Catalysis Today 167 (2011) 107–112

Contents lists available at ScienceDirect

Catalysis Today

journal homepage: www.elsevier.com/locate/cattod

Production of a new second generation biodiesel with a low cost lipase derived from *Thermomyces lanuginosus*: Optimization by response surface methodology

Cristóbal Verdugo[a], Diego Luna[a,*], Alejandro Posadillo[b], Enrique D. Sancho[c], Salvador Rodríguez[c], Felipa Bautista[a], Rafael Luque[a], José M. Marinas[a], Antonio A. Romero[a]

[a] Departamento de Química Orgánica, Universidad de Córdoba, Campus de Rabanales, Edificio Marie Curie, E-14014 Córdoba, Spain
[b] Seneca Green Catalyst, S.L., Campus de Rabanales, E-14014 Córdoba, Spain
[c] Departamento de Microbiología, Universidad de Córdoba, Campus de Rabanales, Edificio Severo Ochoa, E-14014 Córdoba, Spain

ARTICLE INFO

Article history:
Received 4 June 2010
Received in revised form
26 November 2010
Accepted 3 December 2010
Available online 11 January 2011

Keywords:
Biodiesel
Ethanolysis
Lipase
Thermomyces lanuginosus
Sunflower oil transesterification
Lipopan BG

ABSTRACT

Biodiesel production has received considerable attention in the recent years as biodegradable and non-polluting fuel. In this work, an inexpensive purified 1,3-specific lipase from *Thermomyces lanuginosus* (Lipopan 50 BG from Novozymes AS, Denmark) was utilised as biocatalyst in an alternative approach to obtain a novel second generation biodiesel-like biofuel. This novel product, which integrates glycerol as monoacylglycerols (MG) into the biofuels composition, can avoid the removal step/s of such byproduct, mandatory in the production of conventional biodiesel. A multi-factorial design and response surface methodology (RSM) were employed to evaluate the effects of several conditions (temperature, molar ratio of ethanol to oil and pH) on the conversion of sunflower oil into a blend of Fatty Acid Ethyl Esters (FAEE), MG and diacylglycerols (DG). The effects of water content and concentration of lipase on conversion into FAEE and MG were also studied. Results obtained indicate that pH, molar ratio of ethanol to oil and water content were significant factors influencing the conversion in the systems under the investigated conditions. Low temperatures (20 °C), high pH values (close to 12), and an oil/ethanol volume ratios of 3.4/1 were found to be the key controlling parameters which provide optimised results after 1 h reaction (conversions around 70%; kinematic viscosities about 8.5 $mm^2 s^{-1}$).

1. Introduction

The production of biodiesel has become very important in recent years as potential alternative to partially fulfill the expected future energy demands in the transport sector [1,2]. Several methods have been reported for the production of biodiesel from vegetable or waste cooking oils and animal fats, including direct use and blending, microemulsification, pyrolysis, and transesterification [3].

Transesterification is currently one of the most attractive and widely accepted methodologies for biodiesel production [4]. The conventional method for biodiesel production involves the use of homogeneous base catalysts under mild heating (50–60 °C), although there are many others extended methodologies. Main factors affecting transesterification processes include reaction temperature, alcohol/oil molar ratio, type and concentration of catalyst as well as purity of reactants. In any case, an excess of alcohol is normally utilised in the biodiesel production process in order to switch the equilibrium to the production of esters (with glycerol as main by-product) through a stepwise process. In spite of the alkaline impurities generated in the conventional method that need to be removed, glycerol is the main drawback of this method, not only because its generation reduces the atomic yield of the process, but also due to the extensive water cleaning of the biodiesel obtained in order to remove such residual glycerol and alkaline impurities.

We have recently developed a protocol for the preparation of novel types of biodiesel-like biofuels that integrate glycerol into their composition via 1,3-regiospecific enzymatic transesterification of sunflower oil using pig pancreatic lipase (PPL) [5,6]. This protocol predates any conventional method of biodiesel preparation, offering operating conditions remarkably simpler and more efficient and a process virtually waste-free of impurities (acidic or alkaline). No byproduct needs to be removed from the final reaction mixture, simplifying the procedure and reducing the environmental impact of the process [7–10]. These second generation biofuels also exhibit similar physical properties to those of conventional biodiesel and avoid the production of glycerol as by-product. Furthermore, monoacylglycerides (MG) were proved to enhance biodiesel lubricity as demonstrated by recent studies [11–14].

* Corresponding author at: Departamento de Química Orgánica, Universidad de Córdoba, Campus Universitario de Rabanales, Edificio Marie Curie (C-3), Carretera Nacional IV-A, Km 396, E-14014 Córdoba, Spain. Tel.: +34 957212065; fax: +34 957212066.
E-mail address: qo1lumad@uco.es (D. Luna).

0920-5861/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.cattod.2010.12.028

This family of novel biofuels integrating glycerol into their composition were initially prepared taking advantage of the 1,3 selective nature of the pig pancreatic lipase (PPL) [5,6]. However, many other isolated lipases have this inherent 1,3 selective character and their application for biofuels production holds an interesting potential from an economical point of view due to their relative low cost. This is the case of the microbial lipase Lipopan 50 BG (Novozymes AS, Denmark), a purified lipase from *Termomyces lanuginosus* microorganism, widely employed as bread emulsifier [15,16] but never been reported, to the best of our knowledge, as biocatalyst in any chemical process. Many reports have been published using lipases from this microorganism in order to obtain conventional biodiesel, both with free enzymes [17–19] and immobilized on several supports [20–28]. In some cases, central composite design and response surface methodology (RSM) were utilised to optimize the synthesis parameters in biodiesel production [18,19]. Results obtained in most cases pointed out the methanolysis of triglycerides (TG) did not work well as compared to other short chain alcohols such as ethanol [17–28].

In addition to this, a series of other parameters were found to need further investigations to have a better insight into the process for the production of this promising family of novel biofuels via biocatalysis. Water activity (a_w) has been recognised as a key parameter which determines the enzymatic activity [29]. Certain physical properties of enzymes have been shown to change depending on the hydration state of the proteins, influencing the measured reaction rates. Reaction media with $a_w < 0.5$ has been reported to provide optimum conversions to methyl esters in the immobilized lipase-catalysed production of biodiesel from restaurant grease using *Thermomyces lanuginosus* and *Candida antarctica* lipases [30].

Herein, we report an optimisation study of the most important parameters operating in the ethanolysis of sunflower oil using Lipopan 50 BG (Novozymes AS, Denmark), a purified lipase from *T. lanuginosus* microorganism, to obtain novel biofuels integrating glycerol into their composition. These parameters include water content, pH, oil/ethanol ratio and the effect of the temperature and quantity of biocatalyst on the efficiency of the solvent-free enzymatic process. In order to evaluate the influence of several parameters at the same time in the transesterification reaction, a multi-factorial design of experiments and response surface methodology has been developed.

2. Materials and methods

2.1. Materials

Commercial sunflower oil was obtained locally. Chromatographically pure ethyl esters of palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid were purchased from Accustandard, and methyl heptadecanoate was purchased from Sigma–Aldrich and used as such. Other chemicals including absolute ethanol and sodium hydroxide were commercially obtained from Panreac (>99% purity).

2.2. Analytical method

Reaction products were monitored by capillary column gas chromatography, using a Hewlett-Packard 5890 series II equipped with a flame ionization detector (FID) and an HT5 capillary column (25 m × 0.32 mm × 0.10 μm). The injection system was split–splitless. Helium was used as carrier gas was at a flow rate of 1.5 mL/min. The temperature program was an initial temperature of 70 °C, which was maintained for 2 min, and then increased to 200 °C (7 °C/min) and subsequently to 400 °C (20 °C/min), where it was maintained over 5 min.

2.3. Alcoholysis reactions

Sunflower oil for food use, ethanol (Panreac, 99%) and a commercial low cost lipase, Lipopan BG (Novozymes AS, Denmark), were employed in the enzymatic ethanolysis reactions. These reactions were performed according to a previously reported methodology [5,6]. The mixture was stirred at controlled temperature (20–60 °C) in a 50 mL round bottom flask, at varying the pH values (in the 9–12 range) and reaction times of 1 h. This reactor was provided with temperature and stirred speed controllers and equipped with a reflux condenser to avoid ethanol losses. The speed was set at 500 rpm to avoid mass transfer limitations.

pH values were achieved by adding different volumes of aqueous solution of NaOH. In this regard, a blank reaction in the presence of the highest quantity of solution of NaOH was performed to rule out a potential contribution from the homogeneous NaOH catalysed reaction. Less than 10% conversion of the starting material was obtained, so that a homogenous contribution can be considered as negligible under the investigated conditions. The reaction mixture comprises of 9.4 g (12 mL, 0.01 mol) sunflower oil, a variable oil/alcohol volume ratio and different quantities of lipase. The influence of water activity (a_w) was also evaluated by the addition of different quantities of water to the reaction medium. All variable were studied and optimized according to a factorial experimental design and a response surface methodology.

2.4. Viscosity measurements

Viscosity becomes a critical parameter to change in the physical properties of vegetable oils due to its importance in the adequate operation of diesel engines. The transesterification process of oils and fats is currently developed in order to implement the resulting product with reduced viscosity as biofuel in existing diesel engines. Accurate viscosity measurements are critical to assess the quality of biofuels produced, since inappropriate viscosity values can decisively affect the optimum functioning of diesel engines.

Viscosities were determined in a capillary viscometer Oswald Proton Cannon-Fenske Routine Viscometer 33200, size 150. This is based on determining the time needed for a given volume of fluid passing between two points marked on the instrument. It correlates to the movement restriction suffered by the flow of liquid, as a result of internal friction of its molecules, depending on their viscosity. From the flow time (t, seconds), the kinematic viscosity (υ, centistokes, cSt) can be obtained from the equation: $\upsilon \times t = C$, where C is the constant calibration of the measuring system in cSt × s, which is given by the manufacturer (0.040350 $mm^2\ s^{-1}$, at 40 °C) and t the flow time in seconds. The kinematic viscosity is given by the ratio between the dynamic viscosity (h, in Poise, g/cm s) and the density (r, in g/cm^3) $\upsilon = h/r$, in cm^2/s or centistokes, cSt, mm^2/s.

2.5. Experimental design

The effect of process parameters in the enzymatic transesterification reaction and the optimum conditions for the conversion were studied using a multifactorial design of experiments with three factors run by the software StatGraphics® version 5.1. Two of them at three levels and the last one at two levels, giving us 36 runs (Table 1). The experiments were performed in random order. The experimental parameters selected for this study were reaction temperature, oil/ethanol ratio and pH. Table 1 shows the coded and actual values of the process parameters used in the design matrix shown in Table 2. The quantity of lipase in all these experiments

Table 1
Process parameters in factorial design: coded and actual values.

Variables	Unit	Level		
		−1	0	1
Temperature	°C	20	40	60
Oil/ethanol ratio (v/v)	mL/mL	6/1	–	3.4/1
pH	–	8	10	12

was fixed to 30 mg. All experiments were duplicated in order to avoid experimental errors.

2.6. Water content evaluation

A methodology was developed to evaluate the effect of water content in the reaction comprising on a series of experiments in which different and increasing amounts of water were added to the reaction media. All others parameters were fixed at optimum values obtained from previous response surface methodologies (RSM) for temperature (20 °C), pH (constant volume of NaOH solution, pH 12), oil to ethanol volumetric ratio 3.4/1 and 30 mg lipase.

2.7. Effect of the quantity of lipase in the reaction

The effect of the amount of lipase in the reaction media is very important to select the correct reaction conditions. This parameter was evaluated in order to choose the necessary amount of lipase that maximizes conversion without mass transfer limitations. Previous optimized values obtained from RSM (pH 12, oil to ethanol ratio 3.4/1, 15 μL of water added, 20 °C, 1 h reaction) were chosen.

2.8. Statistical analysis

The experimental data obtained from experimental design were analyzed by RSM [34]. A mathematical model, following a second-order polynomial equation, was developed to describe the relationships between the predicted response variable (conversion) and the independent variables of reaction conditions, as it is shown in the Eq. (1), where y_{conv} is the predicted response variable; β_0, β_i, β_{ii}, β_{ij} the intercept, linear, quadratic and interaction constant coefficients of the model, respectively; X_i, X_j ($i = 1, 3$; $j = 1, 3$; $i \neq j$) represent the coded independent variables

Table 2
Experiments matrix of factorial design and the response obtained for conversion and viscosity. Model validation experiments are also shown.

Parameters				Conversion (%)	Kinematic viscosity ($mm^2\ s^{-1}$)
Run	Temperature	pH	Oil/ethanol ratio		
1	1	0	−1	28.9	19.5
2	1	−1	−1	43.7	10.3
3	0	−1	−1	30.7	14.6
4	−1	0	−1	37.5	14.5
5	−1	1	1	67.1	8.9
6	1	1	1	54.3	9.8
7	−1	−1	−1	39.1	14.6
8	0	1	−1	48.8	14.6
9	0	0	1	53.6	9.8
10	0	0	−1	42.8	14.2
11	0	1	1	66.7	9.1
12	1	1	−1	47	14.7
13	1	−1	1	45	9.9
14	1	0	1	49.1	10.6
15	−1	0	1	46.6	10
16	−1	−1	1	48.9	9.2
17	0	−1	1	37.8	10.1
18	−1	1	−1	51.8	16.5
Repeated experiments					
19	0	0	−1	42.9	14.1
20	−1	1	1	68.4	8.7
21	−1	−1	−1	38.7	16.2
22	−1	0	1	45.1	10.5
23	1	−1	1	46.2	9.6
24	0	−1	1	36.2	10.2
25	−1	−1	1	50.1	9.1
26	0	0	1	54.1	9.3
27	1	1	1	55.1	9.4
28	0	−1	−1	28.9	14.7
29	1	1	−1	45.8	14.8
30	−1	0	−1	38.2	14.5
31	1	0	−1	29.2	20.2
32	1	0	1	48.4	10.9
33	0	1	−1	47.4	14.9
34	0	1	1	65.4	9.3
35	1	−1	−1	41.9	10.9
36	−1	1	−1	52.3	14.8

Parameters				Conversion (%)		Kinematic viscosity ($mm^2\ s^{-1}$)	
Run	Temperature	pH	Oil/ethanol ratio	Predicted	Observed	Predicted	Observed
Validation experiments							
37	−0.8	0.3	0.6	51.6	47.4	11.2	10.6
38	0.1	−0.7	−0.2	39.5	34.6	12.6	14.2
39	−0.6	−0.2	−0.4	40.5	31.3	14.0	16.8

Table 3
Analysis of variance (ANOVA) for conversion.

Analysis of variance (ANOVA) for conversion				*F*-value	*P*-value
Source	Sum of squares	df	Mean square		
(A) Temperature	100.86	1	100.86	3.92	0.0585
(B) pH	1393.85	1	1393.85	54.13	0
(C) Oil/ethanol ratio	1139.06	1	1139.06	44.24	0
AA	0.845	1	0.845	0.03	0.8577
AB	87.4225	1	87.4225	3.4	0.0768
AC	2.04167	1	2.04167	0.08	0.7805
BB	215.281	1	215.281	8.36	0.0076
BC	75.9704	1	75.9704	2.95	0.0977
Bloqs	0.7225	1	0.7225	0.03	0.8683
Total error	669.471	26	25.7489		
Total (corr.)	3685.53	35			

$R^2 = 0.898$; R^2 (Adj.) = 0.825.

(reaction conditions):

$$y_{\text{conv}} = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii}X_i^2 + \sum\sum_{i<j=1}^{3}\beta_{ij}X_iX_j \quad (1)$$

Response surface plots were developed using the fitted quadratic polynomial equation obtained from regression analysis, holding one of the independent variables at constant values corresponding to the stationary point and changing the order two variables. The quality of the fit of the polynomial model equation was evaluated by the coefficient of determination R^2, and its regression coefficient significance was checked with *F*-test. Confirmatory experiments were carried out in order to validate the model, using combinations of independent variables which were not part of the original experimental design, but within the experimental region.

3. Results and discussion

3.1. Analysis of variance (ANOVA)

The analysis of variance methods has become very attractive in reaction parameters optimization and in the evaluation of the effects of the parameters in the TG transesterification reaction [31–33] due to its effectiveness in the analysis of variables. The results of factorial design suggested that the major factors affecting the transesterification, for the production of biofuels integrating glycerol as monoacylglycerol, were pH and oil/ethanol ratio (v/v) as shown in Table 3. To improve catalytic efficiency, a three and two level-three-factor multifactorial analysis (ANOVA) was adopted to evaluate the effects of the aforementioned factors on conversion and kinematic viscosity. Results have been summarised in Table 2.

Data was fitted to quadratic polynomial model using the software Statgraphics version 5.1. The quadratic polynomial model was highly significant and sufficient to explain the relationship between conversion/kinematic viscosity and important variables, as summarised in Tables 3 and 4.

Determination coefficients R^2 were 0.898 for conversion and 0.753 for kinematic viscosity, respectively, which imply a good fit between models and experimental data (Figs. 1 and 2). The adjusted correlation coefficients R^2 were 0.825 and 0.738 for conversion and kinematic viscosity, respectively (Table 3). Obtained results pointed out that the pH and oil/ethanol (v/v) ratio were also important parameters influencing the conversion in the systems ($p < 0.05$). Interestingly, that was not the case of the temperature which was in the significance borderline ($p = 0.0585$). Only initial oil/ethanol ratio (and a quadratic level for pH) was significantly important factors ($p < 0.05$) influencing the kinematic viscosity of reaction products (Table 4).

Table 4
Analysis of variance (ANOVA) for kinematic viscosity.

Analysis of variance (ANOVA) for conversion				*F*-value	*P*-value
Source	Sum of squares	df	Mean square		
(C) Oil:ethanol ratio	246.752	1	246.752	92.58	0
BB	13.6503	1	13.6503	5.12	0.0306
Bloqs	0.0367361	1	0.0367361	0.01	0.9073
Total error	85.2931	32	2.66541		
Total (corr.)	345.732	35			

$R^2 = 0.753$; R^2 (Adj.) = 0.738.

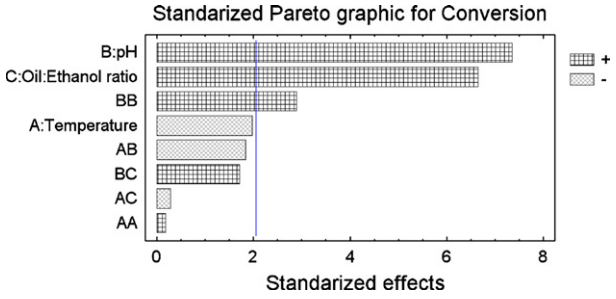

Fig. 1. Pareto graphic for conversion.

After the elimination of non influent parameters in the model for conversion and kinematic viscosity, the R^2 values for conversion and kinematic viscosity were 0.746 and 0.753 respectively, and the equations obtained were remarkably simpler as compared to initial ones.

$$\text{conversion} = 43.03 + 7.62 \times \text{pH} + 5.63 \times \text{ratio} + 5.19 \times \text{pH}^2 \quad (2)$$

$$Y_{\text{Kinem.Visc.}}\ (\%) = 13.18 - 2.62 \times \text{ratio} - 1.31 \times \text{pH}^2 \quad (3)$$

3.2. Optimization of the reaction parameters by RSM

The surface plots described by the regression model were drawn to display the effects of the independent variables on conversion (Fig. 3) and kinematic viscosity (Fig. 4). The influence of the different variables in the conversion of the systems can be clearly seen.

This model showed that the optimum values for the parameters to maximize conversion were low temperatures (20 °C), pH = 12 and oil/ethanol (v/v) ratio = 3.4/1. Conversions around 70% and values of kinematic viscosity about 8.5 $mm^2\ s^{-1}$ could be achieved under these conditions, which in theory will render feasible the utilization of the obtained biofuel in blends with diesel. For example, the addition of 35% of petrodiesel with this biofuel reduces the viscosity to 4.8 $mm^2\ s^{-1}$, falling within the acceptance limits of the EN 14214.

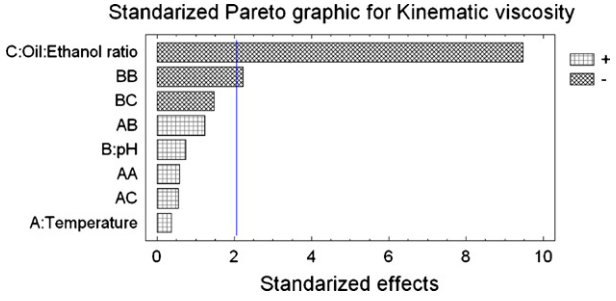

Fig. 2. Pareto graphic for the kinematic viscosity.

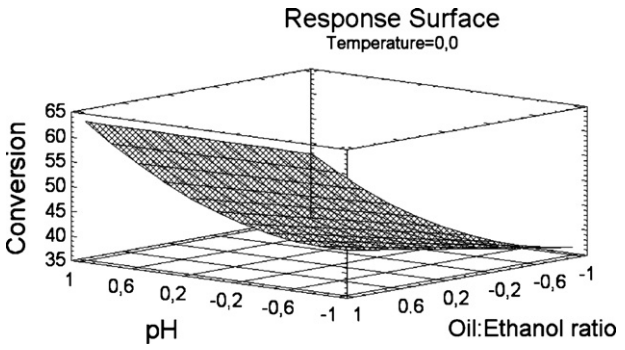

Fig. 3. Conversion vs pH and oil/ethanol ratio response surface plot (more influential parameters).

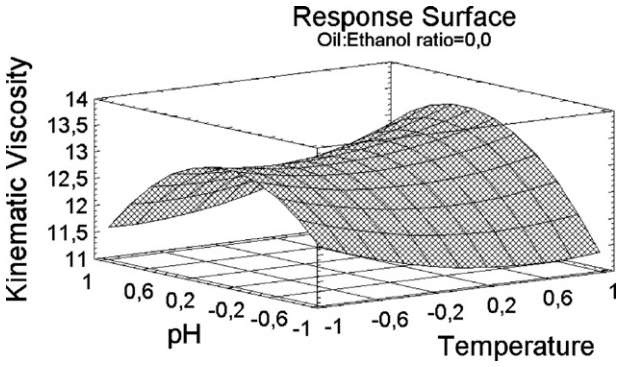

Fig. 4. Kinematic viscosity vs pH and oil/ethanol ratio response surface plot (more influential parameters).

3.3. Effect of water content

The water content is a highly important parameter in enzymatic transesterification, especially in solvent-free systems. A series experiments under optimal conditions for *T*, pH and oil/ethanol ratio, obtained from previous RSM studies, were carried out to evaluate the effect of this parameter.

Fig. 5 shows the effect of water content in conversions obtained in the transesterification reaction. A minimum in kinematic viscosity, which corresponds to a maximum in conversion, was achieved at a concentration of 13.6% of added water. Consequently, the water content is a very important parameter that is necessary to control, in general, in ethanolysis process.

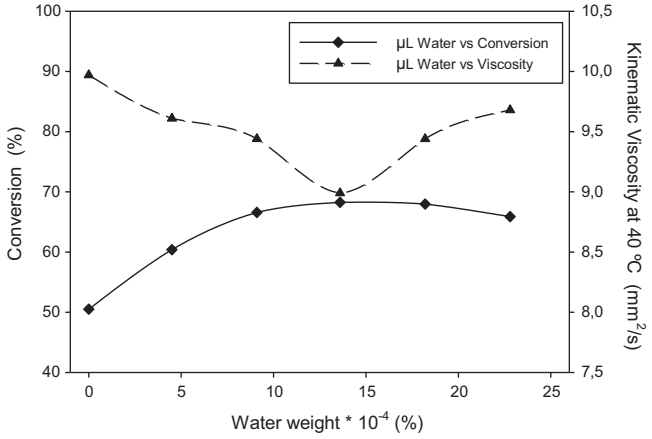

Fig. 5. Influence of water content on conversion and kinematic viscosities.

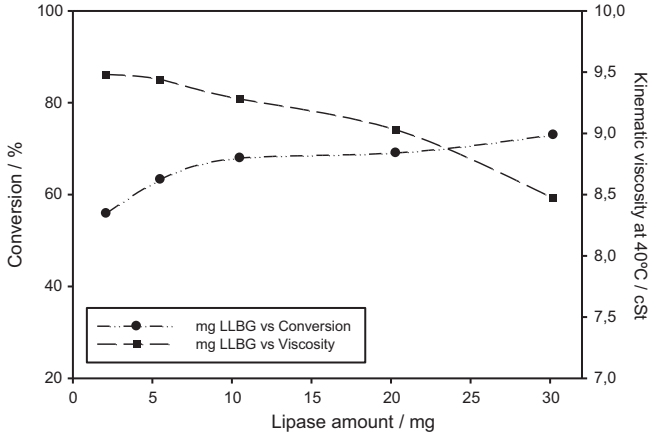

Fig. 6. Influence of the quantity of lipase on conversion and kinematic viscosities.

3.4. Effect of the quantity of lipase

Fig. 6 depicts the effect of the quantity of enzyme utilised on conversion and kinematic viscosity. 20 mg lipase was selected as optimum value in all reactions, as this quantity was shown to be sufficient to provide a combined good conversion and kinematic viscosity values. Improved viscosity values could be obtained using more quantities of biocatalyst but larger amounts of enzymes will have a detrimental effect on the economics of the process.

4. Conclusions

A purified lipase from *T. lanuginosus* microorganism, Lipopan 50 BG (Novozymes AS, Denmark), usually used as bread emulsifier, was successfully evaluated as inexpensive 1,3 selective biocatalyst in the ethanolysis of refined sunflower oil.

Reactions carried out under optimised conditions rendered a novel type of biodiesel-like biofuel comprising of a mixture of monoacylglycerols and FAEEs (1/2 nominally), that can be potentially blend directly with petrodiesel. This new biofuel can be obtained at very short reaction times (50 min) and under mild reaction conditions.

The analysis of variance showed that, in order to obtain an improvement in conversion and kinematic viscosity of the transesterification of sunflower oil with ethanol in solvent free conditions, low temperatures (20 °C), high pH values (close to 12) and an oil/ethanol ratio of 3.4/1 should be the operating parameters. The quadratic models obtained also showed good results in terms of predicting the conversion and kinematic viscosity in the investigated systems.

The proposed methodology offers a comparatively higher atomic yield as compared to conventional biodiesel production (no glycerol byproduct generated), as well as a straightforward processing and end-use after its preparation, avoiding purification steps of residual glycerol.

Acknowledgements

Grants from Consejería de Educación y Ciencia de la Junta de Andalucía (FQM 0162 and FQM-02695), Instituto Andaluz de Biotecnología, Junta de Andalucía (BIOANDALUS 08/13/L35) FEDER funds and Ministerio de Ciencia e Innovación (Project CTQ2008-01330/BQU) are gratefully acknowledged. SUSTOIL (EU program, Topic ENERGY.2007.3.3.3) and Excellence Project of Junta de Andalucía (P09-FQM-4781) are also acknowledged. We are also gratefully to Novozymes AS, Denmark, for the kindly supply of the studied lipase.

References

[1] A. Demirbas, Energy Conv. Manage. 50 (2009) 14–34.
[2] R. Luque, L. Herrero-Davila, J.M. Campelo, J.H. Clark, J.M. Hidalgo, D. Luna, J.M. Marinas, A.A. Romero, Energy Environ. Sci. 1 (2008) 513–596.
[3] A.C. Pinto, L.L.N. Guarieiro, M.J.C. Rezende, N.M. Ribeiro, E.A. Torres, W.A. Lopes, J. Braz. Chem. Soc. 16 (2005) 1313–1330.
[4] J. Van Gerpen, Fuel Process. Technol. 86 (2005) 1007–1097.
[5] V. Caballero, F.M. Bautista, J.M. Campelo, D. Luna, J.M. Marinas, A.A. Romero, J.M. Hidalgo, R. Luque, A. Macario, G. Giordano, Process. Biochem. 44 (2009) 334–342.
[6] C. Verdugo, R. Luque, D. Luna, J.M. Hidalgo, A. Posadillo, E. Sancho, S. Rodríguez, S. Ferreira-Días, F.M. Bautista, A.A. Romero, Bioresour. Technol. 101 (2010) 6657–6662.
[7] S. Al-Zuhair, Biofuels Bioprod. Bioref. 1 (2007) 57–66.
[8] D. Royon, M. Daz, G. Ellenrieder, S. Locatelli, Bioresour. Technol. 98 (2007) 648–653.
[9] A. Salis, M. Monduzzi, V. Solinas, Use of lipases for the production of biodiesel, in: J. Polaina, A.P. MacCabe (Eds.), Industrial Enzymes. Structure, Function and Applications, Springer, Netherlands, 2007.
[10] S. Shah, M.N. Gupta, Process. Biochem. 42 (2007) 409–414.
[11] J. Hu, Z. Du, C. Li, E. Min, Fuel 84 (2005) 1601–1606.
[12] G. Knothe, K.R. Steidley, Energy Fuels 19 (2005) 1192–1200.
[13] G. Knothe, Lipid Technol. 18 (2006) 105–108.
[14] G. Knothe, K.R. Steidley, Fuel 86 (2007) 2560–2567.
[15] S. Moayedallaie, M. Mirzaei, J. Paterson, Food Chem. 122 (2010) 495–499.
[16] V. Stojceska, P. Ainsworth, Food Chem. 110 (2008) 865–872.
[17] Y. Xu, W. Du, J. Zeng, D. Liu, Biocatal. Biotransf. 22 (2004) 45–48.
[18] R.C. Rodrigues, G. Volpato, M.A.Z. Ayub, K. Wada, J. Chem. Technol. Biotechnol. 83 (2008) 849–854.
[19] Y. Wang, H. Wu, M.H. Zong, Bioresour. Technol. 99 (2008) 7232–7237.
[20] M.S. Souza, E.C.G. Aguieiras, M.A.P. daSilva, M.A.P. Langone, Appl. Biochem. Biotechnol. 154 (2009) 74–88.
[21] N. Dizge, B. Keskinler, Biomass Bioenergy 32 (2008) 1274–1278.
[22] N. Dizge, C. Aydiner, D.Y. Imer, M. Bayramoglu, A. Tanriseven, B. Keskinler, Bioresour. Technol. 100 (2009) 1983–1991.
[23] E. Hernandez-Martın, C. Otero, Bioresour. Technol. 99 (2008) 277–286.
[24] W. Du, Y. Xu, D. Liu, Z. Li, J. Mol. Catal. B: Enzym. 37 (2005) 68–71.
[25] R. Costa-Rodrigues, G. Volpato, K. Wada, M.A.Z. Ayub, J. Am. Oil Chem. Soc. 85 (2008) 925–930.
[26] N. Dizge, B. Keskinler, A. Tanriseven, Biochem. Eng. J. 44 (2009) 220–225.
[27] R.C. Rodrigues, J.M. Bolivar, A. Palau-Ors, G. Volpato, M.A.Z. Ayub, R. Fernandez-Lafuente, J.M. Guisan, Enzyme Microbial Technol. 44 (2009) 386–393.
[28] O. Aybastıer, C. Demir, J. Mol. Catal. B: Enzym. 63 (2010) 170–178.
[29] A. Salis, M. Pinna, M. Monduzzi, V. Solinas, J. Biotechnol. 119 (2005) 291–299.
[30] A. Hsu, K. Jones, T.A. Foglia, W. Marmer, Biotechnol. Appl. Biochem. 36 (2002) 181–186.
[31] S.V. Ghadge, H. Raheman, Bioresour. Technol. 97 (2006) 379–384.
[32] A. D'Cruz, M.G. Kulkarni, L.C. Meher, A.K.J. Dalai, Am. Oil Chem. Soc. 84 (2007) 937–943.
[33] A.K. Tiwari, A. Kumar, H. Raheman, Biomass Bioenergy 31 (8) (2007) 569–575.
[34] R.O. Kuehl, Diseño de experimentos, Principios estadísticos para el diseño y análisis de investigación, second ed., Thomson Learning, Mexico, 2001.