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In situ Transesterification of Crambe Seeds with Dimethyl Carbonate Using an Enzymatic Catalyst

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In situ transesterification of crambe seeds oil was investigated to produce fatty acid methyl esters using dimethyl carbonate as acyl acceptor and lipase Novozyme 435® as the catalyst. First, the effects of solvent-to-seeds ratio, seed pretreatment, and extraction time were evaluated on the seed oil removal, comparing the results with conventional Soxhlet extraction. Then, the effects of enzyme loading (5 to 30 wt.% based on the oil mass) and reaction time (60 to 540 min) were evaluated on the simultaneous extraction and reaction. The highest oil extraction yield (ca. 26 wt.%) was achieved at the solvent-to-seeds ratio of 6 mL g^{-1} and 360 min. The oil obtained from seeds that received thermal pretreatment showed a higher concentration of minor compounds. The highest ester content (76.71 wt.%) was obtained with an enzyme loading of 20 wt.%, solvent-to-seeds ratio of 6 mL g^{-1}, for 480 min. The reaction samples showed a predominance of erucate and oleate esters, with identification of the co-products formation (glycerol carbonate and glycerol dicarbonate), and ca. 5 wt.% of acylglycerols.

Keywords: *Crambe abyssinica* Hochst, oil extraction, methyl esters, Novozyme 435®, glycerol carbonate

Introduction

Currently, the European Union countries are responsible for more than 36% of biodiesel production of the world, and rapeseed oil is used as the main raw material for its production.[1] On the other hand, around 12% of the biodiesel production in the world comes from Brazil,[1] and soybean oil comprises about 70% of the raw material used for biodiesel production in the country.[2] To reduce competition between the food and energy sectors, crambe seed oil (*Crambe abyssinica* Hochst) has become an interesting alternative, as it has a short cultivation cycle (ca. 90 days)[3] and contains a high amount of oil in the seeds (26 to 42%).[4,5] Due to the presence of erucic acid (ca. 56%), crambe oil is not suitable for human consumption because this acid is toxic and can cause heart disease by increasing cholesterol levels and lipidosis in heart tissues.[5] Based on these characteristics, the use of crambe oil has been highlighted in several studies for biodiesel production, considering its higher oil productivity *per* hectare, when compared to soybean (traditionally used).[6-9]

Biodiesel is considered a relevant, economically viable, and sustainable substitute for diesel.[10,11] Biodiesel comprises monoalkyl esters of fatty acids, derived from renewable sources, such as vegetable oils, edible or not, and animal fats.[12] The most used method for biodiesel production is the transesterification reaction of a triglyceride and a short-chain alcohol, usually methanol or ethanol, with the formation of esters and glycerol. The by-product generated (glycerol) corresponds to ca. 10 wt.% of total biodiesel production and, despite having wide application in the pharmaceutical, food, cosmetic, polymeric, and oleochemical industries,[13-15] the high volume produced exceeds its demand, causing difficulties for the biodiesel industry regarding its

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destination, since it has become of low economic value in the chemical industry market.[16] Another factor that should be considered is that, when burned, glycerol can result in the emission of acrolein, a toxic and carcinogenic compound.[17]

The glycerol-free process is an alternative to overcome such disadvantages since it produces, instead of glycerol, another component that usually has a higher market value, or is miscible in the biodiesel phase, preserving its physicochemical characteristics and increasing global process yield.[18] The production of glycerol carbonate instead of glycerol, by replacing methanol with dimethyl carbonate (DMC), for instance, is one of the ways found to circumvent or solve glycerol deflation in the market, since glycerol carbonate has been applied as a plasticizer, surfactant, lubricant and considered a green solvent in the industry,[16,19,20] in addition to having attractive properties, such as non-flammability, non-toxicity, high water-solubility, and low evaporation rate.[18,21,22] Furthermore, glycerol dicarbonate can be applied as an additive or chemical intermediate.[16]

The DMC use can provide a strategic means of accelerating the reaction kinetics, since it is miscible with triglycerides,[12] leading to high yields in DMC-based transesterification as the formation of carbon dioxide shifts the equilibrium forward.[23] Besides eliminating the glycerol separation step in the biodiesel production process, DMC also presents advantages to methanol because it does not deactivate enzyme catalysts.[16]

The conventional production of biodiesel comprises several steps, including oil extraction, solvent removal, oil refining, reaction, and product purification, which add high costs to the process.[24] To optimize production reducing drawbacks, the *in situ* process can be considered. In such a process, extraction and reaction occur simultaneously, i.e., oil is not extracted from seeds or sources containing oil before the reaction. Instead, the oil-containing material contacts the acyl acceptor directly in the presence of a catalyst,[25,26] reducing processing time, and solvent and energy consumption.[27]

Among the catalysts frequently applied for biodiesel production, enzymatic heterogeneous ones offer greater ease of separation from the final product, the possibility of reuse, and the application of lower temperatures, which reduces the energy expending.[28,29] *In situ* reactions using enzymatic catalysts have become of great interest in recent studies aimed at biodiesel production.[26] Although only a few research works have been carried out on the topic yet, the enzyme most commonly applied as an *in situ* reaction catalyst for methyl esters production is the commercial lipase Novozyme 435®. This catalyst was used in the *in situ* interesterification with methyl acetate[7] and *in situ* transesterification with methanol[30] and DMC.[31,32]

With that in mind, this study aimed to investigate the *in situ* production of fatty acid methyl esters (FAMEs) from crambe seeds by applying DMC as extraction solvent and acyl acceptor concomitantly, and Novozyme 435® as the catalyst. For that, oil extraction was investigated by evaluating the effects of solvent-to-seeds ratio, seed pretreatment, and extraction time. The contents of active compounds in the oils obtained were determined. Subsequently, the production of FAMEs was investigated using lipase Novozyme 435® as the reaction catalyst, evaluating the effects of enzyme loading and reaction time. Finally, the reaction samples were characterized regarding the methyl esters profile, acylglycerols content, and co-products obtained. The conduct of this research addresses issues of public interest framed in three sustainable development objectives (7-affordable and clean energy; 9-industry, innovation and infrastructure, and 12-responsible consumption and production) established by the United Nations,[33] and in the Brazilian scenario it fits into the strategic area (Sustainable Development) established by The Ministry of Science, Technology and Innovation.[34]

Experimental

Material

Crambe seeds (*Crambe abyssinica* Hochst) provided by Fundação-MS were used, along with dimethyl carbonate (DMC) (Sigma-Aldrich, 99%, Saint Louis, Missouri, United States) as the extraction solvent and acyl acceptor for the reactions. Lipase Novozyme 435® (Novozymes Latin America LTDA, Araucária, Brazil) was used as the catalyst. The reagents used in the characterization of the oil were heptane (Neon, Suzano, Brazil), *N*,*O*-bis(trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (BSTFA with 1% TMCS, Sigma-Aldrich, Saint Louis, Missouri, United States), 5α-cholestane (Sigma-Aldrich, ≥ 97%, Saint Louis, Missouri, United States) and methyl heptadecanoate (Sigma-Aldrich, ≥ 99%, Saint Louis, Missouri, United States). The esters, glycerol carbonate, glycerol dicarbonate, and acylglycerol contents were determined using ethylene glycol (Vetec, 99.5%, Duque de Caxias, Brazil), heptane (Neon, Suzano, Brazil), *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (Sigma-Aldrich, ≥ 98.8%, Saint Louis, Missouri, United States), and the following chromatographic standards (Sigma-Aldrich, ≥ 99%, Saint Louis, Missouri, United States): methyl heptadecanoate, monoolein, diolein, and triolein.

Preparation of the raw material

To improve oil extraction, the whole seeds were submitted to a moistening combined with thermal pretreatment (TT) (120 °C for 1 h), as reported by Mello *et al.*[35] The crambe seeds were subjected to light crushing in an electric blender (Walita, LiqFaz, Itapevi, Brazil) to break the shells. Then, the shells were removed and the seeds were again crushed and submitted to granulometric classification according to the standard series of Tyler sieves (Bertel, Caieiras, Brazil), and the particles retained in the 28-mesh sieve were selected to conduct the experiments. After classification, the seeds were stored in plastic bags wrapped in aluminum foil at room temperature.

Oil extraction

For each test, 3 g of seeds were weighed in an Erlenmeyer with a glass lid (250 mL), then the established amount of solvent was added, and the Erlenmeyer was positioned in the orbital shaker (Marconi, MA 830/A, Piracicaba, Brazil) at 60 ºC and 180 rpm. The temperature was selected based on the results of Postaue *et al.*[36] and Stevanato and Silva.[37] At the end of the extractions, the samples were filtered through qualitative filter paper.

The conventional extraction was carried out in a Soxhlet extractor (45/50), which was coupled to a condenser, using a heating plate (Logen Scientific, Diadema, Brazil). Approximately 5 g of seeds and 150 mL of *n*-hexane were kept at a temperature above 68 °C, with continuous solvent reflux for 8 h of extraction. After that, the solvent excess in the samples was removed, and the oil yield was determined from the correlation between the mass of oil obtained and the mass of seeds fed into the Erlenmeyer.

The characterization of the oil was evaluated simultaneously for the contents of phytosterols, tocopherol, and free fatty acids (FFA) using a gas chromatograph-mass spectrometer (GC-MS) (Shimadzu, model GCMS-QP2010 SE, Tokyo, Japan). The samples were previously treated and analyzed in the chromatographic conditions described by Stevanato *et al.*[38]

In situ reaction and chemical analyses of products

The same procedure previously mentioned for extraction was carried out; however, the Erlenmeyer (reactor) was fed with the seeds, DMC, and lipase. The enzymes, before being placed in the reactor, were activated at 40 °C in an oven with air circulation (Marconi, MA 035, Piracicaba, Brazil). A limitation in the proposed methodology was detected in preliminary tests regarding the separation of the seeds from the catalyst; however, that was solved by placing the seeds in filter paper (without compromising the effectiveness of the process). Therefore, after the reaction time, the filter paper was removed from the extraction medium and the samples were filtered (to remove the enzymes), and the excess solvent in the filtrate was evaporated until constant weight.

To determine the methyl esters content, the reaction was diluted in heptane, methyl heptadecanoate was added to the solution and solutions were analyzed as described by Trentini *et al.*[39] To identify the methyl esters, the samples were diluted in heptane and analyzed in GC-MS (Shimadzu, model GCMS-QP2010 SE, Tokyo, Japan) as reported by Iwassa *et al.*[40] Afterward, identification was performed by comparing the mass spectrum of each compound with the NIST 14 and NIST 14s Mass Spectral Library. The quantification of each methyl ester was performed by area normalization, determining the area percentage of each methyl ester in relation to the total area of the chromatogram.

To determine the glycerol carbonate and glycerol dicarbonate contents, the sample was diluted in ethanol, and ethylene glycol was added to the solution as the internal standard. Sample preparation and gas chromatography analysis conditions were described by Postaue *et al.*[41] The GC-MS (Shimadzu, model GCMS-QP2010 SE, Tokyo, Japan) was used to identify the compounds: for that, the mass detector operated in Scan mode, on a scan range of 30 to 600 *m/z*. The relative intensity of glycerol carbonate fragmentation ions (43/100, 44/95, 31/71, and 87/29), and glycerol dicarbonate (43/100, 45/83, 59/77, 90/35, 31/32, and 77/29), confirmed their presence in the sample.

The acylglycerols content analysis was carried out following the derivatization protocol of Standard UNE EN 14105,[42] analyzed in a gas chromatograph (Shimadzu, GC-2010 Plus, Tokyo, Japan) equipped with a B-5HT Inferno™ capillary column (Zebron, 10 m × 0.32 mm × 0.10 µm, Torrance, USA) and chromatographic conditions described by Trentini *et al.*[43] The quantification of acylglycerols was performed based on calibration curves (correlation coefficients $R^2 > 0.99$) built from at least eight concentration levels by serial dilution. Standard concentrations ranged from 0.05-2.5 mg mL^{-1} for monoolein (monoglycerides), 0.025-2 mg mL^{-1} for 1,3-diolein (diglycerides), and 0.025-2.5 mg mL^{-1} for glyceryl trioleate (triglycerides).

Statistical analyses

All experiments and analyses were conducted in duplicate (mean ± standard deviation (SD)) and the

difference between the variables was estimated from the analysis of variance (ANOVA) and Tukey's HSD (Honestly Significant Difference), adopting $p < 0.05$ as statistically different. For that, it was used the Statistica 8.0 software (StatSoft, Inc., Tulsa, OK, USA).[43] In figures and tables, means followed by identical letters do not statically differ ($p > 0.05$).

Results and Discussion

Oil extraction

Figure 1 shows the oil yield as a function of the variation in the solvent-to-seeds ratio from 4 to 10 (mL g^{-1}) during 60 min of extraction. It is observed that there was an increase of ca. 14% in the oil yield after increasing the ratio from 4 to 6. Considering the small effect on the extraction efficiency when using a solvent-to-seeds ratio between 8 and 10 mL g^{-1}, which is unfeasible for both the cost of the process and environmental factors, the ratio of 6 mL g^{-1} was selected for further experiments.

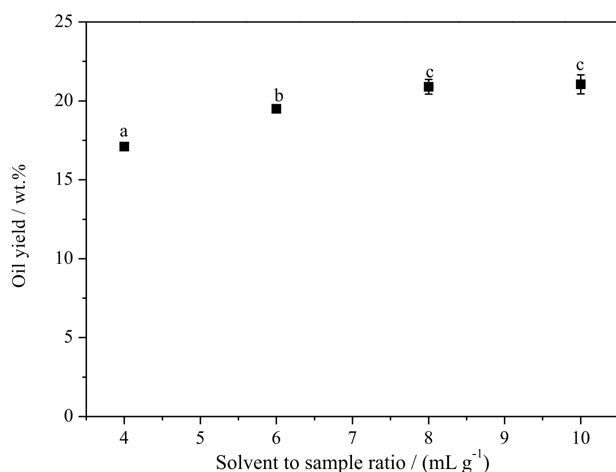

Figure 1. Influence of solvent-to-seeds ratio on extractions performed at 60 °C, 180 rpm, and 60 min.

The increase in the solvent-to-seeds ratio stimulates the solvent permeation in the cell walls of the solute, promoting greater solubility and, consequently, an increase in the diffusion coefficient.[43,44] Additionally, the increase in the concentration gradient between the solvent and the solute implies a variation in the Gibbs free energy, which makes it more negative, as it reduces as the medium becomes more diluted, meaning that the degree of the spontaneity of extraction is favored with an increasing volume of solvent.[45,46]

Another parameter that justifies the increase in the oil yield proportional to the solvent volume is the mass transfer coefficient (k). Chanioti and Tzia[47] reported values from 1.36 to 1.81 min^{-1} for k, when increasing the sample-to-solvent ratio from 1:4 to 1:12 (g mL^{-1}), respectively. The same was observed for studies carried out by Sulaiman *et al.*[48] in coconut oil extraction, in which the k value increased by ca. 33% with the ratio increasing from 1:6 to 1:12 (g mL^{-1}).

Figure 2 shows the oil yield obtained for the solvent-to-seeds ratio of 6:1 (mL g^{-1}) according to the different extraction times evaluated, using seeds after thermal pretreatment (TT) and untreated seeds (UTS). Note that the longer the extraction time, the greater the amount of oil extracted, since the extraction process requires enough time for solvent penetration into the plant matrix.[49]

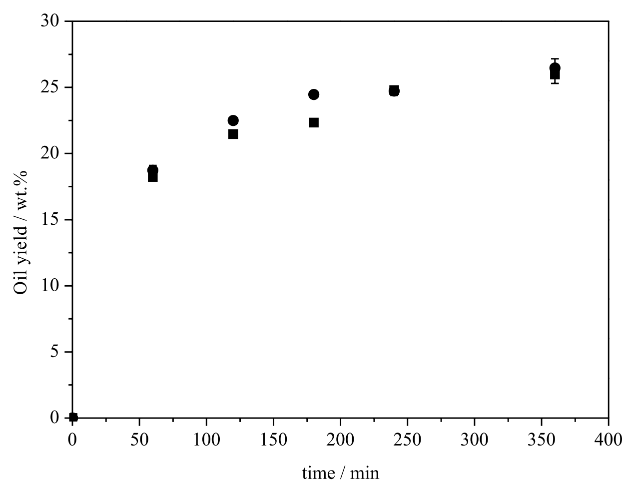

Figure 2. Influence of extraction time on extractions performed at 60 °C and 180 rpm using untreated seeds (■), and seeds after thermal pretreatment (●).

It can be seen from Figure 2 that the time increase from 60 to 360 min influenced the percentage increase by ca. 42% in the oil yield. Moreover, in the initial times, the extraction rate is faster and, during the process, the increase in yield occurs at a slower rate. This variation happens due to the two stages in which the extraction takes place. The first phase consists of a faster extraction speed, in which solute extraction from the surface sites of the seeds prevails, and presents a higher diffusion coefficient; while the second phase consists of a slower speed, as it corresponds to the extraction of the most internal constituents of the plant matrix and, therefore, presents a lower diffusion coefficient.[50,51] That can be confirmed by the high mass transfer coefficients due to the washing process compared to the diffusion ones, reported by Abdullah and Bulent[52] and Chanioti and Tzia[47] in *Nigella sativa* and olive oil extraction, respectively.

It was found 38.83 ± 0.7% of oil in the seeds when the conventional Soxhlet extraction was performed, which corroborates with the reported values (32 to 42%) of oil from crambe seeds.[4,5,53] The thermal pretreatment of the seeds did not influence the oil extraction and, therefore, it

was possible to obtain, in 360 min of extraction, ca. 69% of the total oil content present in the seeds.

Table 1 presents the characterization results, in terms of minor compounds, of the oils obtained at 360 min of extraction.

The oil obtained from seeds without pretreatment (UTS) showed a higher FFA content than the one obtained from seeds that underwent TT. The results obtained were lower than those reported using methyl acetate in ultrasound (1.17%)[4] and orbital shaker (12%).[36]

Regarding the composition of phytosterols, the oils showed a predominance of β-sitosterol, as already reported.[5,36] The TT favored the removal of phytosterols and γ-tocopherol by 20 and 9%, respectively. This increased extraction when TT was applied is associated with the modification and rupture of oleaginous plant cells, allowing greater release of compounds.[54] Iwassa *et al*.[40] reported an increase of 65 and 71% in the removal of γ-tocopherol and phytosterols, respectively, in pressurized propane extraction, when compared to untreated crambe seeds. It is also mentioned that the applied technique proved to be efficient, since Santos *et al*.[55] reached a phytosterol content of only 201 mg *per* 100 g of oil, in the extraction with propane at 40 °C and 160 bar. Trentini *et al*.[56] obtained a similar result, with 272 and 128 mg of phytosterols and γ-tocopherol, respectively, *per* 100 g of oil extracted with DMC at 140 °C and 100 bar.

In situ reaction

Effect of enzyme loading

Figure 3 shows the FAMEs content at 60 °C, reaction time of 360 min, solvent-to-seeds ratio of 6:1 (v/m) (mL g^{-1}) with the enzyme loading varying from 5 to 30 wt.% (based on the oil mass). From this figure, it is noted that when varying the enzyme concentration in the reaction medium from 5 to 20 wt.% (based on the oil mass), the methyl esters content increased from 27.22 to 74.32%. However, it was found that percentages higher than 20 wt.% did not change the yield, as the reaction possibly reached its chemical equilibrium.

The increased production of methyl esters at higher enzyme load can be attributed to the fact that higher amounts of lipases allow more substrate molecules to be absorbed by the active site of the enzyme, favoring catalysis and product formation.[57] Kim *et al*.[30] reported higher methyl esters yield when increasing the percentage of Novozyme 435® lipase from 5 to 50 wt.% (based on the microalgae mass) in the transesterification of *Aurantiochytrium* sp. KRS101 biomass and dimethyl carbonate, as also observed by Nguyen *et al*.[58] in the interesterification of the Black soldier (*Hermetia illucens*) fly larvae (BSFL) fat and methyl acetate, when Novozyme 435® loading increased from 10 to 20 wt.% (based on the substrates mass).

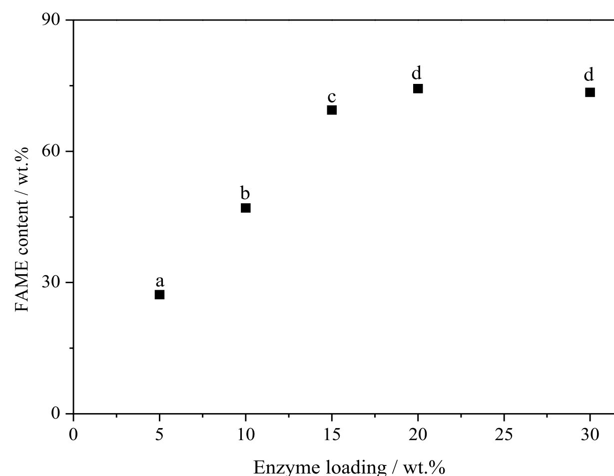

Figure 3. Effect of enzyme loading on methyl esters content in reactions conducted at 60 °C, 180 rpm, and solvent-to-seeds ratio of 6:1 (mL g^{-1}) using thermally treated seeds.

Reactions catalyzed by immobilized lipases occur at the sample/catalyst interfaces, so a high concentration of the enzyme can form a monolayer at the interface, and thus an additional increase in loading would not contribute to the reaction rate.[59] Rusli *et al*.[60] explain that the use of excessive enzyme concentration in the reaction medium usually promotes saturation at the substrate/enzyme interface, directly affecting the reaction rates. Once saturation has been reached and the interface is completely covered by enzyme molecules, additional enzymes do not have access to the substrates and, therefore, there is no increase in the reaction rates.

In addition, an excess of enzyme in the reaction medium can lead to aggregates formation that will consequently block the enzyme active sites available to

Table 1. Characterization of oil obtained from untreated crambe seeds (UTS), and after thermal pretreatment (TT)

Oil	FFA / wt.%	Phytosterol / (mg *per* 100 g of oil)			γ-Tocopherol / (mg *per* 100 g of oil)
		Stigmasterol	β-Sitosterol	Campesterol	
UTS	0.79 ± 0.04[a]	58.30 ± 0.50[a]	111.23 ± 1.53[a]	56.81 ± 0.26[a]	115.14 ± 1.88[a]
TT	0.65 ± 0.02[b]	69.45 ± 2.14[b]	141.24 ± 3.64[b]	61.41 ± 0.09[b]	125.75 ± 1.69[b]

FFA: free fatty acids. Means followed by identical letters do not statically differ ($p > 0.05$).

the substrates, which would lead to a decrease in the total surface area available for the reagents and would not increase the conversion of triglycerides into esters.[30,61] In a research carried out with Novozyme 435®, Tavares *et al.*[7] reported that the 40 wt.% enzyme loading (based on the oil mass) reduced the production of methyl esters in the interesterification of crambe oil and methyl acetate, which was also observed by Jo *et al.*[23] when increasing Novozyme 435® loading in the reaction medium from 30 to 50 wt.% (based on the oil mass).

Despite the increased enzyme demand, the advantages of the simultaneous extraction and reaction process as well as the greater added value of the co-products obtained by the proposed route must be considered. For instance, one advantage of using DMC is the potential for significant cost reduction in catalyst usage due to the longer operational life and reusability of lipases. Therefore, in future work, economic analysis considering all these aspects must be conducted.

Effect of reaction time

Figure 4 shows the influence of the reaction time (60 to 540 min) on the FAMEs content using a solvent-to-seeds ratio of 6:1 (v/m) (mL g^{-1}) and enzyme loading of 20 wt.% (based on the oil mass). By increasing the time from 60 to 360 min, a percentage increase of ca. 194% in methyl esters content is observed. However, from 360 min of reaction on, the formation of the esters became constant, and at 540 min an increase of only 2.46% in esters was observed.

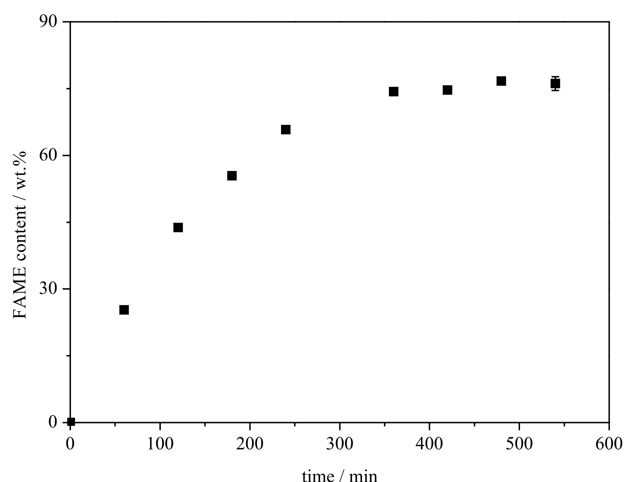

Figure 4. Effect of reaction time on methyl esters content in reactions conducted at 60 °C, 180 rpm, solvent-to-seeds ratio of 6:1 (mL g^{-1}), and enzyme loading of 20 wt.% (based on the oil mass).

In the short reaction times, the oil conversion rates into esters are higher, because the oil extraction rate is favored in the first minutes of extraction, and thus the lipase reacts easily with the constituents present on the surface of the sample. On the other hand, throughout the reaction, the esters formation rates are slower, since the lipase mechanism of interaction with the most internal components of the sample is hampered, due to the diffusion mechanism of oil extraction.

Jo *et al.*[23] and Kim *et al.*[30] report similar findings in the *in situ* transesterification of microalgae using lipase Novozyme 435® as the reaction catalyst, and DMC as solvent. The formation of methyl esters increased proportionally over the reaction time and the equilibrium was reached in 6 and 9 h of process, respectively. Stevanato and Silva[37] reached the reaction equilibrium in 12 h in the *in situ* reaction of crambe seeds and ethanol using the same biocatalyst.

In the proposed method, it was possible to obtain a maximum yield of 76 wt.% in esters, which is higher than that reported by Postaue *et al.*,[9,36] who obtained a maximum content of 60.57 and ca. 54 wt.% of esters, respectively, conducting the process at high pressure and temperature (20 MPa at 325 °C) using crambe oil and methyl acetate as acyl acceptor.

Table 2 presents the methyl esters profile of the samples obtained at times of 360, 420, 480, and 520 min, as well as the levels of glycerol carbonate, glycerol dicarbonate, and acylglycerols detected in these samples.

The samples showed a predominance of unsaturated esters, including linoleate, oleate, and erucate. In general, the reaction time did not influence the ester profile, noting that even the content of the esters that have lower thermal stability due to double bonds and bis-allylic positions (linoleate and linolenate)[9] were not reduced by prolonging the reaction time.

The glycerol carbonate content showed a percentage reduction of ca. 13% when increasing the time from 360 to 480 min, probably due to its consumption in the reaction. On the other hand, in 540 min the highest glycerol carbonate content was reached (ca. 227 ppm). In a study carried out by Postaue *et al.*,[41] the authors reported a maximum content of only ca. 47 ppm for the reaction carried out at 250 °C and 100 bar.

The formation of glycerol dicarbonate was confirmed by GC-MS by comparing the mass spectra of the compound with the literature.[62] The results showed that the glycerol dicarbonate content was reduced with increasing reaction time. Kim and Lee[63] also verified a reduction in the glycerol dicarbonate content and associated this behavior with the conversion of the compound into glycerol carbonate. The results achieved are of high interest since the *in situ* and glycerol-free reaction can significantly increase the profitability of biodiesel production, considering the synthesis of co-products.[64]

Table 2. Composition of the *in situ* reaction product obtained at different reaction times using enzyme loading of 20 wt.% at 60 ºC

Property		Reaction time / min			
		360	420	480	540
FAMEs / %	myristate	0.10 ± 0.01^{a}	0.07 ± < 0.01^{ab}	0.06 ± 0.01^{b}	0.07 ± 0.01^{ab}
	palmitate	2.15 ± 0.06^{b}	2.08 ± 0.17^{b}	2.28 ± < 0.01^{b}	2.68 ± 0.01^{a}
	palmitoleate	0.13 ± 0.02^{a}	0.10 ± < 0.01^{a}	0.14 ± 0.01^{a}	0.13 ± < 0.01^{a}
	stearate	1.14 ± 0.04^{a}	1.24 ± 0.12^{a}	1.17 ± 0.02^{a}	1.31 ± 0.02^{a}
	oleate	18.98 ± 0.02^{a}	18.45 ± 0.80^{a}	19.16 ± 0.03^{a}	19.32 ± 0.05^{a}
	linoleate	7.11 ± 0.04^{b}	6.98 ± 0.46^{b}	7.28 ± <0.01^{ab}	8.07 ± 0.04^{a}
	linolenate	3.90 ± 0.09^{a}	3.69 ± 0.41^{a}	3.86 ± 0.01^{a}	3.74 ± < 0.01^{a}
	arachisate	1.16 ± 0.05^{a}	1.23 ± 0.04^{a}	1.19 ± 0.01^{a}	1.22 ± 0.02^{a}
	gadoleate	5.74 ± 0.02^{a}	5.73 ± 0.40^{a}	5.88 ± 0.02^{a}	5.80 ± 0.13^{a}
	behenate	2.07 ± 0.08^{a}	2.23 ± 0.15^{a}	2.07 ± 0.01^{a}	2.37 ± 0.02^{a}
	erucate	54.51±0.48^{a}	55.18 ± 1.97^{a}	53.93 ± 0.06^{a}	52.34 ± 0.13^{a}
	lignocerate	0.71 ± 0.01^{a}	0.81 ± 0.10^{a}	0.80 ± 0.02^{a}	0.78 ± 0.10^{a}
	nervonate	1.48 ± 0.06^{a}	1.47 ± 0.04^{a}	1.41 ± < 0.01^{a}	1.42 ± 0.02^{a}
	not identified	0.83 ± 0.03	0.83 ± < 0.01	0.79 ± 0.03	0.75 ± 0.06
Glycerol carbonate / ppm		125.77 ± 0.7^{b}	105.49 ± 0.82^{c}	108.90 ± 1.59^{c}	227.16 ± 2.28^{a}
Glycerol dicarbonate / ppm		550.88± 1.63^{a}	291.82 ± 9.96^{b}	184.85 ± 3.34^{c}	130.79 ± 1.25^{d}
Triglycerides / wt.%		5.97 ± 0.28^{a}	3.37 ± 0.30^{b}	2.83 ± 0.32^{b}	1.91 ± 0.33^{c}
Diglycerides / wt.%		4.52 ± 0.45^{a}	2.12 ± 0.25^{b}	2.16 ± 0.18^{b}	1.17 ± 0.16^{c}
Monoglycerides / wt.%		0.19 ± 0.01^{b}	0.36 ± 0.02^{a}	0.36 ± 0.03^{a}	0.22 ± 0.01^{b}

FAMEs: fatty acid methyl esters. Means followed by identical letters do not statically differ ($p > 0.05$).

The triglycerides and diglycerides contents followed a trend similar to that of glycerol dicarbonate, with a reduction when the reaction time was prolonged, while the monoglycerides content varied over time. Other researchers have also reported the acylglycerols content for the reaction with crambe oil, as Postaue *et al*.[36] and Postaue *et al*.,[41] obtaining 4.5 and 3.1 wt.%, in the reaction with methyl acetate (20 MPa at 300 °C) and dimethyl carbonate/ethanol (10 MPa at 250 °C), respectively, but these authors applied severe reaction conditions when compared to those used in this research (low pressure at 60 °C).

Conclusions

In this work, *in situ* transesterification of crambe seeds was performed by replacing methanol with DMC as extractant solvent and acyl acceptor simultaneously, adding Novozyme 435® as the catalyst, and generating glycerol carbonate instead of glycerol, as the main co-product. Approximately, 76.71 wt.% of FAMEs were obtained when 20 wt.% of biocatalyst was added to a solvent-to-seeds ratio of 6 mL g^{-1}, for 480 min at 60 ºC and 180 rpm. The highest glycerol carbonate content was reached in 540 min of reaction in the same conditions. Besides that, glycerol dicarbonate and acylglycerols were also obtained. Hence, *in situ* transesterification of crambe seeds and DMC catalyzed by Novozyme 435® is an efficient alternative to produce glycerol-free biodiesel, along with the synthesis of co-products of interest, which do not need to be separated from the biofuel obtained. The advancement of this technology has a potential impact on the global energy matrix and, for this reason, the next stages of study should include the application of a separation process to increase the purity of the product obtained, as well as the economic and life cycle analysis of the proposed process, to increase scale and industrial application.

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Author Contributions

Camila da Silva conceptualized, wrote, proofread, and edited the work; Thainara B. Massa investigated, executed the methodology, and wrote the work; Bruna Tais F. de Mello wrote the work; Mirian Cristina Feiten edited the work; Natália Stevanato prepared and wrote the first draft; Najla Postaue executed the methodology, and wrote the work. All authors have read and approved the final manuscript.

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