Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Magnetic responsive *Thermomyces lanuginosus* lipase for biodiesel synthesis

Jing Li\(^a\)\(^*,\) Jiandong Zhang\(^a\) Shuguang Shen\(^b\) Bing Zhang\(^a\) William W. Yu\(^c\)\(^*,\)

\(^a\) College of biomedical engineering, Taiyuan University of Technology, Taiyuan 030024, China
\(^b\) College of chemistry and chemical engineering, Taiyuan University of Technology, Taiyuan 030024, China
\(^c\) Department of Chemistry and Physics, Louisiana State University, Shreveport, LA 71115, USA

**ARTICLE INFO**

**Keywords:**

*Thermomyces lanuginosus* lipase  
Magnetic responsive  
Response surface methodology  
Biodiesel production  
Transesterification

**ABSTRACT**

The low cost lipase derived from *Thermomyces lanuginosus* was chosen to conjugate with Fe\(_3\)O\(_4\) nanoparticles as a magnetic responsive lipase (MRL) biocatalyst. The structure of MRL was observed by atomic force microscopy (AFM). The Fourier transform infrared (FTIR) spectroscopy analysis confirmed the lipase conjugated to Fe\(_3\)O\(_4\) nanoparticles. Optimized conditions for the process of biodiesel production by MRL were investigated by the response surface methodology (RSM) and the Box-Behnken design (BBD). The optimized conditions for biodiesel production by MRL were as follows. The molar ratio of methanol to oil was 4.0, water content was 1.5 % as oil weight, the dosage of MRL to oil was 9.0 % (W/W) under 41 °C for 28 h. Under the optimized conditions, the yield of FAMEs by MRL reached 82.20 %. Further experiments showed that the MRL could be used 10 cycles and the yield of FAMEs decreased slightly by 10.97 %. These results indicated that Fe\(_3\)O\(_4\) nanoparticle carrier could efficiently improve the FAMEs synthesis and enhance the MRL stabilization and reusability in the biodiesel production.

1. Introduction

Biodiesel is a mixture of fatty acid methyl esters (FAMEs) or other monoalkyl esters manufactured from the transesterification of triglycerides with short chain alcohols. It has recently attracted considerable attention as a renewable, biodegradable and environment-friendly energy source [1–3]. Compared with the conventional alkali catalysis, biocatalysis by lipases aroused extensive research due to the more gentle reaction conditions, better adaptation to feedstock including waste and inedible oils containing high percentage of free fatty acids or water, less downstream processing and pollutions [4–6].

However, the utilization of this bioprocess is mainly restricted by the cost of the lipases and the difficulty in their reusability and stability. A lot of efforts have been made to reduce the cost of this procedure. Some researches focused on the low cost lipases with good transesterification performance though modification or immobilization lipases to improve the reusability and stability in biodiesel process. *Thermomyces lanuginosus* lipase (TLL), in modified and immobilized forms, has been more and more widely concerned [7–9] due to its relatively low cost and highly adaptable feedback (e.g. waste oil with high water concentration). Other researches concentrated on the optimized conditions to improve the transesterification efficiency [10–12]. Main factors affecting biodiesel process (reaction time, reaction temperature, methanol/oil molar ratio, type and concentration of biocatalyst as well as water content) were observed and optimized by analyzing their interactions. The current studies usually emphasized on either the low-cost lipase preparation or the biodiesel process optimization and few studies involve both of them. However, both sides are equally crucial to accelerate biodiesel industrial by biocatalysts.

In this work, inexpensive magnetic responsive TLL lipase (MRL) was prepared. The secondary structure and three-dimensional images of the as-prepared MRL was obtained by Fourier transform infrared spectroscopy (FTIR) and atomic force microscopy (AFM), respectively. The results also revealed that the structure change was closely related to the stability and catalytic properties of the MRL. Moreover, the MRL was used to synthesize biodiesel and the optimized process was explored by the Box-Behnken design (BBD) of response surface methodology (RSM).

2. Experimental

2.1. Materials

Lipase from *Thermomyces lanuginosus* (Lot# L0777, a liquid preparation with 72.75 ± 0.45 U/mg of protein determined by Ref. [16]), 4-morpholineethanesulfonic acid (MES), n-hexane, methanol, analytical or GC grade FAME (methyl undecanoate, methyl palmitate, methyl...
linoleate, methyl linolenate, methyl oleate, and methyl stearate) as analysis calibration standards were purchased from Sigma-Aldrich. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) were purchased from Thermo Fisher Scientific. Soybean oil (saponification value of 195.0 ± 0.1 mgKOH/g, acid value of 0.1794 ± 0.0009 mgKOH/g, average molecular weight of 864.1 ± 0.5 g/mol) was purchased from a local market (Taiyuan, China). All other chemicals were guaranteed or analytic grade and were used directly. Water used throughout this work was from a Milli-Q ultrapure water system.

2.2. Preparation of magnetic responsive lipase (MRL)

Aqueous monodisperse Fe3O4 nanoparticles with free carboxyl group (Fe3O4−COOH) were synthesized according to references [13,14]. Then with full consideration of factors that effected on the activity and stability of the lipase in the process of conjugation reactions [15], MRL was prepared as follows.

Typically, 1 mL of Fe3O4−COOH nanoparticles aqueous solution was added to a mixture solution of 10 μL EDC (10 mM in MES buffer) and 10 μL Sulfo-NHS (25 mM in the same MES buffer). The reaction liquid was stirred at 20 °C for 20 min to activate the carboxyl groups on the surface of the nanoparticles. After that, excess EDC and Sulfo-NHS in this mixture solution were removed by centrifugal filtration at 2700 g-force for 5 min. The concentrated medium was collected and dispersed in 1 mL water, and then 200 μL of lipase solution (5 g/L in 0.1 M phosphate buffer, pH 8.5) was added into the above solution. MRL was formed at 20 °C with gentle stirring for 2 h. The as-prepared MRL was separated by a permanent magnet and redispers- ed in phosphate buffer. Finally, the solution which got from the above conditions was dried by vacuum freezing drying method, the tan powder of MRL was collected. The specific activity of obtained MRL was 93.18 U/mg, which was increased by 28.08 % compared to the native lipase solution (72.75 U/mg).

2.3. Enzyme activity assays

Hydrolytic activities of native TLL and MRL were determined by olive-oil emulsion method [16]. One lipase activity unit (U) was defined as the amount of enzyme that produced 1 μmol of fatty acids per minute under the assay conditions. The relative activity was defined as the ratio of a certain enzyme activity value to the highest value of the enzyme activity in the same set of experiments. Protein contents (μg/mL) were measured by BCA Protein Assay Reagent kit (Pierce) with the standard protocol. Specific activities (U/mg) of lipases and MRL were expressed as hydrolytic activity per mg protein. All the experiments were performed in triplicate and the standard derivation was calculated.

2.4. Characterizations

2.4.1. AFM observations

The dilute solutions of Fe3O4−COOH nanoparticles, native TLL or MRL solution were prepared in 10% Milli-Q water, and a drop of each solution was placed on freshly cleaved mica surface. The mica sheet was dried gently in vacuum and analyzed by AFM. Using a Nanoscope III Multimode scanning probe microscope (Digital Instruments, Veeco Metrology Group, USA) in the tapping mode, AFM images for Fe3O4−COOH nanoparticles, native TLL and MRL were obtained respectively.

2.4.2. FTIR spectroscopy analysis

All samples were vacuum-dried and ground to form fine homogeneous powders. The ground powders were carried out using an ATR-FTIR spectrophotometer (Nicolet Nexus 670, Thermo Nicolet Corporation, USA) to confirm the MRL conformation. The FTIR spectra were collected for an average of 512 scans at a resolution of 2 cm−1 with a gain of 2 and an aperture of 50 in the range of 4000−400 cm−1.

2.4.3. Measurement of enzyme secondary structure

Secondary structural contents of native TLL and MRL were obtained by analyzing their FTIR spectra in the amide I region from 1700 to 1600 cm−1 in the absorption mode [17,18]. To estimate the secondary structure, the amide I region was performed on the second-derivative analysis by the software of PeakFit v4.12. The positions and number of the peaks were determined from the second-derivative spectra to obtain the α-helix and β-sheet contents of them.

2.5. Biodiesel production by enzymatic transesterification reaction

2.5.1. General procedure and Box-Behnken design for optimized the biodiesel process

The reaction was carried out in a 25 mL shaking flask capped with a septum on a thermostatic oscillator at 200 r/min. Five influence factors, the reaction time, the reaction temperature, the addition mode and content of methanol, the enzyme dosage and the water content, were considered in single factor experiments. A general experiment system was consisted of 5 g soybean oil, 10 % MRL and 1 % Milli-Q water with a three-step methanol addition of 313 μL (approximately 4:1 M ratio of methanol to oil) in each step at 12 h intervals. The mixture reacted at 45 °C for 36 h. The dosage of MRL and water were based on the oil weight.

To further improve the transesterification efficiency of MRL, a five-level-three-factor (53) BBD with the Design-Expert software was adopted. Reaction time (X1, h), reaction temperature (X2, °C), methanol/oil molar ratio (X3), enzyme dosage (X4, % as oil weight) and water content (X5, % as oil weight) were chosen as five independent variables, while the yield of FAMES (Y, %) as the response variable. The variables and their coded and uncoded values are shown in Table 1. A total of 46 experimental sets were made, and 6 replicates at the center point were applied. In order to correlate the relationship between the variables and the response, a quadratic polynomial equation was used for fitting. The general form of the predictive polynomial quadratic equation is given as Eq. (1).

\[ Y = \beta_0 + \sum_{i=1}^{5} \beta_i X_i + \sum_{i=1}^{5} \sum_{j=i+1}^{5} \beta_{ij} X_i X_j \]  

(1)

where Y is the response; β0 is a constant; βi, βii, βij are the coefficients for the linear, quadratic coefficient and interactive effect, respectively.

2.5.2. Determination the yield of FAMES by gas chromatography (GC)

At the end of the reactions, MRL was firstly separated by a magnet. The residual mixture of transesterification was conducted in a decantation funnel; the glycerol phase was separated from the FAME layer. The latter FAME rich-phase was washed with water twice and then was distilled at 60 °C under vacuum to purify the as-prepared FAMEs. The purified FAMEs were then dried by anhydrous Na2SO4 to produce crude biodiesels. 100 μL of the crude biodiesels and 100 μL of methyl un

Table 1

| Variable                        | Coded level | −1  | 0  | +1  |
|---------------------------------|-------------|-----|----|-----|
| Reaction time X1 (h)            |             | 24  | 36 | 48  |
| Reaction temperature X2 (°C)    |             | 40  | 45 | 50  |
| Methanol/oil molar ratio X3     |             | 3.5 | 4  | 4.5 |
| Enzyme dosage X4 (% as oil weight) |         | 8   | 10 | 12  |
| Water content X5 (% as oil weight) |         | 0   | 1  | 2   |
hydrogen flame ionization detector (FID). The different FAME in the sample was separated on a Rtx-5 capillary column (30 m × 0.32 mm, Restek Corporation). The temperatures of injector and detector were set at 280 °C and 300 °C respectively. The initial column temperature was kept at 150 °C for 2 min then increased to 200 °C at a rate of 5 °C/min and kept 2 min; then increased to 240 °C at a rate of 2 °C/min and kept 3 min; finally increasing at 10 °C/min up to 300 °C and then maintained at 300 °C for 10 min. The split ratio was 1:50 and the carrier gas was nitrogen. By comparing the retention times and peak areas of standard FAME peaks, the total quantity of FAMEs in the reaction mixture was calculated by the internal calibration method. The yield of FAMEs was defined as the mass ratio of FAMEs in the reaction mixture to the total theoretical amount of FAMEs measured by saponification of soybean oil as Eq. (2).

\[
\text{Yield of FAMEs(\%)} = \frac{m_{\text{actual}}}{m_{\text{theoretical}}} \times 100\%
\]  

(2)

where \(m_{\text{actual}}\) (g) and \(m_{\text{theoretical}}\) (g) were the masses of FAMEs in the reaction mixture of enzymatic catalyst transformation and saponification, respectively.
3. Results and discussion

3.1. Properties of MRL

The effect of temperature on the hydrolytic activity of native TLL and the as-prepared MRL was studied in olive oil emulsion (pH 7.0) at different temperature. The results are shown in Fig. 1 A; the changing trends of the hydrolysis activities with respect to the temperature were consistent for both native TLL and MRL, the same optimal temperature for both of them was obtained as 40 °C while the MRL showed a superior temperature tolerance compared with native TLL. Subsequently, the pH dependences of the activities in a pH range of 5.0―11.0 at 40 °C were investigated (Fig. 1 B). The optimal pH did not change after the modification by Fe 3O4 nanoparticles but the MRL showed a higher adaptability to the variation of solution pH. The maximum activities for both of them were achieved at pH 8.5.

The same optimal temperature and pH value were observed. The result indicated that the structure of the Fe3O4 nanoparticles bound to the lipase does not have any significant negative effect on the temperature and pH condition of the as-prepared MRL complex. These results are in accordance with the previously reported studies [19–21]. Additionally, the MRL actually retained higher activities than native lipase at different temperature and pH values. Most probably, the formation of covalent bonds between the lipase molecules and Fe3O4 nanoparticles made the configuration of lipase molecules more stable. The stabilization of structure resulted in improved MRL tolerance to different temperature and pH conditions.

3.2. Characterization of MRL

3.2.1. AFM measurements

AFM gave a clear visual insight on the morphologies of Fe3O4−COOH nanoparticles, native TLL and MRL (Fig. 2 A–C). Furthermore, surface roughness and particle diameter analysis were derived from the AFM images by the software of Nanoscope Analysis v 1.20. The Fe3O4−COOH nanoparticles with a regular assemblage shape were observed in Fig. 2 A and the particle size of Fe 3O4−COOH nanoparticles was 17.7 nm, which closely matched the transmission electron microscopy (TEM) analysis [15]. Fig. 2 B demonstrates the 2D profile and 3D topography of native TLL; the size of native TLL was 21.6 nm, which was nearly the same as the reported molecular dimension of TLL [22]. Compared with the regular assemblage shape of Fe3O4 nanoparticles and native TLL particles, the MRL demonstrated different surface appearance in Fig. 2 C. The root mean square roughness value of the MRL was estimated to be 8.29 nm, which is between the value of native TLL particles (1.06 nm) and Fe3O4 nanoparticles (16.2 nm). Those changes indicated that the MRL was successfully formed, furthermore the structure of MRL was speculated in Fig. 2 D.

3.2.2. FTIR analysis and secondary structural study

Fig. 3 A shows the FTIR spectra of the Fe3O4−COOH nanoparticles, native TLL and MRL (A); the curve-fitting results of amide I band for native TLL (B) and MRL (C) in the range of 1600-1700 cm\(^{-1}\).

| Secondary structure            | Content (%) | Native TLL | MRL |
|--------------------------------|-------------|------------|-----|
| α-helix                        | 13.49       | 14.75      |     |
| β-sheet                        | 32.19       | 25.26      |     |
| Unordered (including β-turn and random coil) | 54.32   | 59.98      |     |
| (α-helix + β-sheet)/unordered  | 84.09       | 66.71      |     |
| α-helix/β-sheet                | 41.91       | 58.40      |     |

| Table 2. Quantitative analysis of the secondary structural contents. |

Fig. 3. FTIR spectra of Fe₃O₄−COOH nanoparticles, native TLL and MRL (A); the curve-fitting results of amide I band for native TLL (B) and MRL (C) in the range of 1600-1700 cm\(^{-1}\).
and MRL were analyzed through the absorbance signals of the amide I region in the range of 1600−1700 cm$^{-1}$ which were ascribed to the asymmetric stretching vibration of the C=O, C–N and N–H group in the backbone peptide bonds in proteins [17,18,23]. Fig. 3 also shows the derivative spectra of native TLL (Fig. 3 B) and MRL (Fig. 3 C). The curve fitting analysis demonstrated that the amide I region composed by major peaks that were assigned to individual structural components according to literature [24,25]. With the help of PeakFit, quantitative evaluation of the secondary structures was performed assuming a Gaussian peak shape. The secondary structural contents of them are given in Table 2.

Compared with native TLL, the increase of the contents of unordered structure may be associated to the interaction between the nanoparticles and TLL. Meanwhile, the MRL appeared an increase in α-helix and a decrease in β-sheet content. The changes could be attributed to a more rigid structure so that the lipase tends to adapt a broader temperature and pH environment. Moreover, the increase in α-helix was responsible for the improvement of the enzyme activity as Fig. 4.

Fig. 4. Response surface plots of the interaction of factors on soybean oil conversion. (A) Combined effect of methanol/oil molar ratio and water content; (B) reaction time and reaction temperature; (C) reaction temperature and enzyme dosage; (D) methanol/oil molar ratio and enzyme dosage; (E) enzyme dosage and water content (E); (F) reaction temperature and methanol/oil molar ratio.
Y to the quadratic model in terms of coded factors expressed in Eq. (3).

\[ Y = + 81.85 - 1.18X_1 + 1.92X_2 + 2.12X_3 - 0.53X_4 + 1.48X_5 + 5.01X_1X_2 - 0.75X_1X_3 - 0.46X_1X_4 - 0.50X_1X_5 + 2.48X_2X_3 + 3.70X_2X_4 - 0.47X_2X_5 - 3.44X_3X_4 - 6.36X_3X_5 - 2.86X_4X_5 - 3.44X_4^2 - 5.12X_5^2 - 4.34X_1^2 - 4.68X_2^2 - 4.22X_3^2 + 0.0001 \]  

(3)

The ANOVA of this quadratic equation is shown in the Supplementary material (Table S2). The highly significant regression model was verified by a low P value (P < 0.0001), which should be less than 0.05 [28]. The satisfactory fitting of the quadratic model was confirmed by the determination coefficient \( R^2 = 94.85 \% \), which should be greater than or equal to 80 \% for a good fitting of a certain model [29,30]. The results implied by the model revealed the relationship between the response value and the variables in biodiesel production by MRL.

Table 3
Optimum conditions for transesterification reaction by MRL.

| Biodiesel process parameters | Predicted value | Experimental value |
|-----------------------------|----------------|-------------------|
| Reaction time \( X_1 \) (h) | 27.77 | 28.00 |
| Reaction temperature \( X_2 \) (°C) | 41.74 | 41.00 |
| Methanol/oil molar ratio \( X_3 \) | 3.97 | 4.00 |
| Enzyme dosage \( X_4 \) (% as oil weight) | 9.17 | 9.00 |
| Water content \( X_5 \) (% as oil weight) | 1.44 | 1.50 |
| Yield of FAMEs Y (%) | 83.31 | 82.20 ± 0.77 |

Fig. 5. Handling and stability test of MRL.

3.3. Optimization of the biodiesel synthesis by MRL

3.3.1. Model fitting and Analysis of variance (ANOVA) in the biodiesel process using RSM

The experimental results of the coded factors designed in Table 1 are presented in the Supplementary material (Table S1). The data were fitted to the quadratic model in terms of coded factors expressed in Eq. (3).

3.3.2. Effect of biodiesel process variables

In the biodiesel process, significantly contributed variables were also analyzed (see Table S2 for more details). Four linear terms \( X_1, X_2, X_3 \) and \( X_5 \), six cross products \( X_1X_2, X_1X_3, X_1X_4, X_1X_5, X_2X_3 \) and \( X_4X_5 \), and the five quadratic terms \( X_1^2, X_2^2, X_3^2, X_4^2 \) and \( X_5^2 \) were all remarkably significant model terms at 95 \% confidence level. On the other hand, the independent variable of enzyme dosage \( X_4 \) and the combined effects of reaction time and methanol/oil molar ratio \( (X_1X_3) \), reaction time and enzyme dosage \( (X_1X_4) \), reaction time and enzyme dosage \( (X_1X_5) \), reaction temperature and enzyme dosage \( (X_2X_4) \) had no significant effects on the biodiesel process.

In general, higher F value and smaller P value indicate more significance of the corresponding coefficient [31,32]. Based on the high F values and low corresponding P values in the Supplementary material (Table S2), among the independent variables, methanol/oil molar ratio \( X_3 \) was the most significant variable, then reaction temperature \( X_2 \), water content \( X_5 \) and reaction time \( X_1 \) in the order. Among the interactions, combined effect of methanol/oil molar ratio and water content \( (X_3X_5) \) had the greatest impact on the biodiesel production; then came reaction time and reaction temperature \( (X_1X_2) \), reaction temperature and enzyme dosage \( (X_2X_4) \), methanol/oil molar ratio and enzyme dosage \( (X_3X_4) \), enzyme dosage and water content \( (X_4X_5) \), reaction temperature and methanol/oil molar ratio \( (X_1X_3) \). To graphically explain those main mutual interactions effected on the yield of FAMEs, the 3D response surface plots of them were presented in Fig. 4 (A–F) arranged from the most significant to the least.

Fig. 4 A shows the most significant interactions between methanol/oil molar ratio and water content, because these two factors related to the catalytic performance of enzyme, the transesterification efficiency and the occurring probability of hydrolysis as a competitive reaction. In a certain extent, the higher methanol/oil molar ratio could be more helpful in promoting the yield of FAMEs while the methanol could also result in loss or inactivation of the enzyme activity [33,34]. For the multiple roles and strong influence of water in the reaction system, the activation of enzyme mainly embodied the conformational changes of the lipase molecules due to the exposing and restructuring of the catalytic active sites, which required the presence of oil-water interface [35,36]. So the existence and a considerable concentration of water were in favor of the enzyme structural stabilization to enhance the transesterification efficiency but could accelerate the hydrolysis reaction and decrease the yield of FAMEs [37,38].

3.3.3. Optimal biodiesel process determination and the model validation

Overall, the quadratic model (Eq. 3) exhibited a good fitting with the experimental data as well as the satisfactory predictability. So the quadratic model was taken to determine the optimal parameters for the biodiesel production by MRL using the Design-Expert software. The optimal parameters were statistically predicted in Table 3. In order to verify the accuracy of the predicted model, the suggested optimal conditions were applied to three independent replicates, and the average yield of FAMEs was 82.20 \%, which closely agreed with the predicted value from RSM and hence affirmed the results of the optimization.

Compared the 66 \% of the theoretical yield of FAMEs by native TLL with 1,3-positional specificity lipase [38], the yield of FAMEs increased by 24.55 \% which was assumed that acyl migration occurred during the reaction process due to the native lipase modified by Fe3O4 nanoparticles, the results agreed with previous studies by Mannina [39] and Xu [40].

3.4. Reusability of MRL in biodiesel production

Long lipase lifespan in transesterification reactions will significantly decrease the cost of the process to accelerate the industrial application of MRL in the synthesis of biodiesel [36]. To test the operation stability and reusability of the MRL in biodiesel process, batch transesterification of soybean oil was conducted by the addition of MRL. The reaction conditions were the optimal conditions mentioned in Table 5. The MRL recovered with magnetic separation, washed sufficiently with phosphate buffer (0.1 M, pH 8.5) and dried as described in 2.2, then applied in the next batch with fresh substrates. As expected, the MRL embodied fine transesterification activity retention in repeated use in Fig. 5. Under the optimal conditions, the yield of FAMEs was still up to 73.18...
% and dropped only by 10.97 % after 10 cycles. The decrease of activity was probably caused by the loss of MRL during the magnetic recovery and dropped only by 10.97 % after 10 cycles. The decrease of activity was probably caused by the loss of MRL during the magnetic recovery.

4. Conclusions

As a lower cost and higher efficient biocatalyst, the as-prepared MRL in this work were developed for biodiesel production. The MRL’s morphology was observed by the measurement of AFM and the conjugate behavior of native TLL and Fe3O4−COOH nanoparticles was speculated. The analysis of FTIR spectra and the secondary structures of the MRL showed that TLL successfully conjugated on the surface of Fe3O4−COOH nanoparticles. After modified by Fe3O4−COOH nanoparticles anchored on the MRL and the mild separation method by a magnet, might help to enhance the tolerance to the organic solvent environment and relieve the loss of enzyme dosage.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.mtcomm.2020.101197.