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Simple and Efficient Immobilization of Extracellular His-Tagged Enzyme Directly from Cell Culture Supernatant As Active and Recyclable Nanobiocatalyst: High-Performance Production of Biodiesel from Waste Grease

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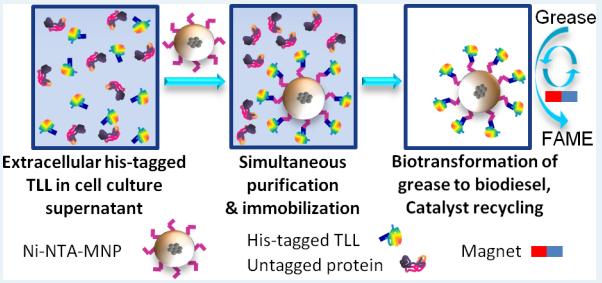
Supporting Information

ABSTRACT: A simple method for immobilizing extracellular enzyme without prepurification of the enzyme was developed. Extracellular His-tagged *Thermomyces lanuginosus* lipase (His-TLL) was immobilized via affinity by direct treatment of core−shell structured iron oxide magnetic nanoparticles containing long-armed nickel-nitrilotriacetic acid surface groups (Ni-NTA-MNPs) with the cell culture supernatant of *Pichia pastoris* (h-TLL), giving high enzyme loading efficiency, specific enzyme loading, and specific enzyme activity. The nanobiocatalyst His-TLL-MNPs (80 nm) (5 wt % loading) catalyzed the one-pot conversion of waste grease (24 wt % FFA) to biodiesel with 94% yield and showed excellent recyclability. Ni-NTA-MNPs were easily regenerated from the recycled biocatalyst and reusable for enzyme immobilization. The immobilization method was proven to be general by the immobilization of extracellular His-tagged *Candida antarctica* lipase B (His-CALB) from the cell culture supernatant of *P. pastoris* (h-CALB).



KEYWORDS: *enzyme immobilization, extracellular enzyme, magnetic nanoparticles, grease, biodiesel, Thermomyces lanuginosus lipase*

Enzyme catalysis has become a useful and green tool for sustainable production of chemicals, fuels, and polymers.[1−6] Although free enzyme often suffers from instability and high cost, immobilized enzymes could enhance enzyme stability and reduce enzyme cost via recycling. Therefore, many immobilization techniques have been developed.[7−11] Among them, the immobilization of enzymes on functionalized magnetic nanoparticles (MNPs) as nanobiocatalyst has attracted much attention due to the unique properties of MNPs, such as a large surface area per volume, high dispersity, less mass transfer limitations, and easy magnetic separation.[12−16] Thus far, most enzyme immobilization methods have required prepurification of the enzyme,[17−19] which is often costly and low-yielding.[20−22] It is especially difficult to purify extracellular enzyme, which is often expressed in cell culture medium at low concentration. Hence, development of a simple and efficient method for direct immobilization of targeted extracellular enzyme from cell culture supernatant without prepurification is highly desirable. Here, we report such a method by the introduction of a His-tag into the target extracellular enzyme and the use of MNPs containing nickel-nitrilotriacetic acid (Ni-NTA) on the surface as a carrier for highly specific attachment of His-tagged extracellular enzyme directly from cell culture supernatant via affinity (Figure 1). The resulting nanobiocatalyst is active and recyclable for the target biotransformation, and the Ni-NTA-MNPs are regenerable from the used nanobiocatalyst (Figure 1).

Thermomyces lanuginosus lipase (TLL)[23−25] is chosen as target extracellular enzyme to demonstrate the simple immobilization method and the practical application of the immobilized enzyme. TLL is known to catalyze the transesterification of vegetable oils with methanol for the production of biodiesel (fatty acid methyl esters, FAMEs),[23,26,27] a clean and renewable alternative to petroleum diesel.[28,29] However, vegetable oils are edible and costly.[30] Recently, waste grease has become an attractive feedstock for biodiesel production since it is nonedible and cheap (0.3 USD/kg). Due to the high free fatty acid (FFA) content (15−40 wt %) of waste grease,[31−33] the conventional base catalysis process is not applicable. Two-step process involving acid-catalyzed esterification of FFA and subsequent base-catalyzed transesterification of triglycerides is not efficient.[34] In our continuous effort to develop one-pot esterification and transesterification process for efficient transformation of waste grease to biodiesel,[35−37] immobilized TLL was shown as suitable biocatalyst for this transformation.[17] Nevertheless, the requirement of the enzyme purification has been a bottleneck in developing cost-effective processes to produce the immobilized enzyme as well as the biodiesel from waste grease.

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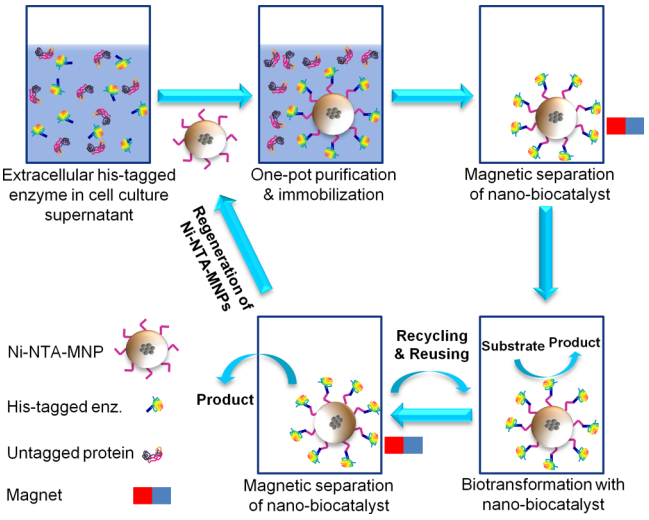


Figure 1. Immobilization of His-tagged extracellular enzyme directly from cell culture supernatant on magnetic nanoparticles (Ni-NTA-MNPs), biotransformation with and recycling of the nanobiocatalyst, and regeneration of Ni-NTA-MNPs from the used catalyst.

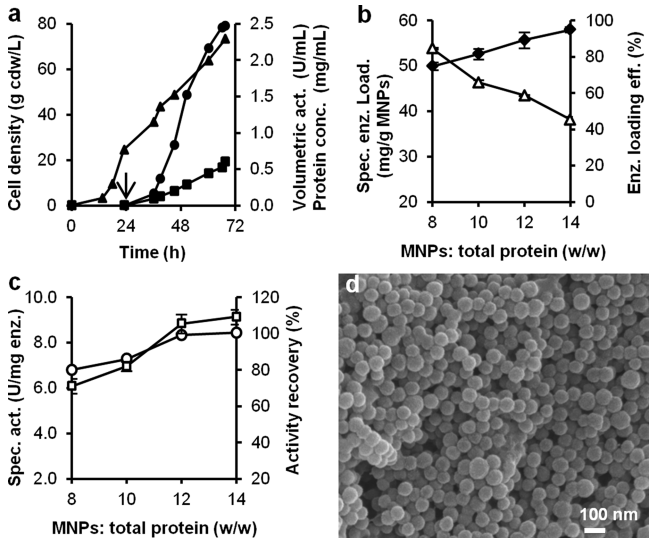
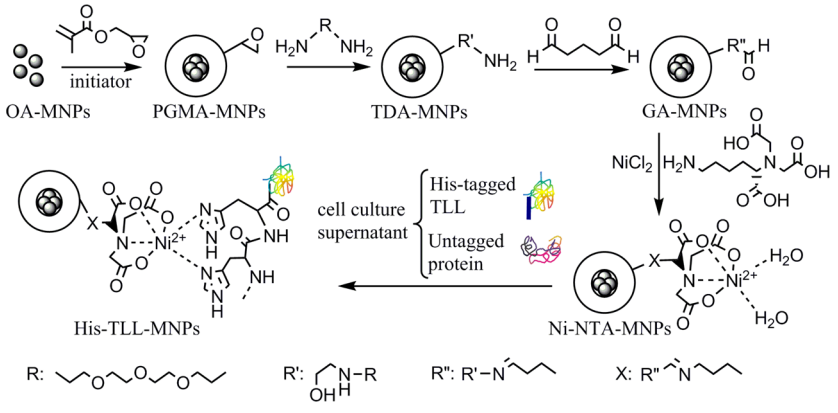


Figure 2. (a) Cell growth, protein secretion, and enzyme activity during the fed-batch cultivation of *P. pastoris* (h-TLL) in bioreactor: cell density (▲), volumetric activity (●), and protein concentration (■). The arrow indicates the starting time point of continuous feeding of methanol for protein induction. (b, c) The key performance parameters of simultaneous purification and immobilization of His-TLL from the culture supernatant at room temperature and pH 5.5 with Ni-NTA-MNPs at different ratios (w/w) of MNPs to total protein (His-TLL: 60%): (b) specific enzyme loading (Δ) and enzyme loading efficiency (♦), (c) specific activity (○) and total activity recovery (□). The data are the mean values with standard deviation from experiments run in triplicate. (d) FESEM image of His-TLL-MNPs.

To demonstrate the immobilization method, Ni-NTA-MNPs were synthesized by the route shown in Scheme 1. PGMA-MNPs (60 nm by TEM) containing an iron oxide core (15 nm by TEM) and poly(glycidyl methacrylate) shell were synthesized according to the published method[19] and then functionalized with a long-chain diamine to give TDA-MNPs (63 nm by TEM). The introduction of the long arm on the surface of the MNPs was to provide more space and flexibility for the enzyme to be attached to retain its enzymatic activity. The aldehyde group was then introduced to form GA-MNPs (63 nm by TEM). Further reaction with N_α,N_α-bis-(carboxymethyl)-L-lysine hydrate and subsequently nickel(II) chloride gave Ni-NTA-MNPs (63 nm by TEM) in 83% overall yield from PGMA-MNPs. The size and morphology of the MNPs synthesized in each step were characterized by TEM, FESEM, and DLS (Figures S1−S3 in the Supporting Information). All MNPs showed the spherical core−shell structure and narrow dispersity.

Extracellular His-tagged *Thermomyces lanuginosus* lipase (His-TLL) was produced by the engineering of *Pichia pastoris* (h-TLL) containing pPICZ*α*A-His-TLL plasmid[38] and the high cell-density cultivation in a bioreactor.[39] As shown in Figure 2a, the cells were in lag phase in first 13.5 h and reached log phase afterward to give 25 g cdw/L at 23 h. At that point, an abrupt rise in the DO value was observed, which implies that glycerol as the carbon source was limited. The protein induction was started by feeding methanol at 23 h, leading to a gradually increased protein concentration and culture supernatant activity. After 13 h of induction, the volumetric activity started to increase with a big and almost constant slop. At the end of the fermentation (67 h), volumetric activity of 2.47 U/mL, protein concentration of 0.6 mg/mL, and cell density of 73.3 g cdw/L were achieved. SDS−PAGE of the cell culture supernatant taken at different time points (Figure S7) showed a clear rising of expression level of His-TLL (at 35 kDa) after induction, and gave a very significant band of His-TLL for the culture supernatant produced at 67 h. The expression level of

Scheme 1. Route for Synthesizing Ni-NTA-MNPs and Preparing Nanobiocatalyst His-TLL-MNPs



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His-TLL was deduced as 60% of the total extracellular protein, on the basis of the purification of His-TLL from a small portion of the 67 h-cell culture supernatant.

Ni-NTA-MNPs were added to the 67 h culture supernatant after removing the cells to selectively immobilize His-TLL via affinity binding between the His-tag and the Ni-NTA function. The immobilization was performed under different conditions, and the enzyme loading efficiency was calculated on the basis of the His-TLL content in the supernatant before and after immobilization. Treatment of the culture supernatant with MNPs at 12:1 mass ratio of MNPs to total protein was examined for 2−8 h, and no significant effect on the immobilization was observed. Obviously, 2 h of incubation time was enough for completing the immobilization because of the high affinity. The temperature effect was also investigated. The specific enzyme loading and enzyme loading efficiency increased from 27.9 to 43.5 mg/g MNPs and from 53% to 89%, respectively, when the temperature increased from 4 to 25 °C (Figure S4). A further increase in the temperature from 25 to 35 °C gave a much lower increase in both performance parameters. Therefore, room temperature (r.t.) was chosen to conduct the later immobilization experiments.

The effect of different mass ratios of MNPs to total protein on the immobilization was investigated at r.t. for 2 h. As shown in Figure 2b,c, an increase in the ratio from 8:1 to 14:1 decreased nearly linearly the specific enzyme loading from 54 to 38 mg/g MNPs and increased linearly the enzyme loading efficiency from 75% to 95%, since more particles were used. An increase in the ratio from 8:1 to 12:1 improved nearly linearly the specific activity of the immobilized enzyme from 6.8 to 8.3 U/mg enzyme determined by using the *p*-NPB assay for 5 min. An increase in the ratio from 8:1 to 12:1 also enhanced the recovery of total enzyme activity from 71% to 106%. The slightly higher than 100% activity recovery is possibly due to the slight increase in the enzyme activity after immobilization. A further increase in the ratio from 12:1 to 14:1 did not significantly further increase the activity recovery and specific activity. The MNPs-to-protein mass ratio of 12:1 seems to be appropriate, in the view of the two key performance parameters. These immobilization experiments were performed at pH 5.5, at which the cells were cultivated.

The immobilization method was then examined at other pH values. The cell culture supernatant was adjusted to a pH of 8.0, followed by immobilization at an 8:1 (w/w) ratio of MNPs to protein and r.t. for 2 h. This gave a specific enzyme loading of 61 mg/g MNPs, enzyme loading efficiency of 84%, specific enzyme activity of 8.3 U/mg His-TLL, and full enzyme activity recovery (Table S1). In comparison with pH of 5.5, these key performance parameters are much better. Considering the advantages of using less MNPs and neutral pH, an 8:1 (w/w) ratio of MNPs to protein and a pH of 8.0 were chosen as the optimal conditions for the enzyme immobilization.

The immobilization procedure was scaled up from 1 to 10 mL of the culture supernatant. The specific enzyme loading of 62 mg/g MNPs, 85% enzyme loading efficiency, specific activity of 8.2 U/mg His-TLL, and 100% total activity recovery were obtained (Table S1). These key performance indicators are nearly the same as those obtained in 1 mL scale, which proved the applicability of using this method for larger scale enzyme immobilization.

The nanobiocatlyst His-TLL-MNPs were analyzed by FESEM. As shown in Figure 2d, His-TLL-MNPs are uniformed spherical particles with a diameter of ~80 nm, which is slightly bigger than Ni-NTA-MNPs (63 nm by TEM). In the FTIR spectra of His-TLL-MNPs and Ni-NTA-MNPs (Figure S5), the NTA groups on the surface gave a characteristic broad absorption at 3400 cm^{-1} (OH) and strong absorption band at 1725 cm^{-1} (C═O).

SDS−PAGE (Figure S7) was used to confirm the selective immobilization of His-TLL with Ni-NTA-MNPs. The protein band at 35 kDa of the supernatant was significantly reduced after immobilization. Nanobiocatalyst His-TLL-MNPs were washed with 10 mM imidazole, and no protein was washed off. Further washing the nanobiocatalyst with 250 mM imidazole gave a single protein band at 35 kDa for His-TLL in the SDS-PAGE of the elute. This indicates that only His-TLL was attached on Ni-NTA-MNPs.

His-TLL-MNPs were used for the production of biodiesel from waste grease containing 24 wt % FFA via simultaneous esterification of FFA and transesterification of triglyceride (TG) with methanol (Figure 3a). The temperature and methanol addition were studied for efficient biotransformation. The reaction was performed by using 1 g of waste grease and 50 mg of His-TLL-MNPs with stepwise (63 μL × 3) or one-shot (189 μL) addition of methanol at 30−50 °C for 24 h. Silica gel microbeads (20 wt %) were added in the reaction system to improve the FAME yield via the absorption of water. The FAME yield was determined by GC analysis (Figure S6), and the results are given in Table 1. One-shot addition of methanol (entry 3, 40 °C) gave 74% FAME yield, whereas stepwise addition of methanol (entry 2, 40 °C) gave 81% FAME yield. The increased yield was due to the reduced toxicity of methanol

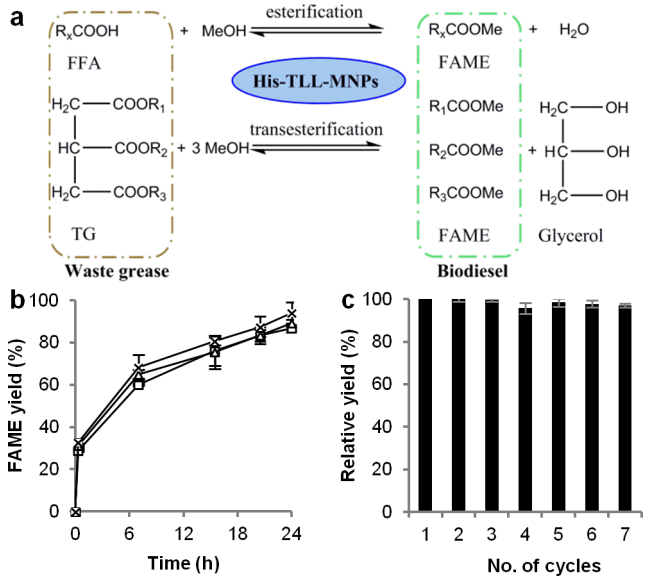

Figure 3. (a) Biotransformation of waste grease (24% FFA) to biodiesel (FAME) by using nanobiocatalyst His-TLL-MNPs via simultaneous esterification of FFA and transesterification of TG with methanol. (b) Time courses of the biotransformations of waste grease (1 g) to FAME by using nanobiocatalyst His-TLL-MNPs at 3 (□), 4 (△), and 5 wt % (×) loading, respectively, with stepwise addition of methanol (at 0, 2, and 6 h with 63 μL in each portion) at 50 °C. (c) Recycling and reuse of His-TLL-MNPs in the biotransformation of waste grease (1 g) to FAME at 5 wt % catalyst loading and 50 °C with stepwise addition of methanol (see part b) for 24 h; All experiments in parts b and c were carried out in triplicate, and the data are the mean values with standard deviation.

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Table 1. One-Pot Biotransformation of Waste Grease to FAME via Simultaneous Esterification of FFA and Transesterification of TG with Methanol Using Nanobiocatalyst His-TLL-MNPs

entry	grease (g)	His-TLL-MNPs (wt %)[a]	methanol addition	reaction time (h)	temp (°C)	FAME yield (%)
1	1.00	5.0	stepwise[b]	24	30	62
2	1.00	5.0	stepwise[b]	24	40	81
3	1.00	5.0	one-shot[c]	24	40	74
4	1.00	5.0	stepwise[b]	24	50	94

[a]Catalyst loading refers to grease. [b]Three equal portions of methanol (63 μL in each portion) were added at 0, 2, and 6 h, respectively. [c]Methanol (189 μL) was added in the beginning.

to the enzyme through stepwise addition. Under stepwise addition of methanol, the FAME yield was increased from 81% at 40 °C (entry 2) to 94% at 50 °C (entry 4). Obviously, 50 °C is better for this reaction. Free TLL was also examined at the same enzyme loading for the same conversion at 50 °C with stepwise addition of methanol, giving 92% FAME yield. Thus, His-TLL-MNPs demonstrated even slightly higher yield than free TLL for the biotransformation of grease.

Different catalyst loadings were examined at 50 °C; the time courses for these biotransformations are given in Figure 3b. The highest FAME yield (94%) was achieved with 5 wt % catalyst loading; nevertheless, at 3 and 4 wt % catalyst loading, FAME was also obtained in 87% and 89% yield, respectively. The average specific activity within the first 20 min for the conversion of grease to FAME at 3 wt % catalyst loading was determined as 10.5 U/mg His-TLL or 0.6 U/mg His-TLL-MNPs. In these biotransformations, no FFA remained in the final reaction mixture, determined by titration.

The recyclability of the nanobiocatalyst His-TLL-MNPs was examined by the reaction of 1 g waste grease and methanol (63 μL × 3) with 50 mg His-TLL-MNPs and 0.2 g silica gel microbeads at 50 °C for 24 h. After each batch, the nanobiocatalyst His-TLL-MNPs were separated from the reaction mixture and silica gel by using a magnet. The catalyst was washed and reused for the next batch of biotransformation of grease to FAME under the same conditions. As shown in Figure 3c, the recyclability of the nanobiocatalyst was excellent, retaining 97% productivity in the seventh cycle. The observed high recyclability of the nanobiocatalyst is partially due to the easy and almost full recovery of the MNPs. Another reason is the strong affinity attachment of enzyme on the MNPs, giving no detectable enzyme leakage during bioconversion and recycling experiments. Moreover, the immobilized enzyme is highly stable, as demonstrated in the 7 days' reactions during recycling experiment with no significant loss of the enzyme productivity. The recycling and reuse of the nanobiocatalyst can significantly reduce the cost of catalyst and, thus, reduce the biodiesel production cost.

The possibility of regenerating Ni-NTA-MNPs from His-TLL-MNPs was also examined. The nanobiocatalyst recovered after 7 cycles of biotransformation with a total reaction time of 7 days was first washed with 500 mM imidazole to remove His-TLL. After further treatment with EDTA to remove imidazole, the MNPs were washed with water and treated with nickel(II) chloride to give Ni-NTA-MNPs in 100% yield. The regenerated MNPs were used again for one-pot immobilization of His-TLL from the cell culture supernatant of *P. pastoris* (h-TLL) under the established optimal conditions, giving the enzyme loading efficiency, activity recovery, and specific enzyme loading of 65%, 80%, and 49 mg/g MNPS, respectively. Thus, the regenerated Ni-NTA-MNPs reached 80% of these key enzyme immobilization performance parameters of the freshly synthesized Ni-NTA-MNPs. The specific activity of the nanobiocatalyst prepared from the regenerated particles reached 8.4 U/mg His-TLL for the hydrolysis of *p*-NPB, which was the same as those of the nanobiocatalyst prepared from the freshly synthesized particles. The efficient regeneration of Ni-NTA-MNPs from the used catalysts and reuse of these MNPs for enzymatic immobilization reduce the cost of the nanocarrier.

To demonstrate the generality of this immobilization method, extracellular His-tagged *Candida antarctica* lipase B (His-CALB) was also directly immobilized from cell culture supernatant of *P. pastoris* (h-CALB) with Ni-NTA-MNPs by using the same procedure described for His-TLL. The immobilization afforded nanobiocatalyst His-CALB-MNPs with a specific enzyme loading of 70 mg/g MNPs, 94% enzyme loading efficiency, a specific enzyme activity of 1.1 U/mg His-CALB (*p*-NPB assay), and 93% activity recovery (Table S1). Because CALB prefers esterification,[36] the nanobiocatalyst His-CALB-MNPs was examined for the esterification of FFA of waste grease with methanol at 4.5 wt % catalyst loading (based on grease) and 30 °C for 12 h, affording 98% conversion of FFA. His-CALB-MNPs were separated and then reused for the new cycle of the esterification of FFA, retaining 98% productivity in the eighth cycle (Figure S8).

In summary, a simple and efficient method for the immobilization of extracellular enzyme without prepurification was developed by using core−shell structured iron oxide magnetic nanoparticles Ni-NTA-MNPs containing long-armed Ni-NTA surface groups to simultaneously purify and immobilize target His-tagged enzyme directly from cell culture supernatant via affinity attachment. Ni-NTA-MNPs (63 nm) were synthesized in high yield (83%), and the use of these particles to immobilize His-TLL directly from the culture supernatant of *P. pastoris* (h-TLL) gave the corresponding magnetic nanobiocatalyst His-TLL-MNPs (80 nm) with high specific enzyme loading (62 mg/g MNPs), high enzyme loading efficiency (85%), and full enzyme activity recovery. Biotransformation of waste grease (containing 24 wt % FFA) with methanol using the nanobiocatalyst (5 wt % based on grease) afforded biodiesel in 94% yield. The nanobiocatalyst was easily recovered under magnet and exhibited excellent recyclability, retaining of 97% productivity in the seventh cycle of biodiesel production. Ni-NTA-MNPs were regenerable from the used biocatalyst and reusable for enzyme immobilization. The production of biodiesel from waste grease using the nanobiocatalyst is green, sustainable, high-yielding, and potentially practical. The immobilization method is generally useful for the immobilization of extracellular enzymes, as demonstrated by the efficient immobilization of extracellular His-CALB from the cell culture supernatant of *P. pastoris* (h-CALB) as an active and recyclable nanobiocatalyst.

■ ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.5b00550.

Chemicals and strains; analytical methods; procedures for the engineering and cultivation of *P. pastoris* (h-TLL), synthesis of Ni-NTA-MNPs, immobilization of His-TLL

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as His-TLL-MNPs, biotransformation of grease to FAME with His-TLL-MNPs, recycling of His-TLL-MNPs, and regeneration of Ni-NTA-MNPs from the used catalyst; procedures for immobilization of His-CALB as His-CALB-MNPs, conversion of FFA of grease to FAME with His-CALB-MNPs, and recycling of His-CALB-MNPs; and supporting figures and table (PDF)

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Notes

The authors declare no competing financial interest.

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