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Evaluation of the Potential of Ethyl Esterification Reaction Catalyzed by Enzyme to Improve the Quality of Bio-Oil

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Abstract. Biomass has been investigated in recent years as an alternative source for fuel production. The use of triglyceride biomass is highlighted and, from the thermal cracking process, solid, liquid and gaseous products with energetic potential are generated. The liquid product, called bio-oil, has characteristics similar those of fossil fuels. Its composition is mainly based on hydrocarbons and carboxylic acids, and the second group generates a limiting characteristic in its processing, refining and use as fuel, increasing the acidity of bio-oil. In this work, bio-oil produced from commercial soybean oil was used in a thermal cracking process at 525ºC. The objective was to carry out ethyl esterification reactions via enzymatic catalysis in order to reduce the acidity index of the crude bio-oil. All reactions were carried out at 40ºC to avoid enzyme deactivation and different conditions of type and amount of catalyst, alcohol mass ratio and reaction time were evaluated. The best result was obtained after 6 hours of reaction with 60% acidity reduction, using Novozym 435 lipase as a catalyst at a concentration of 5% in relation to the substrate mass and 1:3 mass ratio.

Key words. Bio-oil, Novozym 435, acid value reduction, renewable energy, biofuel.

1. Introduction

The investments in energy research and development prioritized in Brazil are distributed in seven segments: hydrogen, nuclear energy, biofuels, energy storage, technologies for sustainable thermoelectric generation, digital transformation and strategic minerals, according to the National Agency for Petroleum, Natural Gas and Biofuels-ANP [1]. Among these, it is worth to highlight the incentive for development of new fuel products.

Bio-oil is a fuel liquid renewable obtained by thermal cracking of different biomass in high temperature, approximately 500ºC, and in the absence of oxygen [2]-[7]. Bio-oil properties vary according to the processing conditions, such as temperature and residence time [8].

The major compounds of bio-oil from thermal cracking of soybean oil are hydrocarbons aliphatic and olefinic, in this mixture that resembling petroleum there is also oxygenated compounds in minor concentration.

About 75% of the compounds of bio-oil are alkanes, alkenes, naphthenes and aromatics and carboxylic acids with carbon number ranging of 4 to 20 [9]. Although bio-oil has similar properties to fossil fuels, it can be called green diesel [10], its properties are analyzed and compared with specific standards, standardized for fuels regulated as ASTM D6751-15 and EN 14214-12.

In order to determine the bio-oil quality, the commonly investigated properties are specific mass, viscosity, calorific value, iodine index and acidity index. The crude bio-oil from soybean presented viscosity 3.24 mm²/s, density 889.89 kg/m^3, iodine index 141.70 mg I_2/ g and acid index of 133.33 mg KOH/g [11].

According to Ramos et al. [11], carboxylic acids were 10% of crude soybean bio-oil that causes high acid index and instability [12]. In addition to the high content of carboxylic acids, the acidity of the bio-oil may be related to the presence of other organic acids, especially formic acid, acetic acid and aldehydes [13].

Upgrading processes are used for reduction on bio-oil acidity, as the addition of solvents, supercritical fluids, reactive distillation, zeolite cracking, emulsification, emulsification with diesel, hydrodeoxygenation, steam reforming, catalytic hydrogenation, hydrogenation, hydrotreating/hydrocracking, addition of antioxidants, esterification, combination of thermal and catalytic cracking, neutralization and esterification [14]-[22].

Lipases can catalyze esterification reactions in organic media and therefore find many applications of interest to industry as in the production of biofuel [23],[27]. Process with lipases are more clean, sustainable and using less energy [25]-[28].

In this study, the effect of enzymatic catalysis in reducing the acidity index of bio-oil was investigated. For this purpose, commercial immobilized lipases were used as catalysts in the ethyl esterification reaction of crude bio-oil from soybean oil. The use of enzymatic catalysts such as immobilized lipases for bio-oil upgrading has not been reported in the literature.

2. Methodology

In this work, the reagents used were ethanol (99.3%) from Quimidrol (Santa Catarina, Brazil), molecular sieve (3 Å, 8–12 mesh) from Sigma–Aldrich (Missouri, EUA).
The commercial soybean oil (brand name "Soya"), produced by Bunge (Santa Catarina, Brazil), was used by Ramos et al. [11] to produce crude bio-oil at 525ºC and 4.5 of residence time. The crude bio-oil properties were viscosity 3.24 mm^2/s, density 889.89 kg/m^3, iodine index 141.70 cg I_2/ g and an acid index of 133.33 mg KOH/g [11].
All other reagents and solvents were of analytical grade and were obtained from Brazilian suppliers.
The enzyme used in this work were *Rhizomucor miehei* (RM-IM), *Thermomyces lanuginosus* and Novozym 435, the form of *Candida antarctica* (now *Pseudozyma antarctica*) lipase B immobilized in a microporous resin (Novozymes).

A. Preliminary studies of lipases to catalysts on ethylic esterification of bio-oil

Esterification reactions were conducted in 10 mL screw-capped flasks containing 250 mg of enzyme (e.g. 5% by mass in relation to the bio-oil), 5 g of de bio-oil and 1.5 g of ethanol mass ratio 3:1 (free fatty acids to ethanol). In these experiments, were evaluated three different enzymes: Novozym 435 (CalB), *Rhizomucor miehei* (RM-IM) and *Thermomyces lanuginosus* (TL-IM). The reaction medium was incubated on a rotary shaker at 200 rpm and 40 ºC at 60 min. At fixed intervals of 10 min, 100 μL samples of the mixture were removed and analyzed for acidity index (AI). The AI of bio-oil was performed according to ASTM D 974 [29].

B. Improvement of the ethylic esterification conditions using a 2^3 factorial design

In this step, to obtain the best condition for enzymatic esterification, a 2^3 factorial design was used with three replicates at center point, Novozym 435, the lipase that stood out in the previous step was used as catalysts. The independent variables evaluated were the concentration of commercial immobilized lipase, the Novozym 435 (1%, 3%, 5% by mass in relation to the bio-oil), mass ratio (a bio-oil to ethanol - 1:3, 1:6, 1:9) and time (2 h, 4 h, 6 h), the response variable was the percentage AI reduction at the end of the esterification reaction.
Esterification reactions were conducted in 10 mL screw-capped flasks containing Novozym 435 (range 1%-5%), 5 g of de bio-oil and the amount of ethanol necessary to obtain the desired mass ratio (MR) (e.g., a bio-oil to ethanol mass ratio in the range of 1:3–1:9). The reaction medium was incubated on a rotary shaker at 200 rpm and 40ºC. Considering that the reaction starts instantly, the first aliquot, which represents time zero, was removed soon after. Immediately after removing the aliquot, the flasks were shaken, with the time defined for each experiment. At fixed intervals of 10 min, 100 μL equal samples of the mixture were removed and analyzed for AI. After the reaction, the Novozym 435 was separated from the reaction medium by filtration using Whatman no 1 filter paper. Molecular sieves were added to the reaction medium, to remove water formed during the esterification reaction, they were previously dried at 300ºC for 3 h and then stored in a desiccator at room temperature.
The data obtained in the factorial design were fitted to a first-order polynomial equation. The goodness-of-fit of the model was evaluated by the coefficient of determination (R^2) and an analysis of variance (ANOVA), to verify the adequacy of the model and response surfaces. The software STATISTICA (version 7.0) was used for regression analyses, statistical tests and response surfaces.

3. Results and discussion

A. Preliminary studies of lipases to catalysts on ethylic esterification of bio-oil

The bio-oil obtained from thermal cracking of soybean oil present an updated acid index of 139.6 mg KOH/g. Lipases were used to the reduction of AI of bio-oil on the ethylic esterification reaction. The results of AI of bio-oil obtained of its esterification catalyzed with three different immobilized enzymes, Novozym 435, *Rhizomucor miehei* and *Thermomyces lanuginosus* as shown in Figure 1.

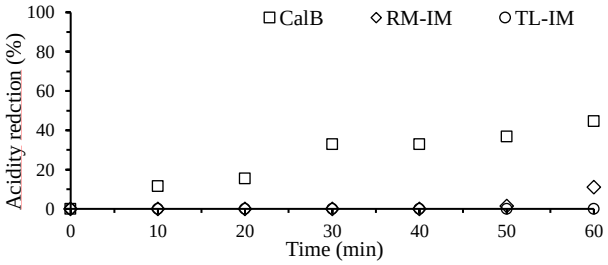

Figure 1. Profile of acid index of bio-oil catalyzed by the lipases on the ethyl esterification reactions. Reaction conditions: 5% of enzyme (wt.%: wt.%) (250 mg) in 5 g of bio-oil and 1.5 of ethanol mass ratio 3:1 (bio-oil to ethanol, at 40ºC and 200 rpm.

AI was measured at the moment of dilution, reaction time "zero", in order to consider the dilution effect, the other measurements were carried along reaction time.
When the Novozym 435 lipase are used as catalyst of esterification of bio-oil, the best profile of AI reduction was obtained. In this time, the difference was of 50% reduction AI of bio-oil using Novozym 435 in relation to enzyme RM-IM. In 1 h of reaction catalyzed with Novozym 435 the AI of bio-oil was 41.92 mg of KOH/g and catalyzed with RM-IM was 81.63 mg of KOH/g. The acidity value of bio-oils from literature ranged between 2.9 and 210.96 mg KOH/g according to Chiarello et al. [22] when considered 16 different feedstocks.
The bio-oil with the best result was with Novozym 435 as catalysts presented reduction AI of 44% at 1 h reaction. At time zero, the AI of the reaction medium containing lipase Novozym 435 was 75.75 mg of KOH/g of sample and at the end of the reaction this value decrease to 41.92 mg of KOH/g of sample. Meanwhile, the RM-IM enzyme showed a reduction of only 11%, lowering its acid value from 91.93 to 81.64 mg KOH/g of sample, and the TL-IM showed no reduction. Thus, according to the results obtained with the tests with the three different enzymes, and from the characteristics already described in the literature, such as mild reaction conditions, performance in different types of

substrates and high stability in solvents, can be is solvent-free system, it is very good properties, the other assays below were performed with lipase Novozym 435 as catalyst [30]-[32]. As seen in the literature review, chemical esterification occurs in reduced reaction times, so it was chosen to conduct the experiment for 60 min in order to verify only the immediate difference related to the lipase species. In this first moment, no other parameters were evaluated, so temperature, concentration and mass ratio were kept constant. With the results obtained from these preliminary tests to lipase that presented the best result in terms of lower bio-oil acidity rate was chosen to perform the factorial planning presented in the sequence. With these findings, the use of RM-IM and *Thermomyces lanuginosus* was not considered in other reactions carried out in this work.

B. Improvement of the ethylic esterification conditions using a 2^3 factorial design

The mass ratio of bio-oil to ethanol is an important variable in ester synthesis [30], and an ethanol excess above stoichiometric ratio is generally used to excess to avoid reversible reactions and increase reaction rates [33].
In this step, the conditions for esterification reactions were optimized for bio-oil and ethanol. The aim of these experiments were AI reduction to improve the bio-oil quality. The independent variables studied were the Novozym 435 concentration (E), mass ratio (MR - bio-oil to ethanol) and time of reaction (t). They were varied according to a 2^3 factorial design with tree replicates at the central point. The response variable was the percentage index acidity reduction. Table 1 shows the real and coded values of the independent variables and the experimental percentage index acidity reduction.
The equation obtained with the independent variables, the linear and quadratic coefficients and the interaction between the amount of catalyst, mass ratio between alcohol and bio-oil and time are shown in Equation 2.

$$\% \text{ IA reduction} = 8{,}485*\%E - 8{,}470*MR + 7{,}437 * t - 8{,}245* \%E*MR - 4{,}942* \%E*t + 5{,}392*MR*t \quad \text{(Eq. 1)}$$

Table 1. Experimental conditions and results according to 2^3 factorial design experiment used for improvement of the esterification of the bio-oil.

Run	Conditions			AI reduction (%)
	E[a] (wt %)	RM[b]	t[c] (h)	
1	1 (-1)	1:3 (-1)	2 (-1)	22.1
2	1 (-1)	1:9 (+1)	2 (-1)	3.7
3	5 (+1)	1:3 (-1)	2 (-1)	58.3
4	5 (+1)	1:9 (+1)	2 (-1)	21.2
5	1 (-1)	1:3 (-1)	6 (+1)	28.9
6	1 (-1)	1:9 (+1)	6 (+1)	46.4
7	5 (+1)	1:3 (-1)	6 (+1)	59.6
8	5 (+1)	1:9 (+1)	6 (+1)	29.8
9	3% (0)	1:6 (0)	4 (0)	43.7
10	3% (0)	1:6 (0)	4 (0)	36.0
11	3% (0)	1:6 (0)	4 (0)	36.0

a: enzyme concentration (Novozym 435); b: mass ratio (bio-oil:ethanol); c: time of the esterification.

The conditions of the esterification reactions that provided the greatest reduction in the acid value of the bio-oil were the concentration of 5% catalyst with a mass ratio of 1:3 at times of 2 and 6 h reaction. With at 6 hours, the reduction was even greater, confirming the statistical dependence of the time factor. Both conditions showed around 60% reduction in the acid index in relation to its value at the beginning of the esterification. Run 3, which represents the condition for 2 h, obtained a value of 34.18 mg KOH/g sample, while experiment 7 reached a value of 29.62 mg KOH/g sample. Run 2, corresponding to the lowest concentration of lipase and the highest alcohol mass ratio, is the one that presents the lowest value of reduction in the acidity index. The acid number of this test was only reduced from 93.42 to 90.0 mg KOH/g sample.
From these results, it was possible to state that the catalyst concentration is extremely relevant in the esterification reaction and when combined with lower alcohol mass ratios, it presents even more positive results.
The average reduction percentage of the triplicate of the central point experiments was 55.23%, and this number represents that the reached value of acidity index was approximately 38 mg KOH/g sample.
The difference obtained between runs 7 (greater reduction in acidity) and experiment 2 (lesser reduction in acidity) was 93%, which confirms the relevance of the factors chosen for analysis.
The percentage values of acidity index reduction obtained from the 2^3 factorial design were submitted to the Analysis of Variance (ANOVA) and the results are presented in Table 2.

Table 2. Analysis of variance for 2^3 factorial design using a acidity reduction (%) as response function.

Font	SS[a]	df[b]	MS[c]	F[d]	F_{tab}
Regression	2564.32	6	427.39	8.91	6.16
Residual	191.80	4	47.95		
Lack of fit	152.48	2	76.24	3.88	19.00
Pure error	39.32	2	19.66		
Total SS	2756.12	10			

a sum of squares; b: degree of freedom; c: mean square; d: variance.

In general, it is possible to verify that the high values of F, found for the regressions in relation to the respective F_{tab}, demonstrate that there are trends when these are modeled by the employed equations. Furthermore, with the low values of F for the lack of fit in relation to the respective F_{tab} value, they indicate that the residuals existing in the respective models can be considered null. These factors are further confirmed by the high value of R^2, which was equal to 0.9304 and the maximum explained variance which corresponds to 98.57.
In the respective Pareto chart was also possible to analyze the numerical significance of the observed trends, expressing the statistical effects in relation to the p value for the degree of freedom of the experiments carried out in a 95% confidence interval, as shown in Figure 2.

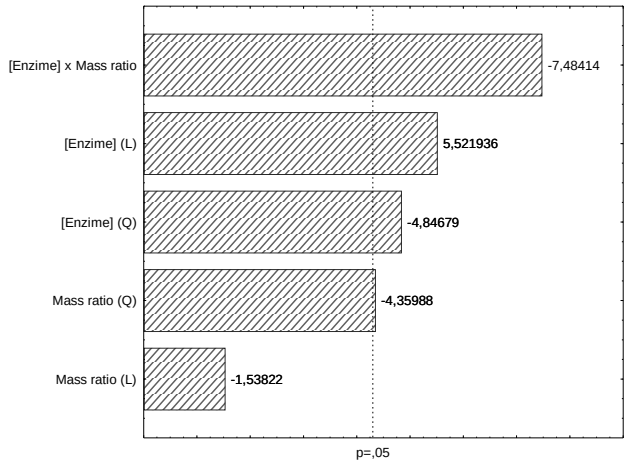

Figure 2. Pareto chart describing the primary and secondary effects of the main reaction variable to acidity reduction (%) of bio-oil according to 2^3 factorial design.

The Pareto chart indicates that the primary effects of catalyst, alcohol and bio-oil mass ratio and reaction time were significant, however, the catalyst concentration exerted a greater influence on the response function. The interaction between the percentage of catalyst and the mass ratio was also statistically significant.

Wang et al. [34] reached results of reduction of up to 88% of the acid value, being this result approximately 20% better than the best condition adopted in the enzymatic process (5% of catalyst and mass ratio 1:3 between bio-oil and ethanol at 72 h reaction time), while Ramos et al. [11] obtained a maximum of 82% reduction of the acid value in an ethyl esterification using the same catalyst.

Comparing the results obtained in this enzymatic study with the others already presented, it can be concluded that the reactions carried out using chemical products as catalysts are more likely to contribute to the reduction of the acidity index. However, post-treatments must still be used in the esterified bio-oil so that its subsequent use is possible.

Therefore, the investigation carried out using enzymatic catalysts in the esterification process is of great value, and the products generated, despite not meeting the parameters for fossil fuels, do not require more unit operations than filtration to remove immobilized lipases. Another convenient fact in the study carried out with enzymes is the fact that high temperatures are not used, and they can be reused in future reactions. In addition, this work is a pioneer investigation in the process of biocatalysis of biofuels, therefore, several new researches can be carried out in the same scope in order to improve the process conditions and further reduce the acidity index.

4. Conclusion

With the ethyl enzymatic esterification reaction was possible to reduce the acidity index. Novozym 435 lipase was the enzyme that presented the best performance in the esterification reactions. With the 2^3 factorial design, it was possible to identify that the condition that most contributed to the reduction of the acidity index was ethyl esterification at 40ºC with a catalyst concentration of 5% and mass ratio (1:3), with reduction of AI 60% of the initial acid value after 6 h reaction. The best operating condition in enzymatic esterification corresponds to the highest enzyme concentration and lowest alcohol mass ratio, resulting in lower reagent consumption. The reduction of the acidity index via enzymatic catalysis was not enough to allow the processing, refining and use of bio-oil as biofuel, other steps will be necessary for the AI to be reduced even further. As already seen, the chemical esterification process using basic and acid catalysts is already widespread. However, it was not possible to identify works that carried out enzymatic esterification in bio-oil and therefore, in this work, there was an opportunity to work using commercial enzymes as catalysts in order to verify the effectiveness of the reaction in reducing the acidity index. Based on this scenario and according to the large existing energy demand, it can be concluded that through the use of appropriate technologies and correct use of biomass it is possible to increase the contribution of renewable energy sources, which will lead to a change, thus contributing, for a society with a better sustainability profile.

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