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PRODUCTION OF METHYL ESTERS BY ENZYMATIC HYDROESTERIFICATION OF CHICKEN FAT INDUSTRIAL RESIDUE

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Abstract - The feedstock cost can comprise more than 75% of the overall biodiesel cost, and then economic issues are the main limitations of biodiesel production. This research focused on the use of alternative feedstock and production processes. The production of fatty acid esters from residues becomes an interesting alternative route since it is possible to reduce the cost of the process. The present work aimed to produce methyl esters from the residual oil of the poultry industry, using a commercial soluble lipase Eversa Transform 2.0® (NS-40116). The effects of methanol (1.2 to 1.8 eqv), water (1 to 2 wt%) and enzyme (0.1 to 0.5 wt%) contents on reaction conversion were evaluated through a 2^3 experimental design with three central points, at 45 ºC, 250 rpm for 16 and 24 h of reaction time. From the results, by using 0.3 wt% enzyme, 1.5 wt% water, and 1.5 eqv of methanol it was possible to reach the most effective esters conversion (90.61%).
Keywords: Agroindustry; FAME; Hydroesterification; Enzyme; Chicken fat.

INTRODUCTION

Brazil is the pioneer in Latin America regarding biofuels production, since ethanol and biodiesel started in the 70's and culminated in 2002 with the Probiodiesel program financed by the Brazilian Federal Government (Ramos et al., 2011). In 2005, biodiesel was officially introduced into the Brazilian energy matrix using the minimum addition percentage of 5% (B5), increasing the percentage to 10% (B10) in 2018, with a new projection to reach 15% (B15) in 2020. According to the RenovaBio law (DOU, 2018), biofuel addition to petroleum diesel has become an incentive to the production due to rising energy consumption and social, economic, and environmental pressure, justifying a larger production of alternative energy sources (Sivakumar et al., 2014; Souza et al., 2016).

Biodiesel can be obtained from vegetable oils such as soybean, castor, palm oil, sunflower, peanut, among others, and also can be produced from animal fats, *in natura* or already processed (Ferrari, 2004; Sivakumar et al., 2014). According to the National Petroleum Agency (ANP), soybean remains the most used raw material for biodiesel production in Brazil (64.84%), followed by bovine fat (15.50%), and other raw materials (19.66%), including chicken fat (0.52%) (ANP, 2017).

For different feedstock, different routes for biodiesel production have been studied to achieve a more efficient and economical approach. Alkaline catalysis is the most used route, with several well-known disadvantages, such as the low-quality glycerin

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produced, production of a large amount of alkaline wastewater, high-energy demand, and the need of a raw material with low free fatty acids levels and water in order to avoid saponification reactions (Sivakumar et al., 2014; Guldhe et al., 2015).

Nowadays one of the most significant factors that affect the economic viability of biofuel production and can comprise more than 75% of the overall biodiesel production cost is the feedstock value (Zhang et al., 2003; Dorado et al., 2006; Haas et al., 2006; Marchetti and Errazy, 2008; Meng et al., 2009). In fact, edible oils with less than 1 wt% of free fatty acids (FFA) have been used as feedstock for industrial biodiesel production, despite the relatively high cost of the raw material.

Hence, the use of unrefined, less expensive, high FFA, lower-grade oils and fats, such as soapstock, acid oils, and deodorized distillates, obtained during the edible oils process, have been suggested as a low-cost feedstock alternative for the reduction of the global cost of enzyme-catalyzed biodiesel production (Gog et al., 2011; Suarez, 2010; Balat, 2011; Lim and Teong, 2011; Haas, 2005).

Related to the enzyme, liquid formulations of free lipases can provide a highly competitive option, making the enzymatic route a welcome possibility for the use of low-grade raw materials, since the biocatalyst has high specificity for the substrates and is quite tolerant to the high levels of acidity and water contents (Nelson and Coks, 2000; Daud et al., 2014). NS 40116 lipase is a thermostable enzyme from the thermophilic fungus *Thermomyces lanuginosus*, with high specificity for raw materials with high content of free fatty acids, high activity in mild process conditions, and was released on the market at a lower cost compared to traditionally used enzymes (USD 20.0.kg^{-1}) according to the manufacturer.

In this context, the present work reports the use of the recently launched Eversa® Transform 2.0, a soluble free-enzyme (NS 40116), for biodiesel production using residual oil from a poultry industry as feedstock, in order to evaluate the influence of methanol, water and enzyme contents on the reaction conversion.

MATERIALS AND METHODS

Material

The NS 40116 lipase used as the catalyst for the hydroesterification reaction was donated by Novozymes/DK. The residual oil was from BRF S.A. Industry (Chapecó-Brazil). All the reagents and chemicals used in this work were analytical grade.

Physicochemical characterization

The physicochemical characterization of the raw material used was carried out following the American Oil Chemists Society (AOCS) methods described by Luque and Melero (2012). The moisture content of the residual oil was determined by the oven-drying method at 105 °C. Briefly, 5.0 g of the sample was weighed into a pre-weighed porcelain capsule and then oven-heated at 105 °C for 3 h. Samples were then cooled in a desiccator and weighed until constant weight.

The fatty acid content was determined according to AOCS methodology Cd 3d-63[8]. Briefly, 1 g of sample and 3-4 drops of phenolphthalein were diluted in 50 mL of 1:1 (v.v^{-1}) ethanol:ether solution. The titration was carried out using 0.1M KOH, under agitation until subtle color change. The acidity was determined with Eq. 1:

$$C_{FFA}(wt\%) = \frac{V_{KOH} \cdot M_{KOH} \cdot MM_{FFA}}{10 \cdot M_S} \quad (1)$$

where C_{FFA} is the content of free fatty acid (FFA, wt%), V_{KOH} is KOH solution volume (mL) used in the titration, M_{KOH} is the molarity of KOH solution (mol.L^{-1}), MM_{FFA} is the average molar mass of fatty acids (FA), around 282 gmol^{-1}, and M_s is the sample mass (g).

The total lipid content of the sample was determined from the Soxhlet extraction. 5.0 g of sample was weighed into an extraction cartridge and then was submitted to Soxhlet extraction apparatus with petroleum ether for 6 h. The solvent was evaporated from the sample from a rotary evaporator and the flask with the extract was placed in an oven at 105 ° C for solvent residues removal. The lipid content was determined from Eq. 2.

$$\text{Lipid content} = \frac{100 \cdot Le}{m} \quad (2)$$

where Le is the amount of lipids extracted (g) and M_s is the sample mass (g).

For the peroxide index determination (Eq. 3), 5 g of the sample was weighed in a 250 mL Erlenmeyer flask, then 30 mL of glacial acetic acid: chloroform solution (3:2) was added to the sample under stirring until complete dissolution. Then 0.5 mL of a saturated potassium iodide solution was added, letting it stand under the light for 1 min; 30 mL of distilled water was added, and the titration was continued with 0.01 M sodium thiosulfate solution until the yellow coloration became clear. Afterwards, 0.5 mL of starch indicator were added and titrated until the blue color disappeared. The standard was prepared using all the reagents except the sample.

$$\text{Peroxide index}\left(\text{meq}\frac{\text{peroxide}}{1000g}\right) = \frac{(S-B)\cdot C \cdot 1000}{m} \quad (3)$$

where S is volume (mL) of sodium thiosulphate spent on titration of the sample, B is the volume (mL) of

sodium thiosulfate used in the standard titration, C is the concentration of sodium thiosulphate solution ($mol.L^{-1}$), and M_s is the mass of sample (g).

The iodine value of an oil or fat is the measure of the unsaturation and is expressed in terms of the number of centigrams of iodine absorbed per gram of the sample (% iodine absorbed). First, the sample was melted and filtered to remove possible solid impurities and traces of moisture. Then, approximately 0.25 g was weighed in a 250 mL Erlenmeyer flask with a cap and 10 mL of cyclohexane were added. Then, 25 mL of Wijs solution was transferred to the Erlenmeyer flask containing the sample, followed by 10 mL of the 15% potassium iodide solution and 100 mL of freshly boiled and cold water. Titration was done with 0.1 M sodium thiosulphate solution until a pale yellow color appeared, adding 1 to 2 mL of 1% starch indicator solution and continuing the titration until the blue color completely disappeared. The standard was prepared using all the reagents except the sample. The iodine value was determined from Eq. 4.

$$\text{Iodine value}\left(gI2/100g\right) = \frac{\left(V_b - V_a\right)\cdot N\cdot f\cdot PM}{M_S} \quad (4)$$

where N is the normality of $Na_2S_2O_3$ ($mol.L^{-1}$) solution, f is the correction factor of Na_2S2O_3, V_b the volume (mL) used in the standard titration, V_a the volume (mL) used in the sample titration, PM is the molecular weight of $Na_2S_2O_3$ and M_s sample mass (g).

The saponification index was determined according to A.O.C.S., Cd 3-25[9] methodology, and the saponification index was determined by Eq. 5.

$$\text{Saponification index} = \frac{56.1\cdot M_{KOH}\cdot(B-A)}{P} \quad (5)$$

where M_{KOH} is the molarity of KOH solution ($mol.L^{-1}$), A is the volume used in the sample titration (mL), B the volume used in the standard titration (mL), and M_s is the sample mass (g).

Experimental design

To investigate the effects of the methanol, enzyme, and water content on the methyl esters conversion from chicken fat residue, a 2^3 factorial experimental design with triplicate at the center point was carried out (see Table 1). Residual poultry oil (100 g) and methanol (MeOH) were employed as substrates in a range from 1.2 to 1.8 equivalents. MeOH was stepwise added (6 dosages, 1 h interval) to prevent enzyme inhibition. Enzymatic reactions were carried out in 250 mL Erlenmeyer flasks closed with a lid and placed in a horizontal incubator shaker (New Brunswick Scientific- Excella E25) at 45 ºC, 250 rpm for 24 h of reaction time. Samples were taken from the reaction mixture at 16 and 24 h, centrifuged (3500 rpm for 10 min) and analyzed. All the assays were carried out in duplicate. The experimental data was statistically evaluated by Statistica Software 6.0 at 95% of confidence level.

Table 1. Experimental planning 2^3 with 3 central points*.

Assay	MeOH (eqv)	E (wt%)	W (wt%)	FAME yield (wt%)	
				16h	24h
1	1.2	0.1	1	80.62	83.92
2	1.8	0.1	1	68.69	73.25
3	1.2	0.5	1	85.95	90.01
4	1.8	0.5	1	64.37	79.38
5	1.2	0.1	2	76.05	80.91
6	1.8	0.1	2	71.51	73.94
7	1.2	0.5	2	82.44	84.32
8	1.8	0.5	2	75.63	83.36
9	1.5	0.3	1.5	90.61	94.08
10	1.5	0.3	1.5	89.96	92.72
11	1.5	0.3	1.5	90.29	93.79

* MeOH is the amount of methanol (eqv); E enzyme content; W the amount of water.

FAME determination

For the determination of the FAME (fatty acid methyl esters) samples were diluted with 2 mL of ethanol and 8 mL of n-heptane. Then, a little amount was transferred to a 1 mL flask in order to obtain a concentration of 1000 ppm and the internal standard was added at a concentration of 250 ppm, using n-heptane as the solvent. Afterward, 1 µL of the solution was injected in triplicate in a gas chromatograph (Shimadzu GC-2010), equipped with FID, auto-injector AOC-20i and a capillary column RTX - Wax (30 m x 0.25 mm x 0.25 µm) serial 1208976. The column temperature was programmed from 120 °C, holding 2 min, heating to 180 °C at 15 $°C.min^{-1}$, holding 3 min, and to 250 °C at 5 $°C.min^{-1}$, holding 2 min. Helium was used as carrier gas, and the injection and detector temperatures were 250 °C with split ratio of 1:50.

RESULTS AND DISCUSSION

Physicochemical characterization of chicken fat residue

Table 2 shows the results of moisture content, lipids, FFA, iodine value, peroxides, and saponification of the raw material used in this work.

As mentioned by Luque and Melero (2012), moisture is a very important factor for oils and fats, since the stability of the oil decreases with increasing humidity. From the results (Table 2) about 1 wt% of moisture was determined, a high content when compared to refined soybean oil (usually around 0.02 wt%). The relatively high moisture can be associated with the tridecantation process, when the residue is

Table 2. Results of physical-chemical characterization of poultry residual oil.

Parameter	Value
Moisture content (wt%)	0.18 ± 0.05
Lipid content (wt%)	99.34 ± 0.11
Free fatty acid content (wt%)	12.00 ± 3.15
Peroxide index (mEq.Kg^{-1})	9.64 ± 0.98
Saponification index (mg KOH.g^{-1})	181.57 ± 6.80
Iodine content (wt%, g100g^{-1})	87.18 ± 1.53

separated into three parts, dry sludge, oil, and water, with part of this water remain trapped in the oil.

The results for moisture content show that approximately 99% of the raw material is composed of lipids. The composition of lipids is a mixture of tri, di, and monoglycerides, free fatty acids, glycolipids, phospholipids, and sterols, most of which are oxidation-susceptible (Silva et al., 1999). The presence of high content of volatile material may lead to a loss of yield of biofuel production by reaction with the catalyst or by the dilution of the raw material (Luque and Melero, 2012).

Because the residue can contain other different undesirable compounds for the hydroesterification reaction, the determination of free fatty acids content comes to complement the analysis of the lipid content. A high amount of FFA, depending on the raw material, may indicate that the residue is in an accelerated degree of degradation, thus releasing fatty acids. In the present case, as there is a great amount of free fatty acids in the raw material, the more beneficial will be the occurrence of the hydroesterification reaction.

The peroxide index determines all substances that oxidize potassium iodide. These substances are generally considered to be peroxides or other similar products that result from the oxidation of the oil, that is, the higher the peroxide content of a substance, the greater its degree of oxidation, which is a complement to the FFA content. In this work, for the evaluated residual oil from the poultry industry, a peroxide index of 9.64 mEq.Kg^{-1} was obtained.

The residual oil from agroindustry follows several processes, including centrifugation at high temperature and rotation, resulting in the modification in the physical and chemical characteristics; the oil then becomes dark, viscous, with an unpleasant odor, and consequently with a high index of peroxides. Rios et al. (2013) evaluated the oxidation process of frying oil by the peroxide index at different times, and the authors observed an index variation from 1.38 mEq. kg^{-1} to 9.96 mEq.kg^{-1} after 4 h, showing that heating is an important parameter in the oil oxidation process.

The saponification index gives an indication of the relative fatty acids molecular weight. However, the saponification index is not used to identify the oil, since the majority of the oils have similar indexes, from 188 to 196 mg KOH.g^{-1} (Chiu and Gioielli, 2002). Kobori and Jorge (2005) reported the quality of residual oils of tomato (172.86 mg KOH.g^{-1}), orange (181.05 mg KOH.g^{-1}), passion fruit (174.97 mg KOH.g^{-1}), and guava (189.91 mg KOH.g^{-1}) from the industrial process, and saponification indexes similar to that obtained in this study (181.57 mg KOH.g^{-1}).

From the iodine results, a reduction was observed in the iodine index (87.18 wt%), probably related to the cleavage of double bonds during the industrial process. As observed in this work, Ferrari et al. (2005) reported the reduction of iodine content from 133.13% in refined oil to 66.92% in frying oil residue, demonstrating the influence of the heating process in the double bond cleavage.

Evaluation of enzymatic esters production from chicken fatty residue

In general, in this work, satisfactory conversions, up to 90% were reached after 24 h of reaction time (see Table 1), suggesting that the use of residual oil may be a viable alternative for ester production. In addition, it should be noted that no previous purification was performed of the raw material before the hydroesterification, and no product polishing was performed at the end of the reaction, which can be considered promising for possible large-scale application. It should be noted that a low-cost feedstock, without any pre-treatment, together with a relatively low-cost enzyme, afforded very satisfactory methyl ester yields. This fact demonstrates the great advantage of this process against the conventional process (Shi et al., 2013) that requires temperature control, purity of the raw material, greater generation of residues with more steps of purification of the final product.

Cesarini et al. (2013) evaluated the production of biodiesel with soluble enzyme Callera Trans L. and crude soybean oil with a high content of free fatty acids (7.8 wt%), and 96% yield of methyl esters was reached after 24 h reaction at 35 °C and 200 rpm. Rivera et al. (2009) studied different types of enzymes (NS 435 and RM IM) and the NS 435 afforded about 75% ethyl ester conversion, while the RM IM enzyme showed 35% ethyl ester conversion. Zenevicz et al. (2017) produced esters via hydroesterification using frying oil by a continuous ultrasound process using Lipozyme TL IM enzyme for the hydrolysis step and the Novozym 435 enzyme for the esterification step, obtaining 79% conversion after 9 min of reaction.

Through experimental design, all investigated variables were shown to be significant in the enzymatic reaction, i.e., by increasing or decreasing the variable values the ester conversion will be significantly modified. The response surface of the experimental design is shown in Figure 1 and it was possible to observe that the best results in terms of conversion

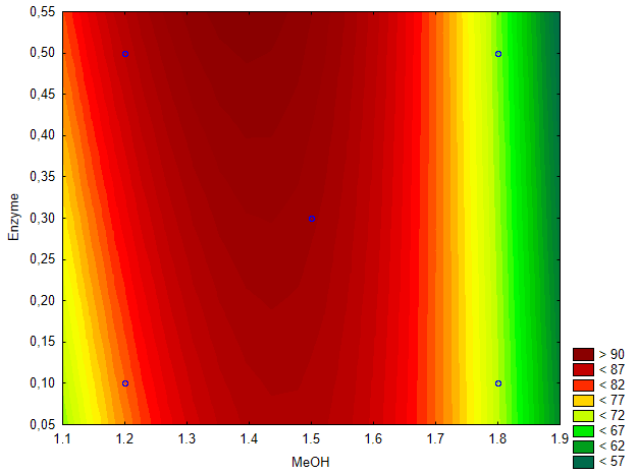

Figure 1. Response surface for FAME production after 24 h of enzymatic reaction.

were reached at high enzyme concentration and the intermediate methanol content. Thus, from the statistical analysis of estimated effects, it was possible to obtain a corresponding correlation that represents the response in terms of ester conversion with a correlation coefficient (R^2) of 0.996 (Eq. 6).

$$\begin{aligned} \mathrm{FAME(wt\%)} &= 99.29 - 6.46\mathrm{MeOH} - 13.17(\mathrm{MeOH})^2 + \\ &+ 1.46\mathrm{E} - 2.13\mathrm{MeOH}\cdot\mathrm{E} + 3.75\mathrm{MeOH}\cdot\mathrm{W} + 1.02\mathrm{E}\cdot\mathrm{W} \end{aligned} \quad (6)$$

CONCLUSIONS

The waste from the poultry industry presented a significant amount of fatty acids that can be used advantageously in the enzymatic esterification reaction to produce methyl esters, both for technical and economic reasons. Reaction conversions as high as 94% were reached in a relatively short time under mild conditions, 45 °C, 0.3 wt% enzyme, 1.5 eqv MeOH and 1.5 wt% water content. Thus, the use of the residual oil from the poultry industry employed in this work constitutes a promising alternative when compared to the traditional base-catalysed process, as it involves a waste feedstock coupled with the low-cost commercially available Eversa Transform 2.0® lipase.

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NOMENCLATURE

A	volume spent on sample measurement (mL)
B	volume of sodium thiosulfate used in the standard titration (mL);
C	concentration of sodium thiosulphate solution ($mol.L^{-1}$)
C_{FFA}	content of free fatty acid (FFA, wt%)
Le	amount of lipids extracted (g)
M	molarity of $Na_2S_2O_3$ solution ($mol.L^{-1}$)
MM_{FFA}	average molar mass of fatty acids (FA), around 282 $gmol^{-1}$
M_{KOH}	molarity of KOH solution ($mol.L^{-1}$)
M_s	sample mass (g)
S	volume of sodium thiosulphate spent on titration of the sample (mL)
V_{KOH}	volume of KOH solution employed in the titration (mL)
V_b	volume used in the standard titration (mL)
V_a	volume spent in sample titration (mL)

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