Immobilized MAS1 Lipase-catalyzed Synthesis of n-3 PUFA-rich Triacylglycerols in Deep Eutectic Solvents

Xiumei Wang¹²³, Xiaoxu Zhao¹², Xiaoli Qin⁴, Zexin Zhao³, Bo Yang³, and Yonghua Wang⁵*

¹ College of Environmental and Biological Engineering, Putian University, Putian 351100, CHINA
² Fujian Provincial Key Laboratory of Ecology-Toxicological Effects & Control for Emerging Contaminants, Putian 351100, CHINA
³ School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, CHINA
⁴ College of Food Science, Southwest University, Chongqing 400715, CHINA
⁵ Guangdong Research Center of Lipid Science and Applied Engineering Technology, School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, CHINA

Abstract: n-3 polyunsaturated fatty acids (PUFA)-rich triacylglycerols (TAG) with many beneficial effects are still difficult to be synthesized efficiently and rapidly by current synthetic techniques. This study reports the fatty acid specificity of immobilized MAS1 lipase and its efficient synthesis of n-3 PUFA-rich TAG by esterification of glycerol with n-3 PUFA in natural deep eutectic solvents (NADES) systems. Immobilized MAS1 lipase showed the highest preference for capric acid [C10:0, the highest specificity constant (1/α)=1] whereas it discriminated strongly against docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) due to their lowest specificity constants (1/α=0.19 and 0.2). Moreover, the highest n-3 PUFA-rich TAG content (55.8%) with similar n-3 PUFA composition to the substrate was obtained in choline chloride/glycerol (CG) system. There was a 1.38-fold increase of TAG content in CG system compared with that in the solvent-free system. Interestingly, immobilized MAS1 lipase exhibited no regiospecificity in the solvent-free and various NADES systems. Besides, the potential reaction mechanism of immobilized MAS1 lipase-catalyzed esterification of glycerol with n-3 PUFA in NADES systems was described. It was found that the use of NADES as solvents could greatly enhance TAG content, and make it easy to separate the product. These results indicated that immobilized MAS1 lipase is a promising biocatalyst for the efficient synthesis of n-3 PUFA-rich TAG by esterification of glycerol with n-3 PUFA in NADES systems.

Key words: immobilized MAS1 lipase, triacylglycerols, n-3 polyunsaturated fatty acids, natural deep eutectic solvents, esterification

1 Introduction

Long-chain n-3 polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), prevent cancer, decrease the risk of cardiovascular disease, and inhibit inflammation¹⁴. Besides, recent studies have shown that n-3 PUFA-rich triacylglycerols (TAG) have higher bioavailability than n-3 PUFA-rich ethyl esters (EE) and are more stable than free fatty acids (FFA)⁵⁶. However, n-3 PUFA-rich TAG content in natural fish oils is very low⁷⁸. Therefore, there is a growing interest in the production of n-3 PUFA-rich TAG for industrial application⁹¹⁰. Enzymatic esterification and glycerolysis reactions have been widely employed to prepare n-3 PUFA-rich TAG. Nevertheless, the synthesized n-3 PUFA-rich TAG content is low and the separation and purification of the complex product are needed when enzymatic reactions are carried out in the solvent-free systems⁸¹⁴¹⁵. Although n-3 PUFA-rich TAG content is relatively high when enzymatic reactions are performed in the organic solvent systems, these organic solvents could cause food safety...
and environmental pollution. Therefore, the exploration of green and more effective solvents for the synthesis of high content of n-3 PUFA-rich TAG and its easy separation is of great importance.

Recently, natural deep eutectic solvents (NADES) have received considerable attention as new generation of solvent due to their easier preparation, lower toxicity, and higher biodegradability when compared with conventional organic solvents. Moreover, many studies have showed that NADES could facilitate the separation of products, enhance the yields, and protect the enzymes from inactivation. Thus, NADES have been widely used as green media in enzymatic reactions for the synthesis of various epoxidized oil, glycolipids, and biodiesel. However, there is little information about using NADES as solvents for the enzymatic synthesis of n-3 PUFA-rich TAG. Therefore, it would be an ideal choice to perform the enzymatic production of n-3 PUFA-rich TAG in some NADES systems.

Immobilized lipases are more desirable because they allow for easy recyclability, good resistant to environmental changes, good durability, high stability, activity and selectivity when compared with free lipases. Moreover, studies have showed that the stability and recovery of immobilized lipase can be enhanced using NADES as reaction solvent. Recently, an immobilized MAS1 lipase using XAD1180 resin as a carrier, which was from marine Streptomyces sp. strain W007, was found to exhibit high catalytic activity during enzymatic esterification, glycerolysis, and transesterification reactions in the solvent-free system. However, the fatty acid (FA) specificity of immobilized MAS1 lipase and its catalytic performances during enzymatic production of n-3 PUFA-rich TAG in NADES systems are generally unexplored. Therefore, the aim of this study was to assess the substrate specificity of immobilized MAS1 lipase and its catalytic performances for the production of n-3 PUFA-rich TAG during the esterification of n-3 PUFA with glycerol in some NADES systems.

First, the fatty acid specificity of immobilized MAS1 lipase was evaluated in a multi-competitive esterification reaction. Subsequently, the abilities of immobilized MAS1 lipase to catalyze the esterification of n-3 PUFA with glycerol in the solvent-free and four types of NADES systems were compared and the analysis of n-3 PUFA compositions of the obtained n-3 PUFA-rich TAG in the different reaction systems were carried out in this study. Finally, the potential reaction mechanism of immobilized MAS1 lipase-catalyzed esterification of glycerol with n-3 PUFA in various NADES systems was discussed.

2 Materials and Methods
2.1 Materials
Lipase MAS1 (PDB ID: 5H6B) was produced by the method described earlier. DHA/EPA-rich EE were sourced from Sinomega Biotech Engineering Co., Ltd. (Zhejiang, China). Standards of 37-component fatty acid methyl esters (FAME, mix(C14-C24), monooleoylglycerol, dioleoylglycerol (15% of 1,2-dioleoylglycerol and 85% of 1,3-dioleoylglycerol), trioleoylglycerol were purchased from Sigma-Aldrich. Xylitol (98%), choline chloride (98%), betaine (98%, anhydrous), glycerol (99%), and urea (99%) were all sourced from Aladdin Chemistry Co. Ltd. (Shanghai, China). Formic acid, n-hexane and 2-propanol of chromatographic grade were obtained from Kermel Chemical Reagent Co., Ltd. (Tianjin, China). All other chemicals were of analytical grade unless otherwise stated. In this study, the composition of n-3 PUFA was the sum of the composition of EPA, docosapentaenoic acid (DPA) and DHA.

2.2 Preparation of immobilized MAS1 lipase
Lipase MAS1 was immobilized using XAD1180 resin (hydrophobic support) as a carrier according to the method described previously. First, the supernatant of crude lipase MAS1 (75 mg/g resin) was mixed with an equal volume of 0.02 mol/L sodium phosphate buffer (pH 8.0). Subsequently, the mixed solutions were transferred to a conical flask containing XAD1180 resin and then the flask was placed in a shaking water-bath with a speed of 200 rpm at a temperature of 30°C for 8 h. After that, the resulting immobilized MAS1 lipase was recovered by filtration of the supernatant and then washed with 0.02 mol/L sodium phosphate buffer (pH 8.0) several times until no protein was detected in the eluate. Finally, the obtained immobilized MAS1 lipase was dried under vacuum at 40°C for 8 h and stored in sealed vials at 4°C until use.

2.3 Determination of the esterification activity of immobilized MAS1 lipase
The esterification activity of immobilized MAS1 lipase was determined by the method described previously. First, the conical flask containing 20 mM lauric acid, 20 mM 1-propanol, and 3% water (w/w, with respect to total reactants) was incubated in a thermostatic water bath oscillator at a temperature of 60°C for 10 min. Then, immobilized MAS1 lipase (15 mg) was added to the flask and mixed with the reaction mixture for 10 min. After that, reaction samples (30 μL) was withdrawn and mixed with n-heptane (970 μL). Finally, gas chromatography (GC) equipped with a column OV351 (60 m × 0.32 mm × 0.10 μm) was used to analyze the propyl ester according to the previous report.

According to the above mentioned method, the esterification activity of immobilized MAS1 lipase was 1500 U/g.

2.4 The FA specificity of immobilized MAS1 lipase
Equimolar amounts of FAs containing DHA (C22:6), EPA (C20:5), linolenic acid (C18:3), linoleic acid (C18:2), oleic acid (C18:1), stearic acid (C18:0), palmitic acid (C16:0),...
myristic acid (C14:0), lauric acid (C12:0), capric acid (C10:0), and caprylic acid (C8:0) were prepared as described previously. The FA specificity of immobilized MAS1 lipase was determined as follows: First, the conical flask containing 11.5 mmol eqiuimolar FAs, 23 mmol glycerol, and 60 μL phosphate buffer (0.1 mol/L, pH 7.0) was incubated at a temperature of 60°C for 10 min. Then, immobilized MAS1 lipase (50 mg) was added to start the reaction. After 10 min of reaction, the reaction was stopped and the reaction mixture was centrifuged at 10,000 g for 10 min. After that, 0.1 g the upper oil solution was added to a 100-nL conical flask containing 25 mL isopropanol and 100 μL 1% phenolphthalein for the acid value determination according to the previous report. Meanwhile, the remaining reaction mixture containing glycerides and FAs was mixed with 20 mL 30% KOH/ethanol solution. After that, n-hexane (10 mL) was added to extract the produced glycerides. Subsequently, the upper phase was collected by a separatory funnel and then was heated under reduced pressure at a temperature of 30°C to remove n-hexane. The obtained glyceride mixtures were methylated to fatty acid methyl esters (FAME) according to the method of Wang et al. Finally, the FA composition of the obtained glyceride mixtures was analyzed using an Agilent 7890A GC equipped with a capillary column CP-Sil 88 (60 m × 0.25 mm × 0.2 μm) as described previously. The conversion degree (Cn) for each FA (%), was calculated as follows:

\[ C_n = \frac{(A_{V_0} - A_{V_t})}{A_{V_0}} \times F_n \]

where \(A_{V_0}\) and \(A_{V_t}\) were acid values of the samples at time zero and time \(t\), respectively. \(F_n\) was the fatty acid composition of the glyceride mixtures determined by GC.

### 2.5 Calculation of the specificity constant

A series of competitive factors (\(α\) values), each of which is proportional to the specificity constant (1/\(α\)), \(K_{cat}/K_{m}\), (where \(K_{m}\) is measure of the specificity/affinity of the enzyme towards the substrate and \(K_{cat}\) is catalytic constant) for a particular substrate, was employed to evaluate the FA specificity of immobilized MAS1 lipase toward competing FAs in the esterification reactions. The competitive factor is determined using Eq. (2) proposed by Rangheard et al.:
2.8 Analysis of n-3 PUFA composition of TAG by GC

Before the analysis, separation of TAG from the reaction mixtures was performed on a thin layer chromatography plate using a mixture of n-hexane, ethyl ether, and acetic acid (80:20:1, v/v/v) as the developing solvent according to the method of Qin et al.\textsuperscript{42} Then, the substrate (n-3 PUFA) and the scraped TAG bands were separately methylated to FAME using the method described earlier.\textsuperscript{30} Finally, the n-3 PUFA composition of the substrate and TAG in the final product was analyzed using an Agilent 7890A GC equipped with a capillary column CP-Sil 88 (60 m × 0.25 mm × 0.2 μm) as described previously.\textsuperscript{30}

2.9 Analysis of the composition of the reaction mixture by HPLC

The detailed composition of the reaction mixture was analyzed using a normal-phase HPLC equipped with a refractive index detector and a Phenomenex Luna column (250 mm × 4.6 mm i.d., 5 μm particle size, Phenomenex Corporation) as described previously.\textsuperscript{33} The mobile phase consisted of n-hexane, isopropanol with formic acid (21:1:0.003, v/v/v) and its flow rate was 1 mL/min. Peaks in HPLC were identified by comparison of their retention times with those known standards. Retention times were 2.9 min (n-6 PUFA), 3.10 min (α-linolenic acid), 3.29 min (linoleic acid), 3.30 min (oleic acid), 3.63 min (C18:2), 3.76 min (DAG), 5.86 min (1,2-DAG), 29.51 min (1,3-DAG), 37.93 min (2-MAG). Waters 2695 integration software was employed to analyze the data and calculate peak-area percentages. In this study, TAG content, DAG content and MAG content were defined as the weight percentage of TAG, DAG and MAG in the reaction mixture, respectively. The percentage of each acylglycerol species was obtained from peak areas in HPLC. Esterification degree was defined as the percentage of initial FA consumed in the reaction mixture as calculated from peak areas.

2.10 Statistical analysis

All experiments were performed in triplicate and the results were presented as the means ± standard deviations (SD). In addition, an ANOVA procedure was employed to determine significant differences among the measured values through significant differences test and variance analysis of Statistical Program for Social Sciences for Windows 13.0.

3 Results and Discussions

3.1 The FA specificity of immobilized MAS1 lipase

The specificity constant (1/α) was used as an indicator to evaluate the FA specificity of immobilized MAS1 lipase. The larger the specificity constant of a fatty acid, the greater the selectivity of immobilized MAS1 lipase for this fatty acid. The specificity constants (1/α) of immobilized MAS1 lipase for eleven fatty acids ranging from C8:0 to C22:6 in the esterification are shown in Fig. 1. Immobilized MAS1 lipase exhibited the highest preference for C10:0 followed by C18:2. However, immobilized MAS1 lipase discriminated strongly against EPA and DHA due to their lowest specificity constants (1/α = 0.2 and 0.19, respectively). The results indicated that the specificity of immobilized MAS1 lipase toward EPA and DHA was very similar. Therefore, the scientists and manufacturers could utilize the specificity of immobilized MAS1 lipase for different fatty acids to design experiments for the modification of fats and oils.

3.2 Esterification of n-3 PUFA with glycerol by immobilized MAS1 lipase in NADES systems

3.2.1 n-3 PUFA-rich TAG content improvement

Among the FAs tested, EPA and DHA were poorer substrates for immobilized MAS1 lipase, as shown in Fig. 1. It was reported that the selectivity of lipase-catalyzed esterification reactions could be affected by NADES.\textsuperscript{34} Moreover, it was demonstrated that n-3 PUFA-rich TAG could prevent cardiovascular disease and cancer, inhibit inflammation, and reduce the risk of atherosclerosis.\textsuperscript{44–47} Therefore, the catalytic performances of immobilized MAS1 lipase-catalyzed esterification of n-3 PUFA with glycerol for the production of n-3 PUFA-rich TAG in the solvent-free and different NADES systems were investigated in this study. The effects of different NADES on esterification degree are shown in Fig. 1. It was observed that the esterification degree in the solvent-free system for the first 3 h was quicker than those in various NADES systems. After that, the esterification degree in the solvent-free system was slower than or equal to those in NADES systems. After 12 h,
Immobilized MAS1 Lipase-catalyzed Synthesis of n-3 PUFA-rich TAG in Deep Eutectic Solvents

Fig. 2 Effects of different NADES on esterification degree during the esterification of n-3 PUFA with glycerol catalyzed by immobilized MAS1 lipase. The control group was performed in the solvent-free system.

Esterification degree separately reached 94.38%, 85.96%, 94.21%, 84.16%, and 96.78% when the esterification reactions catalyzed by immobilized MAS1 lipase were carried out in the solvent-free, betaine/urea (BU), betaine/glycerol (BG), choline chloride/xylitol (CX), and choline chloride/glycerol (CG) systems, respectively. It could be concluded that compared with the solvent-free system, the use of BU and CX as reaction solvents resulted in a lower performance whereas the use of BG and CG as the reaction media showed a similar influence on esterification degree.

The effects of different NADES on TAG, MAG, and DAG contents during the esterification of n-3 PUFA with glycerol catalyzed by immobilized MAS1 lipase are presented in Fig. 3. Figure 3A shows the effects of different NADES on TAG content. It was found that TAG content separately reached 23.44%, 41.77%, 34.62%, 37.72%, and 55.8% after 12 h when the esterification reactions catalyzed by immobilized MAS1 lipase were carried out in the solvent-free, BU, BG, CX, and CG systems, respectively. Moreover, it was observed that in addition to TAG content in BG system, the accumulation rates of TAG in the other NADES systems were quicker than that in the solvent-free system. Although the accumulation rate of TAG in BG system for the first 5 h was slower than that in the solvent-free system, a sudden shift to a higher accumulation rate could be seen for subsequent reactions relative to the reaction performed in the solvent-free system. Therefore, it could be concluded that TAG content was significantly improved in various NADES system when compared with the solvent-free system. In particular, when the esterification reactions were performed in CG system, the maximum TAG content was obtained. However, lower MAG and DAG contents were obtained in various NADES systems than those in the solvent-free system (Figs. 3B and 3C). Besides, it was observed that MAG content showed different trend with DAG.

Fig. 3 Effects of different NADES on TAG (A), MAG (B), and DAG (C) contents during the esterification of n-3 PUFA with glycerol catalyzed by immobilized MAS1 lipase. The control group was performed in the solvent-free system.
content in various NAEDS systems when compared with the solvent-free system. DAG content increased with time and separately reached 49.23%, 36.6%, 46.55%, 40.19%, and 36.63% after 12 h of reaction when the esterification reactions catalyzed by immobilized MAS1 lipase were carried out in the solvent-free, BU, BG, CX, and CG systems, respectively (Fig. 3C). Nevertheless, MAG content in various NADES systems separately increased firstly and then decreased gradually or kept increasing slowly as the reaction proceeded whereas MAG content in the solvent-free system increased dramatically with time (Fig. 3B). After 12 h, MAG content separately reached 21.71%, 7.59%, 13.04%, 6.25%, and 4.35% when the esterification reactions catalyzed by immobilized MAS1 lipase were carried out in the solvent-free, BU, BG, CX, and CG systems, respectively. This was probably because water produced during the esterification process was quickly absorbed by NADES, thus shifting the equilibrium in the positive direction. The results showed that the conversion from glycerol to MAG, from MAG to DAG, and from DAG to TAG could be promoted using NADES as reaction solvents during the esterification of n-3 PUFA with glycerol catalyzed by immobilized MAS1 lipase. Therefore, the use of various NADES as reaction solvents was favorable to synthesize n-3 PUFA-rich TAG.

3.2.2 No change in the selectivity and regiospecificity

The effects of different NADES on 1,3-DAG/1,2-DAG ratio are displayed in Fig. 4. 1,3-DAG/1,2-DAG ratio firstly increased and then decreased gradually with time when the esterification reactions were catalyzed by immobilized MAS1 lipase in the solvent-free and various NADES systems. The 1,3-DAG/1,2-DAG ratio reached the maximum at 3 h and separately decreased to 2.04, 2.19, 2.21, 2.24, and 2.06 after 12 h of reaction when the esterification reactions were carried out in the solvent-free, BU, BG, CX, and CG systems, respectively. It could be concluded that immobilized MAS1 lipase showed similar selectivity between 1,3-DAG and 1,2-DAG no matter whether the esterification reactions were carried out in the solvent-free or in various NADES systems. Besides, it could be seen from Fig. 3A that TAG content in the solvent-free and NADES systems increased as the reaction proceeded. However, the secondary hydroxyl group of 1,3-DAG was difficult to react with n-3 PUFA when compared with the primary hydroxyl group of 1,2-DAG. It could be concluded that TAG was formed mainly through the acylation of 1,2-DAG. Moreover, fatty acids could be transferred not only from sn-1 or sn-3 position to sn-2 position, but also from sn-2 position to sn-1 or sn-3 position. Thus, the decreased 1,3-DAG/1,2-DAG ratio at 12 h indicated that a weak acyl transfer occurred during the reaction process. The results indicated that immobilized MAS1 lipase had no regiospecificity in the solvent-free and various NADES systems.

3.2.3 The n-3 PUFA compositions of the prepared n-3 PUFA-rich TAG

The n-3 PUFA compositions of the substrate and n-3 PUFA-rich TAG in the solvent-free and various NADES systems after 12 h of reaction were analyzed and the results are presented in Table 2. It was found that the substrate mainly consisted of 38.78% EPA, 6.42% DPA, and

| Fatty acids | Composition (%) |
|-------------|-----------------|
| n-3 PUFA    |                 |
| TAG in the solvent-free system | 38.78 ± 0.45 | 38.65 ± 0.24 | 38.51 ± 0.24 | 38.27 ± 0.32 | 38.63 ± 0.34 | 38.91 ± 0.28 |
| TAG in the BU system | 38.51 ± 0.24 | 38.27 ± 0.32 | 38.63 ± 0.34 | 38.91 ± 0.28 |
| TAG in the BG system | 6.42 ± 0.23 | 6.37 ± 0.19 | 6.45 ± 0.19 | 6.76 ± 0.25 | 6.40 ± 0.25 | 6.32 ± 0.21 |
| TAG in the CX system | 45.21 ± 0.32 | 45.10 ± 0.28 | 44.85 ± 0.28 | 43.90 ± 0.38 | 44.87 ± 0.23 | 44.90 ± 0.38 |
| TAG in the CG system | 90.41 ± 0.43 | 90.12 ± 0.61 | 89.81 ± 0.36 | 88.93 ± 0.45 | 89.90 ± 0.46 | 90.13 ± 0.35 |

Table 2: The n-3 PUFA compositions of the substrate (n-3 PUFA) and TAG in the solvent-free and various NADES systems after 12 h of reaction.
45.21% DHA. After 12 h of reaction, no significant difference was observed in n-3 PUFA composition between the substrate and n-3 PUFA-rich TAG in the solvent-free and various NADES systems. Moreover, it was observed that the n-3 PUFA composition was not affected by the addition of various NADES. The results indicated that immobilized MAS1 lipase exhibited similar selectivity to EPA, DPA, and DHA in the different reaction systems.

3.3 The potential reaction mechanism of immobilized MAS1 lipase-catalyzed esterification of n-3 PUFA with glycerol in NADES systems

According to the above results, it could be conferred that the potential reaction mechanism of immobilized MAS1 lipase-catalyzed esterification of n-3 PUFA with glycerol in NADES systems (Fig. 5) could proceed as follows: Glycerol was firstly converted to MAG, followed by the conversion from MAG to DAG, and finally DAG was converted to TAG. At each step, one mole of water was produced and then was quickly captured by NADES during the reaction (Fig. 5A). Thus, the reaction equilibrium was shifted toward the positive direction, resulting in an increase in TAG content. Although the reaction was a heterogeneous system at beginning and then turned to be a pseudo-homogeneous system after stirring, the produced MAG, DAG, and TAG were all not actually soluble in NADES. Thus, the side-reactions were significantly minimized. Moreover, the produced TAG became a single phase at upper layer after the reaction was stopped and the mixture was centrifuged. However, the mixture of NADES, glycerol, and water was remained at the lower layer and immobilized MAS1 lipase suspended at the medium layer (Fig. 5B). Therefore, the separation of products and immobilized lipase from the reaction mixture was simple and effective.

Overall, n-3 PUFA-rich TAG content obtained by immobilized MAS1 lipase was significantly improved in various NADES systems when compared with the solvent-free system. The maximal n-3 PUFA-rich TAG content (55.8%) with similar n-3 PUFA composition to the substrate was obtained at 12 h in CG system. There was a 1.38-fold increase of TAG content in CG system compared with that in the solvent-free system in this study, which was higher than that (1.2-fold at 48 h) obtained by Novozym 435 in the literature. It could be concluded that the catalytic efficiency of immobilized MAS1 lipase was better than that of Novozym 435. Therefore, the results indicated that immobilized MAS1 lipase is a promising and efficient biocatalyst for the synthesis of n-3 PUFA-rich TAG by esterification of glycerol with n-3 PUFA in NADES systems.

4 Conclusions

In this study, immobilized MAS1 lipase exhibited the highest preference for C10:0 whereas it discriminated strongly against EPA and DHA. However, n-3 PUFA-rich TAG content was efficiently improved by immobilized MAS1 lipase-catalyzed esterification of glycerol with n-3 PUFA in NADES systems when compared with the solvent-free system and the maximal n-3 PUFA-rich TAG content reached 55.8% at 12 h in CG system in this study. It was also found that immobilized MAS1 lipase had no regiospecificity and exhibited similar selectivity between 1,3-DAG and 1,2-DAG in the solvent-free and various NADES systems. Besides, n-3 PUFA-rich TAG in the solvent-free and various NADES systems showed similar n-3 PUFA compositions to the substrate. Finally, the potential reaction mechanism of immobilized MAS1 lipase-catalyzed esterification of n-3 PUFA with glycerol in NADES systems was described. It was observed that the use of NADES as reaction solvents not only facilitated product separations, but also shifted the reaction equilibrium in the positive di-
rection due to their absorption of the produced water during the reaction process. These results indicated that immobilized MAS1 lipase is a promising and efficient biocatalyst for the synthesis of n-3 PUFA-rich TAG by esterification of glycerol with n-3 PUFA in NADES systems.

Acknowledgments

This work was supported by the National Key R & D Program of China (2018YFC03111104), National Natural Science Foundation of China (31801462), National Science Fund for Distinguished Young Scholars (31725022), Education and Research Project of Young and Middle-aged Teachers of Fujian Province (JT180467), the Cultivation Program for the Outstanding Young Scientific Research Talents of Fujian Province University (2018), Science and Technology Department of Putian (2018NP2003), Guangdong MEPP Fund (NO.GDOE[2019]A20), Science and Technology Department of Fujian Province (2020J05211, 2018J05063), Science and Technology Planning project of Putian University (2018055), Program for New Century Excellent Talents in Fujian Province University (2017).

Conflicts of Interest

The authors declare no conflict of interest.

References

1) Cleland, L.G.; Caughey, G.E.; James, M.J.; Proudnman, S.M. Reduction of cardiovascular risk factors with long term fish oil treatment in early rheumatoid arthritis. J. Rheumatol. 33, 1973-1979 (2016).
2) Corsetto, P.A.; Montorfano, G.; Zava, S.; Jovenitti, I.E.; Cremona, A.; Berra, B.; Rizzo, A.M. Effects of n-3 PUFA on breast cancer cells through their incorporation in plasma membrane. Lipids Health Dis. 10, 73 (2011).
3) Giudetti, A.M.; Cagnazzo, R. Beneficial effects of n-3 PUFA on Chronic airway inflammatory diseases. Prostag. Oth. Lipid M. 99, 57-67 (2012).
4) Nicholson, T.; Khademi, H.; Moghadasi, M.H. The role of marine n-3 fatty acids in improving cardiovascular health: a review. Food Funct. 4, 357-365 (2013).
5) Lawson, L.D.; Hughes, B.G. Human absorption of fish oil fatty acids as triacylglycerols, free acids, or ethyl esters. Biochem. Biophys. Res. Commun. 152, 328-335 (1988).
6) Valenzuela, A.; Valenzuela, V.; Sanhueza, J.; Nieto, S. Effect of supplementation with docosahexaenoic acid ethyl ester and sn-2 docosahexaenyl monoyglyceride on plasma and erythrocyte fatty acids in rats. Ann. Nutr. Metab. 49, 49-53 (2005).
7) Borg, P.; Binet, C.; Girardin, M.; Rovel, B.; Barth, D. Enzymatic synthesis of triecosapentaenoylglycerol in a solvent-free medium. J. Mol. Catal. B: Enzym. 11, 835-840 (2001).
8) Moreno-Perez, S.; Luna, P.; Señorans, F.J.; Guisan, J.M.; Fernandez-Lorente, G. Enzymatic synthesis of triacylglycerols of docosahexaenoic acid: Transesterification of its ethyl esters with glycerol. Food Chem. 187, 225-229 (2015).
9) Kosugi, Y.; Azuma, N. Synthesis of triacylglycerol from polyunsaturated fatty acid by immobilized lipase. J. Am. Oil Chem. Soc. 71, 1397-1403 (1994).
10) Picq, M.; Bernoud-Hubac, N.; Lagarde, M. Synthesis and Biological Interest of Structured Docosahexaenoic Acid–Containing Triacylglycerols and Phospholipids. Curr. Org. Chem. 17, 841-847 (2013).
11) Li, D.M.; Wang, W.F.; Li, X.H.; Durrani, R.; Yang, B.; Wang, Y.H. Preparation of highly pure n-3 PUFA-enriched triacylglycerols by two-step enzymatic reactions combined with molecular Distillation. J. Am. Oil Chem. Soc. 94, 225-233 (2017).
12) Lyberg, A.M.; Adlercreutz, P. Lipase specificity towards eicosapentaenoic acid and docosahexaenoic acid depends on substrate structure. Biochem. Biophys. Acta 1784, 343-350 (2008).
13) Akanbi, T.O.; Adcock, J.K.; Barrow, C.J. Selective concentration of EPA and DHA using Thermomyces lanuginosus lipase is due to fatty acid selectivity and not regioselectivity. Food Chem. 138, 615-620 (2013).
14) Sun, Z.M.; Xue, C.H.; Guo, Z.X.; Cong, H.H.; Wang, Y.M.; Xue, Y.; Wang, J.F. Preparation of Highly Pure n-3 PUFA-enriched Triacylglycerols by Two-step Enzymatic Reactions Combined with Molecular Distillation. J. Am. Oil Chem. Soc. 94, 225-233 (2017).
15) Bhandari, K.; Chaurasia, S.P.; Dalai, A.K. Lipase-catalyzed esterification of docosahexaenoic acid-rich fatty acids with glycerol. Chem. Eng. Commun. 202, 920-926 (2015).
16) Liu, S.C.; Zhang, C.H.; Hong, P.Z.; Ji, H.W. Lipase-catalyzed acylglycerol synthesis of glycerol and n-3 PUFA from tuna oil: Optimisation of process parameters. Food Chem. 103, 1009-1015 (2007).
17) Smith, E.L.; Abbott, A.P.; Ryder, K.S. Deep eutectic solvents (DESs) and their applications. Chem. Rev. 114, 11060-11082 (2014).
18) Williamson, S.T.; Shahbaz, K.; Mjalli, F.S.; AlNashef, I.M.; Farid, M.M. Application of deep eutectic solvents

J. Oleo Sci. 70, (2) 227-236 (2021)
Immobilized MAS1 Lipase-catalyzed Synthesis of n-3 PUFA-rich TAG in Deep Eutectic Solvents

J. Oleo Sci. 70, (2) 227-236 (2021)

as catalysts for the esterification of oleic acid with glycerol. Renew. Energ. 114, 480-488 (2017).

20) Kim, S.H.; Park, S.; Yu, H.; Kim, J.H.; Kim, H.J.; Yang, Y.H.; Kim, K.J.; Kan, E.; Lee, S.H. Effect of deep eutectic solvent mixtures on lipase activity and stability. J. Mol. Catal. B: Enzym. 128, 65-72 (2016).

21) De Faria, E.J.; Do Carmo, R.S.; Cláudio, A.F.M.; Freire, C.S.; Freire, M.G.; Silvestre, A.J. Deep eutectic solvents as efficient media for the extraction and recovery of cynaropicrin from cynara cardunculus L. Leaves. Int. J. Mol. Sci. 18, 2276 (2017).

22) Zhou, P.F.; Wang, X.P.; Zeng, C.X.; Wang, W.P.; Yang, B.; Hollmann, F.; Yang, Y.H. Deep eutectic solvents enable more robust chemoenzymatic epoxidation reactions. ChemCatChem 9, 934-936 (2017).

23) Pöhnlein, M.; Ulrich, J.; Kirschhöfer, F.; Nusser, M.; Muhle-Goll, C.; Kammengiesser, B.; Brenner-Weip, G.; Luy, A.; Syldatk, C.; Hausmann, R. Lipase-catalyzed synthesis of glucose-6-O-hexanole in deep eutectic solvents. Eur. J. Lipid Sci. Tech. 117, 161-166 (2015).

24) Zhao, H.; Zhang, C.; Critten, T.D. Choline-based deep eutectic solvents for enzymatic preparation of biodiesel from soybean oil. J. Mol. Catal. B: Enzym. 85, 243-247 (2013).

25) Pereira, D.S.; L. Fraga, J.; M. Diniz, M.; C. Fontes-Sant'Ana, G.; F.F. Amaral, P. High catalytic activity of lipase from Yarrowia lipolytica immobilized by microencapsulation. Int. J. Mol. Sci. 19, 3393 (2018).

26) Xing, X.; Jia, J.Q.; Zhang, J.F.; Zhou, Z.W.; Li, J.; Wang, N.; Yu, X.Q. CALB immobilized onto magnetic nanoparticles for efficient kinetic resolution of racemic secondary alcohols: Long-term stability and reusability. Molecules 24, 490 (2019).

27) Huang, Z.L.; Wu, B.P.; Wen, Q.; Yang, T.X.; Yang, Z. Deep eutectic solvents can be viable enzyme activators and stabilizers. J. Chem. Technol. Biot. 89, 1975-1981 (2015).

28) Guajardo, N.; Ahumada, K.; Maria, P.D.; Schreiber, R.A. Remarkable stability of Candida antarctica lipase B immobilized via cross-linking aggregates (CLEA) in deep eutectic solvents. Biocatal. 37, 106-114 (2019).

29) Guajardo, N.; Ahumada, K.; Maria, P.D. Immobilized lipase-CLEA aggregates encapsulated in lentikats as robust biocatalysts for continuous processes in deep eutectic solvents. J. Biotechnol. 310, 97-102 (2020).

30) Yuan, D.J.; Lan, D.M.; Xin, R.P.; Yang, B.; Wang, Y.H. Screening and characterization of a thermostable lipase from marine Streptomyces sp. Strain W007. Biotechnol. Appl. Biochem. 63, 41-50 (2015).

31) Wang, X.M.; Qin, X.L.; Li, D.M.; Yang, B.; Wang, Y.H. One-step synthesis of high-yield biodiesel from waste cooking oils by a novel and highly methanol-tolerant immobilized lipase. Bioresour. Technol. 235, 18-24 (2017).

32) Wang, X.M.; Li, D.M.; Qu, M.; Durrani, R.; Yang, B.; Wang, Y.H. Immobilized MAS1 lipase showed high esterification activity in the production of triacylglycerols with n-3 polyunsaturated fatty acids. Food Chem. 216, 260-267 (2017).

33) Wang, X.M.; Li, D.M.; Wang, W.F.; Yang, B.; Wang, Y.H. A highly efficient immobilized MAS1 lipase for the glycerolysis reaction of n-3 PUFA-rich ethyl esters. J. Mol. Catal. B: Enzym. 134, 25-31 (2016).

34) Lan, D.M.; Qu, M.; Yang, B.; Wang, Y.H. Enhancing production of lipase MAS1 from marine Streptomyces sp. strain in Pichia pastoris by chaperones co-expression. Electron. J. Biotechn. 22, 62-67 (2016).

35) Basso, A.; Froment, L.; Hessele, M.; Serban, S. New highly robust divinyl benzene/acylate polymer for immobilization of lipase CALB. Eur. J. Lipid Sci. Technol. 115, 468-472 (2013).

36) Wang, W.F.; Xu, Y.; Qin, X.L.; Lan, D.M.; Yang, B.; Wang, Y.H. Immobilization of lipase SMG1 and its application in synthesis partial glycerides. Eur. J. Lipid Sci. Technol. 116, 1063-1069 (2014).

37) Qin, X.L.; Lan, D.M.; Zhong, J.F.; Liu, L.; Wang, Y.H.; Yang, B. Fatty acid specificity of Tl lipase and its potential in acylglycerol synthesis. J. Sci. Food Agric. 94, 1614-1621 (2014).

38) Wang, Y.H.; Mai, Q.Y.; Qin, X.L.; Yang, B.; Wang, Z.L.; Chen, H.T. Establishment of an evaluation model for human milk fat substitutes. J. Agric. Food Chem. 58, 642-649 (2010).

39) Qin, X.L.; Wang, Y.M.; Wang, Y.H.; Huang, H.H.; Yang, B. Preparation and characterization of 1,3-dioleoyl-2-palmitoylglycerol. J. Agric. Food Chem. 59, 5714-5719 (2011).

40) Rangheard, M.S.; Langrand, G.; Triantaphylides, C.; Baratti, J.C. Multi-competitive enzymatic reactions in organic media: a simple test for the determination of lipase fatty acid specificity. Biochim. Biophys. Acta 1004, 20-28 (1989).

41) Senanayake, S.P.J.; Shahidi, F. Enzymatic incorporation of docosahexaenoic acid into borage oil. J. Am. Oil Chem. Soc. 76, 1009-1015 (1999).

42) Qin, X.L.; Huang, H.H.; Lan, D.M.; Wang, Y.H.; Yang, B. Typosensitivity of crude Geobacillus sp. T1 lipase fused with a cellulose-binding domain and its use in the synthesis of structured lipids. J. Am. Oil Chem. Soc. 91, 55-62 (2014).

43) Zeng, C.X.; Qi, S.J.; Xin, R.P.; Yang, B.; Wang, Y.H. Enzymatic selective synthesis of 1,3-DAG based on deep eutectic solvent acting as substrate and solvent. Bio-process Bioysst. Eng. 38, 2053-2061 (2015).

44) Nestel, P.J. Fish oil and cardiovascular disease: lipids and arterial function. Am. J. Clin. Nutr. 71, 228S-231S (2000).

J. Oleo Sci. 70, (2) 227-236 (2021)
45) Ghasemi Fard, S.; Wang, F.; Sinclair, A.J.; Elliott, G.; Turchini, G.M. How does high DHA fish oil affect health? A systematic review of evidence. *Crit. Rev. Food Sci. Food Sci.* **59**, 1684-1727 (2019).

46) Ellulu, M.S.; Khaza’ai, H.; Abed, Y.; Rahmat, A.; Ismail, P.; Ranneh, Y. Role of fish oil in human health and possible mechanism to reduce the inflammation. *Inflammopharmacology* **23**, 79-89 (2015).

47) Saraswathi, V.; Gao, L.; Morrow, J.D.; Chait, A.; Niswender, K.D.; Hasty, A.H. Fish oil increases cholesterol storage in white adipose tissue with concomitant decreases in inflammation, hepatic steatosis, and atherosclerosis in mice. *J. Nutr.* **137**, 1776-1782 (2007).

48) Xu, L.; Zhang, L.; Li, D.M.; Liu, P.Z.; Tan, C.P.; Wang, W.F.; Liu, X.Q.; Yang, B.; Lan, D.M.; Wang, X.H. Deep Eutectic Solvents Enable the Enhanced Production of n-3 PUFA-Enriched Triacylglycerols. *Eur. J. Lipid Sci. Tech.* **119**, 1700300 (2017).