before reaction the alkyl esters production and triglycerides consumption increased. Although when the replacing of reactional medium, by 20% of biodiesel from soya oil took place, the water content was important to reaction occurs.

References

[1] P. Nigam and A. Singh, "Production of liquid biofuels from renewable resources.," *Progress in Energy and Combustion Science,* vol. 37, pp. 52-68, 2011.

[2] A. Ashraful, H. Masjuki, Kalam, M. Rizwanul Fattah, S. Imtenan, S. Shahir and H. Mobarak, "Production and comparision of fuel poperties, engine performance, and emission characteristics of biodiesel from various non-edible vegetable oils: A review. Energy Conversion and Management," *80,* pp. 202-228, 2014.

[3] BP, "BP Statistical Review of World Energy," June 2014. [Online]. Available: http://www.bp.com/content/dam/bp/pdf/Energy-economics/statistical-review-2014/BP-statistical-review-of-world-energy-2014-full-report.pdf. [Accessed 12 January 2015].

[4] F. Gírio and C. Matos, "Typical greenhouse gas emissions in the cultivation phase of raw materials for the production of biofuels," LNEG, LIsbon, 2010.

[5] Prio energy, "Prio energy," 10 1 2015. [Online]. Available: http://www.prioenergy.com/produtos-e-servicos/producao-de-biodiesel/.

[6] D. Leung, X. Wu and M. Leung, "A review on biodiesel production using catalyzed transesterification," *Applied Energy,* vol. 87, pp. 1083-1095, 2010.

[7] R. Sim, W. Mabee, J. Saddler and M. Taylor, "An overview of second generation biofuel Technologies.," *Bioresource Technology,* vol. 101, pp. 1570-1580, 2010.

[8] A. Carlos, C. Andrade, E. Souza e Silva and F. Dupas, "Biodiesel production from used cooking oil: A review," *Renewable and sustainable Energy Reviews,* vol. 27, pp. 445-452, 2013.

[9] B. Mustafa, "Potential alternatives to edible oils for biodiesel production – A review," *Energy Conversion and Management,* vol. 52, pp. 1479-1492, 2011.

[10] T. khan, A. Atabani, I. Badruddin, M. Khayoon and S. Triwahyono, "Recent scenario and technologies to utilize non-edible oils for biodiesel production," *Renewable and Sustainable Energy Reviews,* vol. 37, pp. 840-851, 2014.

[11] A. Singh and K. Anbumani, "A comparative study on the use of butyl esters of a soyabean and sunflower oils as biodiesel fuel for compression ignition engine," *Iranian Journal of Mechanical engineering,* vol. 12, March 2011.

[12] D. Barros, L. Fonseca, J. Cabral, C. Weiss and K. Landfester, "Synthesis of alkyl esters by cutinase in miniemulsion and organic solvent media," *Biotechnology Journal,* vol. 4, p. 674, 2009.

[13] X. Zhao, F. Qi, C. Yuan, W. Du and D. Liu, "Lipase-catalyzed process for biodiesel production: Enzyme immobilization, process simulation and optimization," *Renewable and Sustainable Energy Reviews,* vol. 44, pp. 182-197, 2015.

[14] C. Carvalho, J. Cabral and M. Aires-Barros, "Cutinase stability in AOT reversed micelles: system optimization using the factorial design methodology," *Enzyme and microbial Technology,* vol. 24, pp. 569-576, 1999.

[15] S. Badenes, F. Lemos and J. Cabral, "Assessing the use of cutinase reversed micella catalytic system for the production of biodiesel from triglycerides," *Journal chemical Technological Biotechnological,* vol. 85, no. 7, pp. 993-998, 2010.

[16] A. Demirbas, "Biodiesel production from vegetable oils via catalytic and non-catalytic supercritical methanol transesterification methods," *Progress in Energy and Combustion Science,* vol. 31, pp. 466-487, 2005.

[17] D. Barros, L. Fonseca, P. Fernandes, J. Cabral and L. Mojovic, "Biosynthesis of ethyl caproate and other short ethyl esters catalysed by cutinase in organic solvent," *Journal of Molecular Catalysis B: Enzimatic,* vol. 60, pp. 178-185, 2009.

[18] C. Calado, C. Almeida, J. Cabral and L. Fonseca, "Journal of Bioscience and Bioengineering," vol. 96, pp. 141-148, 2003.

[19] S. Badenes, F. Lemos and J. Cabral, "Stability of cutinase wild type and mutants, in AOT reversed micellar system-effect of mixture components of alkyl esters production.," *Journal Chemical Technology & Biotechnology,* vol. 85, pp. 34-41, 2011.