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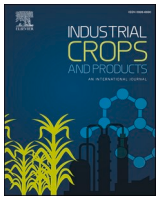

Ethyl biodiesel production from crude soybean oil using enzymatic degumming-transesterification associated process

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ABSTRACT

Brazil is one of the largest soybean producers in the world. Biodiesel production in Brazil (specially soybean oil biodiesel) has been growing every year and demanding more effective and sustainable technologies, which is the case of enzymatic processes. Enzymatic degumming could be an alternative to provide better quality products, before biodiesel production but also, in a one reaction step as a proposal to reduce time and costs. Therefore, this work was aimed at evaluating enzymatic degumming (previously optimized) of crude soybean oil using a phospholipase cocktail associated with transesterification using lipase from *Aspergillus oryzae* for ethyl biodiesel production. For this, transesterification was optimized for ethanol:oil (E:O) ratio, water and lipase % through a central composite rotatable design (CCRD). Optimal conditions were used to evaluate two degumming-transesterification associated processes: i) a one-pot reaction (OPR) where degumming and transesterification were performed at the same reactor; and ii) a two-pot reaction (TPR) where oil was first degummed, followed by transesterification. The optimal transesterification condition were achieved for E:O = 4.48:1, water = 3.41 % and lipase = 2.43 %, where 97 % fatty acid ethyl esters (FAEE) were obtained. Both OPR and TPR provided biodiesels with FAEE > 94 %: TPR was the best with 97.5 % and 99.98 % before and after biodiesel purification. Mineral elements (including phosphorus) and other impurities (anions) were low, and within quality standards. Glycerol produced also presented very low content of impurities which is quite advantageous. Although lipase achieves good conversion to FAEE (95.7 ± 0.29 %) using crude oil (Control), the final biodiesel carries many impurities (P=80.07 ± 0.1 mg/kg), thus requiring subsequent biodiesel purification steps. In addition, the high impurity content generates a biodiesel that does not comply with ANP legislative standards, P<10 mg/kg. The use of enzymatic degumming in the biodiesel production process generates a biodiesel with low impurities and higher final quality, in addition to being a process that generates less effluent. Enzymatic degumming was essential for obtaining high quality biodiesel and its association with transesterification showed to be a great option for decreasing time and costs for biodiesel production.

1. Introduction

Soybean oil and ethanol are raw materials of high production in Brazil and are of great importance to produce biodiesel. Global soybean production will increase by 19.6 percent over the projection period (2031), with Brazil, Argentina, and the United States supplying about 85 percent of the expected growth. Brazil will contribute with about 60 percent of the total production increase (Dohlman et al., 2022). In Brazil, ethanol is industrially produced mainly from sugarcane juice. The National Pró-Alcohol Program was created in the 1970's, with the aim of increasing Brazilian ethanol production and gradually replacing gasoline. Brazil is the second-largest ethanol producer in the world. Pure fuel (hydrated ethanol) and mixed at 27 % v/v (in anhydrous form) with gasoline is the main application of ethanol in Brazil in the transportation

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sector. Most ethanol and biodiesel currently produced and consumed in Brazil are 1 G biofuels from carbohydrates (sucrose and starch), and lipids, mainly TAGs (triacylglycerol), and/or FFAs (free fatty acids) from vegetable oils and animal fats. The National Biofuel Policy (RenovaBio) was implemented in 2017 through the Brazilian Ministry of Mines and Energy (Law 13.576/2017) to set higher goals for biofuel production, and to fulfill agreements signed in the COP21, also called Paris Agreement (Karp et al., 2021; Lima et al., 2020; L. P. Ramos et al., 2017; M. D. N. Ramos et al., 2022; Tecnologias et al., 2021).

Vegetable oils have minor components such as free fatty acids (FFA), metal traces (P, Mg, Ca, Fe), phospholipids and other compounds. The high content of phospholipids in the oil, for biodiesel production, means a high loss of yield in fatty acid methyl esters production, since the fatty acids enclosed in the phospholipid molecules are not free for transesterification, considering the enzymatic route. In this context, the degumming process, generally performed by acid, water or enzyme addition, remove phospholipids, reducing the phosphorus content and other metal traces. Enzymatic degumming uses phospholipases (PL) such as PLA_1, PLA_2 and PLC. After the process, there is a release of FFA (PLA_1 or PLA_2) and DAG (PLC), increasing the available free oil content. Passos et al. (2022a) optimized the degumming process of crude soybean oil using a cocktail of phospholipases (PLA_2, PLC and PI-PLC) and achieved a final product with phosphorus content < 10 mg/kg. Furthermore, the process provided an increase of 0.72 % DAG, thus leading to an increase of the oil yield. Enzymatic degumming is a process in which oil losses are significantly minimized when compared to acid degumming, thus one should conclude that, such a saving energy alternative leads to a lower environmental impact (Cowan and Nielsen, 2009). For biodiesel production, therefore, enzymatic degumming and transesterification combined could also reduce losses, decrease the number of steps, and lead to reduction in energy consumption.

There are three main routes which can be used for biodiesel production, acid, alkaline and enzymatic transesterification. The enzymatic catalysis is quite advantageous for biofuel production when compared to acid and alkaline catalysts mainly considering aspects such as process efficiency, sustainability, and environmental concerns. Enzymes have advantages related to their ability to act under mild reaction conditions, their product selectivity, substrate specificity, and low environmental toxicity (Chapman et al., 2018; Choi et al., 2015; Volpato et al., 2010). Therefore, the fatty acids chains remain almost intact, and the produced glycerin is of pharmaceutical quality (Monteiro et al., 2018), facilitating the purification of both products (biodiesel and glycerin). The environmental advantages also have promoted an increase in the use of lipases as catalysts in the production of biofuels (Monteiro et al., 2021). The industrial implementation of enzymatic processes is limited by some factors such as time, cost to develop enzymes and new technology acceptance/adaptation. Therefore, the progresses in new enzymes development and enzymes optimization will fasten and lower the cost of enzymes development (Guerrand, 2017).

For enzymatic esterification/transesterification soluble lipases can also be used since they work under mild reaction conditions, have a faster reaction time, display higher conversion rates than immobilized enzymes, and allow, without any fatty acid esters yield loss, the presence of water in the process (Cesarini et al., 2013, 2014b; Chen et al., 2008; Nielsen et al., 2008; Tufvesson et al., 2010). Studies on optimization processes for different oleaginous feedstock using lipases have been published (Remonatto et al., 2016, 2018; Wancura et al., 2021).

The works cited previously were developed with refined soybean oil and immobilized lipase, using methanol as the short-chain alcohol for transesterification. It is important to develop processes that use ethanol (abundant raw material in Brazil) and lipases, minimizing the cost of biodiesel production by allowing the use of crude vegetable oils. Association of enzymatic degumming and transesterification in a single step was observed in some works in literature. Cesarini et al., (2014a) used crude soybean oil, 1 % of *Callera Trans L* (lipase), 30 ppm of Lecitase Ultra (PLA_1), 200 ppm of Purifine (PLC), 250 – 500 ppm of LLPL-2 (Lyso-P), methanol at 1.5 eqs and 2–3.5 % of water, obtaining fatty acid methyl esters (FAME) > 95 % and P < 5 ppm in 24 hours process. Steinke et al. (2022) utilized 5 ppm of *Eversa Transform 2.0*, 50 ppm of Quara LowP, 45°C, 2 % water, 1.5 eq. methanol in 72 h process, removing 95 % of phosphorus and producing 86.23 % of FAME in one step. Farobie et al., (2021) produced biodiesel with Eversa Transform 2.0 liquid, methanol and palm oil. They obtained 97.91 % FAEE, 19.33 % FFA with the following conditions: 7:1 molar ratio (methanol: oil) and 40°C. Vieira et al., (2021) used Eversa Transform 2.0 in liquid form, distilled deionized soybean oil and obtained 86.56 % FAEE in 23 hours, under the conditions of 35°C and molar ratio (ethanol: oil) of 3.64:1 and 8.36 % Eversa lipase. In view of the works presented, it is necessary for the market to reduce the cost of biodiesel production and preserve the environment. One way would be to use crude oil without any prior treatment, use the liquid enzyme in its optimized condition, use ethanol as a short-chain alcohol, since it is a raw material widely produced in Brazil and is eco-friendly, optimize the process steps and use enzymatic degumming as a means of preparing the oil for transesterification. The combination of enzymatic degumming and transesterification would generate a biodiesel with few impurities and more profitable for the market, in addition to generating a purer glycerol for the market.

In this way, the objective of this work was to evaluate the production of biodiesel from crude soybean oil associating enzymatic degumming and enzymatic transesterification. For this, the ethyl soybean oil biodiesel production using lipase *Eversa Transform 2.0* was optimized, regarding the FAEE (fatty acid ethyl ester) (%) yield and the optimized condition was applied in the study. Also, the quality of the glycerol side stream generated in the process was also evaluated, providing important highlights to improve quality and decrease costs in biodiesel production.

2. Material and methods

2.1. Materials

Crude soybean oil was kindly provided by Cargill (Uberlandia-MG/Brazil). The phospholipase cocktail Purifine® 3 G (activity of 16900 PLCU/g) was supplied by DSM Food Specialties (Delft, the Netherlands). The cocktail is a system of 3 enzymes: i) PLA_2, from *Aspergillus niger*; ii) PLC, from *Pichia pastoris* and iii) PI-PLC from *Pseudomonas fluorescence*. The *Eversa® Transform 2.0* (derived from genetically modified *Aspergillus oryzae*) (834 ± 40 U/mg of protein, based on the methodology of Carvalho et al., 2015), was supplied by Novozymes (Parana/Brazil). The PLCU is the enzyme activity expressed in phospholipase C units. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 mol of free fatty acid per minute under the assay conditions.

2.2. Methods

2.2.1. Physicochemical analyses

Crude oil was evaluated for FA and TAG profile, partial acylglycerol, phosphorus and metal elements. The FA profile was determined by gas chromatography (Perkin Elmer, Clarus 600, USA) following the official method AOCS Ce 1–62 (American Oil Chemists' Society, 2012d). The conversion of fatty acids from crude soybean oil was performed by a methylation reaction described by Hartman and Lago (1973). The gas chromatograph equipped with a flame ionization detector (FID) and a DB-WAX capillary column (length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm) (Agilent Technologies, U.S.A.); helium as carrier gas at 1.78 mL/min flow rate. The column temperature ramp was programmed from 50 to 250 °C at 10 °C/min. Injector and detector temperatures were set at 250 °C and the injection volume was 1 μL. Individual FA peak identification was performed by comparing retention times of samples with those of FAME standards.

Acylglycerols profile (mono-,di- and triacylglycerols, MAG, DAG and TAG) was determined according to the AOCS method Cd 11b-91

(American Oil Chemists' Society, 2012c) using a gas chromatograph (Agilent Technologies, model 7890 A) equipped with a DB-5HT capillary column (15 m × 0.32 mm i.d.; film thickness: 0.10 μm). Approximately 0.05 g of the analyzed sample was dissolved in 100 μL of tetradecane and 300 μL of N,O-bis((trimethylsilyl) trifluoroacetamide), and the resulting mixture was homogenized and heated to 70 °C for 20 min. After that, 50 μL of the derivatized sample was diluted with hexane (1 mL) and injected into the equipment. Analysis was performed under the following conditions: oven temperature ramp from 50 to 200 °C (15 °C/min), from 200 to 290 °C (3 °C/min; held for 10 min), and from 290 to 360 °C (10 °C/min; held for 15 min); flame ionization detector (380 °C); and He as carrier gas.

The probable TAG composition was estimated from the fatty acid profile, following the methodology described by Antoniosi Filho et al. (1995). These results were used to calculate the average molar mass of each acylglycerol (tri-, di- and mono-) and crude soybean oil.

Phosphorus (P), and other mineral elements (Mg, Fe, and Ca) were determined by inductively coupled plasma – atomic emission spectroscopy according to method Ca 20–99 (American Oil Chemists' Society, 2012b).

The content of phospholipids was determined according to Eq. 1 from the conversion of phosphorus (P) to phospholipids calculated using the ratio of the phosphorus atomic weight (31) to the estimated molecular weight of the phospholipids (PL), which was equal to approximately 25 (Galhardo, Dayton, 2021):

$$PL(\%) = \frac{25 \quad . \quad P(mg/kg)}{10000} \tag{1}$$

2.2.1.1. Biodiesel and glycerol analyses. The total content of TAG, DAG, MAG, FAEE, and ethanol were evaluated using a High-Performance Liquid Chromatography (HPLC) as described by Ferreira et al. (2015). Quantification was performed by peak integration with calibration curves built with known TAG, DAG, MAG, FAEE and ethanol concentrations. Glycerol was determined by stoichiometry according to the methodology described by Bejarano-alva et al., (2020).

The conversion (%) of soybean oil to fatty acid ethyl esters was calculated according to Eq. (2):

$$Conversion(\%) = \left[1 - \frac{3 * N_{TAG,f} + 2 * N_{DAG,f} + 1 * N_{MAG,f}}{3 * N_{TAG,i} + 2 * N_{DAG,i} + 1 * N_{MAG,i}}\right] * 100 \tag{2}$$

where $N_{TAG,i}$, $N_{DAG,i}$, $N_{MAG,i}$, are the initial moles of tri-, di-, and monoacylglycerols, respectively; and $N_{TAG,f}$, $N_{DAG,f}$, $N_{MAG,f}$, are the final moles of tri, di, and monoacylglycerols, respectively.

The D6584–00 ASTM International, (2000) was used to quantify TAG, DAG, MAG and glycerin in biodiesel, which are considered impurities. In this case approximately 100 mg of sample was directly weighed into a 10 mL septa vial. Using microliter syringes, exactly 100 μL of each internal standard and MSTFA was added to the vial and allowed setting for 15–20 min at room temperature. Added approximately 8 mL of n-Heptane to the vial and shaked. Injected 1 μL of the reaction mixture into the cool on-column injection port and started the analysis. Obtained a chromatogram and peak integration report.

Free fatty acid (FFA) content was determined by titration according to the official AOCS Ca 5a-40 method (American Oil Chemists' Society, 2012a). Water content was determined by Karl Fischer titration using an automatic Karl Fischer titrator (Metronm, 870 K F Titrino plus, Switzerland). Oxidative stability index was determined using a Rancimat equipment (Metrohm, 893 Professional Biodiesel Rancimat, Switzerland) at 110 °C and 9 liter/h air flow rate, following the AOCS Cd 12b-92 official method (AOCS, 2017). Ion chromatography was used for quantification of anions (impurities): acetate, formate, chloride, phosphate and sulfate, according to the method described by Silveira, De Caland, and Tubino (2014).

The density of biodiesel was determined using a densimeter (Anton Paar, DMA 4500, Austria) at temperature from 20 °C. The viscosity of biodiesel was measured at temperature from 40 °C using a stress-controlled rheometer (TA Instruments, AR1500ex, England) with stainless-steel cone-plate geometry (diameter = 40 mm; range = 47 mm). The flash point measurements were done according to method ASTM D6450 (ASTM, 2016).

The glycerol side stream was also evaluated for FFA, TAG, DAG, MAG, mineral elements and anions for quality analysis.

Table 1
Levels and factors (independent variables) for the CCRD (2^3).

Variables	Levels				
	-1.68	**-1**	**0**	**+1**	**+1.68**
Ethanol:oil (molar ratio) (X_1)	3.32:1	4:1	5:1	6:1	6.68:1
Water (%) (X_2)	1.32	2	3	4	4.68
Lipase (%) (X_3)	0.32	1	2	3	3.68

2.2.2. Enzymatic Degumming

Prior to biodiesel production, enzymatic degumming was performed according to the optimized conditions determined by Passos et al. (2022a). Process was performed using 300 g of crude soybean oil in jacketed glass cell. The pH of the system was firstly adjusted to 5.5 using a NaOH 0.1 M aqueous solution. Subsequently, the oil was heated to 80 °C using a thermostatic bath (12101–15, POLYSTAT, CHICAGO) during 15 min under mechanical stirring (RW 20 Digital, IKA, GERMANY) at 350 rpm, after which the temperature of the oil mixture was decreased to 60 °C. After temperature stabilization, 200 ppm of Purifine® 3 G and 3 % of water (w/w) were added. The obtained mixture was homogenized under high shear mixing (16000 rpm) (T25 DIGITAL ULTRA-TURRAX, IKA, GERMANY) for 1 min and then maintained at a required temperature under mixing (350 rpm) for 2 h. The reaction was stopped by heating the oil to 85 °C for 15 min at 350 rpm. Thereafter, the oil mixture was centrifuged (2000 g for 15 min, ROTINA 380 R, HETTICH, GERMANY) to separate the degummed oil from the gums. Degummed oil was evaluated for TAG, DAG, MAG, phosphorus, phospholipids, and metal elements content as described in Section 2.2.1.

2.2.3. Ethyl biodiesel production optimization using Aspergillus oryzae lipase and degummed oil

A response surface methodology was adopted to optimize the enzymatic transesterification of the degummed soybean oil. To obtain the maximum fatty acid ethyl ester (FAEE) production, a central composite rotational design (CCRD) 2^3 was used for evaluation of three independent variables: ethanol:oil (molar ratio) (X_1), water (X_2), and lipase concentration (X_3). A set of 18 experiments (with 4 central points) at five different levels (± 1.68) was performed according to Table 1. Reactions were carried out in a 50 mL jacketed glass cell for 36 h using mechanical stirring (RW 20 Digital, IKA, GERMANY) at 900 rpm. Temperature was set at 40 ºC (optimum lipase temperature, (Novozymes, 2016), maintained using a thermostatic bath (12101–15, POLYSTAT, CHICAGO). After reaction, the system was centrifuged (10000 rpm, 10 min, 5 °C, ROTINA 380 R, HETTICH, GERMANY), and separated into two phases: biodiesel and glycerol. The FAEE conversion (methodology described below) in the biodiesel stream was used as the response variable. Using the CCRD results a model was generated using Eq. 3.

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j \tag{3}$$

where Y is the predicted response (FAEE conversion), β are the adjusted constants, and X are the independent variables (uncoded value) The quality of the model was determined by the correlation coefficient (R^2), while the Analysis of Variance (ANOVA) was performed at $p < 0.1$ to evaluate the statistical significance of the model and variables. Model was used to describe the response surfaces, applied to determine the interactions between the independent variables and their effects on the response. Data were analyzed using the Statistica 5.0 software package

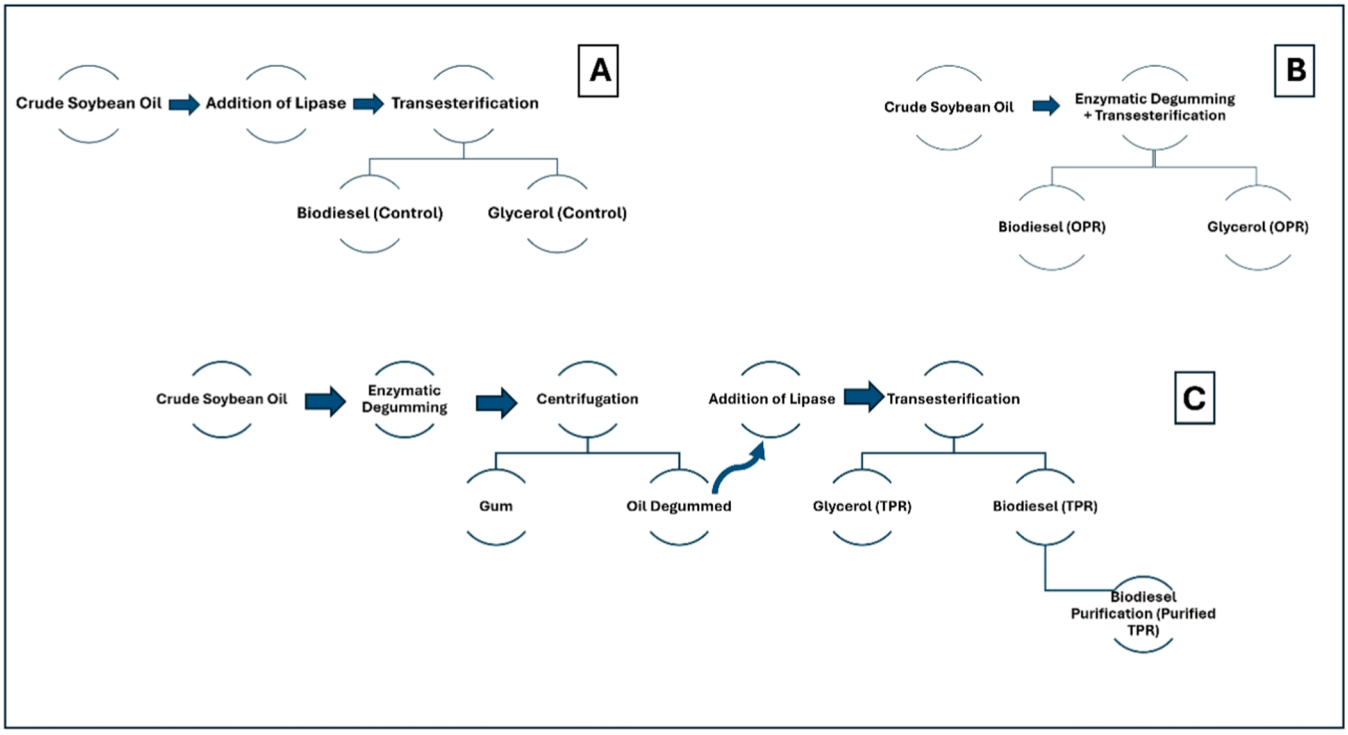

Fig. 1. A) Control Biodiesel Process B) One-Pot Reaction (OPR) Biodiesel Process and C) Two-Pot Reaction (TPR) Biodiesel Process.

(StatSoft Inc., USA).

2.2.4. Biodiesel production using a degumming-transesterification associated process

The Fig. 1 shows the diagram of the biodiesel production processes: Biodiesel Control, One-Pot Reaction (OPR), Two-Pot Reaction (TPR) and Biodiesel Purified.

2.2.4.1. One- pot reaction (OPR) process. After optimization of the biodiesel process, an OPR process was performed. In this case, degumming and transesterification was performed sequentially at the same reaction cell. First, a 33 g sample of crude soybean oil was submitted to enzymatic degumming according to Section 2.2.2. After 2 h of degumming reaction, without phospholipases and gums removal or phospholipase inactivation, enzymatic transesterification was performed. Temperature was conditioned (40 °C), and lipase, water and ethanol were added into the reaction cell under the optimal conditions of the experimental design (CCRD). The system was stirred at 900 rpm, for further 36 hours. After reaction, the system was centrifuged (10000 rpm, 10 min, 5 °C, ROTINA 380 R, HETTICH, GERMANY) for phase separation. Biodiesel phases were evaluated for physicochemical analyses as described in Section 2.2.1.

2.2.4.2. Two-pot reaction (TPR) process. The TPR process was performed such that crude oil was degummed according to described at Section 2.2.2. The separated degummed oil was then submitted to transesterification, in a second pot, according to optimized condition for ethanol:oil ratio, water and lipase concentration, determined in CCRD, during 36 h, at 40°C under mechanical stirring at 900 rpm. Biodiesel, glycerol and lipase streams were separated by centrifugation (10000 rpm, 10 min, 5 °C). Biodiesel and glycerol streams were evaluated as described in Section 2.2.1.

For analysis purpose, TPR biodiesel was further purified according to methodology described by Deboni et al. (2018) for removal of acidity. The acidity decreasing was performed in a jacketed glass cell connected to a thermostatic bath (12101–15, POLYSTAT, CHICAGO) using the following conditions: biodiesel:ethanol (molar ratio) of 1:16, 20 % (by oil mass) Amberlyst A26 OH resin, 50 °C and stirring at 500 rpm for 30 min.

Table 2
Fatty acid (% w/w), TAG and DAG (x, molar fraction) composition for crude soybean oil.

Fatty acid		**Symbol**			**%**
Palmitic acid		P	16:0		10.76 ± 0.01
Stearic acid		S	18:0		3.94 ± 0.05
Oleic acid		O	18:1		24.65± 0.05
Linoleic acid		Li	18:2		52.58 ± 0.07
Linolenic acid		Ln	18:3		7.26 ± 0.06
Arachidic acid		A	20:0		0.39 ± 0.01
Behenic acid		Be	22:0		0.43 ± 0.00
TAG*	**MM (g.mol^{-1})**	***x*100**	**DAG***	**MM (g.mol^{-1})**	***x*100**
LiLiO	881.41	22.60	LiLi	616.96	33.71
LiLiLi	879.39	20.31	OLi	618.98	28.58
OOLi	883.42	13.23	PLi	590.92	12.93
LiLiP	853.35	11.12	PO	592.94	6.46
PLiO	855.37	9.37	OO	621.00	5.94
LiLiLn	877.38	6.47	LiLn	614.94	5.79
SOLi	885.44	4.71	SO	623.01	2.46
OOP	857.39	3.62	SLi	620.99	1.57
PLiLn	851.34	2.73	PP	564.88	1.08
PPLi	827.32	2.21	PLn	588.90	0.91
OOS	887.46	0.98	LnLn	612.92	0.28
PPO	829.33	1.03	PS	594.95	0.23
LnLnLi	875.36	0.84			
POS	859.40	0.70			
MM_{TAG}	**872.05**			**MM_{DAG}**	**612.06**

* Values obtained via a prediction method (Antoniosi Filho et al., 1995).

3. Results and discussion

Table 2 shows the fatty acid and TAG profile for crude soybean oil. Linoleic acid was the FA with the highest content of 52.58 % of the oil composition, followed by oleic acid (24.65 %) and palmitic acid (10.76 %). Besides, lower costs and high productivity, when compared

Table 3
Characterization of crude soybean oil, chemical conditioned and degummed oil.

Lipid profile	Crude Soybean Oil (CSO)	Chemical Conditioned Oil (CC)	Degummed Oil (DO)
FFA (%)	1.29 ± 0.08	0.99 ± 0.016	1.18 ± 0.12
TAG (%)	96.44 ± 0.040	97.46 ± 0.024	96.93 ± 0.07
DAG (%)	1.33 ± 0.002	1.10 ± 0.021	1.67 ± 0.042
MAG (%)	0.23 ± 0.040	0.23 ± 0.003	0.21 ± 0.03
Phospholipids (%)	0.710	0.225	0.018
Mineral elements (mg/kg)			
P	285 ± 5	90 ± 2	7.28 ± 0.05
Fe	1.04 ± 0.01	0.3 ± 0.01	n.d. < 0.1
Ca	58.5 ± 0.8	8.9 ± 0.2	0.38 ± 0.07
Mg	39.0 ± 0.6	8.09 ± 0.18	0.43 ± 0.04

to other oils, soybean oil FA profile is one of the major factors that makes this oil an excellent raw material for biodiesel production. Its high content in unsaturated compounds lead to a biodiesel with low melting and crystallization points, quite suitable for low temperature region (Magalhães et al., 2019). Based on the FA profile, the TAG profile for soybean oil was predicted (Antoniosi Filho et al., 1995) and the DAG profile determined from it. From these results and from total TAG, and acylglycerol contents, the average molar mass was calculated for TAG at 872.05 g/mol, for DAG at 612.06 g/mol and for soybean oil at 847.98 g/mol.

3.1. Enzymatic Degumming

The characterization of crude soybean oil (CSO), and enzymatic degummed oil (DO) are presented in Table 3. For analysis purpose, oil was also characterized after pH conditioning (CC) during degumming. This because, conditioning could already lead to precipitation of phospholipids and other mineral elements. Purifine® 3 G is an enzyme cocktail composed of phospholipase C (PLC), phosphatidylinositol – specific phospholipase C (PI-PLC), and a minor amount of PLA_2, which effectively converts phospholipids into predominantly diglycerides (DAGs), phosphates, FFAs, and lysophospholipids (Nikolaeva et al., 2020). Thus, the potential yield increase from enzymatic degumming with Purifine® 3 G comes from the formation of FFA and DAG while the degumming efficiency is evaluated by the residual P-content in the degummed oil.

The FFA content increase expected after enzymatic degumming is 0.26 %, according to model proposed by Passos et al. (2022a). In this work, enzymatic degumming increased the FFA content by 0.19 %, compared to the chemical conditioned oil, within the expected estimate. After enzymatic degumming, the DAG content increased by 0.58 %, within the estimated levels (0.55 – 0.83 %), also according to Passos et al. (2022a). The Purifine® 3 G caused the reduction of phosphorus content of about 97.44 %, reaching 7.28 mg/kg less than the limit of 10 mg/kg stablished by the Brazilian law (Agência Nacional do Petróleo, 2021). This means a breakdown of phospholipids with a decrease of about 97.46 % of total phospholipids. There was also a decrease in mineral elements such as Fe, Ca, and Mg which are related to the non-hydratable phospholipids. The phosphorus content below 10 mg/kg, as well as reduced mineral elements are required for biodiesel quality, being degumming a necessary step before transesterification.

3.2. Ethyl biodiesel production optimization

The of FAEE conversion (%) (Y_1, dependent variable), was determined for each condition, and the obtained results are presented in Table 4. ANOVA was presented in Table 5. Analysis was performed for 90 % of significance ($p < 0.1$) that, according to Rodrigues et al., (2014) it is a suitable value in case of bioprocesses (involving enzymes and microorganisms). Results showed that the model was also satisfactory such that $F_{calc.(regression/residuals)} > F_{tab}$. According to the evaluation of the effects, the following effects (parameters of the model) presented statistical significance ($p < 0.1$) for explaining the response (FAEE conversion): the linear parameter for ethanol:oil ratio X_1 and lipase % X_3; the quadratic parameter for lipase % X_3^2 and the combined parameter for ethanol:oil ratio and lipase % X_1X_3. However, some non-significant parameters were added to the model, such as X_1^2, X_2 and X_1X_2, as their presence brought greater uniformity to the model. In this case, the following polynomial equation (coded factors) where obtained from the CCRD model for FAEE conversion (%) (Eq. 4). The R^2 value obtained for

Table 5
ANOVA results for the CCRD.

Variation Source	Sum of Squares	Degrees of Freedom	Mean Square	$F_{calc.}$	$F_{tab.}$	p-value
Regression	885.46	7	126.49	7.49	2.41	0.0026
Residuals	168.98	10	16.90			
Total	1054.45	17				
R^2	83.97 %					

Table 4
Experimental and predicted values using the generated model (CCRD 2^3) and their absolute (AD) and relative (RD) deviations.

Trials	Variables			(Exp.) Y_1	Predicted (FAEE %)	AD (in FAEE %)	RD (%)
	X_1	X_2	X_3				
1	4:1	2.00	1.00	96.77	91.76	5.00	5.17
2	4:1	2.00	3.00	96.76	95.32	1.44	1.49
3	4:1	4.00	1.00	95.49	91.58	3.90	4.08
4	4:1	4.00	3.00	95.01	95.52	0.51	0.54
5	6:1	2.00	1.00	78.85	74.93	3.91	4.96
6	6:1	2.00	3.00	91.14	91.64	0.50	0.55
7	6:1	4.00	1.00	82.63	80.67	1.95	2.36
8	6:1	4.00	3.00	96.17	97.77	1.60	1.66
9	3.32:1	3.00	2.00	93.95	98.16	4.21	4.48
10	6.68:1	3.00	2.00	85.30	85.89	0.59	0.70
11	5:1	1.32	2.00	87.13	91.35	4.22	4.84
12	5:1	4.68	2.00	95.76	96.34	0.58	0.61
13	5:1	3.00	0.32	69.12	76.26	7.15	10.34
14	5:1	3.00	3.68	95.98	93.63	2.34	2.44
15 (C)	5:1	3.00	2.00	96.16	96.47	0.30	0.31
16 (C)	5:1	3.00	2.00	96.87	96.47	0.39	0.41
17 (C)	5:1	3.00	2.00	96.96	96.47	0.48	0.50
18 (C)	5:1	3.00	2.00	96.71	96.47	0.24	0.24
						Average	**2.54**

X_1 - ethanol:oil (molar ratio) (%); X_2 - Water (%); X_3 - Lipase (%); Y_1 - FAEE (%). C = Central point.

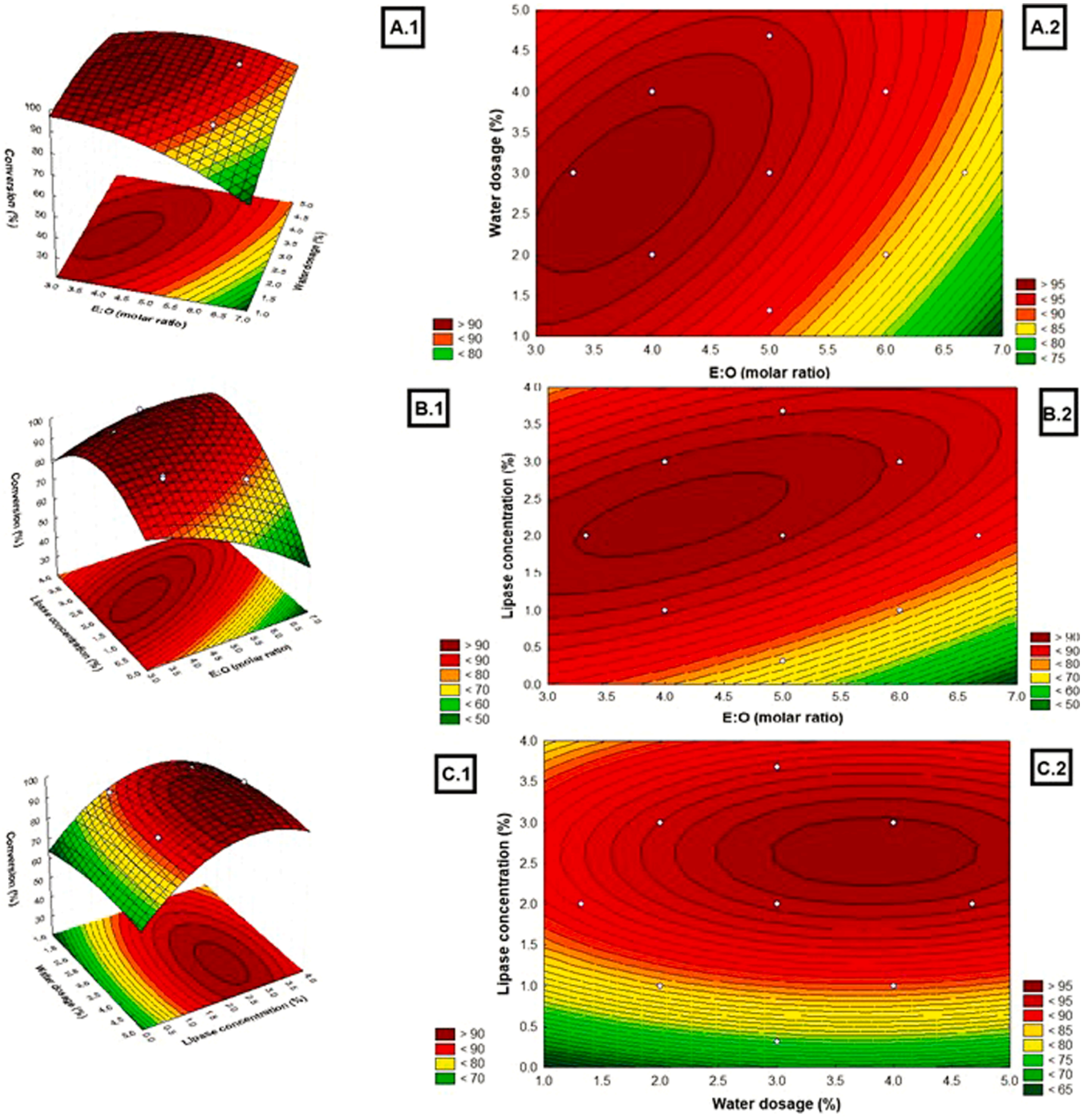

Fig. 2. Response surfaces and contour curves for conversion to FAEE (%) (A, B and C). A) Water (%) and E:O (ethanol:oil/molar ratio); B) Lipase (%) and E:O (molar ratio) and C) Water (%) and Lipase (%).

conversion to FAEE using the mathematical model was 83.97 %.

$$Conversion(\%) = -3.65X_1 - 1.38X_1^2 + 1.49X_2 + 5.16X_3 - 3.88X_3^2 + 1.48X_1X_2 + 3.29X_1X_3 + 95.47 \quad (4)$$

where X_1, X_2, and X_3 are the coded variables for the ethanol:oil (molar ratio), water dosage and lipase concentration, respectively. It can be seen that the parameter X_1(ethanol:oil) (E:O) in Eq. 4 presented a negative effect on the system, as the increase in the ethanol content on the system may probably cause denaturation of lipase, resulting in a decrease of FAEE conversion. It happens because excess ethanol probably solvates the lipase surface, resulting in modifications of its folding structure, decreasing its catalytic activity (Huang et al., 2015; Karmee et al., 2018). Also, enzymatic transesterification is a reversible reaction, and excess ethanol can also provide more acyl accepters, leading to lower FAEE formation (Rachmadona et al., 2020). Moreover, with increased ethanol content, soybean oil in the reaction system was diluted, decreasing its collision frequency with the lipase and, so, the formation rate of enzyme-substrate complexes (Sun et al., 2021a). Thus, in the present work, according to the model, an optimal ethanol:oil molar ratio of 4.48:1 was obtained. This result is lower than other works carried out with molar ratio oil:ethanol (1:5) with the *Rhizomucor miehei* lipase and ratio oil:ethanol (1:7) with *Aspergillus niger* modified lipase

(Huang et al., 2015; Sun et al., 2021b).

The parameter X_2 (water %) in Eq. 4 presented a positive contribution for the system: water increase caused an increase of FAEE conversion. Normally, the active site of this lipase is covered by a "lid" (an amphiphilic peptide loop) to prevent oil and ethanol approaching. When water is added to the reaction system, an interfacial area between water and oil is formed and the "lid" is opened under the action of water (Kuo et al., 2015). Therefore, an appropriate water content was essential for the reaction. Sun et al. (2021a) shows that when water content increased from 4 % to 20 %, no significant change in biodiesel yield (~93 %) was found. However, the acid value increased from 8.9 ± 0.2 mgKOH/g to 11.9 ± 0.1 mgKOH/g. These results showed that in this enzymatic system, transesterification, hydrolysis and esterification took place simultaneously. Among these reactions, excess water might favor the hydrolysis of oil. Furthermore, the presence of water activates the enzyme and protects the active site from the direct action of ethanol, that can cause lipase denaturation. Therefore, even though parameter X_2 is not statistically significant, it is fundamental for the comprehension of the reaction, and, hence, for the model.

The parameter X_3 (lipase concentration) in Eq. 4 presented a positive effect on the reaction: lipase content increase caused an increase in the FAEE conversion. On the other hand, the quadratic term is negative, indicating that lipase excess can also saturates the medium. These results can be attributed to the appearance of more active sites in the presence of high lipase contents, which can increase the formation of lipase-substrate complexes (Abdulla and Ravindra, 2013). In this case, when the system has lipase in excess, the enzymatic activity can decrease due to lipase aggregate formation and mass transfer limitations.

Fig. 2 (A1and A2) shows the response surfaces presenting the X_1X_2 interactions (ethanol:oil ratio and water), as well as its positive effects (Eq.4) leading to increased formation of FAEE. The optimal region for water % was between 2.0 – 3.8 % water dosage, whereas the optimal region for ethanol:oil molar ratio was between 3:1 – 4.5:1. In the present work, according to the model, it was obtained an optimal point for the water % of 3.41 %. This value is lower than those obtained using this lipase in other works (20 %), and for the use of *Candida antarctica* lipase (14 %), and *Callera Trans L* lipase (3–5 %) (Cesarini et al., 2013; Lv et al., 2019; Price et al., 2014; Sun et al., 2021b).

Fig. 2 (B1 and B2) shows the response surfaces presenting the X_1X_3 interactions (ethanol:oil ratio and lipase), as well as its positive effect for increasing FAEE conversion. In this case, the optimal region for lipase was at 1.5 – 3 %, whereas for the ethanol:oil molar ratio (E:O) has an optimal region between 3:1 – 5:1. Fig. 2 (C1 and C2) shows response surfaces presenting the X_2X_3 interactions (water and lipase) as well as its positive contributions to the increase of FAEE conversion. The lipase concentration was in the optimum region between 2 % and 3 % and the water dosage between 3 % and 4.5 %. The presence of water and E:O (ethanol:oil molar ratio) interfere directly in the lipase action as a catalyst in the transesterification reaction, as discussed before. Thus, in the present work, according to the model, the optimal point for the lipase concentration was 2.43 %. The obtained value is lower than other works performed using this lipase (6 %) for the transesterification of *Semen Abutili* seed oil (Sun et al., 2021b).

For model validation, an experimental assay was carried out under the optimal condition (4.48:1 – ratio E:O; 3.41 – water (%) and 2.43 – lipase (%)) and the experimental value of the conversion was compared to the predicted value (Eq. 4). Although there is a difference between the predicted and observed conversion values (98.8 % and 97.11 %, respectively), the absolute and relative deviations (1.68 and 1.73, respectively) were lower than the average absolute and relative deviations in Table 4 (2.18 and 2.54, respectively), confirming model validation.

3.3. Degumming-Transesterification associated process

By applying the degumming parameters optimized by Passos et al., (2022b), together with the optimized parameters for transesterification optimized in this work, it was possible to evaluate two degumming-transesterification associated processes. The first process was called one-pot reaction (OPR) while the second was called two-pot reaction (TPR). Results are presented in Table 6. For comparison purposes, a control system was performed, in which transesterification was carried out without the degumming stage, i.e. using the crude soybean oil. Fig. 3 shows the reaction dynamics for the transesterification step for TPR and OPR reactions, showing the FAEE conversion.

Table 6
Comparison between biodiesel production processes.

Lipids	Control (crude oil biodiesel)	TPR	OPR	Purified TPR[a] Biodiesel
FAEE conversion (%)	95.7 ± 0.29	97.5 ± 0.29	94.1 ± 0.45	99.98 ± 0.001
FFA (%)	3.243 ± 0.052	3.360 ± 0.017	4.33 ± 0.01	0.16 ± 0.001
TAG (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
DAG (%)	0.09 ± 0.01	0.32 ± 0.02	0.225 ± 0.007	0.05 ± 0.02
MAG (%)	0.29 ± 0.01	0.35 ± 0.01	0.31 ± 0.02	0.27 ± 0.01
Glycerin (%)	0.104 ± 0.0001	0.025 ± 0.0015	0.081 ± 0.001	0.04 ± 0.0012
Moisture (%)	0.476 ± 0.025	0.397 ± 0.100	0.405 ± 0.021	0.053 ± 0.015
Mineral elements (mg/kg)				
P	80.7 ± 0.1	1.19 ± 0.10	4.33 ± 0.33	0.54 ± 0.02
Mg	29.12 ± 0.01	0.19 ± 0.03	0.19 ± 0.01	n.d.
Ca	60.6 ± 0.3	0.57 ± 0.06	n.d. < 0.1	n.d.
Fe	1.33 ± 0.04	n.d. < 0.1	0.38 ± 0.01	n.d.
Anions (mg/kg)				
Acetate	0.858 ± 0.054	0.804 ± 0.001	0.906 ± 0.001	0.092 ± 0.000
Chloride	0.141 ± 0.006	0.115 ± 0.001	0.000 ± 0.000	0.120 ± 0.011
Formate	1.200 ± 0.051	3.659 ± 0.023	0.540 ± 0.002	0.011 ± 0.001
Phosphate	0.050 ± 0.021	1.900 ± 0.000	0.307 ± 0.000	0.082 ± 0.002
Sulfate	0.128 ± 0.014	0.349 ± 0.000	0.413 ± 0.014	0.191 ± 0.002
Oxidative stability (h)	6.77 ± 0.05	5.70 ± 0.10	5.25 ± 0.03	5.30 ± 0.03

[a] Biodiesel TPR Purified with resin Amberlyst A26OH

Results shows that the TPR process was that with the higher conversion to FAEE (97.5 %), the lower phosphorus content (1.19 ± 0.10 mg/kg), and the faster conversion when compared to OPR. In TPR, transesterification was performed with degummed oil, i.e. phosphorus content lower than 10 mg/kg. During TPR, also, there are no competition between phospholipase and lipase. On the other hand, in OPR process, after degumming step, gums and phospholipase were not removed for biodiesel conversion, and phospholipase inactivation was performed. Therefore, some enzymatic interference could affect lipase activity – with the phospholipase causing a possible inhibition of lipase action. After 26 h of process, TPR has already obtained its maximum conversion into biodiesel, whereas for OPR, where 36 h was the time with maximum conversion, increasing process energy and costs. The results for the control process (transesterification with crude oil) clearly shows the importance of degumming. Despite this process obtained a significant conversion when compared to others, its high content of mineral elements, led to low-quality biodiesel, as their presence would cause corrosion, oxidation and other problems.

Considering that the TPR trial was those with the maximum conversion, it was further purified for decreasing acidity. It is interesting to

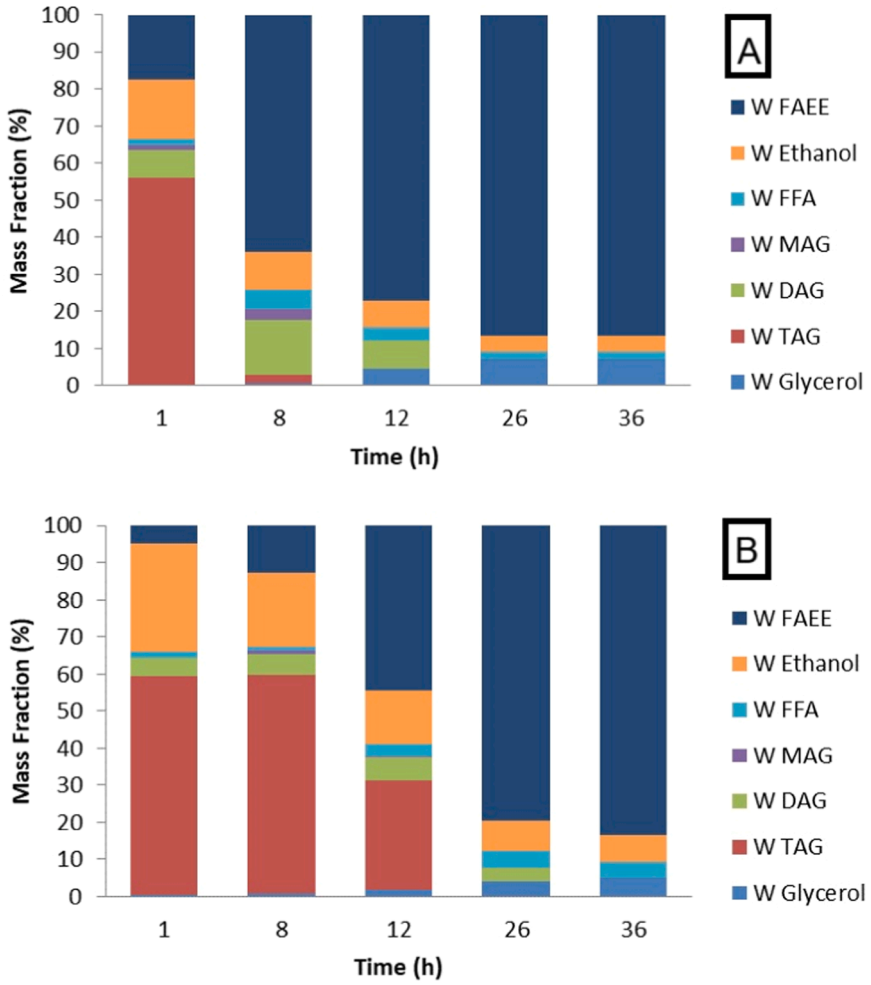

Fig. 3. Reaction dynamics A) TPR biodiesel B) OPR biodiesel. W is for mass fraction (%): FAEE (wt%), Ethanol (wt%), FFA (wt%), MAG (wt%), DAG (wt%), TAG (wt %) and Glycerol (wt%).

note that biodiesel acidity was higher than that observed for crude oil. This because the mechanism of esterification of *Aspergillus oryzae* lipase leads to fatty acid formation during the process, slightly increasing the acidity of the product. After purification, FAEE content increased to 99.98 % and acidity decreased to 0.16 %, lower than crude oil. In relation to other impurities such as non-converted TAG, and partial acylglycerols MAG and DAG levels, all biodiesels presented no traces of TAG and small content of acylglycerols. DAG content in TPR was not only due to transesterification but also due to degumming, that produces partial acylglycerols with breakdown of phospholipids. Purification could also decrease MAG and DAG content. Regarding the content of mineral elements, TPR biodiesel obtained the lowest levels and after purification, minimum amount of mineral elements remained. The purification also acted effectively in reducing other anions (impurities) presented in biodiesel, leading to biodiesel with higher quality. The biodiesel Purified TPR obtained 873.64 ± 0.01 kg/m^3 of density (20°C), 4.30 ± 0.014 mm^2/s of kinematic viscosity (40°C) and 180.6 ± 1.41°C of flash point. The parameters are within the standards required by the ANP (National Agency for Petroleum, Natural Gas And Biofuels) that correspond to the ranges: kinematic viscosity (40°C) = 3 – 5 mm^2/s, density (20°C) = 850 – 900 kg/m^3 e flash point = min. 100°C (ANP, 2023).

The glycerol side stream for TPR, OPR and control processes were also evaluated. Production of high-quality glycerol is also an advantage in biodiesel production, since it could be used in other process such as in chemical, pharmaceutical or cosmetic industries without further purification. Table 7 shows the characterization of the glycerol side streams.

Table 7
Characterization analyzes of glycerol side stream in different processes.

Lipids	Control	TPR	OPR
FFA (%)	1.55 ± 0.007	0.61 ± 0.014	1.02 ± 0.010
TAG (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MAG (%)	0.10 ± 0.001	0.00 ± 0.00	0.11 ± 0.0001
DAG (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mineral elements (mg/kg)			
P	148 ± 4	6.70 ± 0.07	19.02 ± 0.04
Mg	16.1 ± 0.2	0.67 ± 0.01	1.57 ± 0.01
Fe	0.14 ± 0.004	n.d.	n.d.
Ca	20.5 ± 0.4	3.84 ± 0.02	n.d.
Anions (mg/kg)			
Acetate	1.991 ± 0.002	2.613 ± 0.001	2.962 ± 0.006
Chloride	1.493 ± 0.012	1.590 ± 0.009	3.624 ± 0.036
Formate	21.623 ± 0.092	22.880 ± 0.071	20.087 ± 0.085
Phosphate	3.657 ± 0.011	0.961 ± 0.003	8.870 ± 0.028
Sulfate	0.740 ± 0.004	0.508 ± 0.002	0.656 ± 0.004

Glycerol from TPR (Fig. 3A) presented the lower final acidity, and zero content for TAG, DAG, and MAG, showing a higher degree of purity, producing a clearer product when compared to the glycerol from crude oil (Fig. 3B). It also presented lower levels of mineral elements, thus being a purer by-product of the transesterification and with a greater added value, mainly for commercialization in the pharmaceutical and cosmetic industries. On the other hand, glycerol from control process (biodiesel from crude oil, Fig. 3B) presented the higher levels of mineral elements, also highlighting the importance of degumming steps.

In this way, process optimization facilitates the process scale-up for the industrial sector. According to Kamal Pasha et al., (2024) the enzymatic biodiesel production process, using free and immobilized enzyme simultaneously, cost decreased of 13.44 %, decreased energy consumption of 86.8 %, and decreased GHG emissions of 78.86 %. Thus, presenting a process that not only reduces financial costs, but also brings advantages for the preservation of the environment. Therefore, process optimization facilitates the process scale-up for the industrial sector.

4. Conclusion

The use of phospholipase associated with the transesterification process generated a final biodiesel with fewer contaminants, as well as phosphorus content within that required by Brazilian law (ANP) - P < 10 mg/kg. The optimal transesterification parameters for *Aspergillus oryzae* lipase and ethanol as short-chain alcohol were achieved for E:O = 4.48:1, water = 3.41 % and lipase = 2.43 %, where 97 % fatty acid ethyl esters (FAEE) was obtained. The degumming-transesterification associated process for ethyl biodiesel production (TPR and OPR) using both enzymatic processes obtained quite good conversions, with TPR having higher FAEE conversion and lower impurities amount, reaching > 99 % after purification. In addition to a final biodiesel having characteristics closer to those required for its commercial use, the process generated a glycerol side stream with a lower content of impurities when compared to biodiesel from crude oil, which can be used for pharmaceutical or cosmetic industry. Enzymatic degumming associated to enzymatic transesterification with *Aspergillus oryzae* lipase showed to be a good alternative to improve not only sustainability, also considering the use of ethanol, but also to decrease time and cost of high-quality biodiesel and glycerol production.

CRediT authorship contribution statement

Klicia A. Sampaio: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Patrícia T. de Sousa:** Methodology, Formal analysis. **Marcelo A. Morgano:** Methodology, Formal analysis. **Ramon S. B. Ferreira:** Methodology, Formal analysis. **Rafaela Menezes dos Passos:** Writing – original draft, Methodology, Formal analysis, Conceptualization. **Marcela C. Ferreira:** Writing – review & editing, Supervision. **Guilherme J. Maximo:** Writing – review & editing. **Eduardo A. C. Batista:** Writing – review & editing, Supervision. **Antônio J. A. Meirelles:** Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rafaela Menezes dos Passos reports financial support was provided by Coordination of Higher Education Personnel Improvement. Rafaela Menezes dos Passos reports financial support was provided by National Council for Scientific and Technological Development. Rafaela Menezes dos Passos reports financial support was provided by State of Sao Paulo Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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