Immobilized *Rhizopus oryzae* lipase catalyzed synthesis of palm stearin and cetyl alcohol wax esters: Optimization by Response Surface Methodology

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**Abstract**

**Background:** Waxes are esters of long-chain fatty acids and long-chain alcohols. Their principal natural sources are animals (sperm whale oil) and vegetables (jojoba) which are expensive and not easily available. Wax esters synthesized by enzymatic transesterification, using palm stearin as raw material, can be considered as an alternative to natural ones.

**Results:** Palm stearin is a solid fraction obtained by fractionation of palm oil. Palm stearin was esterified with cetyl alcohol to produce a mixture of wax esters. A non-commercial immobilized lipase from *Rhizopus oryzae* was used as biocatalyst. Response surface methodology was employed to determine the effects of the temperature (30-50°C), the enzyme concentration (33.34-300 IU/mL), the alcohol/palm stearin molar ratio (3-7 mol/mol) and the substrate concentration (0.06-0.34 g/mL) on the conversion yield of palm stearin. Under optimal conditions (temperature, 30°C; enzyme concentration, 300 IU/mL; molar ratio 3 and substrate concentration 0.21 g/mL) a high conversion yield of 98.52% was reached within a reaction time of 2 h.

**Conclusions:** Response surface methodology was successfully applied to determine the optimum operational conditions for synthesis of palm stearin based wax esters. This study may provide useful tools to develop economical and efficient processes for the synthesis of wax esters.

**Background**

Palm oil is one of the traditional fats that have been widely used throughout the world in human diet. Global palm oil production was estimated to 42 million ton during 2007/2008, accounting for 40% of the worldwide production of total dietary oils [1]. Palm oil contains a mixture of high and low melting triglycerides. By a simple dry fractionation process under a controlled temperature, palm oil can be resolved into a liquid (olein) and a solid (stearin) fraction [2]. The palm olein fraction is the mostly used in industry due to its low melting point [3]. The high melting point of palm stearin (44-56°C) poses problems in manufacturing of edible fats such as margarine and shortenings as it confers low plasticity to the end product. Many attempts have been carried out to maximise the use of palm stearin by transesterification [4-7]. Among transesterification reactions, alcoholysis of triacylglycerols to produce wax esters is relatively a simple process and rather economically important to the oil and fat industries [8].

Waxes are esters of long-chain fatty acids and long-chain alcohols. The principal natural sources of waxes are from animals and vegetables [9]. They can be composed of saturated and unsaturated wax esters. The saturated wax esters are predominant in beeswax, whereas, traditional raw materials for unsaturated wax esters are sperm whale and jojoba oil. Commercial waxes have a wide range of applications as lubricants, polishes, plasticizers, coating materials in the pharmaceutical and food industries. They are also used as ingredients in the formulation of cosmetics and other chemical products [10].

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Enzymatic synthesis of wax esters from different raw materials have been reported [8,10-12]. In these studies, high yields of palm oil based wax esters have been obtained. Few studies reported the use of palm stearin and oleyl alcohol to generate wax esters [13]. The stearin fraction of palm oil is technologically underutilized because of its high melting point. The objective of our study was to promote the use of the palm stearin fraction of palm oil in wax esters synthesis. Furthermore, this is to our knowledge the first study of using cetyl alcohol and palm stearin to synthesize wax esters by means of response surface methodology.

Enzymatic synthesis of wax esters from palm stearin and cetyl alcohol, a typical fatty alcohol present in natural waxes [11], was carried out using a non commercial immobilised lipase from Rhizopus oryzae [14,15]. A three-level four-factor central composite rotatable design was adopted in our study to determine the optimum conditions. This method is a useful technique to predict the best performance conditions with a reduced number of experiments [16,17].

Methods

Fractionation process
Refined, bleached and deodorized palm oil, of iodine value 53.0, was obtained from the Tunisian Olive Oil Office. It was fractionated in the laboratory using a dry fractionation process, according to the method described by Thiagarajah [18]. Palm oil was melted and kept homogenized at 70°C to destroy all crystals present. The melted oil was agitated with stirring rate of 25 rpm. A fractionation temperature of 30°C was used. After stabilization, two fractions were obtained, namely stearin (solid) and olein (liquid). They were separated by vacuum filtration. The palm stearin fraction was used to produce wax esters.

Production and immobilisation of lipase

Rhizopus oryzae lipase was produced as described by Ben salah et al [14]. The enzyme immobilization was made onto CaCO₃ as described by Ghamgui et al [15]. The activity of immobilized lipase was measured titrimetrically with a pH-stat, under the standard assay conditions described previously by Rathelot et al [19] using olive oil (10%) emulsion as substrate. One international unit (IU) of lipase activity was defined as the amount of lipase that catalyzes the liberation of 1 μmol of fatty acid per min at pH 8.5 and 37°C.

Experimental design

Response Surface Methodology was applied to establish the relationship between the response studied (conversion yield of palm stearin) and the four selected experimental variables (temperature; enzyme concentration; substrate molar ratio (cetyl alcohol/palm stearin) and substrate concentration). A quadratic polynomial model has been postulated and represented by the following equation:

\[
\hat{y} = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2
\]

Where: \(\hat{y}\) is the conversion yield; \(b_0, b_1, b_2, b_3, b_4, b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, b_{11}, b_{22}, b_{33}, b_{44}\) are the estimated model coefficients and \(X_i\) is the coded variables. In this study, the generation and the data treatment of the composite design are performed using Design-Expert 7 software (Stat-Ease Inc., USA).

Transesterification reactions

According to the experimental design (Table 1), the transesterification reactions were carried out in screw-capped flasks containing different amount of cetyl alcohol (from Sigma) and palm stearin (MW = 3 × average of saponification equivalent of palm stearin = 600 g/mol). 3 mL of n-hexane were added in all reaction mixtures. Different concentrations of immobilized lipase were also added in the mixtures with respect to the reaction conditions in Table 1. The reaction mixture was incubated at different temperatures and shaked (200 rpm). A control reaction without adding enzyme was carried out in parallel. After a reaction time of 2 h, 200 μl aliquots of the reaction mixture were withdrawn and the immobilized enzyme was removed by centrifugation at 15,000 rpm (2,400 g) for 5 min. The supernatant was used to analyze wax esters yields. All samples were assayed in duplicates to reduce inter-assay variations and the mean values of the replicates were reported.

TLC Analysis

The reactants were separated by thin-layer chromatography (TLC) at room temperature according to Keng et al [20]. After elution, the plates were dried and stored in an iodine chamber. The presence of the cetyl alcohol, palm stearin fractions and their corresponding esters were detected as brown spots and were identified as compared to authentic standards. Wax standards used were: cetyl oleate, cetyl palmitate, cetyl linoleate and cetyl stearate synthesized in our laboratory. Quantification of the TLC plate was accomplished with the software MCID Analysis v7.0 from Imaging Research (St. Catherine, Ontario, Canada). The ratio: Optical density values/spot area for palm stearin was measured by the MCID software. The initial (t = 0 h) optical density/spot area value corresponds to 100% of the substrate. The residual optical density/spot area value (expressed as percentage of the initial value) measured at the end of the reaction corresponds to the residual substrate (expressed as percentage of the initial substrate...
concentration). The conversion yield corresponds to 
(100 - residual optical density/spot area value).

HPLC Analysis
The identification of wax esters was carried out by HPLC analysis. It was performed on a Perkin Elmer apparatus composed of Series 200 UV/Vis detector and Series 200 Micro Pump. The column was a Lichrospher 100 RP-18, 5 μm (4 × 250 mm), and its temperature was maintained at 45°C. The flow rate was 1.5 mL/min. The mobile phase used was acetonitrile/acetone (50:50, v/v) for a total running time of 35 min. Peak detection was monitored at 206 nm [12].

Results and discussion
Immobilized Rhizopus oryzae lipase was used to catalyze the synthesis of wax esters. The addition of hexane to the reaction medium was found necessary to improve the stability of the immobilized Rhizopus oryzae lipase and the solubility of the substrates (data not shown). Furthermore, the addition of water at the beginning of the reaction failed to improve the conversion yield. In the light of preliminary studies (data not shown), the temperature, the enzyme concentration, the alcohol/TG molar ratio and the substrate concentration which were the most effective on the response, were chosen as operating variables (X1, X2, X3 and X4, respectively) to optimize the synthesis yield.

Model fitting and ANOVA
To carry out a cuboidal composite experimental design, a high and a low level were chosen for each factor (Table 2). Table 1 shows the real experimental conditions of the composite design with the corresponding measured responses. The results were analyzed using a multi regression method [21].

Fitting the data to various models (linear, two factorial, quadratic and cubic) and their subsequent ANOVA showed that reactions of palm stearin and cetyl alcohol were most suitably described using a quadratic polynomial model (Table 3). The second-order polynomial equation generated by the Design-Expert

| Table 1 Experimental conditions of the composite design and the corresponding experimental response |
|---------------------------------------------------------------|
| **Run** | **Temperature (°C)** | **Enzyme Concentration (IU/mL)** | **Molar ratio (mol/mol)** | **Substrate Concentration (g/mL)** | **Actual yield (%)** | **Predicted yield (%)** |
|-------|----------------------|---------------------------------|--------------------------|----------------------------------|----------------------|------------------------|
| 1     | 50 (+1)              | 300 (+1)                        | 7 (+1)                   | 0.06 (-1)                        | 99.51                | 96.52                  |
| 2     | 50 (+1)              | 300 (+1)                        | 3 (-1)                   | 0.06 (-1)                        | 73.15                | 75.97                  |
| 3     | 50 (+1)              | 33.34 (-1)                      | 7 (+1)                   | 0.34 (+1)                        | 44.41                | 41.42                  |
| 4     | 30 (-1)              | 300 (+1)                        | 3 (-1)                   | 0.34 (+1)                        | 52.86                | 55.68                  |
| 5     | 50 (+1)              | 33.34 (-1)                      | 3 (-1)                   | 0.34 (+1)                        | 11.03                | 13.85                  |
| 6     | 30 (-1)              | 33.34 (-1)                      | 7 (+1)                   | 0.06 (-1)                        | 19.54                | 16.55                  |
| 7     | 30 (-1)              | 300 (+1)                        | 7 (+1)                   | 0.34 (+1)                        | 54.45                | 51.46                  |
| 8     | 30 (-1)              | 33.34 (-1)                      | 3 (-1)                   | 0.06 (-1)                        | 32.84                | 35.66                  |
| 9     | 30 (-1)              | 166.67 (0)                      | 5 (0)                    | 0.2 (0)                           | 84.92                | 85.28                  |
| 10    | 50 (+1)              | 166.67 (0)                      | 5 (0)                    | 0.2 (0)                           | 86.87                | 87.23                  |
| 11    | 40 (0)               | 33.34 (-1)                      | 5 (0)                    | 0.2 (0)                           | 200.05               | 20.21                  |
| 12    | 40 (0)               | 300 (+1)                        | 3 (-1)                   | 0.2 (0)                           | 88.61                | 88.97                  |
| 13    | 40 (0)               | 166.67 (0)                      | 3 (-1)                   | 0.2 (0)                           | 53.74                | 42.47                  |
| 14    | 40 (0)               | 166.67 (0)                      | 7 (+1)                   | 0.2 (0)                           | 36.69                | 48.67                  |
| 15    | 40 (0)               | 166.67 (0)                      | 5 (0)                    | 0.06 (-1)                         | 76.41                | 76.77                  |
| 16    | 40 (0)               | 166.67 (0)                      | 5 (0)                    | 0.34 (+1)                         | 42.25                | 42.61                  |
| 17    | 40 (0)               | 166.67 (0)                      | 5 (0)                    | 0.2 (0)                           | 62.45                | 65.97                  |
| 18    | 40 (0)               | 166.67 (0)                      | 5 (0)                    | 0.2 (0)                           | 76.22                | 65.97                  |
| 19    | 40 (0)               | 166.67 (0)                      | 5 (0)                    | 0.2 (0)                           | 66.51                | 65.97                  |
| 20    | 40 (0)               | 166.67 (0)                      | 5 (0)                    | 0.2 (0)                           | 64.78                | 65.97                  |
| 21    | 40 (0)               | 166.67 (0)                      | 5 (0)                    | 0.2 (0)                           | 62.02                | 65.97                  |

| Table 2 Variables values used in the Experimental Design |
|----------------------------------------------------------|
| **Factor** | **Variables** | **Unit** | **level** |
|-------------|---------------|----------|-----------|
| Temperature | X1            | °C       | 30 40 50  |
| Enzyme Concentration | X2    | IU/mL    | 33.34 166.67 300 |
| Molar ratio (Cetyl alcohol/ Palm stearin) | X3   | mol/mol  | 3 5 7    |
| Substrate Concentration | X4  | g/mL     | 0.06 0.2 0.34 |
software is: $$\hat{y} = 65.97 + 0.97 X_1 + 34.18 X_2 + 3.10 X_3 - 17.08 X_4 - 9.29 X_1 X_2 + 8.93 X_1 X_3 + 12.66 X_1 X_4 + 0.98 X_2 X_3 - 7.58 X_2 X_4 + 2.74 X_3 X_4 + 20.28 X_1^2 - 11.18 X_2^2 - 20.40 X_3^2 - 6.28 X_4^2$$, where $X_1, X_2, X_3$ and $X_4$ are the coded variables.

In order to determine the adequacy of the quadratic model, an analysis of variance (ANOVA) was conducted using the usual Fisher’s $F$-tests (Table 4), in which the regression sum of squares is subdivided into two parts. One attributed to the linear regression and the other to the quadratic model [22].

The regression sum of squares was statistically significant when using the $F$-test at a 99% probability level. This suggests that the variation taken into account by the model was significantly greater than the unexplained variation. Likewise, the statistically insignificant lack of fit and the satisfactory levels of R-Squared (0.96) indicated that the model was statistically significant and adequate to represent the relationship between the experimental parameters.

Coefficients of the model were evaluated by regression analysis and tested for their significance (Table 4). $p$-values lesser than 0.0500 indicates that the model terms are significant. Among these parameters, the enzyme concentration ($p$-value = 0.0016) and substrate concentration ($p$-value = 0.0347) have highly significant effects (according to their small $p$-values) on the synthesis of wax esters as compared to the temperature and the alcohol/palm stearin molar ratio. Interactions between temperature and molar ratio alcohol/palm

Table 3 Model Summary Statistics

| Source | Standard deviation | R-Squared | Adjusted R-Squared | Predicted R-Squared | PRESS |
|--------|-------------------|-----------|--------------------|---------------------|-------|
| Linear$^a$ | 17.050 | 0.611 | 0.514 | 0.252 | 91797.93 |
| 2FI | 18.598 | 0.711 | 0.421 | -5.630 | 79274.413 |
| Quadratic$^b$ | 8.884 | 0.960 | 0.868 | -5.867 | 82113.127 |
| Cubic$^b$ | 5.785 | 0.989 | 0.944 | + | |

$^a$: suggested model to be selected; 
$^b$: aliased model not to be selected

+ Case with leverage of 1: PRESS statistic not defined.

Table 4 ANOVA and Regression Analysis of the Quadratic Model

| Source of variation | Sum of Squares | Degrees of freedom | Mean Square | $F$-value | $p$-value | Prob $> F$ | Signif |
|---------------------|----------------|--------------------|-------------|-----------|-----------|------------|--------|
| Model | 11483.454 | 14 | 820.247 | 10.392 | 0.0044 | a |
| $X_1$ | 1.901 | 1 | 1.901 | 0.024 | 0.8818 |
| $X_2$ | 2336.545 | 1 | 2336.545 | 29.603 | 0.0016 |
| $X_3$ | 95.976 | 1 | 95.976 | 1.216 | 0.3124 |
| $X_4$ | 583.453 | 1 | 583.453 | 7.392 | 0.0347 |
| $X_1 X_2$ | 138.198 | 1 | 138.198 | 1.751 | 0.2340 |
| $X_1 X_3$ | 638.138 | 1 | 638.138 | 8.085 | 0.0294 |
| $X_1 X_4$ | 256.492 | 1 | 256.492 | 3.250 | 0.1215 |
| $X_2 X_3$ | 7.742 | 1 | 7.742 | 0.098 | 0.7647 |
| $X_2 X_4$ | 91.839 | 1 | 91.839 | 1.164 | 0.3222 |
| $X_3 X_4$ | 60.006 | 1 | 60.006 | 0.760 | 0.4168 |
| $X_1^2$ | 1050.075 | 1 | 1050.075 | 13.304 | 0.0107 |
| $X_2^2$ | 319.292 | 1 | 319.292 | 4.045 | 0.0910 |
| $X_3^2$ | 1062.246 | 1 | 1062.246 | 13.458 | 0.0105 |
| $X_4^2$ | 100.796 | 1 | 100.796 | 1.277 | 0.3016 |
| Residuals | 473.577 | 6 | 78.929 |
| Lack of Fit | 339.721 | 2 | 169.861 | 5.076 | 0.0799 | b |
| Pure Error | 133.856 | 4 | 33.464 |
| Cor Total | 11957.031 | 20 |

R-Squared = 0.960

$^a$: indicates significant at the level of 99%

$^b$: indicates insignificant at the level of 99%

Estimated standard deviation is equal to Std. Dev. = $\sqrt{473.577/6} = 8.884$
stearin ($p$-value = 0.0294) on one hand and substrate concentration and temperature on the other hand ($p$-value = 0.1215) were found to be modestly significant.

**Graphical Interpretation of the Response Surface Model**

Relationships between the response and the experimental parameters were explored using contour plots. The response surface and the isoresponse curves were plotted against two variables while the two others were held constant at their mean levels.

Figure 1 represents the effect of enzyme concentration and reaction temperature on the conversion yield of palm stearin. Ester synthesis increased rapidly with increasing the enzyme amount. However, the reaction temperature did not have an appreciable influence on ester synthesis. A high conversion yield (>90%) was reached by using a high enzyme concentration (>233 IU/mL) for a very large range of temperatures.

Both enzyme and substrate concentrations seem to play an important role in enhancing synthesis yield of palm stearin based wax esters (figure 2). A significant decrease in the conversion yield was noticed when using a high substrate concentration at any enzyme concentration. On the contrary, a high conversion yield was accomplished by using a low substrate concentration (around 0.06 g/mL) and a high enzyme concentration (>233 IU/mL). These findings are in line with previous reports that the presence of a high substrate concentration could have a negative effect on lipase catalyzed synthesis activity [23,24]. Besides, alcohols are known to be terminal inhibitors of lipases [10,25]. Furthermore, high amount of palm stearin would lead to a greater resistance to mass transfer because of a higher viscosity of the medium [26].

Figure 3 represents the effect of varying enzyme concentration and alcohol/palm stearin molar ratio on the conversion yield of palm stearin. The wax esters synthesis increased markedly with the enzyme concentration while alcohol/palm stearin molar ratio showed parabolic effects on the reaction yield (figure 3). Many lipases exhibit this type of dome shaped plots when catalyzing esterification reactions [27]. The optimum value of alcohol/palm stearin molar ratio was around 5 mol/mol (figure 3). A reaction with a moderate cetyl alcohol/palm stearin molar ratio and a high enzyme concentration (>233 IU/mL) achieved the maximal conversion yields (>80%).

Figure 4 represents the effect of varying substrate concentrations and alcohol/palm stearin molar ratios on the conversion yield of palm stearin. Increasing the alcohol/palm stearin molar ratio from 3 to 5 could enhance the conversion yield. Rising alcohol/palm stearin ratio value beyond 5 caused the reaction conversion yield to decline. This phenomenon also reported by Karboune et al [28] for the lipase-catalyzed transesterification of long-chain triglycerides with phenolic acid, might be attributable to a great resistance of mass transfer due to a high substrate concentration. A high conversion yields could be reached when minimizing the substrate concentration and using an alcohol/palm stearin molar ratio around 5 mol/mol.

**Optimal reaction conditions**

The optimal synthesis conditions of palm stearin based wax esters were predicted using the optimization function of the Design Expert Software. These optimal conditions are presented in table 5 along with their experimental and predicted values. A good correlation was found between predicted and experimental values.
Table 5 Solutions for optimal conditions as generated by the Design Expert Software

| Experiment | Temperature (°C) | Enzyme Concentration (IU/mL) | Molar ratio | Substrate Concentration (g/mL) | Experimental yield (%) | Predicted yield (%) | Standard deviation | texp. Degrees of freedom | Signif |
|------------|------------------|-----------------------------|-------------|--------------------------------|------------------------|---------------------|--------------------|--------------------------|--------|
| 1          | 30               | 131.73                      | 4.92        | 0.06                           | 93.24                  | 94.91               | 8.88               | 0.150                    | 6      |
| 2          | 30               | 300                         | 3.00        | 0.21                           | 98.52                  | 99.45               | 8.88               | 0.081                    | 6      |
| 3          | 50               | 246.07                      | 5.43        | 0.26                           | 95.44                  | 93.93               | 8.88               | 0.131                    | 6      |

α: Indicate insignificant at the level of 99%

Figure 2 Contour and response surface plots showing the effect of enzyme and substrate concentrations on the synthesis yield of palm stearin based wax esters. Temperature and alcohol/palm stearin molar ratio were kept at their zero levels.

Figure 3 Contour and response surface plots showing the effect of enzyme concentration and alcohol/palm stearin molar ratio on the synthesis of palm stearin based wax ester. Temperature and substrate concentration were constant at their zero levels.

Figure 4 Contour and response surface plots showing the effect of alcohol/palm stearin molar ratio and substrate concentration on the synthesis yield of palm stearin based wax ester. The enzyme concentration and temperature were maintained at their zero levels.
implying that empirical models derived from response surface methodology can adequately describe the relationship between the factors and their influence on the palm stearin based wax esters synthesis.

Among the various optimum conditions, the highest conversion yield was obtained for experiment 2, which is carried out at 30°C, using 300 IU/mL of enzyme, an alcohol/triglyceride molar ratio of 3 and a substrate concentration of 0.21 g/mL. Under these optimal conditions, the time course of the alcoholysis reaction between the palm stearin and the cetyl alcohol was presented in figure 5. The conversion yield increased rapidly to reach its maximal value of 98.52 ± 1.76% within 2 h of reaction time. The experimental optimal synthesis yield value was close to the calculated one (99.45%). Rhizopus oryzae lipase immobilized on CaCO₃ used in this work shows an interesting behavior by providing a high esterification yield (98.52%) within 2 h of reaction time. A comparable synthesis yield (92.3%) was obtained by Keng et al [29], but within a longer reaction time (5 h) when using palm oil and oleyl alcohol as substrates and Lipozyme as biocatalyst. Nevertheless, much lower synthesis yields were reported by Gunawan et al [8] (84.6% within 7.38 h) when esterifying oleyl alcohol and palm oil by Lipozyme, used as biocatalyst. It's worth to notice that these studies were also optimized using RSM methodology.

TLC and HPLC analysis of palm based wax esters under optimal conditions

Figure 6a depicts the TLC analysis of palm stearin wax esters under optimal synthesis conditions. Upon a synthesis reaction time of 2 h, spots corresponding to alcohol and triglycerides disappeared, concomitantly with an appearance of a new band corresponding to wax esters. HPLC analysis of palm stearin wax esters (figure 6b) was conducted and identification of peaks was carried out by comparing the chromatograms to those of authentic standard esters. The four main wax esters were cetyl palmitate, cetyl stearate, cetyl oleate and cetyl...
linoleate corresponding to retention times of 15.472, 18.306, 11.871 and 14.488 min, respectively. The peaks corresponding to triglycerides disappeared indicating they were all transesterified with cetyl alcohol to produce wax esters. These results are in agreement with the measured high conversion yields (Table 5).

Conclusions

The enzymatic synthesis of palm stearin based wax esters by means of esterification with cetyl alcohol using an immobilized Rhizopus oryzae lipase as a biocatalyst was carried out in n-hexane. Response surface methodology was successfully applied to determine the optimum operational conditions for maximal conversion yields of palm stearin. The greatest conversion yields were obtained for a high enzyme concentration, a moderate alcohol/triglyceride molar ratio and a low substrate concentration. For example, a high conversion yield of 98.52% was reached within 2 h at 30°C, using an enzyme concentration of 300 IU/mL, an alcohol/triglyceride molar ratio of 3 and a substrate concentration of 0.21 g/mL. This study may provide useful tools to develop economical and efficient processes for the synthesis of wax esters.

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Authors' contributions

MS and IA designed the experiments, carried out the synthesis and the analysis of wax esters and drafted the manuscript; FF carried out the statistical analysis. YG and NM have conceived research and approaches and have given final approval of the version to be published. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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