Integrated Utilization Strategy for Soybean Oil Deodorizer Distillate: Synergically Synthesizing Biodiesel and Recovering Bioactive Compounds by a Combined Enzymatic Process and Molecular Distillation

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ABSTRACT: Soybean oil deodorizer distillate (SODD) is well recognized as a good source of both biodiesel and high-value bioactive compounds of tocopherols, squalene, and phytosterols. To achieve a one-step synthesis of biodiesel and recovery of bioactive compounds from SODD, four commercial immobilized enzymes (Novozym 435, Lipozyme TLIM, Lipozyme RMIM, and Lipozyme RM) and one self-prepared immobilized lipase MAS1-H108A were compared. The results showed that immobilized lipase MAS1-H108A due to the better methanol tolerance and higher catalytic activity gave the highest biodiesel yield of 97.08% under the optimized conditions: molar ratio of 1:2 (oil/methanol), temperature of 35 °C, and enzyme loading of 35 U/g SODD, even after 10 persistent cycles without significant decrease of activity. Simultaneously, there was no loss of tocopherols and squalene in SODD during the enzymatic reaction. Pure biodiesel (characterized by fourier transform infrared (FT-IR) and nuclear magnetic resonance (NMR)) and a high concentration of bioactive compounds could be successfully separated by molecular distillation at 100 °C. In a word, this work provides an interesting idea to achieve environmentally friendly treatment of SODD by combining an enzymatic process and molecular distillation, and it is suitable for industrial production.

1. INTRODUCTION

Soybean oil deodorizer distillate (SODD) is a byproduct obtained in the deodorization step during the refining of soybean oil.1 Each year, million tons of the oil deodorizer distillate is produced and discarded as waste, which brings a heavy burden to the environment as well as to wasting of resources2–3 because SODD contains bioactive compounds such as tocopherols, squalene, and phytosterols.4 Tocopherols are strong antioxidants and active substances with physiological functions and largely applied in food, pharmaceutical, and cosmetic industries.4 Tocopherols contain four isomers, namely, α-, β-, γ-, and δ-tocopherols depending on the number of methyl groups on the chromanol ring in the chemical structure, and three forms of tocopherols (α-, γ-, and δ-tocopherol) are widely found in SODD.8,9 Squalene is a hydrocarbon, C30H50, with six double bonds in its structure and the exact molecular weight is 410.3913 amu,10 originally obtained for commercial purposes primarily from the liver oil of some deep-sea sharks (cartilaginous fishes).11 It has been widely studied in the field of disease prevention, such as cardiovascular diseases and cancer.12 Also, it has applications in the preparation of cosmetics as a natural moisturizer and in the biosynthesis of cholesterol.13 Phytosterol is a triterpene that has a molecular structure similar to cholesterol, with four steroid rings.14 The most important physiological function of phytosterols is to inhibit the absorption of cholesterol in the small intestine and thus reduce the total cholesterol level in the human body.15 In the SODD, the major phytosterol is β-

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phytosterol, accounting for 60–70% of the total phytosterols.\textsuperscript{16} Thus, SODD is a natural source of tocopherols, squalene, and phytosterols (or fatty acid sterol esters, [FASEs]).\textsuperscript{17}

Despite much efforts, the utilization of SODD still remains a challenge either due to the methods that are not environmentally friendly or the recovery and purification yields of the target compounds that are not satisfactory. Normally, the approached 70–95% cost of biodiesel is from raw materials; however, approximately 95% of the raw material is edible oils.\textsuperscript{1,18} SODD is rich in free fatty acids (FFA) and some entrained oil. Therefore, alternative utilization of SODD to produce biodiesel will vastly reduce costs. The methods for the production of biodiesel mainly include the chemical method, enzymatic method, and supercritical liquid method, among which the enzymatic method has obtained the most attention because it requires mild reaction conditions and it is environmentally friendly. Torres et al. reported a two-step enzymatic procedure to obtain fatty acid methyl esters (FAMEs) and FASEs from SODD.\textsuperscript{19} Lee et al. reported over 95% yield of biodiesel from rapeseed oil deodorizer distillate by solid-phase extraction.\textsuperscript{20} Also, supercritical CO\textsubscript{2} extraction and crystallization processes were applied for the isolation of phytosterols from saponified rapeseed oil deodorizer distillate by solid-phase extraction.\textsuperscript{21} Although many studies have focused on the production of biodiesel by SODD or the recovery of bioactive compounds from SODD,\textsuperscript{22,23} the one-step synthesis of biodiesel and recovery of bioactive compounds from SODD were scarcely reported. The generated FAME has a lower boiling point than the corresponding FFA and that results in an easy separation between FAME and the bioactive compounds. Thus, a higher recovery of bioactive compounds will be obtained with a higher conversion of FAME.

The purpose of the present study was to develop a one-step synthesis of biodiesel and recovery of tocopherols, phytosterols, and squalene from SODD by the combined enzymatic process and molecular distillation. Five lipases, including four commercial immobilized enzymes (Novozym 435, Lipzyme TLIM, Lipzyme RMIM, and Lipzyme RM) and immobilized lipase MAS1-H108A prepared in our laboratory were compared for the yield of biodiesel. The effect of reaction conditions on the FAME yield and the contents of bioactive compounds was investigated during this process. Then, FAME and the bioactive compounds could be easily separated by molecular distillation. Finally, the FAME product was characterized by Fourier transform infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy. This work provides an important scientific idea for the industrial production of biodiesel and recovery of bioactive compounds from SODD.

2. RESULTS AND DISCUSSION

2.1. Composition Analysis of SODD. The fatty acid composition, bioactive compound contents, and acide value of SODD are shown in Table 1. The acid value of SODD was 89.50 ± 0.57 mg KOH/g, with the FFA content of 44.7%. Eleven kinds of fatty acids were detected in the SODD, including only 7.60 ± 0.55% of linolenic acid (C18:3), which is way below the 12% limitation of linolenic acid in biodiesel required by the EN14214 standard. The same fatty acid composition of SODD was reported by Sugihara et al.\textsuperscript{12} Besides, the glycerides existing in the SODD were triacylglycerols (around 35%), diacylglycerols (around 2%), and monoacylglycerols (around 1%). The water content was 0.73%.

The qualitative and quantitative analysis of the bioactive compounds contained in the SODD were performed by dual-wavelength UV detection of high-performance liquid chromatography (HPLC). Despite tocopherols, β-sitosterol and squalene could be simultaneously detected at 205 nm (Figure 1A); 296 nm was employed to detect tocopherols due to their maximum absorption at 296 nm (Figure 1B) and the interference on quantitative analysis of other substances (such as FAME) at 205 nm. This HPLC method had a high recovery. The recoveries of α-tocopherol, δ-tocopherol, γ-tocopherol, β-sitosterol, and squalene were 98.33, 97.84, 98.34, 96.89, and 99.02%, respectively.

Based on the optimized analysis method, the contents of α-tocopherol, δ-tocopherol, γ-tocopherol, β-sitosterol, and squalene in SODD were 5.40 ± 0.01, 19.31 ± 0.32, 25.69 ± 0.21, 18.12 ± 0.97, and 15.53 ± 0.54 mg/g, respectively. Similar β-sitosterol and squalene contents in SODD were reported by Yuan et al., who found that there were nearly 20 mg/g β-sitosterol and 13 mg/g squalene in SODD.\textsuperscript{26} However, the contents of tocopherols were different from that recorded in the literature and may be due to the different origin of the SODD. In general, the contents of squalene and δ-tocopherol in SODD are much higher than those of other deodorizer distillates like cottonseed oil deodorizer distillate (CDD), tea seed oil deodorizer distillate (TODD), and rice bran oil deodorizer distillate (RODD).\textsuperscript{27}

2.2. Effect of Lipase on the FAME Yield and Bioactive Compounds’ Retention. For biodiesel production, the most widely used immobilized lipases were Novozym 435, Lipzyme TLIM, Lipzyme RMIM, and Lipzyme RM. To evaluate these four commercial lipases and immobilized MAS1-H108A on the synthesis of FAME under the same reaction conditions, their esterification activities were measured with the same

| Acid Value | 89.50 ± 0.57 mg KOH/g |
|------------|----------------------|
| Lauric Acid (C12:0) | 1.54 ± 0.02 |
| Myristic Acid (C14:0) | 0.36 ± 0.06 |
| Palmitic Acid (C16:0) | 0.13 ± 0.01 |
| Stearic Acid (C18:0) | 5.78 ± 0.15 |
| Oleic Acid (C18:1) | 23.93 ± 0.98 |
| Linoleic Acid (C18:2) | 56.81 ± 1.58 |
| Linolenic Acid (C18:3) | 7.60 ± 0.55 |
| Eicosatetraenoic Acid (C20:4) | 2.43 ± 0.43 |
| Heneicosanoic Acid (C21:0) | 0.11 ± 0.08 |
| Docosanoic Acid (C22:0) | 0.77 ± 0.02 |
| Tetracosanoic Acid (C24:0) | 0.54 ± 0.03 |
| α-Tocopherol | 5.40 ± 0.01 mg/g |
| δ-Tocopherol | 19.31 ± 0.32 mg/g |
| γ-Tocopherol | 25.69 ± 0.21 mg/g |
| β-Sitosterol | 18.12 ± 0.97 mg/g |
| Squalene | 15.53 ± 0.54 mg/g |
| Water Content | 0.73 ± 0.01% |
method. The esterification activities of immobilized MAS1-H108A, Novozym 435, Lipozyme TLIM, Lipozyme RM, and Lipozyme RMIM were 3526, 14094, 545, 4743, and 4617 U/g, respectively.

In this study, four commercial immobilized lipases were compared with immobilized MAS1-H108A on the FAME yield and bioactive compounds’ retention in SODD. The yield of FAME in the reaction process was detected by gas chromatography-mass spectrometry (GC-MS), as shown in Figure 1C. It was found that the maximum FAME yield could be obtained by immobilized MAS1-H108A in all of the reaction periods ($p < 0.05$) when compared to the other four commercial lipases (Figure 2A). Moreover, the FAME yield increased sharply to 81.72% in the initial 4 h, and the maximum FAME yield reached up to 90.42% at 24 h, which was associated with the previous reports.27 Different FAME yields were found in different lipases, although the enzymatic activities of the five immobilized lipases added to the medium were the same at the initial reaction. One reason could be the high methanol tolerance of immobilized MAS1-H108A because methanol as the carbon source was continuously fed during the fermentation process of MAS1-H108A, and there was no effect on the lipase activity of the obtained MAS1-H108A.28 However, the activities of Novozym 435, Lipozyme RMIM, Lipozyme RM, and Lipozyme TLIM were inactivated when a high concentration of methanol was present in the reaction mixture. Similar results were reported by Wang et al.27

The other important reason was that Lipozyme TLIM, Lipozyme RM, and Lipozyme RM with a strong $sn$-1,3-specific property really affected the process of transesterification, while lipase MAS1-H108A had no regiospecific property.30 So immobilized MAS1-H108A showed the highest conversion efficiency during the enzymatic reaction. The changes of the five bioactive compounds were also monitored by HPLC during the reaction (Figure 2B–F). It was found that the retention rates of three tocopherols were all over 99%, catalyzed by MAS1-H108A. The retention rates of the three tocopherols catalyzed by Novozym 435 and Lipozyme RM were significantly lower than those by immobilized lipase MAS1-H108A ($p < 0.05$). Moreover, the retention rates of $\alpha$-tocopherol and $\gamma$-tocopherol in Lipozyme RMIM and Lipozyme TLIM systems were higher than that of Novozym 435, but significantly lower than that of immobilized lipase MAS1-H108A ($p < 0.05$). These interesting phenomena may be attributed to the structural properties of lipase. However, both Lipozyme RMIM and Lipozyme RM were from Rhizomucor miehei (RML) with the same enzyme structure but gave the different level of tocopherols that was possibly due to the different supports of immobilization. Lipozyme RMIM was immobilized on Duolite ES 562, which was a weak anion-exchange resin based on phenol-formaldehyde copolymers.29 Lipozyme RM was immobilized on a hydrophobic acrylic resin by physical adsorption. Research studies indicated that hydrophobic supports immobilize lipases and changed lipase’s conformation and enzymatic activity.31 Besides, a previous study found that the pore size and particle size are critical both
for the loading capacity and for the enzymatic activity. Also, the loss of \( \beta \)-sitosterol could be found by five lipases’ reaction system, which could be reasonable, due to its hydroxide radical easily reacting with FFA presented in the SODD after phytosterols entered the catalytic pocket of lipase. Sengupta et al. reported that phytosterol esters could be synthesized by Lipzyme TLIM using different oils as the sources of particular fatty acids in a stirred tank batch reactor and a packed bed reactor. Besides, the loss phenomena of squalene was not found. The possible reason could be related to the alkene structure of squalene. Therefore, immobilized lipase MAS1-H108A showed a higher retention rate of bioactive compounds, when compared with the four commercial immobilized lipases; thus, it was selected for the following catalysis experiments.

2.3.1. Molar Ratio. The substrate molar ratio is an important variable in enzyme-catalyzed reversible reactions because it affects the equilibrium of the reaction and thus affects the extent of the reaction. To avoid the lipase inactivation caused by the high concentration of methanol, we added methanol equally in three batches (at 0, 2, 4 h). The FAME yield increased with increasing the substrate molar ratio from 1:1 to 1:2 (oil/methanol). The maximum FAME yield of 97.08% with an acid value of 1.83 mg KOH/g was obtained at a molar ratio of 1:2. With the further increase of the molar ratio, the FAME yield decreased gradually (Figure 3A). When the molar ratio was greater than 1:2, although the increase of the methanol content would make the reaction equilibrium point move forward, the phenomenon of methanol denaturation or inactivation of the enzyme was more obvious, so the FAME yield decreased. Therefore, the substrate molar ratio of 1:2 (SODD/methanol) was chosen for the succeeding experiments.

2.3.2. Reaction Temperature. The reaction temperature affects the catalytic activity and stability of the enzyme, as well as the viscosity of the system and the diffusion of substrates and products. To study the effect of temperature on the esterification and transesterification of SODD and methanol catalyzed by immobilized MAS1-H108A, the experiments were carried out at five different temperatures (30, 35, 40, 45, and 50 °C). As can be seen from Figure 4A, the highest yield of

Figure 2. Effect of the five immobilized lipases on the FAME yield and bioactive component contents in the process of catalysis. Yield of FAME (A); content of \( \alpha \)-tocopherols (B); content of \( \delta \)-tocopherols (C); content of \( \gamma \)-tocopherols (D); content of squalene (E); and content of \( \beta \)-sitosterol (F). The reaction was performed at a molar ratio of 1:1 (oil/methanol), a temperature of 35 °C, and enzyme loading of 25 U/g SODD for 24 h. Methanol was added at the beginning of the reaction by one-step.
FAME was obtained at 35 °C in all reaction periods. After 24 h of reaction, the FAME yield could reach 97.08%. It could be understood that the reaction system was viscous at 30 °C, which may provide an insufficient contact between the substrates. As the enzyme activity was inhibited at low temperatures, the synthesis efficiency of FAME was lower than that at 35 °C. However, when the temperature was increased above 40 °C, the synthesis efficiency of FAME began to decrease and could be attributed to the denaturation of the enzyme. After 12 h of reaction, the immobilized MAS1-H108A lost its activity and the FAME yield did not increase above 40 °C. Thus, the optimal reaction temperature was 35 °C.

Similarly, the reaction temperature significantly affected the content of β-sitosterol but did not affect the contents of the three tocopherols and squalene (Figure 4B−F). The minimum content of β-sitosterol was also found at 35 °C in all reaction periods compared to other temperatures. This result further demonstrated that 35 °C was the best condition because β-sitosterol was more liable to produce β-sitosterol esters at the optimal reaction conditions.

2.3.3. Enzyme Loading. Different from the reaction temperature and the molar ratio of the substrate, the enzyme loading does not affect the equilibrium of the reaction but only affects the time to reach a steady-state. To select the optimal enzyme loading, the effects of different additions of immobilized MAS1-H108A ranging from 5 U/g SODD to 45 U/g SODD on the yield of FAME were investigated. As shown in Figure 5A, with an increase in enzyme loading, the FAME yield increased. When the enzyme loading was in the range of 5−45 U/g SODD, the FAME yield at 12 h was 33.76, 84.28, 94.13, 97.08, and 97.86%, respectively. The FAME yield with 25, 35, and 45 U/g enzyme loading was significantly higher than those of 5 and 15 U/g (p < 0.05). Although the FAME yield with 35 and 45 U/g enzyme loading was higher than that of 25 U/g at 12 h, they all reached equilibrium at 24 h, and the FAME yield was all around 97%. When the enzyme loading increased from 35 to 45 U/g, there was no significant difference in the FAME yield due to the saturation of the enzyme binding with the substrate (p > 0.05). For economic consideration of shortening the reaction time simultaneously saves the cost, 35 U/g enzyme loading was a best selection. Consistent with the previous phenomenon, the contents of the three tocopherols and squalene were constant during the reaction (Figure 5B−F).
with the increase of enzyme loading. It was further proved that immobilized lipase played an irreplaceable role in the synthesis of phytosterol esters.

Under the optimal reaction conditions of this study, the maximum FAME yield was 97.08%, which exceeded the minimum limitation (96.5%) required by the EN14214 standard. Moreover, it is noteworthy that the one enzymatic step method used in this work was better than the two enzymatic step (Lipase AY Amano 30 and Novozyme 40013) method and the FAME yield was 96% as reported by Nandi et al.\(^4^0\) Moreover, the acid value decreased from around 89.50 mg KOH/g to below 2 mg KOH/g with no loss of the three tocopherols and squalene.

2.4. Reusability of Immobilized Lipase MAS1-H108A. We further studied the repeatability of immobilized lipase MAS1-H108A by enlarging the original reaction system to 1000 g. Figure 6 shows the FAME yield of every batch catalyzed by immobilized lipase MAS1-H108A. After 10 batches, the FAME yield was still maintained at approximately 95%, which showed no significant difference with the FAME yield of 97.08% obtained in the first batch \((p > 0.05)\). This result may be associated with the rigid structure after immobilization, which provided excellent methanol tolerance. Therefore, the excellent reusability of immobilized lipase MAS1-H108A exhibited great potential in the oil industry.

2.5. Separation and Recovery of FAME and Bioactive Compounds. Separation and recovery of FAME and bioactive compounds from the esterification and transesterification reaction system were performed on molecular distillation. The evaporation temperature significantly affected the FAME yield and bioactive compound contents in both the light and heavy components of molecular distillation. To achieve an effective separation of FAME and bioactive compounds, the experiments were carried out at six different temperatures (80, 90, 100, 110, 120, and 130 °C). Other constant parameters for molecular distillation were set as follows: the feed flow rate was 100 mL/h, the evaporating pressure was 0.1 Pa, and the wiper rolling speed was 380 rpm. The yield of light and heavy components, and the yield and recovery of FAME and bioactive compounds at different temperatures are shown in Table 2. With the increase of the evaporating temperature, the yield of the light component increased rapidly from 60.28 ±
The yield of FAME (A); content of α-tocopherols (B); content of δ-tocopherols (C); content of γ-tocopherols (D); content of squalene (E); content of β-sitosterol (F). The reaction was performed at a molar ratio of 1:2 (oil/methanol) and a temperature of 35°C. Methanol was added in 3 steps at 0, 2, and 4 h.

Figure 6. Reusability of immobilized lipase MAS1-H108A. Reaction conditions: molar ratio of 1:2 (oil/methanol), enzyme loading of 35 U/g SODD, and a temperature of 35°C.
Table 2. Yield and Recovery of Bioactive Compounds and FAME in Both Light and Heavy Components at Different Evaporating Temperatures

|                              | 80 °C     | 90 °C     | 100 °C    | 110 °C    | 120 °C    | 130 °C    |
|------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| yield (%)                    | 60.28 ± 0.93<sup>a</sup> | 72.16 ± 1.84<sup>b</sup> | 73.92 ± 1.82<sup>c</sup> | 76.16 ± 1.48<sup>d</sup> | 78.02 ± 0.23<sup>ab</sup> | 80.30 ± 1.25<sup>d</sup> |
| bioactive compound contents  |<sup>α</sup>-tocopherol |<LOQ>  |<LOQ>  |<LOQ>  |<LOQ>  |<LOQ>  |
| (mg/g)                       | 15.40 ± 1.00<sup>d</sup> | 21.84 ± 2.02<sup>d</sup> | 23.35 ± 3.16<sup>d</sup> | 24.09 ± 0.80<sup>d</sup> | 25.10 ± 1.71<sup>d</sup> | 19.62 ± 1.79<sup>d</sup> |
| FAME yield (%)               | 99.21 ± 0.01<sup>d</sup> | 98.27 ± 0.20<sup>c</sup> | 97.78 ± 0.10<sup>d</sup> | 95.64 ± 1.10<sup>c</sup> | 93.35 ± 0.57<sup>c</sup> | 90.29 ± 1.64<sup>c</sup> |
| FAME recovery (%)            | 81.54 ± 0.01<sup>c</sup> | 92.92 ± 0.20<sup>c</sup> | 99.12 ± 0.10<sup>c</sup> | 99.57 ± 1.15<sup>c</sup> | 99.75 ± 0.61<sup>c</sup> | 98.94 ± 1.97<sup>c</sup> |
| yield (%)                    | 39.72 ± 0.93<sup>c</sup> | 27.84 ± 1.84<sup>d</sup> | 26.08 ± 1.82<sup>d</sup> | 23.84 ± 1.48<sup>c</sup> | 21.98 ± 0.23<sup>c</sup> | 19.70 ± 1.25<sup>a</sup> |
| bioactive compound contents  | 46.94 ± 2.61<sup>c</sup> | 70.13 ± 2.91<sup>c</sup> | 75.32 ± 0.93<sup>d</sup> | 76.22 ± 0.69<sup>d</sup> | 77.06 ± 1.96<sup>d</sup> | 63.84 ± 2.01<sup>d</sup> |
| (mg/g)                       | 36.10 ± 4.30<sup>c</sup> | 50.25 ± 2.09<sup>d</sup> | 48.10 ± 2.05<sup>d</sup> | 32.25 ± 1.33<sup>c</sup> | 21.24 ± 0.99<sup>c</sup> | 11.37 ± 0.08<sup>c</sup> |
| FAME yield (%)               | 21.85 ± 0.98<sup>c</sup> | 30.66 ± 0.87<sup>c</sup> | 32.83 ± 1.04<sup>c</sup> | 34.76 ± 3.80<sup>c</sup> | 36.74 ± 1.95<sup>c</sup> |<LOQ>  |
| FAME recovery (%)            | 29.01 ± 1.25<sup>c</sup> | 10.23 ± 0.96<sup>c</sup> |<LOQ>  |<LOQ>  |<LOQ>  |<LOQ>  |
| bioactive compounds' yield (%) | 97.74 ± 1.06<sup>c</sup> | 96.37 ± 2.30<sup>c</sup> | 93.37 ± 4.08<sup>c</sup> | 83.66 ± 2.78<sup>c</sup> | 75.41 ± 3.86<sup>c</sup> | 57.26 ± 1.96<sup>c</sup> |

<sup>a</sup>Other constant parameters for molecular distillation: the feed flow rate was 100 mL/h, the evaporating pressure was 0.1 Pa, and the wiper rolling speed was set to 380 rpm. The data are expressed as the mean ± standard error. Values with different lowercase letters in one line indicate significant differences (p < 0.05).

The contents of α-tocopherol, δ-tocopherol, γ-tocopherol, β-sitosterol, and squalene in the heavy-component product were 23.35 ± 3.16, 75.32 ± 0.93, 100.92 ± 5.09, 32.83 ± 1.04, and 48.10 ± 2.05 mg/g, respectively. Compared with the initial contents of bioactive compounds, α-tocopherol, δ-tocopherol, γ-tocopherol, β-sitosterol, and squalene were enriched by 4.31, 3.90, 3.92, 1.81, and 3.09 times, respectively.

2.6. Characterization of FAME and SODD by FT-IR and NMR Spectroscopy. FT-IR analysis was applied to investigate the functional groups of the FAME and SODD (Figure 7A). The characteristic peak in the FAME sample that appeared at 1750 cm<sup>−1</sup> indicated a strong band of the carbonyl group (C==O) of methoxy esters (−CO−O−CH3). Another characteristic peak in the FAME sample that was found between 1160 and 1207 cm<sup>−1</sup> was due to the C−O stretching vibrations. Similar results were observed by Ullah et al.41 Because there was about 30% triglycerides in SODD, the SODD sample also had bending vibrations near the wavelength of the above characteristic peaks but the vibration intensity was weaker than FAME.

The FAME and SODD samples were also characterized by <sup>1</sup>H NMR and <sup>13</sup>C NMR spectroscopy and their spectra are shown in Figure 7B. In the <sup>1</sup>H NMR spectrum, a singlet signal of the FAME sample at 3.69 ppm corresponds to the methoxy protons (−OCH<sub>3</sub>) of methyl esters (−COOCH<sub>3</sub>) (Figure 7B). However, this singlet signal was not found in the SODD sample, which confirmed the conversion of triglycerides and FFA into FAME. In the <sup>13</sup>C NMR spectrum, the characteristic peak appeared at 51.23 ppm due to the presence of methoxy carbons (−OMe) of methyl esters in the FAME sample (Figure 7C), and there was no obvious signal in this absorption band in the SODD sample. Kumar et al. have reported the same absorption band in the <sup>13</sup>C NMR spectrum.42 <sup>1</sup>H NMR and <sup>13</sup>C NMR analysis further proved that the successful conversion of SODD to FAME was achieved.

3. CONCLUSIONS

An efficient process was applied for the synthesis of biodiesel and recovery of bioactive compounds (tocopherols, β-sitosterol, and squalene) by a combined enzymatic process and molecular distillation, and a FAME yield over 97% could be obtained that exceeded the minimum limitation (96.5%) required by the EN14214 standard. Moreover, the robust immobilized lipase MAS1-H108A could be used for 10 persistent cycles without significant loss in the catalytic activity that could dramatically decrease the cost by recovery in industrial application. Finally, the total bioactive compounds’ concentration increased from 8.40 to 28.05% and the 97.78% pure biodiesel (characterized by FT-IR and NMR) was obtained. Our study has developed an eco-friendly and simple method combining the enzymatic reaction and molecular distillation to synthesize and purify biodiesel and recover bioactive compounds.

4. EXPERIMENTAL SECTION

4.1. Materials and Chemicals. SODD samples were kindly donated by COFCO ET (Xi’an) International Engineering Co., Ltd. The immobilized lipase MAS1-H108A was prepared in our laboratory.43 MAS1 lipase was from Streptomyces sp. strain W007 and immobilized on a styrene/divinylbenzene copolymer resin AP1090/5753 (Purolite, U.K.).28 Novozym 435, Lipozyme TLIM, Lipozyme RMIM, and Lipozyme RM were purchased from Novozymes (Bagsvaerd, Denmark). Novozym 435 was the immobilized form of the lipase B from Candida antarctica (CALB) and immobilized on the macroporous resin Lewatit VP OC 1600.
Lipozyme TLIM was from Thermomyces lanuginosus (TLL) and immobilized on a cationic silicate resin. Lipozyme RMIM was from R. miehei (RML) and immobilized on Duolite ES 562 resin. Lipozyme RM was also from R. miehei (RML) and immobilized on an acrylic resin.

The chromatographically pure standards of \( \alpha \)-tocopherol, \( \gamma \)-tocopherol, \( \delta \)-tocopherol, \( \beta \)-sitosterol, and squalene were purchased from Aladdin Industrial Corporation (Shanghai, China). The standards of FAME (C14:0–C22:6) and methyl seventeen alkanoate (internal standard) were purchased from Sigma-Aldrich (St. Louis, MO). Methanol, \( n \)-hexane, \( n \)-heptane, 2-propanol, formic acid, and boron trifluoride methanol solution were of chromatographic grade and purchased from Aladdin Industrial Corporation (Shanghai, China). All other commercial chemicals were of analytical grade unless otherwise stated.

4.2. Determination of the Esterification Activity of Immobilized Lipase. The activities of immobilized lipase were determined according to the Novozymes propyl laurate unit (PLU) method EB-SM-1069.02 in the esterification reaction of 1-propanol and lauric acid.

4.3. Screening of Immobilized Lipases. The reaction was conducted in a 100 mL conical flask containing 44.87 g of SODD and 5.12 g of methanol. Enzyme loading was 25 U/g SODD. The flask was shaken with a constant speed of 200 rpm at 35 °C. Samples were withdrawn periodically to analyze the FAME yield and the contents of bioactive compounds during the reaction.

4.4. Optimization of Reaction Conditions. A mixture containing SODD, methanol, and lipase MAS1-H108A were added to a 100 mL conical flask with constant shaking at 200 rpm for 24 h. The reaction parameters, such as the oil/methanol molar ratio (1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:4, and 1:5), enzyme loading (5, 15, 25, 35, and 45 U/g SODD), and temperature (30, 35, 40, 45, and 50 °C), were varied for optimization. In particular, to reduce the inactivation rate of immobilized MAS1-H108A and increase the conversion of FAME, methanol was a three-step average added at 0, 2, and 4 h, respectively. The FAME yield and the contents of bioactive compounds were detected during the reaction.

4.5. Reusability of Immobilized MAS1-H108A. Under the optimal catalytic conditions as given in Section 4.4, the substrate and the enzyme were amplified by 20 times. After 24 h of reaction, the immobilized MAS1-H108A was filtered out and washed three times in \( n \)-hexane. After drying, the new substrate was added for the next batch of reaction. The FAME yield was detected during the reaction in every batch.

4.6. Analysis of the Fatty Acid Composition. The fatty acid composition of SODD was analyzed by a GC (Agilent 7890B) equipped with an Agilent J&W CP-Sil 88 capillary column (60 m × 0.25 mm i.d., 0.20 μm) and a flame-ionization detector. The detailed analysis was carried out according to our previously reported method.35

4.7. Determining the Acid Value. The AOCS Official Method (Ca 5a-40) was used to determine the acid value of SODD before and after the reaction. Phenolphthalein solution was used as the end point indicator, and the calibrated KOH solution was used as a titration solution to detect the acid value of samples.

4.8. Analysis of the FAME Yield. The FAME yield was defined as the ratio of the conversion of the sample during the reaction to the conversion of the sample methylated completely in theory.

For complete methylation, about 6 mg of the sample (M1) was methylated to FAME, according to the method described by Wang et al. with some modifications.46 The difference was
that 1 mL of n-hexane and 1 mL of 1 mg/mL methyl seventeen alkanoate (internal standard) were added in the end for extraction. About 6 mg of the sample (M2) was added to a 1 mL volumetric flask. Then, added 0.5 mL of 1 mg/mL methyl seventeen alkanoate was added, a constant volume to 1 mL with n-hexane.

Samples were analyzed by a GC-MS (SHIMADZU TQ8050) equipped with an automatic sampler (AOC-20i+s). A capillary column SH-Rtx5MS (crossbond 5% diphenyl/5% dimethyl polysiloxane) with dimensions of 30 m × 0.25 mm I.D × 0.25 μm film thickness (SHIMADZU) was used for the separation of the five representative FAME in the samples during the reaction. The column oven was initially held at 150 °C for 1 min, heated from 150 to 190 °C at a rate of 10 °C/min, and then increased to 210 °C at a rate of 5 °C/min. After that, it was increased to 250 °C at a rate of 10 °C/min and held for 1 min, and then again increased to 285 °C at a rate of 5 °C/min and held for 1 min, and increased to 290 °C at a rate of 5 °C/min and held for 1 min. Finally, increased to 310 °C at a rate of 5 °C/min and held for 5 min. Helium was used as the carrier gas and the flow was 50 mL/min. The temperatures of the injector and the detector were 280 and 300 °C, respectively. Detection was performed in the full scan mode from m/z 50 to 320.

The conversion was calculated by the internal standard method as follows:

\[
\text{FAME yield (\%) = \frac{C_2}{C_1} \times 100}\%
\]

The conversions of M1 and M2 were C1 and C2, respectively.

4.9. Analysis of Bioactive Compounds. Bioactive compounds were performed by HPLC on an Agilent Liquid Chromatograph, equipped with a quaternary pump, an autosampler, a thermostatted column compartment, and a UV-detector (205 and 296 nm), according to the method described by Yuan et al. with some modifications. The optimized chromatographic column was C18-00G-4375-E0 (250 mm × 4.6 mm; Phenomenex) and mobile phase was 90:10:0.001 (v/v/v) methanol/isopropanol/formic acid at a flow rate of 1 mL/min. The column was kept at a constant temperature (30 °C), and 10 μL of the sample was injected in all of the analyses. The standard curve was drawn for each compound and quantified by the external standard method.

4.10. Analysis of Water Content. The water content analysis was performed by the Karl Fischer titration method using a Karl Fischer 830 coulometer equipped with a 728 stirrer (fully automated coulometric method, Metrohm, Switzerland). Around 1 g of the feedstock was required for each test.

4.11. Separation of FAME and Bioactive Compounds by Molecular Distillation. The VLK70-5 FDRR short path distillation plant (VTA, GMBH & Co. KG, Germany) was used for the separation of FAME and recovery of bioactive compounds. After removing methanol with a rotary evaporator at 70 °C, the mixture of FAME and bioactive compounds was put into the feed vessel, which was surrounded by a heating jacket (60 °C). The feed flow rate was 100 mL/h, the evaporating pressure was 0.1 Pa, and the wiper rolling speed was set to 380 rpm. The evaporating temperature (80–130 °C) was investigated on the contents and recovery of FAME and bioactive compounds.

4.12. Characterization of FAME and SODD by FT-IR and NMR. FT-IR analysis was carried out using a Nicolet ISS0 FT-IR spectrometer (ThermoFisher Scientific). The FT-IR spectra were recorded in the wavelength ranging from 4000 to 400 cm⁻¹ during 128 scans, with the resolution at 2 cm⁻¹. The sample preparation method was according to the method described by Wang et al. The 1H and 13C NMR analysis of the FAME and SODD were carried out at 600 MHz using a Bruker AVANCE III 600HD spectrometer with 5 mm BBO probes. Deuterated chloroform was used as an internal standard and a solvent. The 1H NMR spectrum was acquired with a recycle delay of 1.0 s and 16 scans. The 13C NMR spectrum was obtained with a recycle delay of 2.0 s and 160 scans. MestReNova software (Mestrelab Research SL, Santiago de Compostela, Spain) was employed to analyze the spectrum.

4.13. Statistical Analysis. All experiments were carried out in triplicate and the results were reported as mean ± standard deviation (SD). The difference of measured values in the esterification and transesterification process of immobilized MAS1-H108A and the process of molecular distillation was analyzed by the One-way analysis of variance (ANOVA) procedure (p < 0.05).

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Notes

The authors declare no competing financial interest.

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