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Enzymatic Esterification for Acidity Reduction of Poultry Fat

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This work studies the acidity reduction of poultry fat through enzymatic esterification with ethanol to convert free fatty acids to ethyl-esters. Poultry fat samples were collected in a Portuguese company and characterized for their acid value (3.3-6.3 mg KOH/g fat), iodine value (73-109 g iodine/100 g fat), density (0.918-0.924 g/cm^3 at 15 ºC), kinematic viscosity (36.67-39.87 mm^2/s at 40 ºC) and moisture content (0.2-0.4 wt%). For the esterification, four commercial enzymes were tested as catalyst (Novozym 435, Lipozyme CALB L, Lipozyme RM IM, Palatase 20000 L, all from Novozymes). It was selected the one that contributed to the highest acidity reduction of poultry fat, in this case Novozym® 435, a CALB lipase immobilized in a hydrophobic carrier or acrylic resin. Different operating conditions were studied: reaction temperature (35, 45 and 55 ºC), reaction time (from 0 to 5 h), enzyme/fat mass ratio (0.0012 and 0.0024 wt/wt) and ethanol/FFA mass ratio (1.5, 1.6 and 3.1 wt/wt). Results show that at the best operating conditions (2 h of reaction time, 55 ºC of temperature, enzyme/fat mass ratio of 0.0024 and ethanol/FFA mass ratio of 1.6 wt/wt) at least 57 % reduction of free fatty acids can be achieved in just one reaction step. The reaction conditions can be further optimized, or a second esterification step can be performed for further reducing the free fatty acids content.

1. Introduction

The world production of animal fats is above 6.8 million tons, with more than half produced in North America (NRA, 2009). In particular, the poultry rendering industry produces not only fat, but also poultry, feather and blood meals, which are normally incorporated as ingredients for aquaculture, livestock and poultry feeds and pet food. These are produced using mostly slaughter by-products that represent about 30 % of the live poultry weight. Briefly, in the dry rendering process the crushed raw material is heated, cooked and dried in a batch digester. Then, the content is discharged in a percolator and pressed to separate the liquid fat from the protein solids. One indicator of the fat quality is its free fatty acid (FFA) content, as it is known that increased levels of FFAs reduce the digestibility and energy content of fats (NRA, 2009). The presence of FFAs indicates that the fat was exposed to water, acids and enzymes that hydrolyse triglycerides. The acidity level depends upon the rendering time and temperature, but also on the storage duration and temperature before the fatty stock is rendered (Mata et al., 2014). Thus, it is critical for the poultry industry to obtain a fat with reduced acidity, for which a well-designed process is required. To reduce the acidity and increase their commercial value, various options are available (Caetano et al., 2017; Mata et al., 2013), being the enzymatic esterification one of the most interesting (Caetano et al., 2014, 2017). It can be applied to fats from various sources, such as mammalian, poultry or fish oil, and has several important advantages when compared to purely chemical processes (Mata et al., 2012a,b). It is not necessary to use strong acids or alkalis, the operating conditions are milder (at temperatures below 60 ºC and atmospheric pressure), and less hazardous reagents such as ethanol can be used, while generating water as by-product (Caetano et al., 2012, 2013). Hence, this work aimed to study the acidity reduction of poultry fat through enzymatic esterification with ethanol to convert FFA to ethyl-esters.

2. Methods

2.1 Poultry fat characterization

The poultry fat samples were collected in a Portuguese company that converts by-products from animal processing industries into stable, value-added materials, such as fats or oils and meals that are used as ingredients for animal feeds. At the laboratory the poultry fat samples were characterized for the following parameters: density (kg/m^3), kinematic viscosity (mm^2/s), acid value (mg KOH/g fat), iodine value (g iodine/100 g fat), and moisture content (wt %). Table 1 shows the properties evaluated in the characterization of the poultry fat used in the enzymatic esterification reaction assays.

Table 1: Characteristics of the poultry fat samples

Parameter	Sample 1	Sample 2	Sample 3
Density (g/cm^3)	0.918	0.918	0.924
Kinematic viscosity (mm^2/s)	36.67	39.87	38.66
Acid value (mg KOH/ g fat)	3.3 - 4.5	4.1 - 6.3	6.1 - 6.3
Iodine value (g iodine/100 g fat)	109	73	87
Moisture content (wt %)	0.2	0.2	0.4

Table 1 shows that the three poultry fat samples have high acid values between 3.3 and 6.3 mg KOH/g oil meaning that a high amount of free fatty acids is present in the fat. The acid value depends on the nature of the fat sample and its FFA content. A slight modification of the acid value of the samples occurred in time, during storage in the laboratory, which may be associated to several factors, such as temperature, humidity and exposure to light and atmospheric air. The degree of unsaturation is measured by the iodine value, ranging between 73 and 109 g iodine/100 g fat. The iodine value is related to the halogenation reactions, which assumes that each double bond present in the unsaturated fatty acids can easily react with two halogen atoms, in this case iodine. Thus, the higher the iodine content the higher the amount of unsaturated fatty acids. The poultry fat moisture content is relatively low, below the maximum acceptable of 1 wt% moisture for being commercialized.

2.2 Enzyme selection for the esterification reaction

For the enzyme selection, four commercial lipase enzymes were tested that are known to be able to catalyse the esterification reaction of oil and fats i.e. that are capable of converting fatty acids into esters. The enzymes that were tested have different characteristics and activity, as shown in Table 2.

Table 2: Characteristics of the lipases tested

Enzyme	Origin	Activity *	Optimum pH	Optimum T	Specific substrate
Novozyme 435	*Candida antartica B*	10000 PLU/g	5-9	30-60 ºC	Esters and alcohols
Lipozyme CALB L	*Candida antartica B*	5000 LU/g	5-9	30-60 ºC	Esters and alcohols
Lipozyme RM IM	*Rhizomucour miehei*	275 IUN/g	7-10	30-50 ºC	esters
Palatase 20000 L	*Rhizomucour miehei*	20000 LU/g	7-10	30-50 ºC	esters

*LU – Lipase unit; PLU – Propyl laurate unit; IUN – Interesterification unit.

First, it was estimated the minimum amount of each enzyme needed to perform the esterification reaction, considering their activity. Then, it was determined the amount of alcohol required for the esterification reaction, considering the stoichiometric ethanol to react with the FFA, i.e. by knowing that the FFA content or acidity (%) of a fat is approximately half of its acid value (mg KOH/ g fat). Therefore, for the esterification reaction, it was weighed approximately 10 grams of poultry fat into each reaction vial and it was added the estimated volume of enzyme and ethanol 99.8 %. The vials were then placed in a thermostatic bath at the desired temperature with magnetic stirring and allowed to react during the defined time. At the end of the reaction time, three washes were carried out in a separatory funnel, discarding the aqueous phase that mainly contains water, enzyme and alcohol. The esterified fat was then collected, the fat mass weighed and the final acid value determined by titration with a potassium hydroxide solution of known concentration. Then, the acid value reduction percentage was calculated by knowing the initial acid value, previously determined and the final acid value of the fat, after the reaction. This way, it was possible to know the most suitable conditions to perform the esterification that conducted to a higher reduction of the acid value.

For the enzyme selection, the esterification was conducted at a temperature of 45 °C, with an ethanol/FFA mass ratio of 1.5 wt/wt, during 2 and 3 hours of reaction time. Table 3 shows the acid value reduction achieved by esterification at the two reaction times, for each of the enzymes tested.

Table 3: Acid value reduction for each of the enzymes tested

Enzyme	Acid value reduction at 2 h reaction time (%)	Acid value reduction at 3 h reaction time (%)
Lipozyme RM IM	24	39
Novozyme 435	36	39
Palatase 20000 L	9	30
Lipozyme CALB L	9	12

Table 3 shows that the enzymes that allow the greatest acidity reduction of poultry fat are Lipozyme RM IM and Novozyme 435. However, for a 2 h of reaction time, Novozyme 435 was more efficient. Based on these results, Novozyme 435 was selected for the study of the enzymatic esterification operating conditions.

2.3 Experimental planning for the study of the best esterification conditions

After the enzyme selection, the experiments were planned in order to vary five parameters so as to optimize the FFA esterification: reaction temperature, alcohol/FFA mass ratio, enzyme/fat mass ratio, reaction time and alcohol type. The baseline conditions, considered as a starting point for the study of the best operating conditions to perform the esterification reaction, were set as 45 ºC of temperature, 1.5 wt/wt of alcohol/FFA mass ratio and 0.0012 wt/wt of enzyme/fat mass ratio (corresponding to the assay 2 of Table 4). For each series of experimental assays four parameters were set with the values of the baseline conditions, varying only the parameter under study. Table 4 shows the planning of experiments for the study of the enzymatic esterification conditions.

Table 4: Experimental planning for the study of the esterification reaction conditions

Assay	Temperature (ºC)	Alcohol/FFA mass ratio (wt/wt)	Enzyme/fat mass ratio (wt/wt)	Time (h)	Alcohol type
1	35	1.5	0.0012	3	Ethanol
2	45	1.5	0.0012	3	Ethanol
3	55	1.5	0.0012	3	Ethanol
4	45	1.6	0.0012	3	Ethanol
5	45	3.1	0.0012	3	Ethanol
6	45	1.5	0.0024	3	Ethanol
7	45	1.5	0.0012	5	Ethanol
8	45	2.6	0.0012	3	Butanol

In order to study the reduction of the fat acidity over time, for the same operating conditions, 8 reaction flasks were placed at the same time in the thermostatic bath and one flask at a time was withdrawn from the bath and its acid value determined.

3. Results and Discussion

In order to determine the best operating conditions to conduct the esterification reaction, for a higher reduction of the poultry fat acidity, experiments were planned to vary some parameters such as temperature, enzyme/fat mass ratio and alcohol/FFA mass ratio. The enzyme used in these assays is Novozyme 435, selected previously, which conducted to a higher reduction of the poultry fat acidity. The starting conditions were those of the standard assay, identified as assay 2 in Table 4 of the experimental planning. All the reaction parameters were maintained constant, except the one that is intended to be studied.

3.1 Reaction temperature

For the study of the reaction temperature, the enzymatic esterification was performed with Novozyme 435 and absolute ethanol (99.8 % v/v) at three different temperatures (assays 1, 2 and 3 of Table 4), within the range considered to be optimal for the enzyme (30-60 ˚C). Figure 1 shows the results of the acid value (AV) variation over time for the esterification with 1.5 wt/wt of alcohol/FFA mass ratio and 0.0012 wt/wt of enzyme/fat mass ratio, at the temperatures of 35, 45 and 55 ˚C. Table 5 shows the acidity reduction obtained at the three temperatures studied.

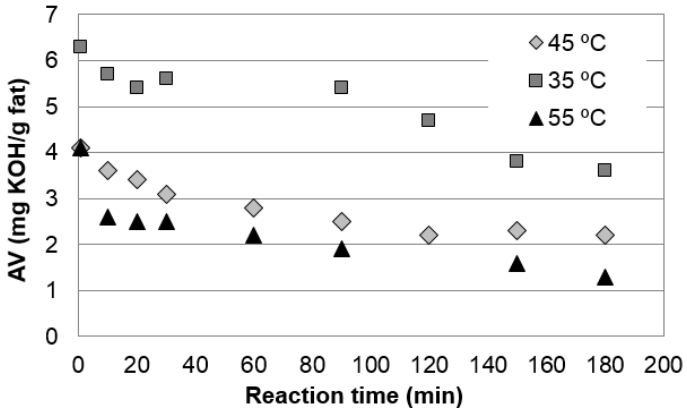

Figure 1: Acid value (AV) variation over time for the esterification at three temperatures.

Table 5: Acidity reduction for each temperature studied after 3 h reaction

Temperature (ºC)	Acidity reduction (%)
35	43
45	46
55	68

A greater reduction of the acid value was achieved at the temperature of 55 °C. At this temperature not only the conversion of FFA to esters was more efficient as the rate of this conversion is higher. Therefore, up to a certain limit (60 °C), the higher the temperature, the greater the amount of product formed in less time. This is justified because the reaction speed increases with temperature, allowing the molecules to move faster, with more energy and higher probability for collisions among molecules.

3.2 Ethanol/FFA mass ratio

In order to test the effect of the alcohol/FFA mass ratio, the enzymatic esterifications were performed with Novozyme 435 at 45 °C of reaction temperature and enzyme/fat mass ratio of 0.0012 wt/wt. The alcohol/FFA mass ration tested were 1.5, 1.6 and 3.1 wt/wt (assays 2, 4 and 5 of Table 4). Figure 2 shows the results of the variation of the acid value over reaction time, with the alcohol/FFA mass ratio of 1.5, 1.6 and 3.1 wt/wt. Table 6 shows the acidity reduction for each alcohol/FFA mass ratio studied.

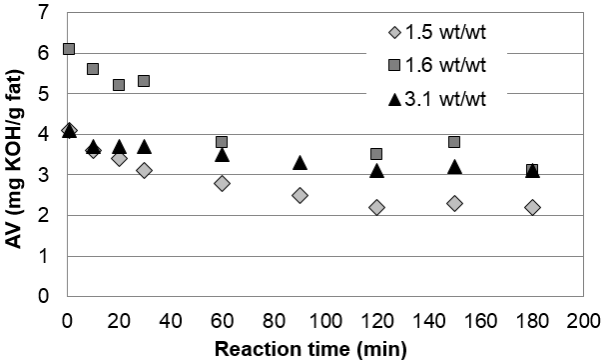

Figure 2: Acid value (AV) variation over time for the esterification with the three alcohol/FFA mass ratios tested.

Table 6: Acidity reduction for each alcohol/FFA mass ratio after 3 h reaction

Alcohol/FFA mass ratio (wt/wt)	Acidity reduction (%)
1.5	46
1.6	49
3.1	24

Table 6 shows that the acidity reduction is lower for larger amounts of ethanol, while for the alcohol/FFA mass ratio of 1.6 wt/wt, the acidity reduction was greater. These results show that increasing the amount of alcohol to double has a detrimental effect. This is possibly due to enzyme precipitation and deactivation in the presence of higher amount of alcohol, showing that the reaction is very sensitive to abrupt changes in ethanol.

3.3 Enzyme/fat mass ratio

For the study of the enzyme/ fat mass ratio, the enzymatic esterification was performed with Novozyme 435 and absolute ethanol (99.8 % v/v) with two different enzyme/fat mass ratios (assays 2 and 6 of Table 4), corresponding to respectively, the minimum amount of enzyme required for the reaction to occur and to twice this amount. Figure 3 shows the results of the acid value (AV) variation over time for the esterification at 45 °C of temperature, with an alcohol/FFA mass ratio of 1.5 wt/wt and enzyme/fat mass ratio of 0.0012 and 0.0024 wt/wt. Table 7 shows the acidity reduction for each enzyme/fat mass ratio studied.

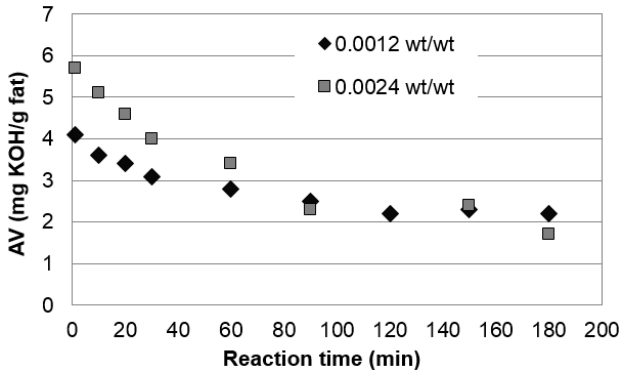

Figure 3: Acid value (AV) variation over time for the esterification with the two enzyme/fat mass ratios tested.

Table 7: Acidity reduction for each alcohol/FFA mass ratio after 3 h reaction

Enzyme/fat mass ratio (wt/wt)	Acidity reduction (%)
0.0012	46
0.0024	70

From the analysis of results it is verified that the enzyme/fat mass ratio that contributed to a higher acidity reduction is 0.0024 wt/wt, which is twice the minimum amount required for the esterification reaction to occur. Also, it is verified that the enzyme/fat mass ratio is the reaction parameter with a greater influence in the acidity reduction.

3.4 Reaction time

In order to test the effect of the reaction time, esterification assays were performed with Novozyme 435 during the reaction time of 5 hours, alcohol/FFA mass ratio of 1.5 wt/wt, enzyme/fat mass ratio 0.0012 wt/wt and 45 °C of reaction temperature (assay 7 of Table 4). At these conditions, an acidity reduction of 64 % was achieved. This result is similar to that obtained in the remaining esterifications carried out with a reaction time of 3 hours. Also, in all the performed esterification assays it was verified that for a reaction time above 2 hours the gain in the acidity reduction is not significant.

3.5 Alcohol type

Experimental assays were carried out with butanol, at 45 °C and for 3 h of reaction time, with alcohol/FFA mass ratio of 2.6 wt/wt, and enzyme/fat mass ratio of 0.0012 wt/wt. Figure 4 shows the results of the acid value (AV) variation over time for the esterification with the two alcohols tested (butanol and ethanol) under the same reaction conditions (assays 2 and 8 of Table 4).

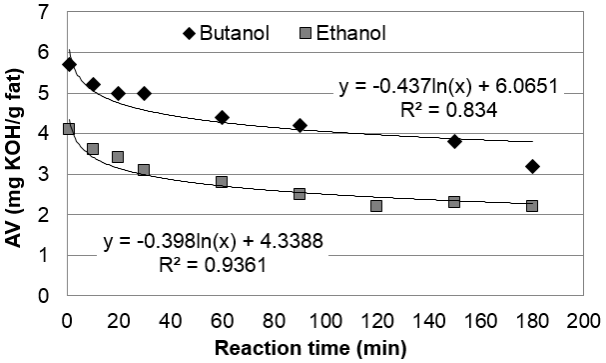

Figure 4: Acid value (AV) variation over time for the esterification with the two alcohol types tested

The assays carried out with butanol allowed an acidity reduction of 44 %. As expected, ethanol is more efficient for reducing the fat acidity (46 %) than butanol. This can be explained by the fact that butanol has a longer carbon chain than ethanol. It is known that the activity of the alcohol decreases with the increase of the carbon chain, due to the decrease of polarity, which reduces the activity of the molecule and thus, the conversion of FFA to esters. In addition, ethanol has the advantage of being less toxic to the enzyme than butanol.

3.6 Best operating conditions assay

After analyzing the results obtained from the previous esterification assays, it was performed the reaction at the best conditions selected from the previous assays, which are the following: ethanol 99.8 % v/v, reaction

temperature of 55 °C, reaction time of 120 min, enzyme/fat mass ratio of 0.0024 wt/wt and alcohol/FFA mass ratio of 1.6 wt/wt. The esterification assays performed for the best operating conditions selected contributed to an acidity reduction of 57 %, which is lower than the results obtained in the tests previously performed for temperature of 55 ˚C (68 %) and with an enzyme/fat ratio of 0.0024 wt/wt (70 %). This can be justified by the fact that the sample used to test these parameters in the selected best conditions (sample 3 of Table 1) has more impurities than the sample used in the previous assays performed (sample 2 of Table 1).
Although not directly comparable to this work, the study of Véras et al. (2011) looked at biodiesel production through simultaneous esterification and transesterification, without co-solvents and avoiding inhibition of the enzyme by ethanol and glycerol, concluding that the enzymatic production of biodiesel performed faster than most processes presented in literature, even those that include stepwise addition of alcohol or co-solvents.

4. Conclusions

This work performed a parametric analysis of the best reaction conditions to perform the acidity reduction of poultry fat by enzymatic esterification. It is concluded that the enzymatic esterification is an effective method for the poultry fat acidity reduction, with reduced product losses in comparison to the alkali neutralization, it is less sensitive to the presence of moisture in the fat than the chemically catalysed reaction and the milder temperatures used (< 55 ºC) allow preserving the fat nutritional properties with reduced energy input and costs. Novozyme 435 is an efficient catalyst for this purpose, having the advantage of being an immobilized enzyme, which potentially can be reused 5-10 times without activity loss. The esterification reaction is very sensitive to the presence of alcohol, since for higher amounts of alcohol, the enzyme can precipitate and lose its activity. For the range of values studied to perform the esterification, the best operating conditions are: a reaction temperature of 55 °C, an alcohol/FFA mass ratio of 1.6 wt/wt, an enzyme/fat mass ratio of 0.0024 wt/wt, a reaction time of 2 hours and ethanol as the alcohol type. Under these conditions, a minimum acidity reduction of 57 % can be achieved.

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