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Performance of Liquid Eversa on Fatty Acid Ethyl Esters Production by Simultaneous Esterification/Transesterification of Low-to-High Acidity Feedstocks

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Abstract: Liquid Eversa was evaluated in hydrolysis of acylglycerols from soybean oil deodorizer distillate (SODD), as well as simultaneous esterification/transesterification of SODD with low-to-high free fatty acids (FFAs) content using ethanol as acyl acceptor. Hydrolysis of SODD at mild temperature (37 °C) and without pH control (water:SODD mass ratio of 4:1) increased its FFAs content from 17.2 wt.% to 72.5 wt.% after 48 h reaction. A cold saponification of SODD allowed a saponification phase (SODD-SP) to be recovered with 93 wt.% saponification index and 2.25 wt.% FFAs content, which was used to find the experimental conditions for simultaneous esterification/transesterification reactions by experimental design. Temperature of 35 °C, enzyme concentration of 8.36 wt.%, and molar ratio of 3.64:1 (ethanol:SODD-SP) were found as the best conditions for fatty acid ethyl esters (FAEEs) production from SODD-SP (86.56 wt.% ester yield after 23 h reaction). Under the same reaction conditions, crude SODD (17.2 wt.% FFAs) and hydrolyzed SODD (72.5 wt.% FFAs) yielded products containing around 80 wt.% FAEEs. Caustic treatment could increase the ester content to around 90 wt.% and reduce the FFAs content to less than 1 wt.%. Our results show the good performance of liquid Eversa in aqueous (hydrolysis reactions) and organic (esterification/transesterification reactions) media.

Keywords: liquid Eversa; SODD; hydrolysis; simultaneous esterification and transesterification; substrate low to high acidity

1. Introduction

There is in the market a low-cost liquid lipase (Eversa Transform, a variant lipase from *Thermomyces lanuginosus*) specially formulated for the biodiesel industry [1–4]. It has been reported that this enzyme exhibits excellent performance in both liquid and immobilized forms, with both methanol and ethanol as acyl acceptors, and using refined and acid feedstocks (alkyl esters yields up to 99%) (Table A1 in Appendix A) [1,5–19].

Among the several fatty-rich industrial co-products, the soybean oil deodorizer distillate (SODD) is an interesting low-cost raw material as source of fatty acids. The SODD is a by-product of the soy oil refining, generated in the oil deodorization step [20] that is carried out to remove volatile compounds responsible for unacceptable odor, color and taste in the quality standard of oils for commercialization [20,21]. This by-product is mostly composed

of FFAs, acylglycerols (monoglycerides (MAGs), diglycerides (DAGs) and triglycerides (TAGs)) and smaller amounts of tocopherols, free sterols and scalene [22–24].

Due to the high content of saponifiable materials (up to 90 wt.%), deodorizer distillates (DDs) of vegetable oils (soy, palm, rapeseed, etc.) have been exploited as raw material for biodiesel production (Table A2 in Appendix A) [25–30], reaching ester yields from 88% to about 98%, mainly using commercial immobilized lipases (Lipozyme IM, Novozym 435 and Lipozyme RM-IM) [25–30]. According to our research in the scientific literature, there are still no works on the production of fatty acids ethyl esters (FAEEs) from SODD using liquid Eversa.

This work aimed to evaluate the performance of liquid Eversa in the simultaneous esterification of FFAs and transesterification of acylglycerols in feedstocks containing low-to-high FFAs content, employing SODD as model substrate. For this purpose, crude SODD (containing around 17 wt.% FFAs, as described below) was pretreated by two processes, aiming to reduce (cold saponification) or increase the FFAs (Eversa hydrolysis) content in the SODD. The experimental conditions for obtaining high ester yields by simultaneous esterification/transesterification using liquid Eversa as biocatalyst were evaluated by statistical design. At the defined conditions, FAEEs were produced from SODD containing low, medium, and high FFAs content and using ethanol as acyl acceptor. Finally, as an attempt to increase the FAEEs content in our product, a caustic treatment was adopt under the previously reported conditions [1,5].

2. Results and Discussion

2.1. Soybean Oil Deodorizer Distillate (SODD) Characterization

As the SODD is a by-product of oil refining, its composition depends on the oil source and the processing steps. In this way, its physical-chemical characterization is important to verify the particularities of the raw material under study. The physical-chemical properties of the SODD (Table 1) show a saponification index (181.62 ± 0.96 mg KOH/g) close to that reported by Yin et al. [31,32] (154.87 mg KOH/g ± 2.62), who used SODD for biodiesel production. The saponification index of refined soybean oil is in the range 180–200 mg KOH/g [33], therefore, our results were very close to these values, indicating that the SODD had similar characteristics to refined oil in terms of that index.

Table 1. Physicochemical properties of the soybean oil deodorizer distillate (SODD).

Parameter	Results	Method
Acidity index (mgKOH/g) (25 °C)	34.19 ± 0.52 (17.18 wt.%)	[34]
Iodine index by the Wijs method (gI_2/100 g) (25 °C)	112.98 ± 0.32	[35]
Saponification index (mgKOH/g) (25 °C)	181.62 ± 0.96 (91.27 wt.%)	[36]
Kinematic viscosity (mPa.s) (40 °C)	32.74 ± 0.01	Note 1
Kinematic viscosity (mPa.s) (25 °C)	57.10 ± 0.01	Note 1
Moisture (%) (130 °C)	1.33 ± 0.04	[37]
Saponifiable matter as fatty acid methyl esters (FAME) (wt.%)	85.15 ± 0.53	[38,39]
α-Tocopherol (g/100 g) (%)	0.37 ± 0.001	[40]
β-Tocopherol (g/100 g) (%)	0.09 ± 0.005	[40]
γ-Tocopherol (g/100 g) (%)	1.01 ± 0.003	[40]
δ-Tocopherol (g/100 g) (%)	0.36 ± 0.003	[40]
Total of tocopherols (g/100 g) (%)	1.83	[40]
Palmitic acid (C16:0) (g/100 g) (%)	3.19 ± 0.01	[41]
Stearic acid (C18:0) (g/100 g) (%)	0.99 ± 0.03	[41]
Oleic acid (C18:1) (g/100 g) (%)	5.43 ± 0.04	[41]

Table 1. *Cont.*

Parameter	Results	Method
Linoleic acid (C18:2) (g/100 g) (%)	7.91 ± 0.15	[41]
Linolenic acid (C18:3) (g/100 g) (%)	0.95 ± 0.06	[41]
Total of free fatty acids (g/100 g) (%)	18.47	[41]

Note 1: Rheometer (Brookfield DV-III Ultra with bath TC-650. Brookfield Brazil. Middleboro. MA. EUA) and spindle SC4–27. program Rheocalc V3.3 Build 49–1.

Regarding the FFAs content, the SODD acidity index (34.19 ± 0.52 mg KOH/g) was lower than those previously reported for deodorizer distillates of soybean oil (107.64 ± 228 mg KOH/g) [31,32], palm oil (191.69 mg KOH/g) [27], rice oil (163.66 ± 0.57 mg KOH/g) [42], and rapeseed oil (97.61 ± 1.87 mg KOH/g) [43]. In general, the content of FFAs of vegetable oil by-products depends on the composition of the original oil, as well as the deodorization conditions; thus, the physical-chemical parameters of deodorizer distillates can vary according to the oilseed [20].

The iodine index is a parameter related to the degree of the oil unsaturation [35]. The value obtained for the SODD (112.98 ± 0.32 gI_2/100 g) was about twice higher than that obtained for palm oil deodorizer distillate (63.8 gI_2/100 g) [27], since soybean oil is rich in polyunsaturated fatty acids, while palm oil is rich in saturated fatty acids. However, for biodiesel production it is recommended an iodine index less than 115 gI_2/100 g [44]. The moisture of SODD (1.33 ± 0.04%) was higher than those of refined oils (less than 0.5%) [45], but this parameter can be corrected for biodiesel production, since the presence of water may favor the hydrolysis of the esters produced, thus reducing the reaction yield. The kinematic viscosity of the SODD at 25 °C (57.10 ± 0.01 mPa.s) was very close to that of refined soybean oil [46] that is consistent with the high content of acylglycerols (MAGs, DAGs and TAGs) in the SODD (~70 wt.%).

The total content of FFAs and acylglycerides was also quantified by gas chromatography in terms of fatty acid methyl esters (FAMEs). For that, all glyceridic matter was previously submitted to an alkaline transesterification with methyl alcohol, converting it totally into FAMEs. Table 1 shows that the SODD is mainly composed of saponifiable matter (85.15 ± 0.53 wt.%), which makes it an excellent raw material to produce biodiesel.

Soybean oil is a good source of tocopherols (α-, β-, γ- and δ-tocopherols) [24], but part of these compounds are lost in the oil deodorization, making the SODD a good source of tocopherols too. The total of tocopherols in the SODD under study (around 1.8 wt.%) was around 10-fold lower than those reported in the literature (16.3–18.2%) [20]. This difference can be attributed to the fact that the degradation of tocopherols occurs quickly [23]. Tocopherols are prone to degradation at alkaline conditions and at high temperature (up to 61% degradation at 300 °C during the oil distillation process) [47].

The FFAs in the SODD were majority composed of linoleic, oleic and palmitic acids, since soybean oil is composed of linoleic acid (51%), oleic acid (23%), palmitic acid (10%), linolenic acid (7–10%) and stearic acid (4%) [48]. However, the total content of FFAs (18.47 wt.%) found in this work was lower than those reported by Kasim et al. [49] and Gunawan et al. [22], 41.15 ± 0.39% and 45.38 ± 2.13%, respectively. However, this parameter depends on the operational conditions of the deodorization process (temperature and pressure).

2.2. SODD Saponification

A cold saponification method was adopted in this study aiming to recovery the saponifiable matter of the SODD. The saponifiable phase (93.03 wt.% saponification index, 2.25 wt.% FFA and 1.33 wt.% tocopherols) was selected to carry out the statistical design to define better reaction conditions to produce FAEEs (discussed below). The saponification method adopted here degraded a large amount of tocopherols, mainly α-tocopherol, as will be discussed below. Tocopherol's degradation during saponification processes was also reported by Maniet et al. [50].

2.3. SODD Hydrolysis Reaction

SODD was hydrolyzed using free Eversa as biocatalyst. As the *Thermomyces lanuginosus* lipase, Eversa (a genetically-modified variant of *Thermomyces lanuginosus* lipase) is a 1,3-specific lipase [1] that mainly releases acyl moieties linked to the sn-1 and sn-3 positions at the glycerol backbone, being the hydrolysis of TAGs to DAGs faster than the hydrolysis of MAGs [51,52].

Figure 1 shows that after 48 h hydrolysis, a FFA yield of 72.48 ± 2.91 wt.% was reached, remaining approximately constant after 72 h reaction (FFA yield of 72.64 ± 3.76 wt.%). This FFA yield represents a reaction conversion of around 80% (based on the initial saponification index, 91.27%). Other works using *Thermomyces lanuginosus* lipase also report hydrolysis conversions in the same magnitude order, viz. 89% [53] and 94% [54], using soybean oil and waste cooking oil, respectively. However, the hydrolysis conversions are not directly comparable because of different reaction conditions. Besides FFAs, the hydrolysis product also contained non-converted acylglycerols, esters and tocopherols, as follows (in wt.%): 8.02 ± 0.05 MAGs, 7.12 ± 0.01 DAGs, 8.62 ± 0.03 TAGs, 3.76 ± 0.01 esters, and 0.28 total tocopherols (0.09 ± 0.004 α-tocopherol, 0.12 ± 0.11 β-tocopherol, 0.04 ± 0.001 δ-tocopherol, and 0.03 ± 0.001 γ-tocopherol).

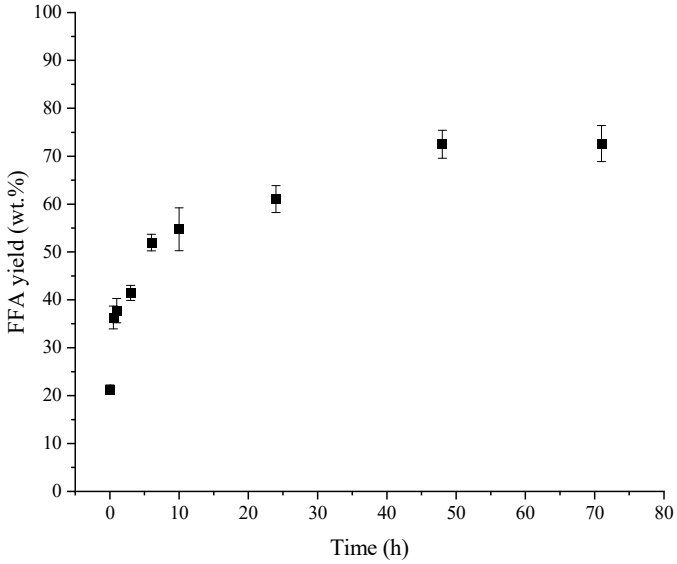

Figure 1. Free fatty acid (FFA) yield profile (wt.%) vs. time for the hydrolysis of SODD by the enzyme Eversa Transform 2.0. Reaction conditions: up to 72 h reaction; 37 °C; 4:1 mass ratio (H_2O:SODD) and 5% enzyme (m/mSODD).

2.4. Simultaneous Transesterification and Esterification Reactions

As commented above, the SODD saponifiable phase (SODD-SP, 93.03% saponification index) was used as substrate in the statistical design assays. The levels of the variables of the statistical design were based on reaction conditions previously reported for the synthesis of biodiesel using Eversa Transform (Table A2 in Appendix A) to establish a range of parameters that encompassed a large part of those works. The response variable was analyzed in terms of the reaction ester yield (FAEEs, mass basis).

The experimental runs resulted ester yields (in wt.%) ranging from 59.30 to 82.06% (Table 2 showing experimental and predict ester yields). Analysis of variance (ANOVA) (Table A3 in Appendix A) allowed determination of the significant parameters. The F-values of model and lack of fit were 24.448 (p-value < 0.0002, 95%-confidence level) and 14.402 (p-value > 0.08) (Table A3 in Appendix A), respectively, indicating that the fitted model (Equation (1)) represents well the behavior of our system (R-squared value of 0.9692). The difference between adjusted and predicted R-squared is recommended to be less than 0.20. The adjusted R-squared and predicted R-squared values were 0.9295 and 0.8890,

indicating good representativity of experimental dataset and capability of extrapolating the model, respectively.

Table 2. Experimental design for the influences of three independent variables (real and coded) on the ester yield of the esterification/transesterification reaction with SODD saponifiable phase (SODD-SP) (experimental and predicted values).

Assays	Molar Ratio (Ethanol:SODD-SP)	Enzyme Concentration (wt. %)	Temperature (°C)	Ester Yield Experimental (wt.%)	Ester Yield Predicted (wt.%)
1	2.3:1 (−1)	3 (−1)	30 (−1)	73.00 ± 2.55	72.38
2	3.3:1 (+1)	3 (−1)	30 (−1)	70.77 ± 0.79	70.46
3	2.3:1 (−1)	7 (+1)	30 (−1)	81.91 ± 0.80	82.40
4	3.3:1 (+1)	7 (+1)	30 (−1)	80.10 ± 1.37	80.48
5	2.3:1 (−1)	3 (−1)	40 (+1)	59.30 ± 1.60	61.14
6	3.3:1 (+1)	3 (−1)	40 (+1)	69.36 ± 1.03	69.70
7	2.3:1 (−1)	7 (+1)	40 (+1)	67.57 ± 1.68	67.28
8	3.3:1 (+1)	7 (+1)	40 (+1)	74.40 ± 1.34	75.84
9	1.96:1 (−1.68)	5 (0)	35 (0)	67.98 ± 0.72	69.70
10	3.64:1 (+1.68)	5 (0)	35 (0)	73.90 ± 2.06	75.28
11	2.8:1 (0)	1.64 (−1.68)	35(0)	67.99 ± 4.44	68.36
12	2.8:1 (0)	8.36 (+1.68)	35 (0)	82.06 ± 4.87	81.93
13	2.8:1 (0)	5 (0)	26.6 (−1.68)	72.94 ± 1.61	76.42
14	2.8:1 (0)	5 (0)	43.4 (+1.68)	65.14 ± 4.32	63.08
15	2.8:1 (0)	5 (0)	35 (0)	72.03 ± 0.45	72.49
16	2.8:1 (0)	5 (0)	35 (0)	72.74 ± 1.01	72.49
17	2.8:1 (0)	5 (0)	35 (0)	72.76 ± 0.25	72.49

A second order (Equation (1)) model was fitted to the experimental data of ester yield vs. the coded independent variables X_1 (ethanol:SODD-SP molar ratio), X_2 (enzyme concentration, in wt. %) and X_3 (temperature, in °C):

$$Yield = 72.50 + 1.67X_1 - 0.50{X_1}^2 + 4.04X_2 + 0.94{X_2}^2 - 3.67X_3 - 0.98{X_3}^2 - 0.35X_1X_2 + 2.62X_1X_3 - 0.62X_2X_3 \quad (1)$$

A good agreement was observed between experimental and predicted (from Equation (1)) responses (Figure A1 in Appendix A).

Figure 2 shows the response surfaces constructed from the model (Equation (1)). As expected, both enzyme concentration (more expressive) and molar ratio (ethanol:SODD-SP) influenced positively the ester yield, i.e., an increase in these parameters leads to higher ester yields. In particular, as SODD has a high content of FFAs and its esterification generates water as a by-product, the reaction was favored using an excess of ethanol, which shifts the reaction equilibrium towards the products [17,55]. On the other hand, temperature negatively affected the ester yield, i.e., the reaction was favored at lower temperatures because high temperatures can inactivate the enzyme. These effects and their magnitudes are clearly shown in the Pareto chart (Figure A2 in Appendix A).

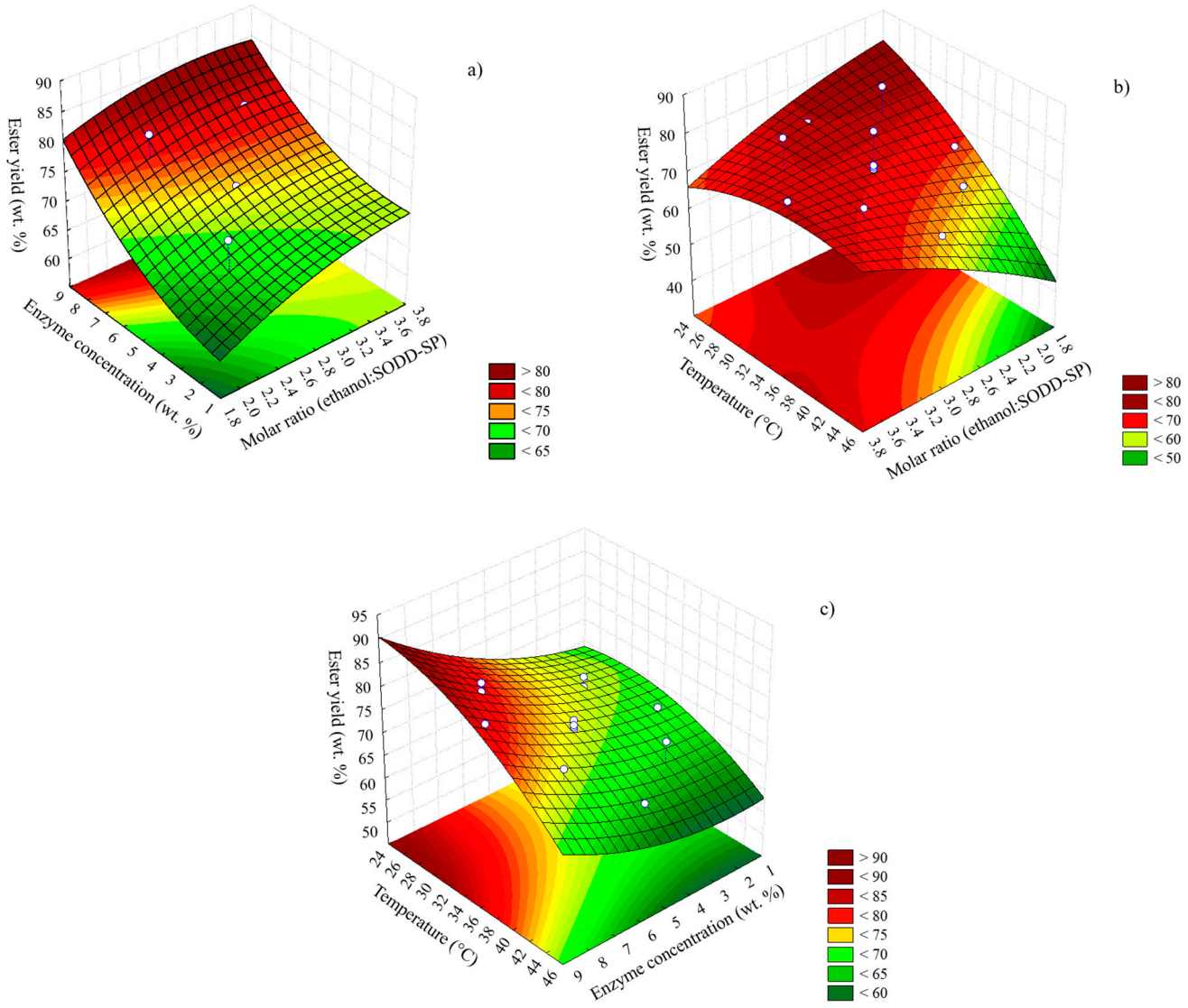

Figure 2. Response surfaces of biodiesel reaction with SODD saponifiable phase (SODD-SP). (**a**) Enzyme concentration vs. ethanol:SODD-SP molar ratio. (**b**) Temperature vs. ethanol:SODD-SP molar ratio. (**c**) Temperature vs. enzyme concentration. Z-axis indicates the ester yield (wt. %).

The highest FAEE yield was reached at 35 °C, 8.36 wt.% enzyme concentration and 3.64:1 molar ratio (ethanol:SODD-SP) (Figure A3 in Appendix A). Under these conditions, the model predicted an ester yield of 83.31 wt.%, which could be experimentally validated in an independent assay (83.25 ± 1.11 wt.% ester yield after 16 h reaction, Figure 3). Figure 3 shows that the reaction equilibrium was reached after 23 h reaction (86.56 wt.% ester yield), after which the ester yield remains practically constant (86.84 wt.% after 48 h reaction). This result is very close to that reported by Wancura et al. [7] (85.08%) using methanol, deacidified cattle tallow, and liquid Eversa.

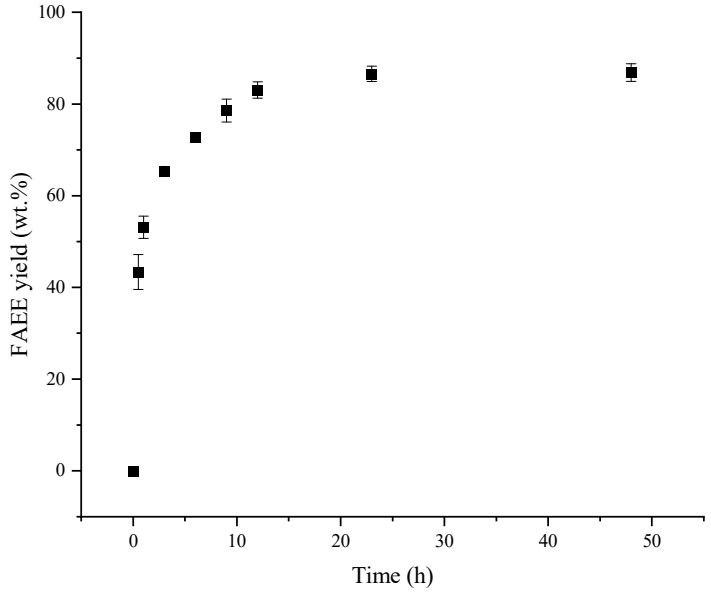

Figure 3. Profile of fatty acid ethyl ester (FAEE) yield (wt.%) vs. time for the esterification/transesterification of SODD saponifiable phase (SODD-SP) (15 g) with ethanol (8.10 g). Reactions conditions: up to 48 h reaction; 35 °C; molar ratio of 3.64:1 (ethanol:SODD-SP) and 8.36 wt.% enzyme concentration.

2.5. Performance of Liquid Eversa in Simultaneous Transesterification and Esterification of Fatty Material with Different Free Acidity

Under the best conditions described above, an experiment was carried out using SODD-SP (2.25% FFAs), crude SODD (17.18% FFAs), and hydrolyzed SODD (72.48% FFAs). Table 3 shows a mass percentage of the main components (FAEEs, FFAs, acylglycerides and tocopherols) for each step. Eversa similarly converted all substrates (with low, medium, and high FFAs content) in FAEEs (86.56, 76.85 and 80.02 wt.% ester yields, respectively); only a slightly higher ester yield (less than 10 wt.%) was observed for a substrate with low free acidity. However, for a highly acidic substrate, the product contained a higher content of FFAs and non-converted acylglycerides than the other substrates (low and medium acidity).

Table 3. Mass percentage of main components of producing FAEEs from low-to-high acidity SODD.

FAEEs Production (Reaction Step)				**Caustic Treatment**	
Inputs		**Outputs**		**Outputs**	
Component	**Value (wt.%)**	**Component**	**Value (wt.%)**	**Component**	**Value (wt.%)**
Crude SODD		**Crude FAEEs**		**Crude FAEEs**	
SI	91.27 ± 0.88	Saponifiable		Saponifiable	
FFAs	17.18 ± 0.26	FAEEs	76.85 ± 0.65	FAEEs	82.42 ± 0.78
Acyl-gly [a]	74.09	FFAs	5.08 ± 0.01	FFAs	1.08 ± 0.08
Toc-total	1.83	Acyl-gly	1.59	Acyl-gly	0.94
α-tocopherol	0.37 ± 0.001	Glycerol	0.04 ± 0.01	Glycerol	0.04 ± 0.004
β-tocopherol	0.09 ± 0.005	MAGs	0.73 ± 0.01	MAGs	0.42 ± 0.0
γ-tocopherol	1.01 ± 0.003	DAGs	0.79 ± 0.09	DAGs	0.45 ± 0.05
δ-tocopherol	0.36 ± 0.003	TAGs	0.03 ± 0.01	TAGs	0.03 ± 0.003
		Toc-total	1.39	Toc-total	0.41
		α-tocopherol	0.06 ± 0.01	α-tocopherol	0.002 ± 0.001
		β-tocopherol	0.07 ± 0.001	β-tocopherol	0.005 ± 0.001
		γ-tocopherol	0.99 ± 0.01	γ-tocopherol	0.19 ± 0.003
		δ-tocopherol	0.27 ± 0.001	δ-tocopherol	0.22 ± 0.01
SODD–SP		Crude FAEEs		Crude FAEEs	
SI	93.03 ± 1.08	Saponifiable		Saponifiable	

Table 3. *Cont.*

FAEEs Production (Reaction Step)				Caustic Treatment	
Inputs		Outputs		Outputs	
Component	**Value (wt.%)**	**Component**	**Value (wt.%)**	**Component**	**Value (wt.%)**
Crude SODD		**Crude FAEEs**		**Crude FAEEs**	
FFAs	2.25 ± 0.13	FAEEs	86.56 ± 0.31	FAEEs	90.83 ± 0.82
Acyl-gly [a]	90.78	FFAs	2.77 ± 0.08	FFAs	0.82 ± 0.08
Toc-total	1.33	Acyl-gly	1.89	Acyl-gly	1.03
α-tocopherol	0.34 ± 0.01	Glycerol	0.05 ± 0.01	Glycerol	N.d.
β-tocopherol	0.01 ± 0.003	MAGs	0.90 ± 0.05	MAGs	0.50 ± 0.02
γ-tocopherol	0.84 ± 0.01	DAGs	0.87 ± 0.23	DAGs	0.53 ± 0.09
δ-tocopherol	0.14 ± 0.001	TAGs	0.07 ± 0.03	TAGs	N.d.
		Toc-total	1.01	Toc-total	0.67
		α-tocopherol	0.20 ± 0.05	α-tocopherol	0.06 ± 0.02
		β-tocopherol	0.01 ± 0.001	β-tocopherol	0.01 ± 0.002
		γ-tocopherol	0.67 ± 0.02	γ-tocopherol	0.49 ± 0.03
		δ-tocopherol	0.13 ± 0.001	δ-tocopherol	0.11 ± 0.002
Hydrolyzed SODD		Crude FAEEs		Crude FAEEs	
SI [b]	96.24	Saponifiable		Saponifiable	
FFAs	72.48 ± 2.91	FAEEs	80.02 ± 0.11	FAEEs	88.83 ± 0.85
Acyl-gly	23.76	FFAs	8.07 ± 0.19	FFAs [c]	9.16 ± 0.001
Glycerol	N.d.	Acyl-gly	11.91	Acyl-gly	1.82
MAGs	8.02 ± 0.05	Glycerol	N.d.	Glycerol	0.73 ± 0.08
DAGs	7.12 ± 0.01	MAGs	2.41 ± 0.09	MAGs	1.09 ± 0.07
TAGs	8.62 ± 0.03	DAGs	7.64 ± 0.01	DAGs	N.d.
Toc-total	0.41	TAGs	1.86 ± 0.01	TAGs	N.d.
α-tocopherol	0.10 ± 0.003	Toc-total	0.21	Toc-total	0.29
β-tocopherol	0.10 ± 0.08	α-tocopherol	0.01 ± 0.005	α-tocopherol	0.06 ± 0.05
γ-tocopherol	0.06 ± 0.01	β-tocopherol	0.07 ± 0.003	β-tocopherol	0.07 ± 0.004
δ-tocopherol	0.15 ± 0.02	γ-tocopherol	0.03 ± 0.04	γ-tocopherol	0.04 ± 0.02
		δ-tocopherol	0.10 ± 0.005	δ-tocopherol	0.12 ± 0.01

SODD–soybean oil deodorizer distillate; FAEEs–fatty acid ethyl esters (by gas chromatography using EN-14103 method [39]); FFAs–free fatty acids (by gas chromatography using Agilent method [41]); SI–saponification index (by AOCS method Cd 3–25) [36]; Toc-total–sum of α-, β-, γ-, and δ-tocopherol (by liquid chromatography, AOCS method Ce 8–89 [40]); SODD-SP–SODD saponifiable phase; Acyl-gly–sum of monoglycerides, diglycerides, triglycerides, and free glycerol (by gas chromatography using ASTM D 6584 method [56]); [a] values indirectly calculated as (SI–FFAs); [b] values indirectly calculated as FFAs+total acylglycerides; [c] measured by AOCS method Ca 5a-40 [34]; reaction conditions: 24 h reaction; 35 °C; ethanol:SODD-SP molar ratio of 3.64:1, 8.36 wt.% enzyme, and 6.74 g of molecular sieves (only for FAEEs production from hydrolyzed SODD).

In general, even using raw material with different acidities, the Eversa performance was very close. In relation to the initial saponifiable matter (91–96 wt.% saponification index), the conversion of saponifiable matter to FAEEs was up to around 90%, showing the good performance of this enzyme for lowly and highly-acid raw materials, as already demonstrated with other fatty materials [1,4,7,8,10,11,13,14,19,55,57–59]. For example, Miranda et al., [5] reported FAEE yields around 90 wt.% after 48 h of reaction in the presence of 6.0% water, using liquid Eversa and refined soybean oil. After a caustic treatment, the ester yield could be increased to 98.2 wt.%. Other authors, using the same enzyme and methanol as acyl acceptor, obtained similar yields: 96% [8], 96.7 % [19], and 97.5 % [1].

Regarding the SODD as acyl donor, Facioli and Barrera-Arellano [30] reported 88% conversion of SODD to FAEEs; however, the enzyme used was the *Mucor miehei* immobilized lipase (Lipozyme^{IM}). Wang et al., [29] and Du, Wang and Liu [28] reported ester yields of 97% and 95% (calculated as the percentage of methyl esters measured in relation to the theoretical methyl esters amount), respectively, using SODD, Novozym 435 (immobilized *Candida antarctica* lipase B) and methanol as acyl acceptor. This brief review shows that our results are very closed to those previously reported using other systems acyl donors-acyl acceptors-enzyme.

As an attempt to reduce residual FFAs in our product, a caustic treatment of the product was used. For the product from SODD-SP, the FAEEs content increased to 90.83 wt.%

and the non-converted acylglycerides and FFAs decreased to 1.03 and 0.82 wt.% (Table 3), respectively. Although our final product has still not met the values recommend to biodiesel for some parameters (such as FAEEs, min. 96.5 wt.%) [60], a deep study focused on this matter could adjust it as a biofuel and even recover other value-added compounds. A deep study of separating FAEEs, FFAs, tocopherols, and other compounds, as well as the economic analysis of these processes is in progress in our group.

3. Materials and Methods

3.1. Materials

Soybean oil deodorizer distillate (SODD) was supplied by COCAMAR (Maringá, PR, Brazil). Eversa® Transform 2.0 (Novozymes A/S, Bagsværd, DK), chromatography standards (α-, β-, γ- and δ-tocopherols, methyl heptadecanoate, monoolein, diolien, triolein, butanethiol, tricarpine, free fatty acids) and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Molecular sieves (3Å) were obtained from JT Baker (New Jersey, NJ, USA). All other chemicals were analytical grade and were used as received.

3.2. Characterization of the SODD

SODD was characterized in terms of acidity index by AOCS method Ca 5–40 [34], iodine index by AOCS method Cd 1–25 [35], saponification index by the AOCS method Cd 3–25 [36], density by AOCS method Cc 10a-25 [61] and humidity by the AOCS method Ca 2b-38 [37]. Viscosity at 25 and 40 °C was measured in a Brookfield Rheometer (Brookfield DV-III Ultra with TC-650 bath, Brookfield Brazil, Rio de Janeiro, RJ, Brazil) with a SC4–27 spindle.

3.3. SODD Saponification

The saponifiable matter from SODD was obtained by a cold saponification reaction according to the AOCS method Ca 6a-40 [62] with adaptations. SODD (100 mL), 10% alcohol solution (600 mL) and 50% KOH solution (100 mL) were added in an Erlenmeyer flask, and the mixture was stirred (in a magnetic stirrer) for 1 h at room temperature. Afterwards, 55 mL of ethyl ether were added, and the material was transferred to a separation funnel. Both phases were titrated with 0.1 M HCl for neutralization. The saponifiable phase was washed twice with hot distilled water (volume ratio 1:1), dried overnight in an oven at 60 °C, and used for enzymatic esterification/transesterification.

3.4. SODD Hydrolysis Reaction

The hydrolysis of SODD saponifiable matter was carried out at 37 °C in a thermostatically controlled closed reactor with mechanical stirring. The reaction medium was composed of 50 g of SODD, 200 g of distilled water (1:4 SODD/water mass ratio) and 2.5 g of free Eversa (5%, m/m_{SODD}). The reaction was monitored by measuring free fatty acids (FFAs) released in the reaction medium by gas chromatography [41]. For that, samples were withdrawn, immediately cooled in an ice bath, and centrifuged. The light phase (oily phase) was washed twice with hot distilled water (1:1 volume ratio), dried overnight in an oven at 60 °C, and used for FFAs analysis by gas chromatography. The heavy phase containing the enzyme was discarded. At the end of the reaction, the reaction product was washed twice with hot distilled water (1:1 volume ratio), dried overnight in an oven at 60 °C, and used for simultaneous enzymatic esterification and transesterification of FFAs and non-hydrolyzed acylglycerides (MAGs, DAGs, and TAGs).

3.5. Esterification/Transesterification Reaction Using SODD Saponifiable Phase (SODD-SP) and Ethanol

Firstly, a statistical design was performed to define the reaction conditions: ethanol: SODD-SP molar ratio, enzyme concentration and temperature (Table 4). The ethanol:SODD-

SP molar ratio (in terms of saponifiable material) was calculated based on the saponification index (SI) using Equations (2) and (3):

$$\text{SODD} - \text{SP (mol)} = m_{\text{SODD-SP}}(\text{g}) \times SI\left(\frac{\text{mg}_{\text{KOH}}}{\text{g}_{\text{SODD-SP}}}\right) \times \frac{1}{MM_{\text{KOH}}}\left(\frac{\text{mol}}{\text{g}}\right) \times 10^{-3}\left(\frac{\text{g}}{\text{mg}}\right) \quad (2)$$

$$\text{Ethanol} : \text{SODD} - \text{SP molar ratio}\left(\frac{\text{mol}_{\text{ethanol}}}{\text{mol}_{\text{SODD-SP}}}\right) = m_{\text{ethanol}}\ (\text{g}) \times \frac{1}{MM_{\text{ethanol}}}\left(\frac{\text{mol}}{\text{g}}\right) \times \frac{1}{\text{mol}_{\text{SODD-SP}}} \quad (3)$$

Table 4. Coded values of the input variables for statistical design.

Variables		−1.68	−1	0	+1	+1.68
Molar ratio (ethanol:SODD-SP)	X1	1.96:1	2.3:1	2.8:1	3.3:1	3.64:1
Enzyme concentration (wt.%)	X2	1.64	3	5	7	8.36
Temperature (°C)	X3	26.6	30	35	40	43.4

The data were analyzed and represented graphically using the software Statistica version 7.0 (Stat Soft), with a significance level of α = 0.05. In the optimization stage, all tests were performed in closed flasks in an orbital shaker (Model MA832, Marconi, Piracicaba, SP, Brazil) at 250 rpm for 16 h. After defined the best conditions, the esterification/transesterification reactions were conducted in a batch reactor (50 mL working volume, thermostated and mechanically stirred at 2000 rpm) to construct the ester yield profile with the time (0–48 h) using SODD-SP. Samples were withdrawn, immediately cooled in an ice bath, and centrifuged. The light phase (oily phase) was washed twice with hot distilled water (1:1 volume ratio), dried overnight in an oven at 60 °C, and used for chromatography analyses (FFAs, FAEEs, MAGs, DAGs, TAGs, and free glycerol). The heavy phase containing the enzyme was discarded.

3.6. Caustic Treatment

A volume of 4% NaOH solution (*w/v*) was added to the esterification/transesterification product to reach 1.15 moles of base per mole of residual FFAs. The reaction mixture was stirred in a shaker (SL–222, Solab, Piracicaba, SP, Brazil) at 60 °C, 60 rpm, for 1 h. Then, the mixture was decanted for 10 min at 60 °C, and centrifuged at 8000 rpm for 10 min at 25 °C. The light phase (upper oily phase) was recovered, washed twice with hot distilled water (volume ratio 1:1), and dried overnight in an oven at 60 °C.

3.7. Tocopherol Quantification by Liquid Chromatography

Tocopherols were analyzed according to the AOCS method Ce 8–89 [40] with adaptations. The liquid chromatography system was a Waters E2695 chromatograph (Waters Co., Milford, MA, USA) equipped with UV detector (Photodiode Array Detector, Waters Co., Milford, MA, USA). The chromatographic separation was performed in a Luna® Silica 100 column (250 × 4.6 mm × 5 µm, Phenomenex INC., Torrance, CA, USA) at room temperature. The mobile phase was a mixture of n-hexane:isopropanol (98:2, *v/v*) at a flow rate of 1 mL/min, 20 µL injection volume, 12 min analysis time.

3.8. Quantification of Esters by Gas Chromatography

The yield of FAEEs (in wt.%) was determined by gas chromatography according to EN-14103 method [39], with modifications. An Agilent chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) was used, equipped with a flame ionization detector (FID-250 °C) and a Rtx-Wax column (30 m × 0.25 mm × 0.25 µm, Restek Corporation, Bellefonte, PA, USA) at a temperature of 210 °C, with helium as carrier gas and methyl heptadecanoate as an internal standard. The samples were centrifuged at 9000 rpm for 10 min at 5 °C, the light phase was washed with hot distilled water and centrifuged (three times), and dried overnight in an over at 60 °C. For quantification, 50 mg of sample were

diluted in 1 mL of methyl heptadecanoate solution (10 mg/mL, in heptane) and 1 µL was injected in the equipment.

3.9. Quantification of Glycerol, Triglycerides (TAGs), Diglycerides (DAGs), and Monoglycerides (MAGs) by Gas Chromatography

The content of free glycerol, TAGs, DAGs, and MAGs (in wt.%) was determined by gas chromatography in an Agilent chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) equipped with a Select Biodiesel column (glycerides, UM + 2 m RG, 15 m × 0.32 mm × 0.1 µm, Agilent Technologies, Santa Clara, CA, USA) and a flame ionization detector. The temperature ramp was 50 °C for 1 min, heating to 180 °C at 15 °C/min, 230 °C at 7 °C/min and 380 °C at 10 °C/min, maintained for 10 min. The detector temperature was 380 °C and helium was used as the carrier gas. The calibration curves were constructed with diolein, monoolein and triolein standards, butanethiol and tricaprine as internal standards, and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) as derivatization reagent. Sample preparation, analysis and quantification were performed according to the ASTM D 6584 method [56].

3.10. Quantification of Free Fatty Acids (FFAs) by Gas Chromatography

FFAs were quantified by gas chromatography according to methodology adapted from the Agilent Catalog [41]. A gas chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) was used, equipped with a flame ionization detector set at 250 °C, a split-splitless injector (250 °C, split ratio 40:1) and an Rtx-WAX column (30 m × 0.25 mm × 0.25 µm, Restek Corporation, Bellefonte, PA, USA). The oven temperature was set at 120 °C for 1 min, heating to 250 °C at 10 °C/min, and 250 °C for 5 min. Helium was used as carrier gas (42 cm/s, 24 psi at 120 °C, 1.8 mL/min). The samples were dissolved in dichloromethane at a concentration of 0.016 g/mL, and the standards were prepared in five different concentrations (0.25, 0.5, 1.0, 1.5 and 2.0 g/L) to adjust the calibration curve.

4. Conclusions

This study showed that liquid Eversa is a versatile lipase, serving multi-purposes, namely hydrolysis and simultaneous esterification and transesterification of feedstocks with low-to-high acidity. Besides that, soybean oil deodorizer distillate was shown to be an interesting source of unsaturated fatty acids (oleic, linoleic and linolenic acids), as well as a potential feedstock to produce fatty acid ethyl esters. Under the adopted conditions, products with a high content of free fatty acids (72.5 wt.%, by enzymatic hydrolysis) and fatty acid ethyl esters (up to 90 wt.%, by simultaneous esterification and transesterification, followed by caustic treatment) could be prepared.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

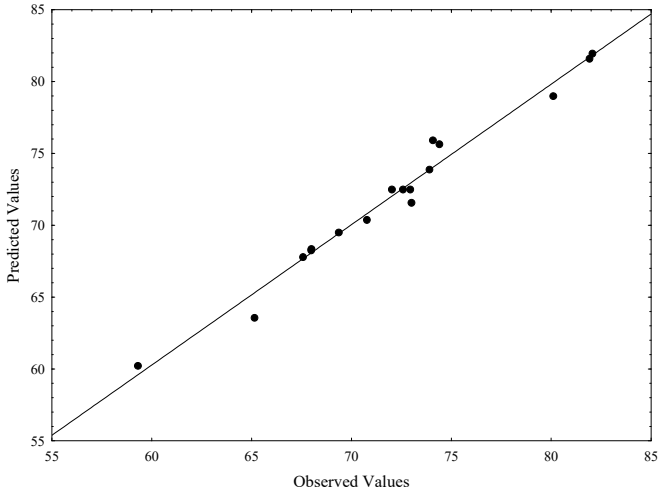

Figure A1. Correlation between experimental (**observed values**) and fitted (**predicted values**) ester yields (wt.%) of the esterification/transesterification with SODD saponifiable phase and ethanol using Eversa Transform 2.0 as biocatalyst.

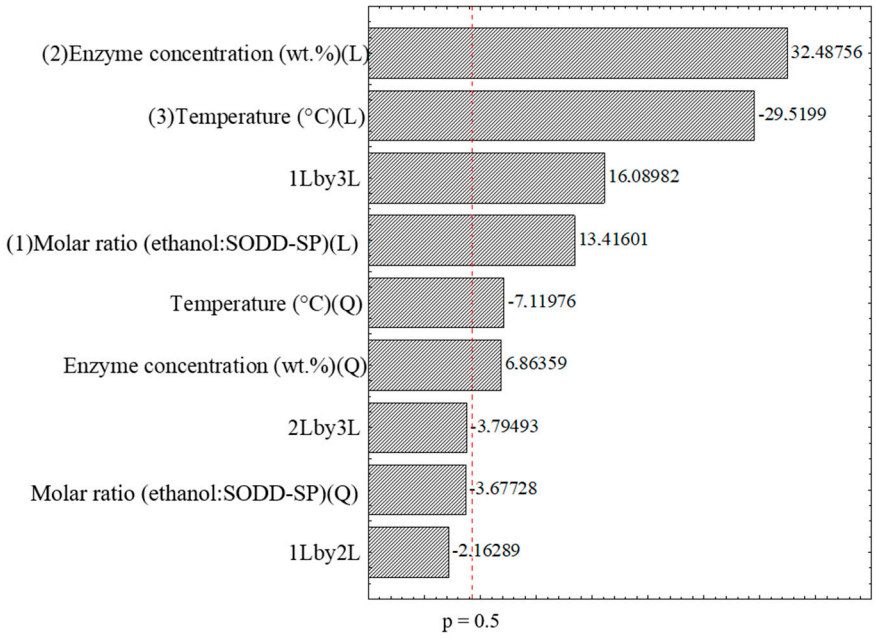

Figure A2. Pareto chart with the effects of the parameters and their interactions for the experimental design of the esterification/transesterification with SODD saponifiable phase and ethanol using Eversa Transform 2.0 as biocatalyst.

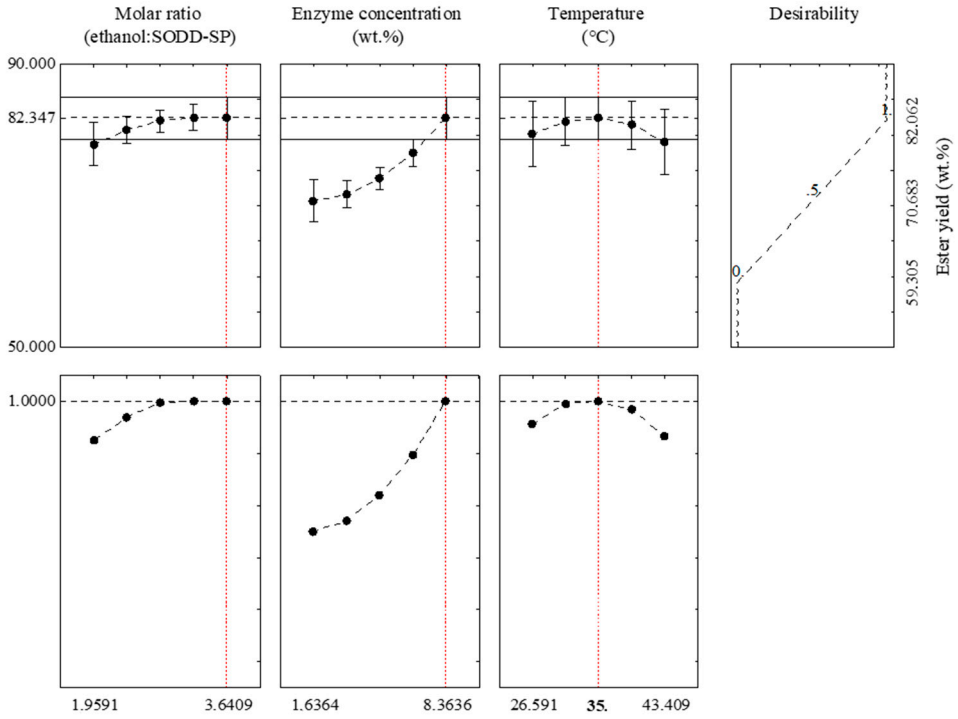

Figure A3. Profiles for predicted values and desirability of the esterification/transesterification with SODD saponifiable phase and ethanol using Eversa Transform 2.0 as biocatalyst.

Table A1. Studies of biodiesel production using Eversa® Transform lipase (liquid or immobilized) as biocatalyst.

Acyl Donor	Acyl Acceptor	Reaction Conditions	Biocatalyst Form	Yield (%)	Reference
Soy oil	Ethanol	6:1 alcohol: oil (molar ratio) 12 U esterification/g oi 40 °C, 1500–1700 rpm, and 24 h	Magnetic CLEAs [a]	98.9	[5]
Glyceril trioleate	Fusel oil	5:1 alcohol:oil (molar ratio) 2 wt.% enzyme 35 °C, 250 rpm, and 24 h	Liquid	>97%	[6]
Deacidified cattle tallow	Methanol	4.5:1 alcohol: oil (molar ratio) 1.0 wt.% enzyme 35 °C, 300 rpm, 6.0 wt.% water, and 8 h	Liquid	85.08	[7]
Soy oil	Methanol	550 kg oil and 2.2 kg methanol 0.2 wt.% enzyme, 45 °C, 20.0 wt% water, 100 ppm NaOH, and 24 h	Liquid	96	[8]
Castor oil	Methanol	6:1 alcohol: oil (molar ratio) 5.0 wt.% enzyme, 35 °C, 5.0 wt.% water, and 8 h	Liquid	83	[9]
Residual oil from a poultry industry	Methanol	100 g oil and 1.5 eqv. alcohol 0.3 wt.% enzyme, 45 °C, 1.5 wt.% water, 250 rpm, and 24 h	Liquid (NS 40116 trademark)	90.61	[10]
Cotton seed oil	Methanol	6:1 alcohol: oil (molar ratio) 5 wt.% enzyme, 35 °C, 6 wt.% water, 250 rpm, and24 h	Liquid	98.5	[11]

Table A1. *Cont.*

Acyl Donor	Acyl Acceptor	Reaction Conditions	Biocatalyst Form	Yield (%)	Reference
Sunflower oil	Ethanol	1:4 alcohol: oil (molar ratio) 4.1 mL hexane, 10 wt.% enzyme, 40 °C, 150 rpm, and 3 h	Immobilized on Sepabeads	99	[12]
Oleic acid	Methanol	3.44:1 alcohol:acid (molar ratio) 11.98% enzyme, 35.25 °C, and 2.5 h	Liquid	96.73	[13]
CTO [b] of the kraft pulping process	Methanol	1.5:1 alcohol: oil (molar ratio) 1 wt.% enzyme, 500 rpm, 40 °C, and 16 h	Liquid	96.57	[14]
Castor oil	Methanol	6:1 alcohol: oil (molar ratio) 5 wt.% enzyme, 5 wt.% water, 750 rpm, 35 °C, and 8 h	Liquid	94.21	[15]
Castor oil	Methanol	6:1 alcohol: oil (molar ratio) 5 w.t% enzyme, 5 wt.% water, 750 rpm, 35 °C, and 8 h	Liquid	Not informed	[16]
Bleached sardine oil	Ethanol	8:1 alcohol: oil (molar ratio) 60 U enzyme, 10 wt.% water, 25 °C, and 4 h	Liquid	93.98	[17]
Castor oil	Methanol	6:1 alcohol: oil (molar ratio) 10 wt.% enzyme, 750 rpm, 35 °C, and 8 h	Liquid	94.21	[18]
Soy oil	Methanol	1.5 eqv. methanol 1 wt.% enzyme, 2.5 wt.% water, 250 rpm, 35 °C, and 16 h	Liquid	96.7	[19]
Soy oil	Methanol	1.5 eqv. methanol 0.2 wt.% enzyme, 3 wt.% water, 500 rpm, 35 °C, and 24 h	Liquid	97.5	[1]
SODD-SP [c]	Ethanol	3.64:1 ethanol:SODD-SP (molar ratio) 8.36 wt.% enzyme 35 °C and 48 h	Liquid	90.83	This study

[a] Crosslinked enzyme aggregates, [b] Crude tall oil, [c] Soybean oil deodorizer distillate saponifiable phase.

Table A2. Research focused on the production of biodiesel by the enzymatic route from deodorizer distillates (DD) of vegetable oils.

DD Source Oil	Alcohol	Reaction Conditions	Biocatalyst	Yield (%)	Reference
Soy	Methanol	Methanol:DD 2.3:1 (molar ratio), 53.6 °C, and 2 h	Lipozyme IM [a]	88	[30]
Soy	Methanol	Methanol:DD 3.6:1 (molar ratio), 40 °C, and 24 h	Novozym 435 [b]	97	[29]
Soy	Methanol	Methanol:DD 3.9:1 (molar ratio), 40 °C, and 24 h	Novozym 435	95	[28]
Palm	Ethanol Methanol	2 g of alcohol added in two steps to 8 g of DD, 60 °C, and 2.5 h	Novozym 435 Lipozyme RM-IM [c] Lipozyme TL-IM [d]	93	[27]

Table A2. *Cont.*

DD Source Oil	Alcohol	Reaction Conditions	Biocatalyst	Yield (%)	Reference
Rapeseed	Ethanol	Ethanol:DD 4:1 (molar ratio), 40 °C, and 30 h	Lipase from *Rhizopus oryzae* immobilized on hydrophobic macroporous resin NKA [e]	98.23	[26]
Rapeseed	Methanol	Methanol:DD 167 µL:2 g, 34 °C, and 6 h	Lipase from *Rhizopus oryzae*	98.16	[25]

[a] Lipozyme IM - *Mucor miehei* lipase immobilized on a macroporous ion exchange resin, [b] *Candida antarctica* lipase immobilized on acrylic resin, [c] Lipozyme RM-IM-*Rhizomucor miehei* lipase immobilized on a macroporous ion exchange resin, [d] Lipozyme TL-IM-*Thermomyces lanuginosus* lipase immobilized on a macroporous ion exchange resin, [e] NKA-Neurokinin A.

Table A3. Analysis of variance (ANOVA) for ester yield (wt. %) from SODD saponifiable phase (SODD-SP) (response variable) as function of the independent variables (ethanol:SODD-SP molar ratio, enzyme concentration and temperature).

Factor	SS *	DF *	MS *	F $_{Calculatted}$	*p*-Value
Model	527.9898	9	58.6655	24.4483	0.000176
(1) Molar ratio (ethanol:SODD-SP)(L)	38.0718	1	38.0718	179.989	0.005510
Molar ratio (ethanol:SODD-SP)(Q)	2.8603	1	2.8603	13.522	0.066644
(2) Enzyme concentration (wt.%)(L)	223.2497	1	223.2497	1055.441	0.000946
Enzyme concentration (wt.%)(Q)	9.9646	1	9.9646	47.109	0.020575
(3) Temperature (°C)(L)	184.3259	1	184.3259	871.424	0.001146
Temperature (°C)(Q)	10.7223	1	10.7223	50.691	0.019162
1L by 2L	0.9895	1	0.9895	4.678	0.163034
1L by 3L	54.7595	1	54.7595	258.882	0.003841
2L by 3L	3.0462	1	3.0462	14.402	0.062951
Lack of Fit	12.0879	5	2.4176	11.429	0.082403
Pure Error	0.4230	2	0.2115		
Total SS	549.5108	16			

* DF: degree of freedom; SS: sum of squares; MS: mean square; R-squared = 0.9692; adjusted R-squared = 0.9295; predicted R-squared = 0.8890.

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