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Optimisation de la production des esters éthyliques d'acide gras à partir des graines d'*Adansonia grandidieri* par une transestérification *in situ* catalysée par sa lipase endogène.

Optimization of fatty acid ethyl esters production from the Adansonia grandidieri seeds using an in-situ transesterification process catalyzed by its endogenous lipase.

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RÉSUMÉ :

La transestérification *in-situ* suscite un intérêt grandissant dans l'optique de simplifier la production de biodiesel. En raison de sa teneur élevée en lipides et de son activité lipasique, la graine d'*Adansonia grandidieri* a un potentiel élevé pour une production de biodiesel en utilisant la transestérification *in situ* sans l'ajout d'un catalyseur supplémentaire. Les effets du solvant, de la température, du rapport solvant / poudre et du rapport molaire éthanol / huile sur la capacité de la lipase endogène à catalyser cette réaction ont été étudiés. Parmi les solvants testés, l'hexane était le meilleur. Avec ce solvant et en utilisant l'approche de la méthodologie de surface de réponse, un modèle quadratique a été développé pour la synthèse des esters éthyliques d'acides gras et l'analyse de variance du modèle a révélé 7 termes significatifs. Les conditions optimales étaient : température de réaction, 39 ° C ; rapport hexane / poudre, 3,9 : 1 et rapport molaire éthanol / huile, 2,7 : 1. Dans ces conditions, un rendement de 92 % a été obtenu après 15 h de réaction. L'activité lipasique résiduelle a été évaluée après cette transestérification *in-situ* : trois réactions successives de transestérification éthanolique avec un rendement supérieur à 53 % ont été réalisées. Ces résultats mettent en exergue le potentiel de la graine d'*Adansonia grandidieri* pour la production de biodiesel par une transestérification *in-situ.*

Mots clés : Ester éthylique d'acide gras, Transestérification *in-situ*, Graine d'*Adansonia grandidieri*, Lipase végétale.

ABSTRACT:

In-situ transesterification methods have gained an increased interest with the aim of simplifying the production of biodiesel. Due to high lipid content and lipase activity, *Adansonia grandidieri* seed has a high potential to produce biodiesel using *in-situ* transesterification without any additional catalyst. The effects of solvent, temperature, solvent to powder ratio, and ethanol to oil molar ratio on the ability of endogenous lipase to catalyze this reaction were investigated. Among the solvent tested, hexane was the best. With this solvent and using the response surface methodology approach, a quadratic model was developed for the fatty acid ethyl esters synthesis and the analysis of variance of the model revealed 7 significant terms. The optimal conditions were: temperature of reaction, 39 °C; hexane to powder ratio, 3.9:1 and ethanol to oil molar ratio, 2.7:1. Under these conditions, a yield of 92 % was obtained after 15 h of reaction. Residual lipase activity was assessed after this *in-situ* transesterification: three successive ethanolic transesterification reactions with a yield that exceeded 53 % were performed. These results highlight the potential of *Adansonia grandidieri* seed for biodiesel production through *in-situ* transesterification.

Keywords: Fatty acid ethyl esters, *In-situ* transesterification, *Adansonia grandidieri* seed, Plant lipase.

1. INTRODUCTION

The industrialization of the society leads to a growing demand for clean energy (Agarwal, 2007; Nigam and Singh, 2011). Currently, fossil fuels provide the major part of the energy requirement (Rodionova et al., 2017; Voloshin et al., 2016). However, their limited reserves, high price volatility on the market and impact on the environment have led to the search for alternatives to these fuels (Ajanovic, 2011; Gasparatos et al., 2015; Gupta and Verma, 2015; Naik et al., 2010). Responding to this energy concern, biodiesel, a mixture of fatty acid alkyl esters (FAAE) usually produced by transesterification of triacylglycerols from vegetable oils with a short chain alcohol (methanol, ethanol, etc.) in the presence of either a chemical or an enzymatic catalyst, has been increasingly used as a substitute for petroleum diesel worldwide (Nguyen et al., 2020). The processing steps of oil feedstock used for the biodiesel production have been estimated to account for 70 - 80 % of the total cost of biodiesel production (Shuit et al., 2010; Tuntiwiwattanapun et al., 2017; Zeng et al., 2009). Recently, to reduce this production cost, many authors have shifted their interest to *in-situ* transesterification (Jiang et al., 2013; Kim and Yeom, 2020; Martinez-Silveira et al., 2019; Nguyen et al., 2020; Pascoal et al., 2020; Sendzikiene et al., 2020). This process is a direct transesterification of the oil contained in the biological material without prior extraction of the oil. Consequently, the oil-bearing material is the reaction substrate instead of pre-extracted refined oil used for conventional method for the biodiesel production.

The *in-situ* transesterification may be classified into catalytic and noncatalytic reactions (Go et al., 2016). The most frequently used process is usually done through chemical catalysis. Owing to the difficulties including, products separation, equipment corrosion and wastewater pollution encountered during chemical catalyzed *in-situ* transesterification, enzymatic *in-situ* transesterification could be more suitable (Jiang et al., 2013; Leung et al., 2010), but this approach has been only exploited by a few (Gu et al., 2011; Jiang et al., 2013, 2012; Jo et al., 2014; Sendzikiene et al., 2020; Su et al., 2009, 2007). However, the major bottleneck of enzymatic biodiesel production is high cost of lipases. Lipase enzymes, triacylglycerol hydrolase E.C. 3.1.1.3, can be of microbial, animal and vegetable sources (Barros et al., 2010). Many oilseeds (e.g., castor, ricin, *Jatropha curcas*) which have a high lipid content have also lipases therefore, they can be used both as oil feedstock and biocatalyst to produce biodiesel. This approach has a great potential because it eliminates the cost of extraction and purification of lipases. Gu et al. (2011) and Jiang et al. (2013) were used germinated castor seed and germinated *Jatropha curcas* L. as substrate and catalyst for the production of biodiesel, respectively. Under optimized conditions, there obtained a biodiesel yield of 87,6 % and 87,41 % illustrating the great potential of germinated oilseed self-catalyzed reactive extraction for biodiesel production.

Recently, our previous works have shown that *Adansonia grandidieri* seeds have a lipid content of 61.8 %, but also that crude lipase powder made from these germinated seeds can catalyze the synthesis of fatty acid ethyl esters (FAEE) (Kouteu et al., 2017; Nanssou Kouteu et al., 2016). To the best of our knowledge, there is no report on the application of *A. grandidieri* seeds for *in-situ* transesterification. Hence, the main objective of this study was to evaluate the practicable options to produce FAEE from germinated *A. grandidieri* seeds by *in-situ* transesterification without additional catalyst. The germinated seeds of *A. grandidieri* were first characterized and then the influence of the nature of the solvent during

transesterification of native seed lipids was evaluated. Finally, the synthesis of FAEE was optimized using the response surface methodology and the residual lipase activity in *A. grandidieri* seed after *in-situ* transesterification operated under optimal conditions was assessed.

2. MATERIALS AND METHOD

2.1. Materials

A. grandidieri seeds have been obtained from a PhileoL plantation located in Androy region (Madagascar). Sunflower oil (Palmitic acid, 6.4 %; Palmitoleic acid, 0.2 %; Stearic acid, 2.7 %; Oleic acid, 37.1 %; Linoleic acid, 53.6 %) was purchased in a supermarket in Montpellier (France). High-performance thin layer chromatography silica plates (HPTLC, 20 × 10 cm, silica gel 60 F254) were purchased from Merck (Darmstadt, Germany). All chemicals and solvents were of analytical or higher grade and were purchased from Sigma-Aldrich (Saint-Quentin, France).

2.2. Methods

2.2.1. Physicochemical characterization of seeds

The physicochemical analysis done on the dormant and germinated seed included: moisture content, ash content, protein content, total lipids content, carbohydrate content and fatty acid profile. The *A. grandidieri* seeds were germinated and dried according to the protocol established by Kouteu *et al.* (2016).

According to the NF V03-909 standards, the moisture content was determined by measuring the mass loss of a sample incubated at 105 °C in an oven (WTB Binder, Germany) until a constant mass was attained.

Crude ash content was carried out based on the French standard NF V18-101. Four grams (4 g) of crushed seeds was calcined in an electric oven at a temperature of 815 ± 10 °C until a constant mass it was obtained.

Protein content was determined according to the Dumas method by a CHN analyzer (Vario MACRO cube, Elementar, Germany). It involves combustion of 125 mg of ground seed previously encapsulated in tin foil in an oven (≈ 960 ° C) under an oxygen-enriched atmosphere The gases resulting from combustion (N_2, O_2, CO_2, H_2O, NO_x, SO_2, etc.) were trapped except for nitrogen and nitrogen oxides. Entrained by a flow of helium, the nitrogen oxides were then reduced to elemental nitrogen through the intermediary of tungsten, which is then detected by a thermally conductive detector which emits an electrical signal proportional to its content. The result obtained was multiplied by a nitrogen-protein conversion factor of 6.25 to obtain the protein content (Yi et al., 2013).

Total lipids content was determined according to the method described by Folch et al. (1957).

The carbohydrate content was evaluated by the difference of total lipid content, ash content and protein content from the raw material (carbohydrate content = 100 – Total protein – Total ash – Total lipid).

The procedure used for the determination of fatty acids profile was divided in two successive stages, the transformation of fatty substances into methyl esters and the analysis of the methyl esters by gas chromatography. The methyl esters were prepared according to the French standard NF T60-233. The relative percentage of each fatty acid is calculated from the peak area of the corresponding methyl ester.

All analyses for physicochemical characterization of seeds were done in triplicate for each sample.

2.2.2. In-situ transesterification reaction

The reactions were carried out in a 50 mL flask at 40 °C, whilst orbital stirring (250 rpm), with a solvent ratio (hexane or 2-methyltetrahydrofuran, ethanol) / seed of 3: 1 (v/w) and a molar ratio of oil / ethanol of 3: 1 (mol/mol) for 24 hours. Samples (50 µL) were taken regularly to monitor the kinetics. After dilution in hexane, the solutions were filtered (Millipore 0.45 microns) and analyzed by High-Performance Thin-Layer Chromatography Coupled with Densitometry.

2.2.3. *High-Performance Thin-Layer Chromatography Coupled with Densitometric* Analysis.

The quantification of FAEE in the reaction medium were performed according to Kouteu et al.(2017). The various automated steps and the data processing were carried out using the winCATS-Planar Chromatography Manager software (V1.4.4.6337 CAMAG, Switzerland). Under nitrogen atmosphere, different quantities of FAEE standards and test samples were deposited on a silica plates (HPTLC, 20 × 10 cm silica gel Plate 60) using an automatic depositor (ATS4 Camag, Muttenz, Swiss). The mixtures hexane / diethyl ether / acetic acid (80: 20: 2, v / v / v) and copper sulphate: phosphoric acid: methanol: water (10: 8: 5: 78, v / v / v / v) were used for elution and revelation, respectively. Finally, the carbonization of the plates was done in an oven at 180 °C for 10 minutes. The area of the different compounds revealed was determined by scanning the plate at 550 nm with a TLC3 scanner (CAMAG, Muttenz, Switzerland). The area of each spot is proportional to the amount of compounds it contains. From the standards, a calibration curve was established to calculate the amount of FAEE of the different reaction media.

2.2.4. Experimental design

Modelling and optimization of the yield of FAEE obtained during *in-situ* transesterification reactions were performed using an experimental design. The variables of this study were temperature (° C), solvent / powder ratio (v/w) and oil / ethanol (mol / mol) molar ratio. To make a direct comparison of the effects of the variables between them and to eliminate the bias generated by their respective units, a transformation of the variables was previously necessary. These new variables are dimensionless and referred to as coded variables. The transition from natural variables to coded variables was based on the formula below.

$$X_n = 2\frac{(X-\underline{X})}{(X_{max}-X_{min})} \quad (1)$$

X_n is coded variable which could be the temperature (X_1), solvent/powder ratio (X_2), and oil/ethanol molar ratio (X_3). X_{max} and X_{min} are the highest and the lowest value of the variable in its original unit, respectively (**Table 1**).

The experimental design used is a central composite circumscribed (CCC) design with 18 experiments (6 axials and 4 central points). The experiments were completely randomized to limit the systematic error.

To adjust the experimental data, a polynomial of second degree with interaction whose expression is presented below was used.

$$y = \alpha_0 + \sum_i \alpha_i x_i + \sum_{ii} \alpha_i x_i^2 + \sum_{ij} \alpha_{ij} x_i x_j + \varepsilon \quad (2)$$

Table 1: Domain of variation of the factors used in experimental design

	-1.41	-1	0	1	+1.41
X_1: Temperature (°C)	25.9	30	40	50	54.1
X_2: Oil to ethanol molar ratio	1:1.6	1:2	1:3	1:4	1:4.4
X_3: Hexane to powder ratio	2.6 :1	3 :1	4 :1	5 :1	5.4 :1

In this equation, y is the yield of FAEE, and x_i and x_j are the factors, α_0 is the constant of the equation, α_j is the linear coefficient, α_{ii} is the quadratic coefficient, α_{ij} is the interaction coefficient and ε, the error.

$$\text{Yield } (\%) = \frac{\text{Concentration of FAEE} \times \text{Volume}}{\text{Theoretical Quantity of FAEE}} \times 100 \quad (3)$$

The quality of the fit of the postulated model was evaluated by determining the coefficients of determination (R^2) and adjusted determination $\left(R^2_{adj}\right)$ and performing the test of the lack of fit of the model.

The statistical signification of the coefficients included in the model on the yield of FAEE was determined using analysis of variance (ANOVA). A coefficient is statistically significant if its p-value is less than 0.05.

All calculations were performed with the Statgraphics Centurion software version 16. Determination of optimal conditions to maximize the yield of esters was performed using the optimization function available in this software. The response surface curves were plotted using Sigmaplot software version 12.

3. RESULTS AND DISCUSSION

3.1. Chemical composition of germinated and dormant *Adansonia grandidieri* seeds.

Our previous work on the influence of germination on the ethanolysis activity of *A. grandidieri* seed showed that germination is a key step to have an important activity (Nanssou Kouteu et al., 2016). Therefore, the seeds were germinated (96 h), shelled, then dried and crushed (635 µm). The proximal composition of these powders is shown in Table 2.

Table 2: Proximal composition of germinated and dormant seed of *A. grandidieri*

	Moisture	Ash	Proteins	Lipid	Carbohydrates
	(g/100 g wet matter)	(g/100 g dry matter)			
Dormant	4.8 ± 0.4	5.5 ± 0.5	19.1 ± 0.1	61.8 ± 0.7	13.6±0.3
Germinated	3.0 ± 0.1	5.1 ±0.3	18.7 ±0.2	60.4±0.3	15.8±0.3

The protein and lipid contents of germinated seeds are 18.7 ± 0.2 % and 60.4 ± 0.3 %, respectively. They are therefore only very slightly influenced by a germination of 96 h (dormant seeds: Protein 19.1 ± 0.1 % and lipids 61.8 ± 0.7 %). It is generally accepted that lipids are used to provide the energy needed for plant growth during germination of oilseeds. This small variation would mean that the lipids are sparingly

mobilized during the first 96 hours of germination. During germination of a similar duration, Jiang et al. (2013) and Gu et al. (2011) also observed a small variation in the lipid content of seed kernel of *R. communis* and *J. curcas*, respectively.

The analysis of the oil from germinated seeds have shown that the oil is very acidic. Indeed, it contains about 24.3 % of free fatty acids (FFA) (relative to the mass of total lipids). This presence is probably related to a high lipolytic activity of the endogenous lipase once the seed germinated. Consequently, the transformation of the endogenous lipids of the seed into biodiesel would correspond, on the one hand, to the transesterification of the native triacylglycerols (TAG) and, on the other hand, to a simultaneous esterification of the FFA present. Therefore, to simplify the discussion on the results obtained, the term "(trans) esterification" which corresponds to the two reactions, the transesterification of TAG and the esterification of FFA, would be more appropriate.

The type and nature of the fatty acid of germinated seeds (Table 3) is identical to that of dormant seeds. However, in quantitative terms, it is worth noting a decrease in the palmitic acid content (41.7 to 35.7 %), the major fatty acid, associated with an increase in those of oleic acid (23.5 to 27.2 %) and linoleic acid (15.4 to 19.9 %). Our previous works have shown that the crude lipase extract from *A. grandidieri* seeds showed no typo-selectivity with respect to the carbon chain length of an ethyl ester and a triacylglycerol (Nanssou Kouteu et al., 2016).

Table 3: Relative percent composition of fatty acids in dormant and germinated *A. grandidieri* seeds oil

	Dormant	Germinated
Fatty acids	(%)	(%)
Myristic acid	0.2	0.1
Palmitic acid	41.7	35.7
Palmitoleic acid	0.4	0.5
Margaric acid	0.2	0.2
Margaroleic acid	0.5	0.3
Isostearic acid	0.8	0.7
Malvalic acid	2.0	2.1
Stearic acid	4.0	4.3
Oleic acid	23.5	27.2
Linoleic acid	15.4	19.9
α-Linolenic acid	2.2	1.6
γ-Linolenic acid	1.6	1.4
Dihydrosterculic acid	1.6	0.7
Arachidic acid	0.9	0.4
Paullinic acid	0.2	0.7
Behenic acid	0.3	0.2

Previous works on the influence of the water activity (a_w) on powder of *A. grandidieri* in organic medium (Kouteu et al., 2017) have shown that a pre-conditioning at a water activity of 0.33, corresponding to a water content of 3.5 %, was required for optimal lipase activity. The water content of the germinated *A. grandidieri* seed used in this work was measured at 3.0 % which corresponds to a water activity of 0.29. This value being close to that considered optimal, therefore it is not useful to perform a pre-conditioning.

3.2. Influence of solvent on ethanolic transesterification yield of endogenous lipid of *Adansonia grandidieri* seeds.

The (trans)esterification reactions of the native lipids of a seed can generally be carried out in the presence or absence of a solvent. However, the use of a solvent helps to overcome diffusion problems (external and internal) (Barekati-Goudarzi et al., 2016; Makareviciene et al., 2020). When the (trans) esterification reactions of the native lipids are catalyzed by a lipase, the solvent must meet two major criteria i) have a good extractive capacity of the seed oil and ii) guarantee the stability and the activity of the lipase. Hexane is known to be a good solvent for extracting seed oil, and its influence on lipase is a function of the nature of the latter. Recently, the work of Sicaire et al., (2015) have shown that 2-methyltetrahydrofuran, a biobased, biodegradable, and non-toxic solvent, could be an alternative to hexane (solvent of fossil origin), for extracting oil from oilseeds. In order to determine the best solvent for (trans)esterification of the oil of the *A. grandidieri* seeds, reactions were conducted with these two solvents at a solvent / enzyme powder ratio (3:1, v/w) at 40 °C and with an amount of ethanol corresponding to an oil / ethanol mole ratio of 3: 1. The yields obtained were compared with a control medium where the solvent was replaced by ethanol.

The kinetics of reactions were dependent on the nature of the solvent (**Figure 1**). In fact, the reaction rates with 2-methyltetrahydrofuran and using ethanol as acyl acceptor and extraction solvent at the same time were lower than with hexane. With the latter, a maximum yield of ethyl esters (69 %) is rapidly obtained after 15 hours of reaction. With only ethanol in the medium, the maximum yield of FAEE is also reached, but for a reaction time of 20 h. However, the maximum yield obtained with this medium is only 18 %. With 2-methyltetrahydrofuran and 20 h, yield is around 30 %, but there continues to be a significant increase of FAEE yield after 20 hours of reaction. These results are probably related to the polarity of the solvents with respect to their partition coefficient of the organic solvent in an octanol/water two-phase system (log P) 4, 1.85 and -0.30 for hexane, 2-methyltetrahydrofuran and ethanol, respectively (Lide, 2005). It is common for the most apolar solvent to lead to better results during enzymatic transesterification (Gagnon and Vasudevan, 2011). Our results are therefore consistent with most of the work. For this reason, hexane was retained to perform the other tests.

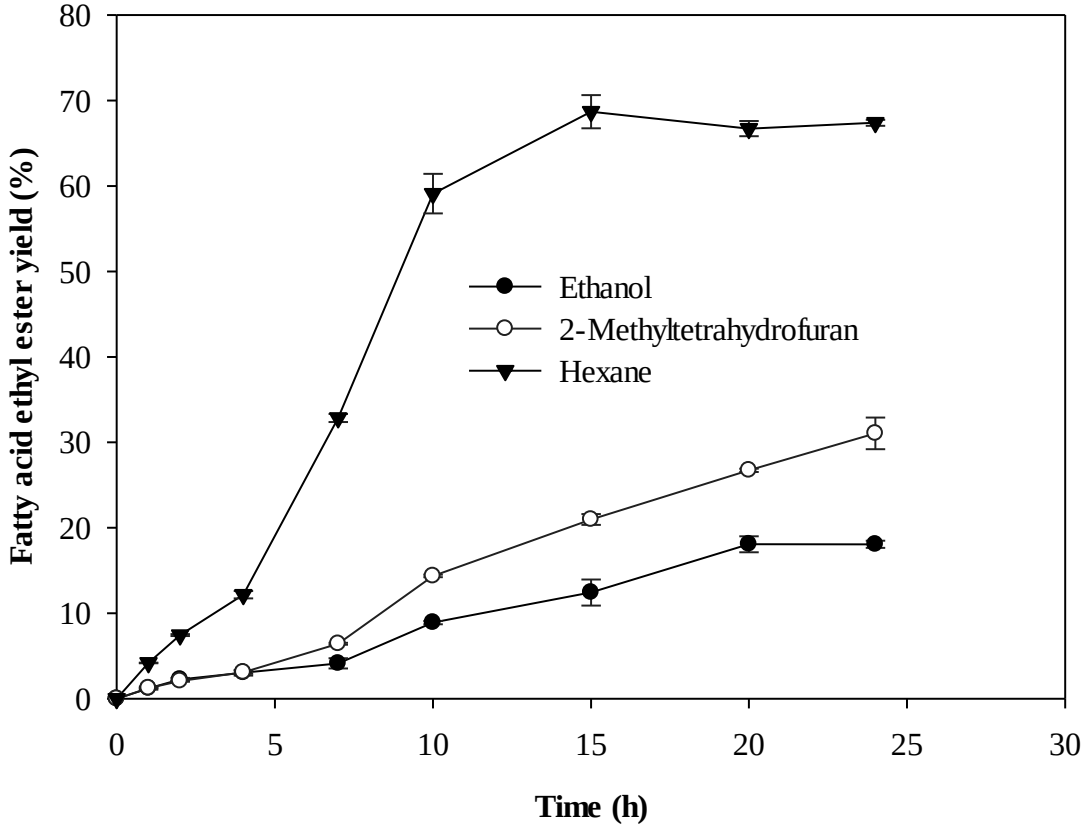

Figure 1: Kinetic transesterification of germinated *Adansonia grandidieri* seeds oil. Reaction conditions: 40 °C. solvent (2-Methyltetrahydrofuran. Hexane. Ethanol) to powder (3:1 v/w). oil to ethanol (3:1 mol/mol) under orbital agitation (250 rpm) during 24 h.

3.3. Modelling of ethanolic transesterification of endogenous lipid of *Adansonia grandidieri* seeds: Influence of temperature, Hexane to powder ratio and Ethanol to oil molar ratio.

The response surface methodology was used to study the influences of temperature, oil / ethanol molar ratio, hexane / powder ratio and potential interactions of these factors on the yield of FAEE. It is in this perspective that a central composite circumscribed design was used. This plan includes 18 test points including 4 in the center of the plan making it possible to estimate the experimental error, 8 test points of a factorial plan and 6 test points in star configuration. The study initially involved developing a model to predict the variation of the yield according to the factors studied, followed by the determination of the qualitative adjustment indicators of the model. Finally, the significance of the various factors and the optimal conditions were also determined.

3.3.1. Empiric model of ethanolic transesterification of endogenous lipid of Adansonia grandidieri seeds.

The experimental yields and theoretical yields of FAEE obtained for the experiments are presented in Table 4. These data permitted the development of a quadratic model using Statgraphics Centurion XVI software. This model has 10 terms: one constant, 3 linear effects (X_1, X_2, X_3) and three quadratic (X_1^2, X_2^2, X_3^2) factors and 3 combinations (X_1X_2, X_2X_3, X_1X_3) between the factors representing the interactions.

Table 4: Factors (real and coded values) and responses of experimental design of the transesterification of germinated *Adansonia grandidieri* seeds oil.

	Factors						Responses	
	Coded value			Real Value			FAEE yield (%)	
N°	X_1	X_2	X_3	X_1	X_2	X_3	Experimental	Theoretical
1	0	-1.41	0	40	1.6	4	67.5 ± 1.0	69.7
2	-1	-1	-1	30	2	3	71.7 ± 0.3	67.3
3	1.41	0	0	54.1	3	4	62.8 ± 0.6	60.6
4	0	0	0	40	3	4	89.9 ± 1.1	89.1
5	1	1	-1	50	4	3	34.4 ± 0.9	33.1
6	1	-1	1	50	2	5	56.5 ± 0.4	55.8
7	0	1.41	0	40	4.4	4	45.1 ± 1.3	45.8
8	-1	1	-1	30	4	3	46.3 ± 0.7	45.5
9	-1	-1	1	30	2	5	51.6 ± 0.8	51.3
10	0	0	0	40	3	4	87.6 ± 1.0	89.1
11	1	-1	-1	50	2	3	55.6 ± 0.6	56.8
12	0	0	-1.41	40	3	2.6	65.6 ± 0.8	68.5
13	0	0	0	40	3	4	91.5 ± 1.2	89.1
14	0	0	0	40	3	4	90.3 ± 0.3	89.1
15	1	1	1	50	4	5	40.9 ± 0.5	43.8
16	0	0	1.41	40	3	5.4	64.8 ± 0.6	64.7
17	-1	1	1	30	4	5	43.9 ± 1.5	41.2
18	-1.41	0	0	25.9	3	4	61.1 ± 0.2	66.2

The signs + and - in front of the coefficient of the different terms of the model mean that the parameter influences positively or negatively the FAEE yield, respectively.

$$\text{FAEE Yield} = 89.10 - 1.97X_1 - 8.47X_2 - 1.34X_3 - 12.84X_1^2 - 0.48X_1X_2 + 3.75X_1X_3 - 15.66X_2^2 + 2.91X_2X_3 - 11.23X_3^2 \quad (3)$$

In most of the published works (Amalia Kartika et al., 2013; Wu et al., 2014; Zeng et al., 2009) which used experimental designs, the authors found that the adjusted coefficient of determination is a good indicator to judge whether the model explains all the results. According to these authors, a value of R_{adj}^2 close to 1 indicates a better model. The R_{adj}^2 of the model obtained was 0.964 meaning that the model explains up to 96.4 % of the relationship between the experimental parameters and the FAEE yield. Karam (2004) advocated adding to R_{adj}^2 the lack of fit test of the model to avoid hasty conclusions. This test confirmed that the model appeared adequate for describing the observed data because the probability value (0.0745) is greater than 0.05.

3.3.2. Determination of significant factors during ethanolic transesterification of endogenous lipid of Adansonia grandidieri ***seeds.***

The model developed had 9 parameters that can have a significant influence on the FAEE yield. In order to determine them, the analysis of the variance of the model was carried out, and the results are presented in **Table 5**. Considering a risk of 5 %, a factor is considered significant when its p-value is less than 0.05. Of the nine terms, seven are significant.

The oil / ethanol molar ratio is the most influential factor of those studied. Its contributions for the variation of the yield of ethyl esters were 14.4 % and 26.7 % for its linear and quadratic effect, respectively, for a total of 40.2 %. This result is not surprising in the light of the importance of oil/ethanol molar ratio for the studies on biodiesel production by enzymatic transesterification. Its linear (P (X_2) = 0.0004) and quadratic (P (X_2^2) = 0.0001) effects were found to be significant and to negatively influence the yield of ethyl esters. This means that the molar ratio at its lowest and highest value contributes to a decrease in yield. These effects of ethanol could be explained by the fact that at low ratios, there would not be enough alcohol to synthesize the ethyl esters and high molar ratios would lead to inhibition or deactivation of the *A. grandidieri* seed lipase. Gu et al. (2011) have also highlighted a similar phenomenon during a methanolysis of *J. curcas* seed oil by its lipase.

Table 5: Analysis of variance of quadratic model of the FAEE yield of ethanolic transesterification of endogenous lipid of germinated *A. grandidieri* seed

Variance Source	Sum of squares	Degrees of freedom	Mean square	F-Value	P value
X_1	46.79	1	46.79	18.23	0.0236*
X_2	859.85	1	859.85	336.02	0.0004*
X_3	21.95	1	21.95	8.45	0.0622
X_1^2	1304.74	1	1304.74	515.26	0.0002*
X_1 X_2	1.71	1	1.71	0.72	0.4595
X_2 X_3	111.75	1	111.75	43.85	0.0070*
X_2^2	1945.83	1	1945.83	766.35	0.0001*
X_3 X_1	67.86	1	67.86	26.49	0.0142*
X_3^2	993.83	1	993.83	394.25	0.0003*
Lack of fit	84.96	5	16.99	6.68	0.0745
Pure error	8.35	3	2.78		
Total (corr.)	5447.66	17			

*All coefficients having superscript asterisk are significant.

As previously observed with the oil / ethanol molar ratio, the quadratic (P (X_1^2) = 0.0002) and linear (P (X_1)= 0.023) effects of the temperature are significant and negatively influence (decrease) the yield of FAEE. The temperature in an extractive reaction can have an impact on the properties of the medium and the stability of the catalyst. High temperatures could cause a decrease in the viscosity of the medium thus facilitating the solubility and the diffusion of the reagents inducing an increase in the reaction rate. However, this increase is not always beneficial since it could also lead to evaporation of the solvent and /

or destabilization of the three-dimensional structure of the enzyme resulting in thermal denaturation of the lipase. In view of the reaction conditions used, the negative influence of the quadratic effect of temperature would reflect more a thermal denaturation of the lipase. Jiang et al. (2013) also noted a reduction in the lipase activity of *Ricinus communis* seeds during an extractive reaction of seed oil at temperatures above 35 ° C. They indicated that the use of high temperatures (40-45 ° C) would promote the hydrolytic activity of the lipase which is undesirable for transesterification reactions.

Variance analysis have also shown that the quadratic effect of the hexane / powder mass ratio (P (X_3^2) = 0.0003), that is to say high values (> 5 : 1), is significant and causes a decrease in the FAEE yield, probably due to excessive dilution of oil and ethanol (Gu et al., 2011; Jiang et al., 2013). The hexane / powder ratio is the only factor with a non-significant linear effect (P (X_3) = 0.0622). However, its effect is a function of the value of the other parameters since its interactions with the temperature and the oil / ethanol molar ratio are (P (X_1X_3) = 0.0142 and P (X_1X_3) = 0.007). The 3D and 2D graphs illustrating these interactions are shown in **Figure 2**. For example, for a temperature of 30 ° C, a decrease of 10 % of the yield of FAEE (72 % vs 62 %) is observed when the ratio hexane / powder increases from 3 to 5 (**Figure 2**b). On the other hand, at a temperature of 50 °C, for this same variation of the hexane / powder ratio (3 to 5), an increase of 5 % is observed (61 % vs 66 %) (**Figure 2**b). For low temperatures (30 °C), low hexane / powder (3: 1) mass ratios should be used for high yields (72 %) while at high temperatures (50 °C) they are high hexane / powder (5: 1) mass ratio which are preferable. Since thermal denaturation may occur at high temperatures (50 °C), the use of high hexane / enzymatic powder ratios at these temperatures would mitigate this effect (Miroliaei and Nemat-Gorgani, 2002).

The interaction between hexane / powder ratio and the oil / ethanol molar ratio are illustrated in **Figure 3** a&b. For an oil / ethanol molar ratio of 2, when the hexane / powder ratio increases from 3 to 5, the yield decreases by 8 % (75 % vs 67 %) while at a molar ratio of 4, it increases by 3 % (53 vs 56 %). These results would mean that to obtain high yields with low oil / ethanol molar ratios, low hexane / powder ratios should be used, and when higher oil / ethanol molar ratios are used the hexane / powder ratio should be increased by the same magnitude. These trends may be explained by the fact that enough hexane in the medium would be required to protect the lipase against its inhibition by ethanol. In addition, large amounts of hexane in the medium would cause a dilution of ethanol. For a better activity of the lipase, that is to say reduce these two effects (dilution and inhibition) these factors must be maintained at values in the center of the domain, that is to say at intermediate values: molar oil / ethanol ratio (3: 1) and solvent / enzymatic powder ratio (4: 1) (**Figure 3**a).

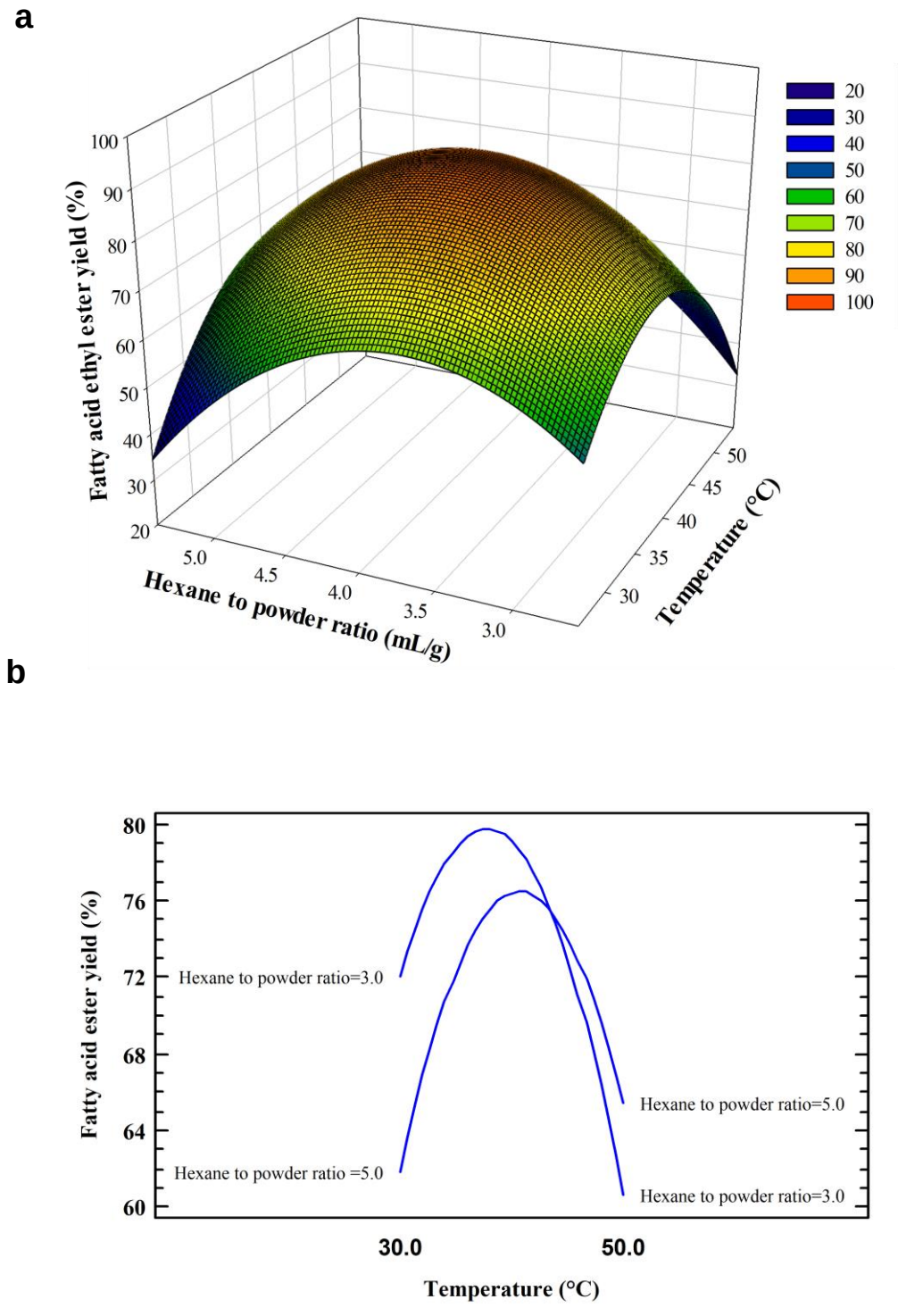

Figure 2: 3 D (a) and 2D (b) graphical representation of interaction between temperature and hexane to powder ratio

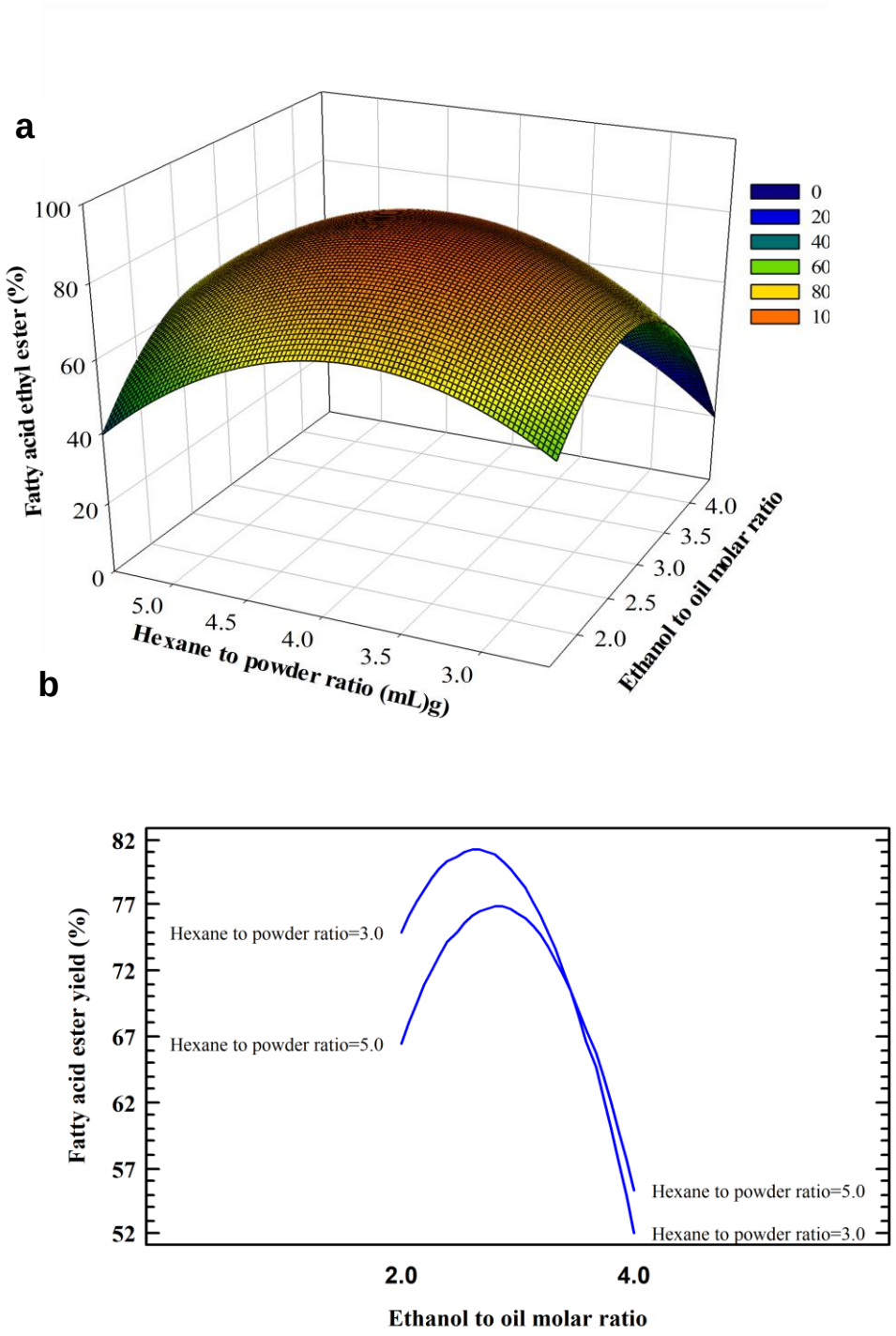

Figure 3: 3 D (a) and 2D (b) graphical representation of interaction between ethanol to oil molar ratio and hexane to powder ratio

3.4. Optimization of FAEE yield

Knowing the effects of the various factors on the (trans)esterification of the oil of germinated *A. grandidieri* seed, the optimal conditions for this synthesis were then determined. The determination of the value of the

experimental factors to maximize the yield of ethyl esters was carried out using the STATGRAPHICS centurion version IV software. The optimal point obtained was within the experimental domain (**Table 6**).

Table 6: Optimal conditions for ethanolic transesterification of endogenous lipid of *Adansonia grandidieri* seeds

Factors	Domain	Optimal point	FAEE yield
Temperature (°C)	25.9 - 54.1	39.1	
Ethanol to oil molar ratio	1.6 - 4.4	2.7	91 %
Hexane to powder ratio (mL/g)	2.6 - 5.4	3.9	

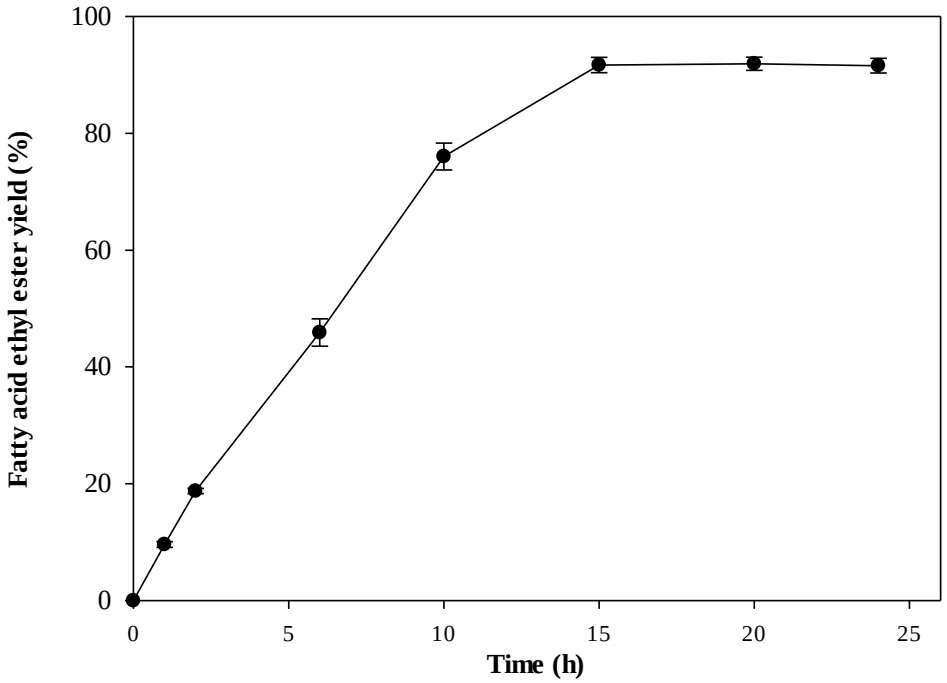

Figure 4: Kinetic transesterification of endogenous lipid of *Adansonia grandidieri* under theoretical optimal conditions. Reaction conditions: Temperature, 39.1 °C, ethanol to oil molar ratio 2.7, Hexane to powder ratio 2.7, under orbital agitation (250 rpm) during 24h

An optimal yield of 91 % is predicted by the model under the following conditions: temperature 39.1 ° C., 2.7 molar ratio oil / ethanol and hexane ratio / enzymatic powder 3.9 mL/g (**Table 6**). The optimum temperature is close to the result obtained with the delipidated powders (40 ° C) during previous works (Kouteu et al., 2017; Nanssou Kouteu et al., 2016). Kinetics were performed under the predicted conditions in order to validate them (**Table 6**). A 92 % yield is obtained after 24 hours of reaction (**Figure 4**). However, this reaction time can be reduced since there is a non-significant difference of yield ethyl esters after 15 hours of reaction (92 %). The reported time for the lipase catalyzed *in-situ* transesterification ranged from

8h to 48h with a yield between 86.1 and 95.9 % (Gu et al., 2011; Jiang et al., 2013, 2012; Jo et al., 2014; Sendzikiene et al., 2020; Su et al., 2009, 2007). The results of this study are in agreement with previous studies. This yield obtained is close to that predicted by the model and confirms that the model explains very well the formation of FAEE during the (trans)esterification of the oil of the seeds of *A. grandidieri* by its lipase.

3.5. Evaluation of residual activity of powder after ethanolic transesterification.

It is important to know if after the *in-situ* (trans)esterification the lipase retained a residual activity. To evaluate it, the reaction medium obtained after a (trans)esterification reaction under the previously determined optimum conditions (**Table 6**) was used for transesterification of sunflower oil, oil used in previous works to assess the activity of these lipase in ethanolic transesterification. Sunflower oil corresponding to the amount of oil initially contained in the seed were introduced into this medium, as well as anhydrous ethanol with ratio of oil / ethanol of 3: 1 (mol/mol).

A FAEE yield of 88 % is obtained after adding ethanol in the medium, reflecting a residual catalytic activity of *A. grandidieri* seed lipase (**Figure 5**). Two other additions (sunflower oil and ethanol) were made every 15 hours of reaction. FAEE yield obtained (87 %) is not significantly different from that observed after the first addition, but after the third addition, it is only 53 %. This decrease would probably be due to its inhibition by the alcohols present in the medium, that is the glycerol formed or unreacted ethanol during the various reactions (Choi et al., 2018). These results therefore highlight a residual catalytic activity of the lipase *A. grandidieri* at the end of the conversion of the oil of its seed.

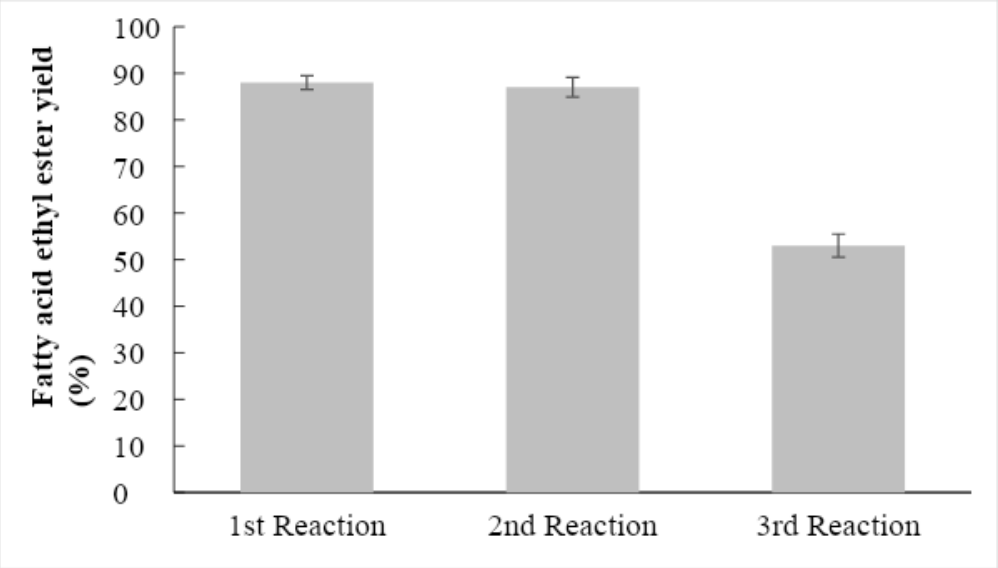

Figure 5: FAEE yields of three ethanolic transesterification reactions of sunflower oil catalyzed by residual activity of *A. grandidieri* seed lipase after optimal conversion of endogenous lipid of *Adansonia grandidieri* seed. Reaction conditions: 39 °C, ethanol to oil molar ratio 3:1, under orbital agitation (250 rpm) during 15h.

4. CONCLUSION

The production of fatty acids ethyl esters from the germinated *Adansonia grandidieri* seed by an *in-situ* transesterification process through the exploitation of its endogenous lipase without a prior extraction therefrom was successfully evaluated. An optimal yield of 92 % using a response surface methodology approach was obtained for the following conditions: Temperature of the reaction, 39 °C; hexane to powder ratio, 2.9:1; ethanol to oil molar ratio, 2.7:1 and time of reaction, 15h. Therefore, *Adansonia grandidieri* seed can be considered a valuable seed for industrial FAEE production by *in-situ* transesterification.

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6. CONFLICTS OF INTEREST

No potential conflict of interest was reported by the authors.

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