ValORIZATION OF AGRO-INDUSTRIAL RESIDUES: BIOPROCESSING OF ANIMAL FATS TO REDUCE THEIR ACIDITY

António A. Martins 1, Soraia Andrade 1, Daniela Correia 1, Elisabete Matos 2, Nídia S. Caetano 1,3* and Teresa M. Mata 4,*

1 LEPABE-Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto (FEUP), R. Dr. Roberto Frias 5/7, 4200-465 Porto, Portugal; amartins@fe.up.pt (A.A.M.); soraiaavandrade@hotmail.com (S.A.); elisabete.correia@04@gmail.com (D.C.); nsc@isep.ipp.pt (N.S.C.)
2 B2E Associação para a Bioeconomia Azul—Laboratório Colaborativo, Av. Liberdade s/n, 4450-728 Leça da Palmeira, Portugal; ematos@b2e.pt
3 CIETI, Department of Chemical Engineering, School of Engineering (ISEP), Polytechnic of Porto (P.Porto), R. Dr. António Bernardino de Almeida 431, 4249-015 Porto, Portugal
4 INEGI-Institute of Science and Innovation in Mechanical and Industrial Engineering, R. Dr. Roberto Frias 400, 4200-465 Porto, Portugal
* Correspondence: tmata@inegi.up.pt; Tel.: +351-22-508-1400 (ext. 1404); Fax: +351-22-508-1449

Received: 12 August 2021
Accepted: 27 September 2021
Published: 29 September 2021

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland.
This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Abstract: Adding value to agro-industrial residues is becoming increasingly important, satisfying needs to promote resources’ use efficiency and a more sustainable and circular economy. This work performs a parametric and kinetic study of enzymatic esterification of lard and tallow with high acidity, obtained by the rendering of slaughter by-products, allowing their use as a feed ingredient and increasing their market value. After an initial analysis of potential enzyme candidates, a Candida antarctica lipase B was selected as a biocatalyst for converting free fatty acids (FFA) to esters, using excess ethanol as the reagent. Results show that the fat acidity can be reduced by at least 67% in up to 3 h of reaction time at 45 °C, using the mass ratios of 3.25 ethanol/FFA and 0.0060 enzyme/fat. Kinetic modelling shows an irreversible second-order rate law, function of FFA, and ethanol concentration better fitting the experimental results. Activation energy is 54.7 kJ/mol and pre-exponential factor is 4.6 × 10^6 L mol⁻¹ min⁻¹.

Keywords: animal fats; acidity reduction; circular economy; Lipozyme CALB L; industrial conditions; reaction kinetics

1. Introduction

In the EU, about 17 million tonnes of slaughter by-products are generated each year. These represent approximately 45% of the animal “live weight killed” (LWK), e.g., bones, fat, blood, feathers, hoofs, offal, hide, horns, and hair [1]. These residues must be sustainably managed or subsequently processed to obtain other products, thus reducing disposal costs and associated environmental impacts and creating a source of revenue [2].

Though the meat processing industry has significant impacts, it is also fundamental for sustainable development, as it satisfies the basic needs of human beings. This fact is recognized by the European Union, in its “Farm to Fork strategy” established by the “European Green Deal” as one of its fundamental pillars [3], whose main objective is to make food systems fairer, healthier, and with less negative environmental impacts. Thus, for an easier transition to a more circular economy, it is essential to find alternative and successful ways of recycling industrial by-products and to manage resources more sustainably.

The rendering process was developed over the nineteenth century as a direct offshoot of the candle-making and soap industries. Until 1950s, little fat was used in the production of animal feed, and the protein meal by-product was applied to the land as a fertilizer. This was because the nutritional benefits of the rendered by-products were not well known,
and neither were the nutritional needs of domestic animals [1,4]. The main purpose of the rendering industry is to provide an essential flow for animal by-products, transforming them into a wide variety of products and thus creating a source of potential revenue. This way, they contribute to reducing resource use, avoiding health problems and disposal costs of these bio-wastes, and creating environmental credits for the entire system [4].

In rendering, the animal residues are dried, yielding a fat and a protein meal [1]. These co-products may have several potential applications, such as electricity and heat generation [5], biofuel production [6], biopolymers [7], olefins [8], fertilizers [9], biochar [10], or bioflocculant [11], and/or other value-added products such as ingredients for livestock feed [12] or nutritional supplements [13]. Moreover, rendering of animal residues can significantly contribute to the 2030 UN Agenda for Sustainable Development [14], particularly to the UN sustainable development Goal 12 of responsible consumption and production, by promoting and facilitating the efficient use of natural resources throughout their life cycle and sustainable management, minimizing negative impacts on human health and environment, substantially reducing waste generation, and contributing to a more circular economy [15].

Today, animal fats are widely used for feed applications, but their utilization requires a high-quality standard [16], otherwise the fat will be used preferentially for lower-value applications, such as biodiesel production [17]. The important characteristics are the fatty acids composition and the fat melting point. In particular, for producing high-quality feed pellets, the fat needs to have a low acidity or free fatty acid (FFA) content [18]. Due to inadequate storage, transportation, and industrial processing of animal by-products, the fat can deteriorate by hydrolysis, oxidation, fermentation, or hydrogenation, causing FFAs release and increasing the fat acidity and rancidity. This is accompanied by changes in the fat flavor, aroma, color, and consistency [19]. Other relevant factors influencing the decomposition of fats include storage temperature, moisture and oxygen content, and fat composition.

Rancidity is normally caused by oxidation reactions that may be coupled with hydrolysis, induced by lipases existing in the original raw material or from microorganisms [20]. Fats degradation by oxidation can be prevented by adding antioxidants. However, this can sometimes be ineffectual and represent an additional operating cost. Additionally, as some antioxidants may be toxic, it makes the fat unusable for some applications. Therefore, in order to increase the fat quality, the FFAs can be converted into esters that are more stable compounds. Short chain alcohols are normally used as reagents for esterification reactions, allowing higher reaction yields [21].

The acidity reduction of animal fat with high acidity, essential to increasing its market value as an ingredient for animal feed [22], can be performed by enzymatic esterification, using lipases as catalysts and ethanol as the reagent [21]. Hence, this work aims to perform a parametric and kinetic study of the enzymatic esterification of a real mixture of lard and tallow with high acidity. The fat mixture was obtained from an industrial rendering facility, operating in northern Portugal. In practice, the fatty material obtained from the rendering process shows a wide variation of the acid value, depending on how meat was processed, original animal species, storage conditions, and even climatic conditions, as changes in animal fats are strongly dependent on the temperature. To the authors’ best knowledge, there are no other studies published in the literature involving the enzymatic esterification of mammalian fat with high acidity, obtained from a true industrial process, nor using the same enzymes as catalyst. Additionally, there are very few studies involving enzymatic esterification of animal fats and/or they focus on different types of fat or different fat mixtures [18,19,23,24]. Furthermore, the majority of studies deal with vegetable oils and/or aim at biodiesel production [6,21,25–33]. In the case of this study, the aim is to improve the quality, as a feed ingredient, of a mixture of mammalian fats, obtained from a real industrial rendering process. As the intended application of the resulting mammalian fat mixture is the production of animal feed, catalysts and reagents with low or no toxicity should be used in the esterification reaction, as is the case of lipases and bioethanol.
2. Materials and Methods

2.1. Sampling and Fat Characterization

Samples of a fat mixture, with lard and tallow, of about 1 kg each, were collected during summer and autumn in a Portuguese rendering company. The fat samples were kept in the dark to avoid degradation before characterization. Even under these conditions, the samples had to be used quickly, because the acidity and moisture content tend to increase during storage. Additionally, if the experiments are performed shortly after the collection of the fat samples, the results are more representative of the real conditions. If this process is implemented in actual industrial practice, the fat materials will be processed to reduce acidity immediately after rendering to reduce quality losses and storage costs.

At the laboratory, according to standard methods described in Caetano et al. [26], the fat samples were characterized for the following parameters: acid value (mg KOH/g fat), kinematic viscosity (mm²/s), density (kg/m³), moisture content (% wt/wt), and iodine value (g iodine/100 g fat). The fatty acids profile of the mixture of lard and tallow was determined by gas chromatography, according to standard methods and using conditions and equipment described in Mata et al. [24].

2.2. Reactants and Enzymes

Ethanol (Panreac, 96% v/v) was purchased locally. Other reactants used for chemical analysis were of analytical grade.

Four commercial enzymes (Lipozyme® CALB L, Lipozyme® TL 100L, Novozym® 435, and Lecitase® Ultra) were tested in this work, kindly provided by Novozymes A/S (Denmark). Table 1 presents the activity and optimum usage conditions provided by Novozymes A/S for these four enzymes.

Table 1. Description and optimum usage conditions for lipases provided by Novozymes A/S.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Formulation</th>
<th>Optimum Temperature</th>
<th>Activity ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipozyme® CALB L liquid</td>
<td>30–60 °C</td>
<td>5000 LU/g</td>
<td></td>
</tr>
<tr>
<td>Lipozyme® TL 100 L liquid</td>
<td>20–50 °C</td>
<td>100,000 LU/g</td>
<td></td>
</tr>
<tr>
<td>Novozym® 435 immobilized</td>
<td>30–60 °C</td>
<td>10,000 PLU/g</td>
<td></td>
</tr>
<tr>
<td>Lecitase® Ultra liquid</td>
<td>35–60 °C</td>
<td>10,000 LU/g</td>
<td></td>
</tr>
</tbody>
</table>

¹ PLU = Propyl Laurate Unit, LU = Lipase unit. 1 PLU = amount of enzyme activity that generates 1 µmol of propyl laurate/min at the standard conditions. 1 LU = amount of enzyme activity that releases 1 µmol of tritratable butyric acid from the substrate glycerol tributyrate/min at the standard conditions. It is assumed PLU = LU = IUN (IUN is other measure of enzyme activity).

These enzymes are known to be able to efficiently catalyze the esterification of oil and fats [27,34]:

- Lipozyme® TL 100 L, a 1,3 lipase originating from Thermomyces lanuginosus provided in liquid formulation. It is recommended by the producer as a very effective catalyst for transesterification, interesterification, ester hydrolysis, and desymmetrisation of esters, exhibiting a high degree of substrate selectivity.
- Lipozyme® CALB is a lipase from Candida antarctica B provided in liquid formulation. It is stable over a broad pH range, especially alkaline, exhibiting a high degree of substrate specificity.
- Novozym® 435 is a CALB lipase from Candida antarctica B immobilized on a hydrophobic carrier, an acrylic resin Lewatit VP OC 160. The physical appearance is white spherical beads with a particle diameter in the range of 252–687 µm.
- Lecitase® Ultra, a phospholipase A1 from Thermomyces lanuginosus provided in liquid formulation. It has inherent activity towards both phospholipid and triglyceride structures and is commonly applied for degumming and reducing the vegetable oil phosphatides.
2.3. Procedure for the Esterification Assays

Each enzymatic esterification assay (Figure 1) started with the determination of the initial acidity of each mixture of lard and tallow. Approximately 10 g of the fat sample were placed in 50 mL reaction flasks. Then, the required weight/volume of enzyme and ethanol 96% (v/v) for each assay were measured and added to perform the esterification. This was done in duplicate. The reaction flasks were tightly closed with a screw cap and placed in a thermostatic bath, with magnetic stirring, at the desired temperature, and allowed to react. At pre-established times, a sample was taken from each bottle to determine its acidity and assess the reduction in acidity over time. Esterification assays were performed in duplicate and the average values reported. At the end of the established reaction time, the fat was washed by adding distilled water three times in a separating funnel. The aqueous phase was discarded, consisting mainly of water, enzyme, and excess alcohol. The esterified fat was then weighed and the final acidity determined. The overall acidity reduction was determined by knowing the initial acid value of the fat, previously determined, and the final acid value, after the reaction.

Figure 1. Esterification assays scheme: fat sample bottle (left), thermostatic bath with reaction flasks and magnetic stirring (middle), and fat washing in separating funnel (right).

In this work, experimental assays were carried out using an industrial mixture of lard and tallow obtained from a real industrial rendering process. These fat mixtures are very complex and may contain contaminants that limit the enzymatic esterification or even make it unviable. Additionally, there are currently no fast and cheap analytical methods to determine the amount of FFA in situ and characterize their profile in animal fats in order to more accurately estimate the amounts of enzyme and ethanol that should be used in the esterification reaction to reduce the acidity below a level that ensures the best selling price [2]. Moreover, from an industrial point of view, it is important to increase the reaction rate as much as possible, thus reducing the reaction time needed to fulfill the quality requirements of the final product while ensuring lower process operating costs.

Esterification reactions are equilibrium controlled [35]; therefore, using excess ethanol in relation to FFA will increase the reaction rate and move the equilibrium towards higher values of acidity reduction, ensuring that the final reaction products have adequate quality and the highest selling prices [2,6]. The higher the alcohol/FFA mass ratio, the lower the reaction time for achieving a suitable acidity reduction. On the other hand, the longer the reaction times, the greater the probability of occurring a reverse hydrolysis reaction, leading to a decrease in esters formation, thereby decreasing reaction yield and increasing process costs. In industrial practice, the alcohol/FFA mass ratio should also ensure a stoichiometric excess of alcohol in relation to free fatty acids to guarantee that the equilibrium is shifted to the reaction products [18,23]. Thus, the required volume of ethanol was estimated considering an excess over the stoichiometric amount needed to react with the FFA, taking
into account the esterification reaction stoichiometric relations: one mole of ethanol reacts with one mole of free fatty acid. Thus, an adequate molar excess of ethanol of at least 10 should be considered to perform the experiments, deemed sufficient to ensure that an adequate reduction of acidity is attainable in, at most, three hours of reaction time [28,29]. Thus, an ethanol/fat molar ratio of 20:1 was considered, corresponding to an ethanol/FFA mass ratio of 3.25 [24], based on the fact that the FFA content/concentration or acidity (%) of a fat is approximately half of its acid value (mg KOH/g fat).

To estimate the required amount of enzyme to be used in each experiment, information was obtained from the literature [36]. Generally, biological catalysts are sensitive to the medium conditions (e.g., amount and type of alcohol, and temperature), which can lead to catalyst inactivation and thus to lower reaction yields [18,23]. Moreover, the quantity of enzyme used should be enough to ensure no variations of its distribution in the reaction media, as for example due to mixing problems [36]. Thus, for Lypozone TL IM, a lipase used in the transesterification of palm olein in a batch reactor with an activity of 450 IUN/g (IUN Interesterification Unit), it was used a mass ratio of 0.045 between the mass of enzyme and the mass of palm olein [36]. Considering that IUN=PLU=LU, the values of the ratio enzyme/fat mass were calculated assuming that the required amount is inversely proportional to the enzyme amount and using the ratio enzyme/fat mass found in the literature [36]. To ensure that the enzyme availability is not a limiting factor, as the reaction mixture is quite heterogeneous and viscous, the values used experimentally were at least 10 times larger.

To select the temperature values to perform the experiments, the optimal operating temperature range of the enzymes (Table 1) was considered. Generally, a temperature raise is beneficial, as the enzyme activity and consequently the reaction rate increase with temperature. Moreover, increasing the temperature lowers the mixture viscosity, facilitating the reactants contact and ensuring that all FFA, alcohol, and enzyme are available for the esterification reaction. However, caution is necessary, as high temperature values may be inadequate due to the fact that enzymes degrade at high temperatures, making it impossible to catalyze the esterification reaction [37]. Moreover, at an industrial scale, higher temperatures may lead to alcohol losses from the reacting mixture due to evaporative losses, reducing the available quantity of reactants and increasing the operational risk.

2.4. Parametric Study Experimental Strategy

In order to study the possibility of industrial implementation of the new enzymatic esterification process, aiming to reduce the fat acidity and increase its market value, a sequential experimental strategy was adopted in this work:

(i) First, the best enzyme was identified from a set of four commercial lipases.
(ii) Then, for the selected enzyme, the most adequate operating conditions to perform the esterification were evaluated, aiming to maximize the acidity reduction.
(iii) Finally, the reaction kinetics were determined, focusing on using simple reaction rate models, more suitable to design industrial scale processes, where the reactant mixtures are very complex and difficult to characterize.

For the enzyme selection experiment, different enzyme/fat mass ratios were tested, depending on the enzyme activity and trying to identify if the quantity of enzyme influences the acidity reduction. The reaction temperature was set at 40 °C and the ethanol/FFA mass ratio at 3.25, for a maximum reaction time of 2 and 3 h. After the experiment and based on the results, the enzyme that yielded the greatest reduction in acidity was selected, thus ensuring a higher market value for the fat.

After selecting the enzyme with the best potential results, experiments were performed to identify the best operating conditions to perform the esterification reaction of the industrial fat mixture for the chosen enzyme. Of the various parameters that can influence the esterification reaction, in this work, the following three were considered:

- Enzyme/fat mass ratio,
- Alcohol/FFA mass ratio, and
• Reaction temperature.

As no information is available regarding the expected process conditions for the esterification of real industrial fat mixtures, it was not practicable to perform a design of experiments. Therefore, for the parametric study, the experimental assays were performed sequentially, varying one parameter at a time, for a fixed reaction time of 3 h, and the optimization criteria were the percentage of acidity reduction.

1) Thus, a first set of six experimental assays was carried out at different reaction temperatures, keeping fixed the alcohol/FFA and the enzyme/fat mass ratios.

2) Then, a second set of four experiments was performed, varying the alcohol/FFA mass ratio and fixing the temperature and the enzyme/fat mass ratio, for which the percentage of acidity reduction determined in the previous set of experimental assays was higher.

3) Finally, a third set of four experiments was performed, varying the enzyme/fat mass ratio and keeping fixed the temperature and alcohol/FFA mass ratio, for which the percentage of acidity reduction determined in the previous sets of experimental assays was higher.

This procedure is equivalent to perform a sequential optimization study of the operating conditions, based on the fat acidity reduction criterion. Although it does not ensure the identification of optimal reaction conditions, as it is not an optimization procedure, the set of values obtained is close enough to the optimum and adequate for an industrial implementation of an acidity reduction process.

2.5. Kinetic Modeling

Besides the determination of the overall acidity reduction, the variation of the acidity as a function of time was also determined during the experiments. Thus, it was possible to characterize the esterification reaction kinetics, in particular to identify an adequate rate law and calculate the values of the corresponding kinetic constants. This information is important for process design, in particular to properly design the system: the reactor and supporting equipment that will perform the fat acidity reduction in the rendering company.

As the fat material is a complex mixture of fatty acids and other compounds that may mimic their chemistry and also react with the enzyme, the identification of a reaction mechanism is very difficult. Besides that, the utilization of excess ethanol in the process, to ensure a more complete esterification of the fatty acids and corresponding acidity, also makes it harder to ascertain what the real effects due to each reactant are [18,23]. Thus, only simple rate laws were considered in this work, focusing on those that are a function of FFA concentration (represented as C18:1 or oleic acid concentration), due to the fact that the ethanol concentration does not significantly change during the course of the experiments performed, at most by 10%. A similar approach is considered in the literature when modelling the kinetics of esterification reactions from experimental data [38,39]. Taking into account the specific reaction stoichiometry and the molar excess of alcohol normally used, most authors have proposed simple rate laws, in particular second-order reversible laws, considering the concentration of all reactants and reaction products for either enzymatic or acid/alkali catalyzed esterification. Other rate laws were also considered for heterogeneous catalysis [40] or even complex enzymatic catalysis mechanisms [41].

It was assumed that the reaction medium is homogeneous, and the reaction occurs in batch mode, in liquid phase, with stirring at constant temperature. Even for Novozym 435, the assumption of a homogeneous medium is valid, as the combination of very small particles with strong mixing ensures that the external and internal diffusional resistances are minimal and the reactional system is under chemical control (catalyst efficiency equal to one). As considered above, the free fatty acids present in the lard and tallow mixture are considered to be well-represented by oleic acid (C18:1), supported by the analysis of the fatty acids profile of fat sample performed in this study, and others available in
literature [42–44]. Thus, as a proxy, it was assumed that the FFA concentration is equivalent to the same concentration of oleic acid [42–45], calculated as follows.

\[ C_A = \frac{\left( \frac{A}{100} \right) \times m}{V \times M_{\text{OleicAcid}}} \]  

(1)

where \( C_A \) is the FFA concentration, equivalent to the oleic acid concentration (mol.L\(^{-1}\)), \( A \) is the acidity (\%) or the FFA content/concentration calculated by dividing the fat acid value (mg KOH/g fat) by 1.99, i.e., acid value (mg KOH/g fat) = 1.99 fat acidity (%), \( m \) is fat mass in grams, \( M_{\text{OleicAcid}} \) is the molecular mass of oleic acid (g mol\(^{-1}\)), and \( V \) corresponds to the reacting mixture (fat plus alcohol) volume in liters.

Therefore, assuming that the reaction volume is constant, the following mass balance equation describes the variation of the fatty acids concentration, \( C_A \), in the reaction vessel [46].

\[ \frac{dC_A}{dt} = r_A \]  

(2)

where \( r_A \) is the rate law (the rate of disappearance of FFA) and \( C_A \) is the FFA concentration. \( r_A \) can be a function of just oleic acid, or alcohol, or both. In this work, both simple irreversible and reversible rate laws were considered. In particular: first- and second-order irreversible reactions, a function only of the oleic acid concentration, and reversible second-order reaction, a function of both the oleic acid and ethanol concentration (with the goal of identifying any potential effect of the ethanol concentration on the fitting of the experimental data to the kinetic data); the Michaelis-Menten rate law; and the second-order reversible reaction of both oleic acid and ethanol [46].

The fitting of the rate law to the experimental results took into consideration the possibility that Equation (2) can be integrated analytically or not, and assumes that for \( t = 0 \), the initial concentrations of alcohol and fat are known or can be calculated independently. The first case occurs for most of the rate laws considered in this work. The analytical expressions presented below are expressed in a form that simplifies the task of identifying which is the most adequate rate law and the calculation of the equation constants, as from the plot of the left side vs. right side of each equation, the kinetic constants and the adequacy of the rate law can be evaluated [46].

- First-order kinetics, a function of \( C_A \), taking into account that the concentration of alcohol varies little in the course of the reaction and that, after a reaction time of three hours, the chemical equilibrium is still far away.

\[ -r_A = kC_A \Rightarrow ln \left( \frac{C_{A0}}{C_A} \right) = kt \]  

(3)

- Second-order kinetic function of \( C_A \), again considering the small variation of the alcohol concentration during the assays and the possibility that the equilibrium impacts of the acidity reduction are negligible.

\[ -r_A = kC_A^2 \Rightarrow \frac{1}{C_A} - \frac{1}{C_{A0}} = kt \]  

(4)

- Second-order kinetics function of oleic acid and ethanol concentration, \( C_B \), where \( M = \frac{C_{B0}}{C_{A0}} \) is the ratio between the initial concentrations of oleic acid and ethanol, to better account for reaction stoichiometry and the potential influence of alcohol concentration in the reaction kinetics.

\[ -r_A = kC_AC_B \Rightarrow ln \left( \frac{C_B}{C_A} \right) = (C_{B0} - C_{A0})kt + lnM \]  

(5)
- Michaelis-Menten kinetics: 
\[ r_A = \frac{r_{\text{max}} C_A}{K_M + C_A}, \] 
function of \( C_A \), where \( r_{\text{max}} \) is the maximum reaction velocity, \( K_M \) is the Michaelis-Menten constant, and \( M = \frac{C_B}{C_A} \), to verify if the reaction kinetics flow.

\[
r_A = \frac{r_{\text{max}} C_A}{K_M + C_A} = \frac{C_{A0} - C_A}{\ln \left( \frac{C_{A0}}{C_A} \right)} = -K_M + \frac{t}{\ln \left( \frac{C_{A0}}{C_A} \right)} M \tag{6}
\]

- The second-order reversible rate law is expressed as in Equation (7).

\[
r_A = k_1 C_A C_B - k_2 C_W C_E \tag{7}
\]

where \( C_W \) and \( C_E \) represent the water and ester concentrations, respectively, and \( k_1 \) and \( k_2 \) represent the kinetic constant of the forward and reverse reactions, respectively. In this work, the concentration of oleic acid, \( C_A \), is limiting, and assuming that, at \( t = 0 \), the concentration of ester and water in the solution are very small and can be considered to be zero, Equation (7) can be re-written in the form of Equation (8).

\[
r_A = k_1 C_{A0}^2 (1 - X_A)(M - X_A) - k_2 C_{A0}^2 X_A^2 \tag{8}
\]

where \( X_A \) is the oleic acid conversion, directly related to the acidity reduction and defined by the relation \( X_A = 1 - \frac{C_A}{C_{A0}} \), and \( C_{A0} \) is the initial concentration of oleic acid that can be determined directly from the sample initial acidity. To use the rate law defined by Equation (2), the general mass balance must be written in terms of \( X_A \), in the form of Equation (9).

\[
C_{A0} \frac{dX_A}{dt} = -r_A \tag{9}
\]

Thus, substituting Equation (8) in Equation (9) and simplifying results in the differential Equation (10).

\[
\frac{dX_A}{dt} = k_1 C_{A0} (1 - X_A)(M - X_A) - k_2 C_{A0} X_A^2 \tag{10}
\]

Equation (10) must be solved for the initial condition \( t = 0 \Rightarrow X_A = 0 \). At equilibrium, \( \frac{dX_A}{dt} = 0 \) and the equilibrium constant \( K_e \) and the equilibrium conversion \( X_e \) can be expressed as shown in Equation (11).

\[
K_e = \frac{k_1}{k_2} = \frac{X_e^2}{(1 - X_e)(M - X_e)} \tag{11}
\]

Although it is possible to integrate analytically the second-order reversible rate law given by Equation (10) [46], the resulting expression is too complex to be used graphically. Thus, in this work, a different approach was considered to determine the kinetic constants. Equation (10) was integrated numerically, and the values of the kinetic constants were determined by minimizing the sum of the residues between the experimental and predicted values for the times in which the experimental points were obtained.

All kinetic constants in Equations (3) to (8) represent apparent kinetic constants, as the fatty acids were assumed to be represented by oleic acid, when in reality it is a complex mixture of molecules. In the calculations, as each experiment was done in duplicate, the kinetic constants were calculated separately, and the average values are reported.

Some experiments were performed varying the temperature and keeping the other operating conditions fixed. Under these conditions it is possible to describe the temperature dependence of the apparent kinetic constant, \( k \), assuming that it depends only on the temperature, using the Arrhenius equation in the form shown in Equation (12)

\[
k = k_0 \exp \left( \frac{-E_a}{RT} \right) \tag{12}
\]
where \( k_0 \) is the pre-exponential factor, \( E_a \) is the activation energy, \( R \) the ideal gas constant and \( T \) the absolute temperature. Linearizing Equation (12), the expression in Equation (13) is obtained.

\[
\ln k = \frac{-E_a}{RT} + \ln k_0
\]  

(13)

Plotting \( \ln k \) as a function of \( \frac{1}{T} \), if Equation (4) is valid, the value of \( E_a \) is determined from the slope of the linear fit line, and \( \ln k_0 \) is the y-intercept. The constants of Arrhenius equation were determined only for the rate law that better fits the experimental data.

3. Results
3.1. Fat Characterization

The following average values were obtained for the lard and tallow mixture analyzed, being the variations around the average values reported in literature [30,45].

- 4.43 mg KOH/g fat of acid value
- 0.15 wt % of moisture content
- 2.2% of acidity or FFA content
- 49.8 mm²/s kinematic viscosity at 40 °C
- 920 kg/m³ density at 20 °C
- 94.5 g I₂/100 g fat of iodine value.

The acid value and acidity are relatively low, below the threshold value that the market accepts without a price penalty [2]. Hence, when the fat acidity is above the threshold, a reduction of the fat acid value can increase the fat market value, justifying this study interest. Moreover, as it is expected that the future requirements imposed in the raw materials used in the animal feed will be ever more stringent, rendering companies will need to adapt, in which case the results presented in this work are relevant.

Iodine value measures the fat unsaturation degree. Literature values show for pork fat an iodine value of 77 g I₂/100 g of fat [30]. In this study, a higher iodine value for a mixture of pork and cow fat was obtained, showing more susceptibility to this fat degradation. For density and kinematic viscosity, the values obtained in this study agree with those reported in the literature [30,45]. The moisture content is low since water is removed during the rendering process. The fatty acid profile of the lard and tallow mixture is presented in Table 2.

The monounsaturated fatty acids (MUFA) group achieved the major percentage of the total fatty acids (43.9%), C18:1 being the most abundant with 36.7%. The saturated fatty acids (SFA) group summed 39.8% of the total fatty acids, C16:0 being the most abundant with 23.0% followed by C18:0 with 14.2%. The polyunsaturated fatty acids (PUFA) group summed 14.2% of which C18:2n6 is the most abundant fatty acid with 11.9%. The trans fatty acids (TFA) are in lower percentage with just 1.7%. This fatty acid composition agrees with data published for mammalian fats. For example, Dias et al. (2009) analyzed the fatty acids profile of waste lard, finding that the most abundant is also oleic acid (C18:1) with 40.6%, followed by palmitic acid (C16:0) with 21.6% and then linoleic acid (C18:2) with 21.2%, and stearic acid (C18:0) with 11.0%. Enser et al. [45] analyzed the fatty acid composition of muscle from beef, lamb, and pork, finding that the most abundant is C18:1 (32.5–36.1%) followed by C16:0 (22.2–25:0) and then C18:0 (12.2–18.1%). Ning et al. (2018) analyzed the composition of long-chain fatty acids in raw swine slaughterhouse waste, showing that C18:1 is the most abundant with 40.47 ± 2.36, followed by C18:2 (22.97 ± 1.12), C16:0 (20.29 ± 2.94), and C18:0 (10.46 ± 1.79).
Table 2. Fatty acids composition of a lard and tallow mixture.

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>g/100 g fat sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA, of which</td>
<td>39.8 ± 0.9</td>
</tr>
<tr>
<td>C14:0</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>C16:0</td>
<td>23.0 ± 0.9</td>
</tr>
<tr>
<td>C18:0</td>
<td>14.2 ± 0.2</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>MUFA, of which</td>
<td>43.9 ± 0.6</td>
</tr>
<tr>
<td>C16:1</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>C18:1</td>
<td>36.7 ± 0.5</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td>PUFA, of which</td>
<td>14.2 ± 0.3</td>
</tr>
<tr>
<td>C18:2n6</td>
<td>11.9 ± 0.2</td>
</tr>
<tr>
<td>C18:3n3</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td>C20:2n6</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>C20:4n6</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>C20:5n3</td>
<td>n.d.</td>
</tr>
<tr>
<td>C22:5n3</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>C22:6n3</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>TFA</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Unsaponifiables</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, SFA = saturated fatty acids, TFA = trans fatty acids. Bold values represent the total values of MUFA, PUFA, SFA, and TFA. n.d. = not detected.

3.2. Enzyme Selection

Table 3 presents the results obtained for the enzyme selection. Average values are reported for each assay performed in duplicate. Good reproducibility was observed for all the assays.

Table 3. Acidity reduction and enzyme/fat mass ratios for the enzymes tested.

<table>
<thead>
<tr>
<th>Enzyme/Fat Mass Ratio</th>
<th>% Acidity Reduction, 2 h Reaction Time</th>
<th>% Acidity Reduction, 3 h Reaction Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novozym 435</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0010</td>
<td>24%</td>
<td>27%</td>
</tr>
<tr>
<td>0.0011</td>
<td>10%</td>
<td>2%</td>
</tr>
<tr>
<td>0.0113</td>
<td>9%</td>
<td>10%</td>
</tr>
<tr>
<td>Lipozyme CALB L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0100</td>
<td>68%</td>
<td>64%</td>
</tr>
<tr>
<td>0.0200</td>
<td>67%</td>
<td>72%</td>
</tr>
<tr>
<td>Lipozyme TL 100 L *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0050</td>
<td>−90%</td>
<td>−55%</td>
</tr>
<tr>
<td>0.0100</td>
<td>−133%</td>
<td>−146%</td>
</tr>
</tbody>
</table>

* Negative values mean an increase of the fat’s acidity in the experiments with Lipozyme TL 100 L.

As shown in Table 3, the larger acidity reduction (60–70%) was achieved with Lipozyme CALB L. Thus, this enzyme was selected as the best enzyme to perform the parametric and kinetic study. Moreover, it was observed that for reaction times larger than 2 h, the rate of acidity reduction reduces significantly; as the values presented for 2 and 3 hours’ reaction time are similar, the variations are mainly due to variations in the samples characteristics. Furthermore, as the reaction is nearing equilibrium conditions, the rate of acidity reduction naturally reduces, or the FFA may be completely consumed, and the remaining acidity is due to other compounds that also consume KOH. Only a more complete characterization of the fat samples, in particular an identification of the compounds present, will allow a more complete explanation of the results.

Although the enzymes under study are considered to be adequate for the esterification and formation of esters from the FFA, in the presence of moisture they can reverse the
reaction, leading to hydrolysis of glycerides and thus forming FFA [47]. This happened with Lipozyme TL 100 L, which instead of reducing increased the fat acidity, as shown in Table 3. This occurred because, according to the producer (Novozymes A/S, Denmark) specifications [48], this enzyme is also a very effective catalyst for the hydrolysis of triacylglycerols in the presence of moisture. However, under anhydrous conditions, the reaction reverses, and this enzyme is able to synthesize new molecules by esterification.

The results show some variability between the experiments performed for the same enzyme using different enzyme/fat mass ratios. Yet, the variations are small and may be due to variations in the characteristics of the fat samples used, as the raw materials arriving for rendering varied from day to day. Therefore, it can be concluded that the effects due to changes in the value of the enzyme/fat mass ratios are not relevant and the quantities of enzyme used do not limit the esterification reaction.

### 3.3. Reaction Conditions Parametric Study

The experimental conditions and results of the parametric study are presented in Table 4. In all assays, the reaction time was equal to three hours. In most experiments, lower values of the enzyme/fat mass ratio were used when compared with those used in the previous section. The results of the previous section showed that lower values may be adequate to achieve a similar acidity reduction, as no significant differences were observed.

<table>
<thead>
<tr>
<th>Experimental Assays</th>
<th>Temperature (°C)</th>
<th>Alcohol/FFA Mass Ratio</th>
<th>Enzyme/Fat Mass Ratio</th>
<th>% Acidity Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st set</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>32.5</td>
<td>3.25</td>
<td>0.0060</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>35.0</td>
<td>3.25</td>
<td>0.0060</td>
<td>49</td>
</tr>
<tr>
<td>3</td>
<td>40.0</td>
<td>3.25</td>
<td>0.0060</td>
<td>62</td>
</tr>
<tr>
<td>4</td>
<td>42.5</td>
<td>3.25</td>
<td>0.0060</td>
<td>62</td>
</tr>
<tr>
<td>5</td>
<td>45.0</td>
<td>3.25</td>
<td>0.0060</td>
<td>67</td>
</tr>
<tr>
<td>6</td>
<td>47.5</td>
<td>3.25</td>
<td>0.0060</td>
<td>63</td>
</tr>
<tr>
<td>2nd set</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>45.0</td>
<td>2.44</td>
<td>0.0060</td>
<td>42</td>
</tr>
<tr>
<td>8</td>
<td>45.0</td>
<td>4.06</td>
<td>0.0060</td>
<td>38</td>
</tr>
<tr>
<td>9</td>
<td>45.0</td>
<td>4.88</td>
<td>0.0060</td>
<td>43</td>
</tr>
<tr>
<td>10</td>
<td>45.0</td>
<td>6.50</td>
<td>0.0060</td>
<td>36</td>
</tr>
<tr>
<td>3rd set</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>45.0</td>
<td>3.25</td>
<td>0.0030</td>
<td>39</td>
</tr>
<tr>
<td>12</td>
<td>45.0</td>
<td>3.25</td>
<td>0.0048</td>
<td>55</td>
</tr>
<tr>
<td>13</td>
<td>45.0</td>
<td>3.25</td>
<td>0.0090</td>
<td>58</td>
</tr>
<tr>
<td>14</td>
<td>45.0</td>
<td>3.25</td>
<td>0.0120</td>
<td>65</td>
</tr>
</tbody>
</table>

Results were divided into three sets of experiments, as described in Table 4. The three sets of experiments were carried out consecutively and not simultaneously, so that the best operating conditions found in the first set were used in the second and so on.

The first experiments set analyzed different temperatures, from 32.5 to 47.5 °C, to perform the esterification reaction. Results showed that up to 45.0 °C, the acidity reduction increased with the temperature, reaching 67% at 45.0 °C and then decreased to 63% at 47.5 °C. This is explained by the increase of the reaction rate and of the equilibrium constant with temperature, which results in higher conversion of the reactants in both cases. However, at higher temperatures, and although it is still within the range recommended by the manufacturer, some loss of enzyme activity may occur. Thus, the best operating temperature was set at 45.0 °C.

The second experiments set analyzed the alcohol/FFA mass ratio, by fixing the best temperature of 45.0 °C, found in the first set of experiments, and the enzyme/fat mass ratio of 0.0060, just varying the alcohol/FFA mass ratio. Results showed that below the
alcohol/FFA mass ratio of 3.25 (or ethanol/FFA molar ratio of 20, being the FFA expressed as oleic acid), the alcohol added as reagent was not enough to move the reaction equilibrium to the products, and limitations on the availability of ethanol may have occurred. On the other hand, above 3.25 of alcohol/FFA mass ratio, the excess ethanol may have contributed to the enzyme denaturation or precipitation [31]. Thus, the best operating alcohol/FFA mass ratio was set at 3.25.

The third experiments set analyzed the enzyme/fat mass ratio by fixing the temperature to 45.0 °C and the alcohol/FFA mass ratio to 3.25. Results show that for the lowest values of enzyme/fat mass ratio, 0.0030 and 0.0048, a lower acidity reduction was observed, signaling that the availability of enzyme is inadequate and limits the reaction rate. For enzyme/fat mass ratios higher than 0.0060, a greater acidity reduction was obtained, reaching 65% of acidity reduction for 0.0120. However, the variation observed experimentally, for an enzyme/fat mass ratio of 0.0060 and of 0.0120, is small and probably due to differences in the sample composition, showing that a value of 0.0060 is adequate and ensures a proper distribution of enzyme throughout the reaction media. Moreover, as the enzyme is one of most important process cost factors [2], the optimal amount of enzyme should be as small as possible, yet ensuring the largest acidity reduction possible.

Since the three sets of experiments were done consecutively and not simultaneously, and the fat samples used in different sets could present small differences in their composition, because it is a mixture of fats from an industrial process, the results are better compared within each set of experiments and not between different sets. This justifies any possible variation in the results for different sets; for example, it explains why in Experiment 13, in which the amount of enzyme used is greater than in Experiment 5, the reduction in acidity was not higher if the remaining conditions are the same. On the other hand, in the case of Experiments 7 and 8, the reason for the lower acidity reduction compared to Experiment 5 is the higher concentration of alcohol used that may have contributed to the enzyme denaturation or precipitation [31].

3.4. Kinetic Rate Laws

Each kinetic rate law was fitted to each experiment results of Table 4. As similar results were obtained for all experiments, the results obtained for the best reaction conditions were used: 45 °C of temperature, 3.25 of alcohol/FFA mass ratio, and 0.0060 of enzyme/fat mass ratio.

As an enzyme catalyzed the process, it was deemed appropriate to test the Michaelis-Menten kinetics model [49]. However, results presented in Figure 2a showed that this rate law is not adequate to describe the esterification reaction kinetics.

If a first-order rate law is assumed, a better fit is observed, as shown in Figure 2b. Yet, there are significant deviations between the linear fitting and experimental points, and the y-intercept is not close to zero [46,50]. Thus, it can be concluded that a first-order rate law is also not adequate to describe the acidity reduction kinetics.

Better fits are observed when the second rate laws are used, as shown in Figure 2c,d. Comparing the two variants, both show the same behavior with similar correlation coefficients, both closely verifying the limit condition for the y-intercept [46,50]. Although the correlation coefficients are not very close to 1, it must be stressed that the experiments were performed using real life samples in a simple experimental setup; thus, no much better fittings than those reported in this work are expected. Hence, it can be concluded that irreversible second-order rate laws are reasonably good to describe the enzymatic esterification reaction kinetics.

Comparing the two variants of the irreversible second-order kinetics (Equations (4) and (5)), results show higher correlation coefficients for the irreversible second-order rate law, a function of both the oleic acid and alcohol concentrations. Additionally, it is expected that a second-order rate law, a function of both oleic acid and alcohol concentrations, is more adequate to describe the kinetics, as results are sensitive to the alcohol concentration. Furthermore, according to the reaction stoichiometry and expected reaction mechanism, it
is predictable that both oleic acid and alcohol influence the reaction kinetics, thus justifying the utilization of Equation (5) [33,51,52].

![Graphs and equations illustrating the kinetics of esterification reactions](image)

**Figure 2.** Kinetics determined for the best operating conditions of enzyme/fat mass ratio of 0.0060 and alcohol/FFA mass ratio of 3.25, 45 °C, and 3 h reaction time: (a) Michaelis-Menten kinetics; (b) irreversible first-order kinetics function of oleic acid concentration; (c) irreversible second-order kinetics function of oleic acid concentration; (d) irreversible second-order kinetics function of oleic acid and alcohol concentrations; (e) reversible second-order kinetics function of oleic acid and alcohol concentrations; and (f) fitting of experimental data to the Arrhenius plot in the tested temperature range.

As described above, esterification reactions of FFA are reversible [35]. Even though results obtained showed that irreversible second-rate laws are adequate from an industrial perspective, a second-order reversible rate equation as defined in Equation (7) was considered. As described above, instead of using the analytical expression obtained by integrating Equation (10), as linearizing it is not possible, the first-order equation was integrated numerically using the Euler method [53] and the constants determined by the
least squares method. The integration was performed with a time step small enough to ensure that the integration error is negligible, and the application of the methodology to the other rate laws yielded the same results. The correlation coefficient $R^2$ is calculated using the standard statistical formula [54].

For all experimental data sets, similar behavior was observed when fitting the second-order reversible rate law to the experimental data. Figure 2e presents the results for the experiments performed using the optimized reaction conditions, showing a worse fit when compared with the fittings given in Figure 2c,d, as the correlation coefficient is significantly lower. In particular, it can be observed that the first two points deviate substantially for the theoretical rate law. This may be due to an inadequacy of the model, or different reaction mechanisms depending on the concentration of the reactants involved. As the reaction medium is a complex mixture of FFA and other chemicals, the initial acidity reduction may be due to the reaction between the enzyme and/or the ethanol with those compounds.

Concerning the equilibrium conditions, values of $K_e = 0.155$ and $X_e = 0.789$ were obtained. As expected, the maximum conversion obtained for three hours of reaction time is smaller than the equilibrium conversion, and a tendency to reach an equilibrium can be seen in Figure 2e. Yet, the fitting is poor; thus, it can be concluded that irreversible second-order rate laws, in particular as a function both of oleic acid and ethanol, are more adequate to the experimental data reported in this work. Other kinetic models could be more adequate to this reacting system, but, as no additional information is available for the enzymatic esterification of mammalian fat, simple models were preferentially selected in this work.

Figure 2f shows the fitting of the Arrhenius law to the experimental values (Equation (12)), considering the apparent kinetic constant for the second-order rate law function of the oleic acid and alcohol concentrations. A good fit is observed, showing that the temperature dependence is described by Equation (12). The values of the activation energy and the pre-exponential factor are, respectively, 54.7 kJ/mol and $4.6 \times 10^6$ L·mol$^{-1}$·min$^{-1}$.

### 4. Discussion

#### 4.1. Adequacy of the Homogenous Media Assumption

The results of the kinetic analysis allow an evaluation of the adequacy of the homogenous media assumption, in particular for Novozym® 435, which is used in immobilized resin, in which diffusional resistances may be important. Please note that, as the remaining enzymes are used in the liquid formulation, there are no diffusional effects that may limit the chemical reaction. The relative importance of the diffusional effects can be discovered by estimating the value of the effectiveness factor $\eta$, that is a function of the Thiele Modulus, $\phi$, assuming a first-order and a spherical-shaped particles $\phi = R_P \sqrt{k/D_{eff}}$, where $R_P$ is the average particle diameter, and $D_{eff}$ is the effective diffusivity [55]. The value of $R_P$ can be estimated from the particle size distribution, which is highly poly-dispersed. From the literature, a value of $R_P = 100 \mu m$ is considered [56]. For the $D_{eff}$, a value of the $10^{-9}$ m$^2$/s was assumed [55], using the value of first-order kinetic constant reported above (Figure 2b), $\phi \approx 1$. For spherical particles, $\eta = (3/\phi^2) (\phi \coth \phi - 1)$ [56], where $\coth$ is the hyperbolic cotangent function. For the set of parameters considered above, $\eta \approx 0.94$; although confirming that there are diffusional resistances in the catalytic system, it can be assumed that the process behavior is controlled by the chemical reaction, as the resistances are small. Therefore, it can be considered that even for Novozym® 435, the assumption of homogeneous reaction media is valid.

#### 4.2. Considerations on the Economic Feasibility of the New Process

The new esterification process for the fats acidity reduction would be implemented downstream of the rendering process, and it consists of a stirred reactor with a heating jacket, a tricanter to wash the treated fat, and auxiliary equipment (e.g., pump, piping, valves, and temperature control system). After the acidification reduction, the treated fat is stored under a nitrogen atmosphere before being sent to costumers.
A detailed analysis of the processes has already been published in the literature [2], and here a brief description is made. The investment cost in new equipment was considered to be zero, as the company has the internal resources to build the new process units using already-existing assets. The operational costs and revenues directly depend on the fat total quantity and fat acidity range. Thus, the new process operating costs include: the consumption of electricity (0.11 €/kWh) and steam (15.67 €/ton), the treatment of water (0.16 €/m³) and wastewater (2.50 €/m³), the consumption of reagent (commercial ethanol, 800 €/m³), and catalyst (Lipozyme® CALB L, 52.11 €/kg).

The revenues generated by the new process were calculated using information provided by the company [2], concerning the annual amounts of fat processed and the acidity range. Results of the economic analysis showed that, although the reduction in the mammalian fat acidity allows its market value to be increased from 360 €/kg (>9.5% acidity) to 600 €/kg (<5% acidity), the total costs are still greater than the revenues. This is mainly due to the high costs of commercial ethanol and Lipozyme CALB L, which correspond to the main two factors limiting the process economic feasibility [2].

4.3. Enzyme-Catalyzed Esterification vs. Other Approaches

The enzyme-catalyzed processes have advantages when compared to purely chemical processes [35]. First, the low toxicity of the catalyst and reagent used, enzymes and bioethanol, respectively, allow the resulting fat to be used as an ingredient in animal feed. In the case of chemical esterification, normally used for biodiesel production, concentrated sulfuric acid is used as a catalyst and methanol as a reagent, which are toxic and harmful to health. Moreover, bioethanol of renewable origin can be used to perform the reaction as it is recommended as a green solvent in biomass processing. Bioethanol can be obtained from sugar-rich and/or lignocellulosic renewable materials [57], from microalgae [58], or from industry’s by-products, such as brewer’s spent grains [59,60] or spent coffee grounds [61], contributing to the transition to a more bio-based and renewable economy [62]. Furthermore, due to its low toxicity, bioethanol is suitable for the esterification reaction of animal fats aimed to be further used in the animal feed industry or for other applications where the raw materials’ toxicity may be a relevant issue.

Second, the esterification reaction occurs at milder temperatures (30–50 °C) and atmospheric pressure [63], preserving the fat properties with lower energy input and costs in comparison to the alternative chemically catalyzed reactions that normally require higher temperatures (>60 °C) [22]. Third, reactions employing enzymes, such as lipases, are more selective and efficient than alkali-catalyzed reactions, reducing product losses, e.g., as a result of FFAs saponification [20]. Additionally, enzymatic esterification can be applied in oils or fats from different sources, either oil seeds, such as palm, rapeseed, soybean, sunflower, corn [25,28], or animal fats [24].

Therefore, processes based on reactions catalyzed by enzymes have reduced environmental and health impact, minimizing energy consumption and waste generated, fulfilling environmental sustainability requirements, integrated with economic growth and social well-being [32]. As far as the authors of this work are aware, no studies can be found in the literature concerning the enzymatic esterification for the acidity reduction in raw mammalian fat with high acidity, obtained from a real industrial rendering process, and using a Candida antarctica B lipase as a catalyst.

5. Conclusions

This study demonstrated the possibility to carry out the enzymatic esterification of a mixture of mammalian fat, obtained from a real industrial rendering process, aiming to reduce its high acidity and allowing its use as ingredient for animal feed with higher quality and market value. The enzymatic catalysis was chosen in this work to perform the esterification reaction because it offers a better alternative to chemical catalysis to improve the quality of the final product (ingredient for animal feed), due to the selectivity and specificity of the enzymes and the possibility of using moderate reaction conditions.
Therefore, at least 67% of the acidity reduction was achieved at a mild temperature of 45 °C, thus allowing the preservation of the fat properties for the intended application. Despite the high cost of lipases, which often restrict their use as biocatalysts, they are currently considered an interesting option for high commercial value applications, due to their many advantageous characteristics, such as high substrate specificity, reaction under moderate conditions, stability in organic solvents, and reduced toxicity. Among the four lipases tested in this study, the most effective to perform the esterification reaction was a *Candida antarctica* B lipase, with the commercial designation of Lipozyme® CALB from Novozymes A/S. Concerning the kinetic modelling, an irreversible second-order rate law expressed as a function of both the oleic acid and alcohol concentrations fitted the experimental results better, the activation energy and the pre-exponential factor being, respectively, 54.7 kJ/mol and $4.6 \times 10^6$ L·mol$^{-1}$·min$^{-1}$.

As future work, the possibility of recovering enzymes for reuse is a topic that should be addressed, as it has the potential to significantly reduce costs and even make the process profitable. In addition, continuous water removal can improve the reaction rate and reduce processing time, reducing energy consumption and consequently production costs. These improvements can contribute to the implementation of more ecological and bio-based processes, which are essential for the development of more sustainable processes.

**Author Contributions:** E.M. supplied the fat samples. S.A. and D.C. prepared and analyzed the fat samples, designed the experimental set-up, contributed with lab work, and performed data analysis and figures design; T.M.M., N.S.C. and A.A.M. approved the experimental set-up and experimental design, contributed to data analysis and figures design, and revised and wrote the manuscript. T.M.M., N.S.C., A.A.M. and E.M. critically reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was financially supported by project PP-IJUP2014-SOJA DE PORTUGAL; Base Funding—UIDB/00511/2020 of Laboratory for Process Engineering, Environment, Bio-technology and Energy—LEPABE—funded by National funds through the FCT/MCTES (PID/BDAC); Base Funding—UIDB/04730/2020 of Center for Innovation in Engineering and Industrial Technology, CIEI—funded by national funds through the FCT/MCTES (PID-DAC); António Martins thanks FCT (Fundação para a Ciência e Tecnologia) for funding through program DL 57/2016—Norma transitória.

**Acknowledgments:** To Novozymes A/S, for their kind offer of the enzymes used in this work. The authors thank Susana Casal from LAQV/REQUIMTE for the analysis of fatty acids composition.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

**References**

1. Alao, B.O.; Falowo, A.B.; Chulayo, A.; Muchenje, V. The potential of animal by-products in food systems: Production, prospects and challenges. *Sustainability* 2017, 9, 1089. [CrossRef]
27. Zhou, Y.; Li, K.; Sun, S. Simultaneous esterification and transesterification of waste phoenix seed oil with a high free fatty acid


57. Morais, W.G.; Pacheco, T.F.; Corrêa, P.S.; Martins, A.A.; Mata, T.M.; Caetano, N.S. Acid pretreatment of sugarcane biomass to obtain hemicellulosic hydrolysate rich in fermentable sugar. *Energy Rep.* 2020, 6, 18–23. [CrossRef]


