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Highly Efficient Production of Diacylglycerols via Enzymatic Glycerolysis Catalyzed by Immobilized MAS1-H108W Lipase

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Abstract

Developing highly efficient and cost-effective immobilized biocatalysts is essential for optimizing diacylglycerol (DAG) production via biotransformation of natural oil. To address this, the 1,3-regiospecific MAS1-H108W lipase, derived from marine *Streptomyces* sp. strain W007, was produced through high-density fermentation (20 °C, pH 7.0, 132 h). This lipase was immobilized by XAD1180 resin adsorption, yielding an immobilized MAS1-H108W lipase with a lipase activity of 4943.5 U/g and a protein loading of 201.5 mg/g under selected conditions (lipase/support ratio 100 mg/g, initial buffer pH of 8.0). After immobilization, the lipase maintained its optimal temperature at 70 °C and shifted its optimal pH from 7.0 to 8.0, along with enhanced thermostability. The immobilized MAS1-H108W lipase demonstrated superior efficiency in DAG synthesis compared to non-regiospecific immobilized MAS1 lipase and commercial lipases (Novozym 435 and Lipozyme RM IM). Under the optimized reaction conditions (reaction temperature 60 °C, olive oil/glycerol molar ratio 1:2, adding amount of immobilized MAS1-H108W lipase 1.0 wt.%), a maximum DAG content of 49.3% was achieved within 4 h. The immobilized lipase also exhibited excellent operational stability, retaining 81.9% of its initial production capacity after 10 reuse cycles. Furthermore, in the glycerolysis of various vegetable oils (corn oil, rapeseed oil, peanut oil, sunflower oil, and soybean oil), the DAG content catalyzed by immobilized MAS1-H108W lipase consistently exceeded 48%. This work provides a highly efficient and economical immobilized biocatalyst for DAG production, and highlights the significant potential of regioselective lipases in promoting efficient DAG synthesis via glycerolysis.

Keywords: immobilized MAS1-H108W lipase; diacylglycerol; enzymatic glycerolysis; biocatalysis

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1. Introduction

Diacylglycerol (DAG), a structural lipid, naturally exists as 1,2-DAG, 2,3-DAG and 1,3-DAG [1,2]. DAG exerts multiple physiological benefits in humans due to its distinct metabolic pathways compared to conventional dietary fats and oils, including suppression of body fat accumulation, reducing the levels of postprandial cholesterol and glucose, and enhancement of bone mineral density [3,4]. Consequently, the demands of DAG are expanding rapidly in the fields of foods and nutraceuticals [3–5]. Vegetable oils are the most abundant natural sources of oil, but DAG occurs naturally only as a minor component

in them (the maximum content of 9.5%) [6,7]. This limited natural abundance requires the development and application of efficient DAG production technologies to satisfy growing market requirements.

Current industrial-scale DAG production primarily employs chemical catalysis and enzymatic bioconversion strategies [8]. Chemically, DAG synthesis relies on alkaline catalysts and high temperatures (220–260 °C) [9], which pollute the environment and waste energy. In addition, the non-directional nature of chemical transesterification hindered the formation of specific glyceride [3]. In comparison with the chemical method, enzymatic strategies are preferred for DAG synthesis, owing to their milder reaction conditions, higher catalytic selectivity, and environmental compatibility [9–11]. The common industrial DAG production employs enzymatic esterification [12]. However, this process requires high levels of free fatty acids (FFA), leading to higher production costs [13]. Enzymatic partial hydrolysis generates substantial quantities of FFA, which results in the relatively low DAG yield at the end of DAG production [14,15]. Relative to these methods, glycerolysis of triacylglycerols (TAG) is a promising process for DAG production. Compared to glycerolysis reactions run in tert-butanol or ionic liquids, solvent-free conditions present a distinct advantage for specific systems by eliminating post-processing of the medium [16], thereby enhancing process sustainability through reduced solvent consumption, waste generation, and energy demand associated with solvent removal and recovery. This aligns with the principles of green chemistry by minimizing environmental impact and simplifying downstream operations [17]. However, the glycerolysis in solvent-free systems requires relatively high enzyme loadings ($\geq$4 wt.% relative to substrates) and long reaction times (approximately 10–24 h) to reach equilibrium [18], which limits DAG synthesis efficiency. Therefore, the development of a highly efficient immobilized biocatalyst capable of reducing the required enzyme amount and shortening the reaction time could enable an environmentally friendly, economically viable, and industrially scalable production of DAGs.

Recently, a robust and non-regiospecific lipase, named MAS1, was mined and characterized with marine *Streptomyces* sp. strain W007 [19]. MAS1 has been immobilized onto macroporous resin XAD1180 by physical adsorption and employed to catalyze esterification and glycerolysis reactions for synthesizing triacylglycerols rich in n-3 polyunsaturated fatty acids. Immobilized MAS1 lipase displayed superior catalytic efficiency compared to Novozym 435 and Lipozyme RM IM, demonstrating its great potential for oil modification by esterification/transesterification [20,21]. However, its catalytic efficiency in the glycerolysis of natural oil for producing DAG remains unknown. Moreover, since non-regiospecific lipases can transesterify any acyl group in TAG molecules to any position in the glycerol, while 1,3-regiospecific lipases only catalyze the transesterification of acyl groups at the sn-1 and/or sn-3 positions of TAG to the corresponding positions of glycerol [22], the regioselectivity of lipases is likely another critical factor governing the efficiency of DAG synthesis via enzymatic glycerolysis. Although systematic studies in this area remain limited, recent structural studies have elucidated the basis for the regiospecificity of MAS1 lipase [23]. Notably, its mutant MAS1-H108W lipase exhibited strict sn-1,3 regiospecificity, along with a twofold increase in hydrolytic activity [24]. Thus, MAS1 and MAS1-H108W lipases constitute a well-suited pair of study templates for this investigation. Macroporous resin XAD1180 exhibited excellent protein loading capacity (106 $\pm$ 1.02 mg/g), esterification activity (2546 $\pm$ 13 U/g), and specific activity (24 $\pm$ 0.72 U/mg) during the immobilization of MAS1 [22], and was therefore selected as the immobilization support.

This work is intended to develop a novel and highly efficient immobilized biocatalyst for DAG production via enzymatic glycerolysis. The key novelty of this study lies in the construction and application of the engineered lipase MAS1-H108W immobilized on macroporous resin XAD1180, which demonstrated superior catalytic performance compared to

both immobilized MAS1 lipase and commercial lipases (Novozym 435 and Lipozyme RM IM). Specifically, MAS1-H108W lipase was produced via high-density fermentation and immobilized onto hydrophobic resin XAD1180 by physical adsorption. Subsequently, characterizations of free and immobilized MAS1-H108W lipase were investigated. The DAG production catalyzed by immobilized MAS1-H108W lipase was compared with that of immobilized MAS1 lipase and commercial lipases (Novozym 435 and Lipozyme RM IM). Then, the reaction conditions of enzymatic glycerolysis, including reaction temperature, olive oil/glycerol molar ratio, and the added amount of immobilized lipase were optimized to achieve the highest production efficiency, as it showed the best performance. Additionally, the reusability and applicability of immobilized MAS1-H108W lipase were assessed. The findings presented in this study offer a promising and scalable approach for the industrial production of DAGs, highlighting the potential of immobilized MAS1-H108W lipase as a robust, cost-effective, and environmentally friendly biocatalyst that is suitable for future food and nutraceuticals applications.

2. Materials and Methods

2.1. Materials

The X33/MAS1-H108W strain with 60% glycerol solution (v/v) was preserved by our laboratory at −80 °C. The XAD1180 resin (particle size 0.4 mm, specific surface area 700 m^2/g, pore volume 40 nm, density 1.015–1.025 g/cm^3) was purchased from the Rohm and Haas Company (Philadelphia, PA, USA). Glycerol, of analytical grade, was obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). The olive oil brand was Olivoila (Shanghai, China), and the sunflower oil brand was Fulinmen (Beijing, China). The brands of peanut oil, soybean oil, rapeseed oil, and corn oil were all from Jinlongyu (Shanghai, China). n-Hexane, isopropanol, and formic acid, all of which were of chromatographic grade, were sourced from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Anhydrous sodium sulfate, of analytical grade, was procured from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The commercial enzymes (Novozym 435 and Lipozyme RM IM) were sourced from Novo Nordisk (Copenhagen, Denmark). All other reagents of analytical purity were obtained from Sinopharm Group (Shanghai, China).

2.2. Preparation of MAS1-H108W Lipase

High-density fermentation was carried out in a 5 L bioreactor, according to the method reported previously [25]. After fermentation, the broth was centrifuged (10,000 g, 30 min, 4 °C) to collect the supernatant. The supernatant was filtered through 0.45 μm filter membranes and then concentrated through a 10 kDa molecular mass membrane (Sartorius, Hamburg, Germany). The obtained supernatant was stored at 4 °C until use. The supernatant was analyzed by SDS-PAGE. The hydrolytic activities of lipase were detected by the olive oil emulsion method [26].

2.3. Immobilization of MAS1-H108W Lipase

MAS1-H108W lipase was immobilized onto XDA1180 resin, according to the method reported previously [21]: Firstly, the XDA1180 resin (10 g) was sequentially pretreated with 30 mL of 95% ethanol (v/v), 30 mL of 5% HCl (v/v) acid, and 30 mL of 2% NaOH (w/v). After that, the resin was equilibrated with 100 mL of sodium phosphate buffer (0.1 M, pH 8.0). Then, MAS1-H108W lipase solution (100 mg protein/g resin) and an equal volume of phosphate buffer (0.1 M, pH 8) were added to a flask containing 4 g of the pretreated resin. After that, this flask was stirred by a thermostatic water bath oscillator (30 °C, 200 rpm) for 8 h. Subsequently, immobilized MAS1-H108W lipase was recovered

and repeatedly washed with buffer until no protein was detected in the eluate. Finally, immobilized MAS1-H108W lipase was dried under vacuum at 40 °C for 8 h with designated as MAS1-H108W @XAD1180. The immobilized lipase was stored at 4 °C until use.

The immobilization conditions, such as lipase/support ratio (25–150 mg/g resin) and initial pH (5.0, 6.0, 7.0, 8.0, 9.0, 10.0) of buffer, were varied for optimization. The following buffers were employed: 0.1 M phosphate buffer (pH 5.0–8.0) and 0.1 M Tris-HCl buffer (pH 9.0–10.0). The concentration of the protein was determined by a Bradford assay [27]. The hydrolytic activities of lipase were detected by the olive oil emulsion method [26]. The lipase/support ratio was calculated by:

$$lipase/support\ ratio(mg/g) = \frac{(C_0V_0 - CV)}{m} \quad (1)$$

In this equation, C_0 and V_0 are the protein concentration (mg/mL) and volume (mL) of enzyme solution before immobilization, respectively. C and V are the protein concentration (mg/mL) and volume (mL) of the eluate after immobilization, respectively. m is the mass (g) of the XAD1180.

The immobilization yield was defined as follows:

$$Immobilization\ yield\ \% = 1 - \frac{A_{supernat} \cdot V_{supernat}}{A_{free} \cdot V_{free}} \quad (2)$$

In this equation, $A_{supernat}$ (U/mL) and $V_{supernat}$ (mL) are the activity and volume of the supernatant after immobilization, respectively. A_{free} (U/mL) and V_{free} (mL) are the activity and volume of enzyme solution before immobilization.

2.4. Characterizations of Free and Immobilized MAS1-H108W Lipase

The Fourier transform infrared (FT-IR) analysis of free and immobilized MAS1-H108W lipase was carried out, referring to the method of Liu et al. [28]. The effects of temperature on the hydrolytic activity of free and immobilized MAS1-H108W lipase were evaluated at temperatures varying from 50 to 90 °C, at pH 7.0. The relative activity of each lipase was calculated as the ratio of its hydrolytic activity measured at specific temperatures to the maximum activity, measured as described above. The effects of pH on the hydrolytic activity of the lipases was investigated at various pH values, ranging from 5.0 to 10.0 at 70 °C. The buffers used were 0.1 M phosphate buffer (pH 5.0–8.0) and 0.1 M Tris–HCl buffer (pH 9.0–10.0). The relative activity of each lipase was calculated as described above. The thermostability of the lipases was assessed by measuring the residual hydrolytic activities of lipases after incubation at 60 °C and 70 °C for 4 h, respectively. During incubation, samples were withdrawn at selected times and were assayed for hydrolytic activity. The hydrolytic activity of free and immobilized MAS1-H108W lipase was determined according to the olive oil emulsion method [26].

2.5. Production of DAG by Enzymatic Glycerolysis

DAGs were prepared by the enzymatic glycerolysis catalyzed by immobilized lipases in the solvent-free system: olive oil and glycerol (a molar ratio of 1:2) were mixed in a flask and MAS1-H108W @XAD1180 of 1.0 wt.% (accounting for the mass of olive oil and glycerol) were added. This mixture was stirred at a speed of 200 rpm in a heat-collection constant temperature type magnetic stirrer at 60 °C. Reaction mixtures were withdrawn at selected times for high-performance liquid chromatography (HPLC) analysis. Here, the effects of reaction conditions on DAG synthesis in enzymatic glycerolysis were investigated, including reaction temperature (40, 50, 60, and 70 °C), the molar ratio of olive oil to glycerol

(2:1, 1:1, 1:2, and 1:3), and the added amount of MAS1-H108W @XAD1180 (0.5, 1.0, 1.5, and 2.0 wt.%).

2.6. Reusability and Applicability of MAS1-H108W @XAD1180

To investigate the reusability of MAS1-H108W @XAD1180, glycerolysis reactions were conducted at 60 °C with an added amount of enzyme of 1.0 wt.% and a substrate molar ratio of olive oil to glycerol at 1:2 for 4 h. After each reaction, MAS1-H108W @XAD1180 was recovered from the reaction mixtures via centrifugation, washed thrice with n-hexane, and placed at room temperature to remove residual n-hexane for subsequent reuse. The reaction mixtures from each run were withdrawn for HPLC analysis after each cycle. Additionally, to verify the applicability of MAS1-H108W@XAD1180, glycerolysis reactions with different vegetable oils were carried out under chosen conditions.

2.7. Analysis of the Composition of the Reaction Mixtures

According to the previous method [29], the composition of the reaction mixtures was analysis by HPLC (Shimadzu Corporation, Kyoto, Japan), equipped with a parallax refractive index detector and a Phenomenex Luna column (250 mm × 4.6 mm i.d., 5 μm particle size, Phenomenex Corporation, Torrance, CA, USA). The samples were homogenously dispersed in the mobile phase, consisting of formic acid, isopropanol and n-hexane (0.003:1:20, $v/v/v$), filtered through the filter membrane and chromatographically separated at a flow rate of 1 mL/min. Peaks in high-performance liquid chromatography were identified through comparing their retention time with those of known standards. The retention times were as follows: 3.1 min (TAG), 4.6 min (1,3-DAG), 5.8 min [1,2(2,3)-DAG], 29.5 min [1(3)-MAG], and 37.9 min (2-MAG). The content of DAG was analyzed with the area normalization method.

2.8. Statistical Analysis

All experiments were performed in triplicate. The significance of differences among measured values was evaluated using IBM SPSS Statistics 29.0.1.0. and the data were expressed as the means ±standard deviations.

3. Results and Discussions

3.1. Expression and Immobilization of MAS1-H108W Lipase

High-density fermentation was carried out in a 5 L bioreactor. The results indicated that under the determined optimal induction conditions (20 °C, pH 7.0) for 132 h, the lipase activity reached the maximum value (620 U/mL), with a protein concentration of 2.4 g/L and a wet cell weight of 569 g/L (Figure 1a). The SDS-PAGE analysis for crude MAS1-H108W lipase (~27 kDa) was presented in Figure 1b.

The immobilization conditions for MAS1-H108W lipase were investigated. As shown in Figure 1c, the protein loading increased with increasing lipase/support ratios, while the immobilization yield decreased. Lipase activity increased as the ratio increased from 25 mg/g to 100 mg/g, reaching a maximum value of 4940.62 U/g at 100 mg/g. However, beyond this point, further increases in the ratio led to a decline in activity. This reduction may be attributed to excessive protein loading, which could hinder substrate access to the active sites of lipases [22]. As shown in Figure 1d, protein loading, immobilization yield, and lipase activity initially increased and then declined as the initial buffer pH increased. The maximum values of 201.5 mg/g and 4943.5 U/g were achieved at a pH of 8. Therefore, the optimal immobilization conditions were identified as a lipase/support ratio of 100 mg/g and an initial buffer pH of 8.0.

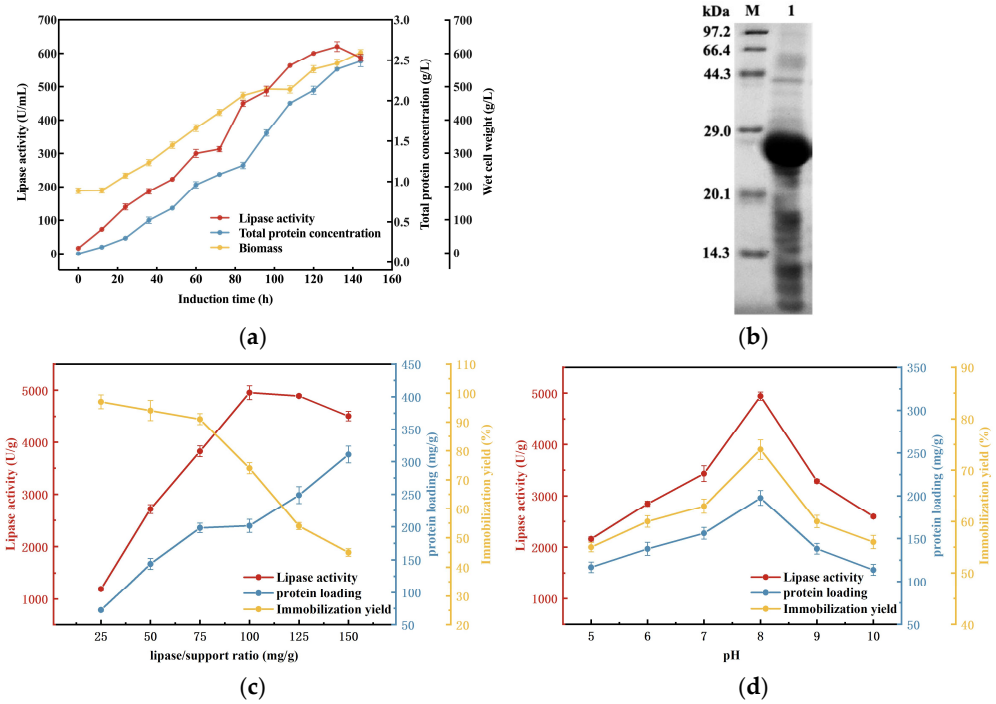


Figure 1. (**a**) Total protein concentration, lipase activity, and biomass during high-density fermentation of recombinant strain X-33/ MAS1-H108W. (**b**) SDS-PAGE analysis for crude MAS1-H108W lipase. M: molecular weight reference, Line 1: the culture supernatant of lipase MAS1-H108W. (**c**) Effect of lipase/support ratio on the immobilization of MAS1-H108W. (**d**) Effect of initial buffer pH on the immobilization of MAS1-H108W.

3.2. Characterizations of Free and Immobilized MAS1-H108W Lipase

The binding proof of MAS1-H108W on XAD1180 resin was confirmed by FT-IR analysis. As shown in Figure 2a, the absorption band associated with C–H stretching of benzene ring (2850–3000 cm^{-1}) in the spectra of XAD1180 resin and MAS1-H108W@XAD1180 demonstrates the presence of the resin. Furthermore, the absorption band associated with C–O stretching of primary alcohols (1000–1100 cm^{-1}) in the spectra of MAS1-H108W and MAS1-H108W@XAD1180 indicated the presence of the lipase [30,31]. These findings indicated that the lipase was successfully immobilized onto the XAD1180 resin.

The effects of temperature on the hydrolytic activities of free and immobilized MAS1-H108W lipase were investigated within the range of 50 to 90 °C (Figure 2b). Both free and immobilized lipases exhibited progressive activity increases as the temperature ranged from 50 to 70 °C. Above 70 °C, both forms of lipase showed a progressive decrease in hydrolytic activity. Therefore, the optimal temperature for both free MAS1-H108W lipase and MAS1-H108W@XAD1180 was 70 °C. Notably, the MAS1-H108W@XAD1180 exhibited significantly higher hydrolytic activity at temperatures exceeding 70 °C and retained 52% of the maximal activity at 90 °C, while the relative activity of free MAS1-H108W lipase plummeted to 25% at 90 °C. These results were probably attributed to the protective effect of the XAD1180 resin against the thermal denaturation of MAS1-H108W lipase [29,32].

The effects of pH on hydrolytic activity of free and immobilized MAS1-H108W lipase were investigated at various pH values, ranging from 5.0 to 10.0 (Figure 2c). Free MAS1-H108W lipase exhibited higher activity in the range of pH 5.0–7.0, with the maximal activity at pH 7.0. The activity above the optimal pH decreased rapidly, resulting in only 31% relative activity at pH 10.0. However, MAS1-H108W@XAD1180 demonstrated higher activity in the range of pH 8.0–10.0, retaining over 90% of its maximal activity with peak performance at

pH 8.0. These results present that MAS1-H108W lipase showed enhanced alkaline tolerance after immobilization. Similar results were also reported previously [33,34].

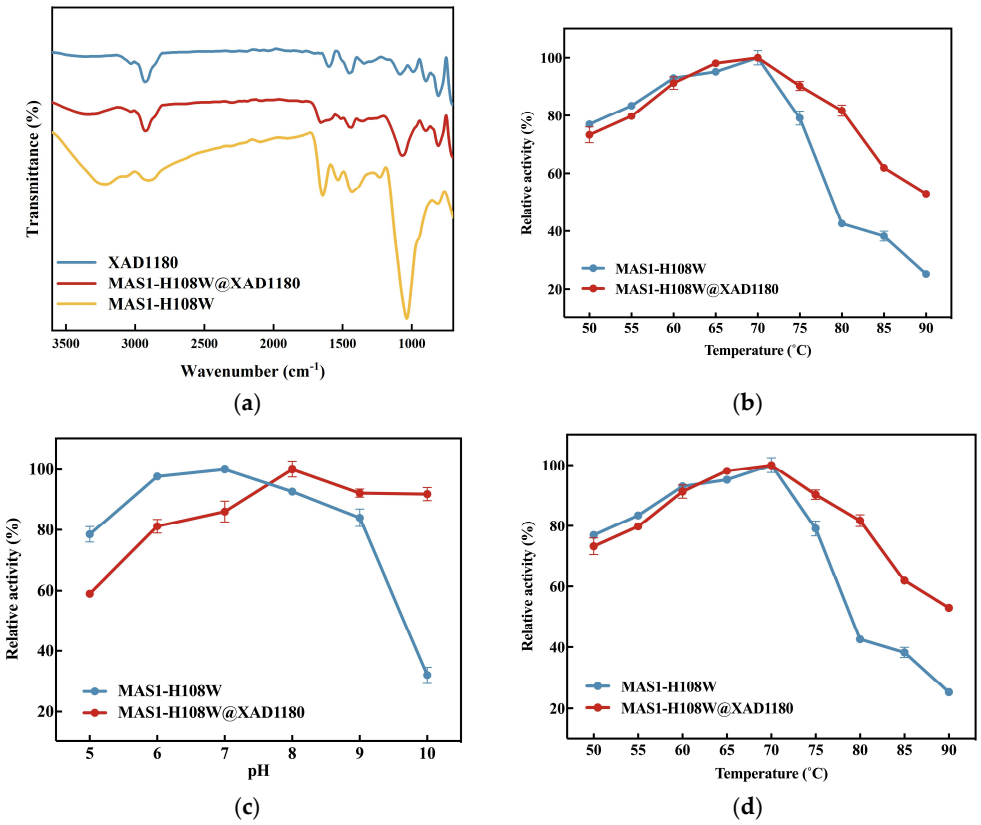


Figure 2. (**a**) FT-IR spectra of XAD1180 resin, MAS1-H108W@XAD1180, and free MAS1-H108W lipase. (**b**) Effects of temperature on the hydrolytic activity of free and immobilized MAS1-H108W lipase. (**c**) Effects of pH on the hydrolytic activity of free and immobilized MAS1-H108W lipase. (**d**) Thermostability of free and immobilized MAS1-H108W lipase.

The results of the thermostability of free and immobilized MAS1-H108W lipase are shown in Figure 2d. Both forms of lipase exhibited negligible activity loss during 0.5 h incubation at 60 °C. With the time prolonged to 4 h, the relative activity of the free MAS1-H108W lipase decreased dramatically to 12.16%, while the MAS1-H108W@XAD1180 maintained 95.60% of its initial activity. When incubated at 70 °C, free MAS1-H108W lipase was nearly inactivated (0.92% residual activity) within 1 h, while the MAS1-H108W@XAD1180 retained 71.52% of its initial activity, even after 4 h incubation. These results indicate that immobilization significantly enhanced the thermostability of MAS1-H108W lipase, which could be attributed to increased structural rigidity of the immobilized lipase [33,35].

3.3. Comparison of DAG Production by Different Immobilized Lipases-Catalyzed Glycerolysis Reaction

The immobilized MAS1 lipase (MAS1@XAD1180) was obtained from previous work [22]. The comparison of DAG production by different immobilized lipases-catalyzed glycerolysis reactions were evaluated. As shown in Figure 3a,b, reactions catalyzed by MAS1@XAD1180 and MAS1-H108W@XAD1180 reached equilibrium within 8 h, achieving DAG content of 45.01% and 42.55%, respectively. However, the reactions catalyzed by Novozym 435 and Lipozyme RM IM reached equilibrium within 36 h (46.82% of DAG content) and 24 h (44.71% of DAG content), respectively. In the available literature, glycerolysis reactions of soybean oil and palm oil catalyzed by Novozym 435 reached equilibrium within 24 h (48.5% of DAG content) and 22 h (43.5% of DAG content), respectively [14,19].

Glycerolysis reactions of lard oil and fish oil catalyzed by Lipozyme RM IM reached equilibrium within 11 h (46.72% of DAG content) and 6 h (20.76% of DAG content), respectively [36,37]. Discrepancies in DAG synthesis efficiency by Novozym 435 and Lipozyme RM IM between previous reports and the present works are possibly due to differences in reaction conditions and substrate. Notably, both commercial lipases exhibited lower production efficiency than MAS1-H108W@XAD1180. These results suggest that the glycerolysis for DAG production catalyzed by MAS1-H108W@XAD1180 exhibited the highest efficiency of four immobilized lipases. To further explore the relationship between DAG synthesis efficiency and lipase regioselectivity, Figure 3c shows the composition of the reaction mixtures at equilibrium for glycerolysis catalyzed by the different lipases. The TAG content of glycerolysis catalyzed by non-regiospecific MAS1@XAD1180 (42.76%) was higher than those obtained with the 1,3-regiospecific lipases MAS1-H108W@XAD1180 (37.87%), Novozym 435 (35.84%), and Lipozyme RM IM (41.10%). Correspondingly, the DAG content produced by the non-regiospecific lipase was lower than those generated by the three 1,3-regiospecific lipases. These could be explained as being because any acyl group in TAG molecules can be transesterified to any glycerol position by non-regiospecific lipases, whereas transesterification by 1,3-regiospecific lipases is restricted to sn-1 and/or sn-3 acyl groups and their corresponding glycerol positions [23]. These results further illustrated that the regioselectivity of lipases could be a critical factor for highly efficient DAG synthesis and 1,3-regiospecific MAS1-H108W@XAD1180 is a more suitable immobilized biocatalyst than non-regiospecific MAS1@XAD1180 for DAG production.

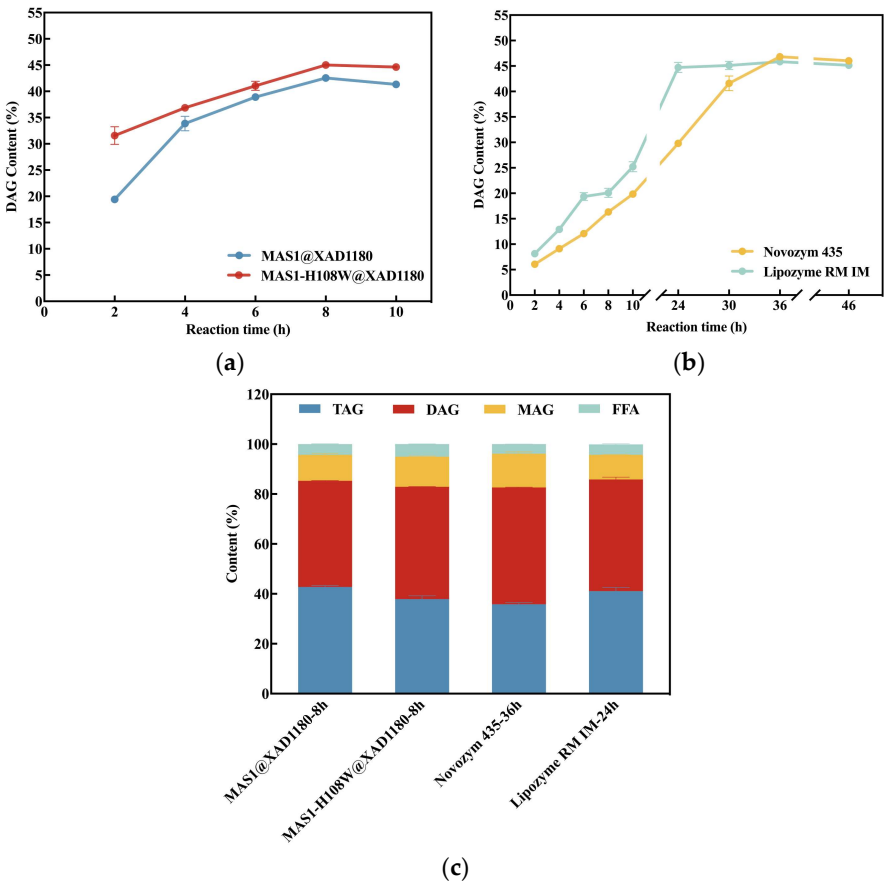


Figure 3. (**a**) Time course of enzymatic glycerolysis catalyzed by MAS1@XAD1180 and MAS1-H108W@XAD1180. (**b**) Time course of enzymatic glycerolysis catalyzed by Novozym 435 and Lipozyme RM IM. (**c**) Composition of reaction mixtures at equilibrium in glycerolysis catalyzed by different lipases. Reaction conditions: temperature, 50 °C; olive oil /glycerol, 2:1 (mol/mol); immobilized lipase, 1.5 wt.% (accounting for the mass of olive oil and glycerol).

3.4. Optimization of DAG Production by MAS1-H108W@XAD1180-Catalyzed Glycerolysis Reaction

3.4.1. Reaction Temperature

The effects of different reaction temperatures on enzymatic glycerolysis for DAG production are presented in Figure 4. The reaction reached equilibrium within 4 h at 60 °C, achieving a DAG content of 44.31%. However, the reaction required 6, 8, and 6 h to reach equilibrium at 40, 50, and 70 °C, achieving DAG contents of 39.14%, 45.51%, and 42.23%, respectively. These results indicate that the production efficiency of DAG at 60 °C was significantly higher than that at other temperatures. Hence, 60 °C was identified as the optimal reaction temperature and employed in subsequent experiments. In previous studies, the optimal temperature for enzymatic glycerolysis of vegetable oils in solvent-free systems was consistently observed within the range of 45–60 °C [19]. Distinctions in optimal temperatures may be attributed to differences in the thermal stability of the immobilized enzymes employed in these reactions [19,38].

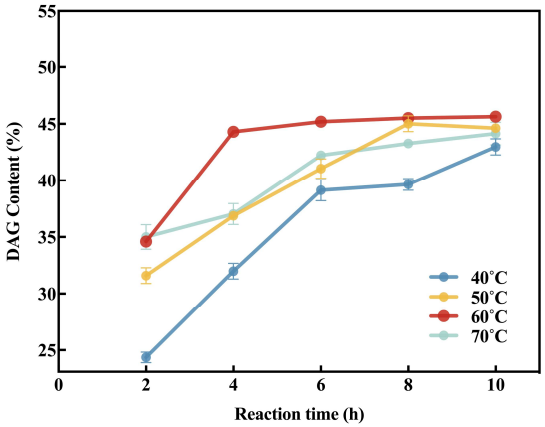


Figure 4. Effect of temperature on DAG production. Reaction conditions: temperature, 40, 50, 60, 70 °C; olive oil /glycerol, 2:1 (mol/mol); immobilized lipase, 1.5 wt.% (accounting for the mass of olive oil and glycerol).

3.4.2. Molar Ratio of Substrate

Figure 5 shows the effects of the substrate molar ratio (olive oil to glycerol) on enzymatic glycerolysis for DAG production. As the molar ratio decreased from 2:1 to 1:2, the DAG content at equilibrium increased from 44.31% to 48.97%, and the time required to reach equilibrium was reduced from 6 h to 4 h, indicating a gradual improvement in production efficiency. This could be attributed to enhanced mass transfer efficiency due to the moderate increase in glycerol concentration, thus facilitating facilitated molecular interactions between glycerol and olive oil [39]. However, a further decrease in the molar ratio to 1:3 resulted in a lower DAG content of 47.34%, although the reaction time remained at 4 h, indicating a potential inhibition of the reaction at higher glycerol concentrations. These results may be due to excessive glycerol concentrations increasing the viscosity of the reaction system, which decreased mass transfer efficiency and ultimately diminished the DAG content [32]. Therefore, the most cost-effective substrate molar ratio was identified as 1:2. In contrast, the optimal molar ratio reported for the glycerolysis of soybean oil catalyzed by immobilized TLL, CALB, and RML under solvent-free conditions was 2:1 (oil to glycerol), yielding DAG contents of 59.19%, 54.19%, and 59.03% after 24 h, 12 h, and 24 h, respectively [40–42]. This requirement for a higher oil content, coupled with the lower DAG synthesis efficiency, resulted in lower cost-effectiveness compared to that observed in the present study.

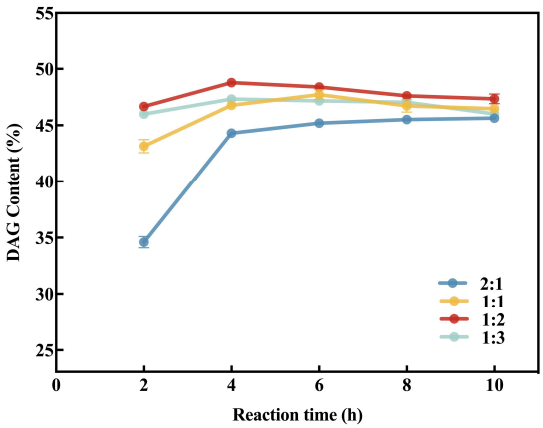


Figure 5. Effect of substrate molar ratio on DAG production. Reaction conditions: temperature, 60 °C; olive oil/glycerol, 2:1, 1:1, 1:2, and 1:3 (mol/mol); immobilized lipase, 1.5 wt.% (accounting for the mass of olive oil and glycerol).

3.4.3. Adding Amount of MAS1-H108W@XAD1180

The effects of the added amount of MAS1-H108W@XAD1180 on enzymatic glycerolysis was described in Figure 6. All reactions reached equilibrium within 4 h. The DAG content at equilibrium increased from 44.32% to 49.3% as the added amount of MAS1-H108W@XAD1180 increased from 0.5 wt.% to 1.0 wt.%. However, when the added amount of MAS1-H108W@XAD1180 was 1.5 wt.%, there was no significant influence on the DAG content. But, the further increasing added amount to 2.0 wt.% led to a decrease in the DAG content at equilibrium to 46.76%. These results suggested that more enzymes promoted the glycerolysis reactions within a certain range of added amount, while excessive enzymes could induce enzyme agglomeration and diffusion problems [43,44], reducing the production efficiency of DAG. Considering both the cost and production efficiency, as well as the results from Sections 3.4.1 and 3.4.2, 1.0 wt.% was determined to be the optimal added amount of MAS1-H108W@XAD1180, and 4 h was identified as the optimal reaction time. Although enzymatic glycerolysis of vegetable oils in solvent-free systems has been reported to achieve DAG contents mostly between 50% and 60%, higher enzyme loadings (4–10 wt%) and longer reaction times (approximately 10–24 h) were required to reach equilibrium [19]. These results demonstrate that MAS1-H108W@XAD1180 exhibited higher catalytic efficiency in the solvent-free glycerolysis reaction compared to other immobilized biocatalysts.

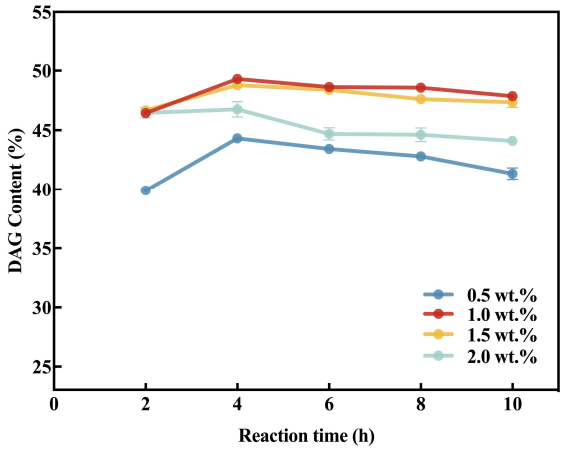


Figure 6. Effect of added enzyme amount on DAG production. Reaction conditions: temperature, 60 °C; olive oil/glycerol, 2:1 (mol/mol); immobilized lipase, 0.5, 1.0, 1.5, and 2.0 wt.% (accounting for the mass of olive oil and glycerol).

3.5. Reusability and Applicability of MAS1-H108W@XAD1180

Figure 7 presents the reusability study of MAS1-H108W@XAD1180 for DAG production in the glycerolysis reaction. The DAG content of 49.3% obtained in the first reaction was set to 100%, with the subsequent cycle calculated relative to this baseline. MAS1-H108W@XAD1180 retained 81.9% of its initial production capacity after 10 repeated reactions, demonstrating progressive activity decline with repeated use. This decline might be attributed to thermal denaturation or the desorption of lipase from the support [45,46]. According to the available literature, CALA@NKA-9 remained 57.82% of the initial glycerolysis activity after nine consecutive applications [47]. PS@LXTE-1000 remained 78.3% of its initial catalytic ability after seven cycles of reuse [39]. TL-PEGDGE-LX remained 62.34% of the initial bioactivity after ten cycles of reuse [40]. These results indicated that MAS1-H108W@XAD1180 has a greater operational stability during glycerolysis for DAG synthesis.

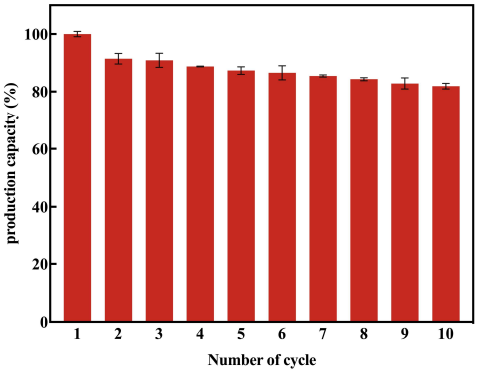


Figure 7. Results of reusability of MAS1-H108W@XAD1180. Reaction conditions: temperature, 60 °C; olive oil/glycerol, 2:1 (mol/mol); immobilized lipase, 1.0 wt.% (accounting for the mass of olive oil and glycerol), reaction time, 4 h.

Under the optimal reaction conditions determined above, MAS1-H108W@XAD1180 was employed to catalyze glycerolysis of five other common vegetable oils (corn oil, rapeseed oil, peanut oil, sunflower oil, and soybean oil). As shown in Table 1, the initial DAG content of all the vegetable oils was low, ranging from 0.820 ± 0.011% to 3.465 ± 0.06%. After the glycerolysis catalyzed by MAS1-H108W@XAD1180, the DAG content significantly increased and exceeded 48% in each product. These results demonstrated the broad and efficient catalytic ability of MAS1-H108W@XAD1180 for producing DAG from various vegetable oils via glycerolysis. The observed differences in DAG content during the glycerolysis of five vegetable oils could be attributed to the differences in initial DAG concentrations among the oils.

Table 1. DAG content (%) of five vegetable oils (Initial DAG) and DAG synthesized by MAS1-H108W@XAD1180-catalyzed glycerolysis reactions of five vegetable oils [1].

Vegetable Oils	Olive Oil	Corn Oil	Rapeseed Oil	Peanut Oil	Sunflower Oil	Soybean Oil
Initial DAG	1.752 ± 0.034	3.465 ± 0.06	1.527 ± 0.087	2.670 ± 0.091	1.692 ± 0.052	0.820 ± 0.011
DAG in the glycerolysis reactions	49.041 ± 0.144	48.446 ± 1.01	48.749 ± 0.663	49.455 ± 0.886	49.753 ± 0.446	50.021 ± 0.115

[1] Each value represents the mean ± SD (n = 3).

4. Conclusions

In this study, the 1,3-regiospecific MAS1-H108W lipase was produced by high-density fermentation and successfully immobilized onto XAD1180 resin. The resulting immobilized

biocatalyst, MAS1-H108W@XAD1180, maintained its optimal temperature while exhibiting a shift in optimal pH from 7.0 to 8.0, along with enhanced thermostability. Furthermore, MAS1-H108W@XAD1180 demonstrated a superior performance in DAG production compared to both the non-regiospecific MAS1@XAD1180 and commercial lipases (Novozym 435 and Lipozyme RM IM). Under the optimal reaction conditions in the solvent-free system (60 °C, 1:2 of olive oil/glycerol molar ratio, MAS1-H108W@XAD1180 of 1.0 wt.%, and 4 h of reaction time), a maximum DAG content of 49.3% was achieved. MAS1-H108W@XAD1180 also showed good reusability, retaining 81.9% of its initial production capacity after 10 reuse cycles. Moreover, MAS1-H108W@XAD1180 proved effective in the glycerolysis of various vegetable oils (corn oil, rapeseed oil, peanut oil, sunflower oil, and soybean oil), consistently yielding DAG contents above 48% in all cases. These findings underscore the potential of MAS1-H108W@XAD1180 as a highly efficient, cost-effective, and robust immobilized biocatalyst that is suitable for sustainable and industrial-scale production of DAG. However, the reproducibility of the immobilization efficiency and catalytic performance under scaled-up conditions remains to be fully validated. In addition, although the immobilized biocatalyst showed promising reusability, gradual enzyme leakage or deactivation may still occur over extended operational cycles, which could affect long-term economic feasibility. A more comprehensive cost–benefit analysis comparing the production and immobilization expenses of MAS1-H108W@XAD1180 with those of commercial alternatives would be necessary to conclusively demonstrate its industrial advantage. Future work needs to focus on large-scale process optimization and detailed techno-economic assessment to facilitate practical application.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

DAG	diacylglycerol
FFA	free fatty acids
TAG	triacylglycerols
FT-IR	Fourier transform infrared
HPLC	High Performance Liquid Chromatography

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