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Study on the enrichment of palmitoleic acid in Sn-2 monoester from sea-buckthorn fruit oil

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Abstract

Palmitoleic acid (C16:1n7, POA), a 16-carbon ω-7 monounsaturated functional fatty acid, has been demonstrated a variety of beneficial properties to human health and a wide range of applications in nutrition, medical and chemical industries. Lipozyme® RM IM (Rhizomucor Miehei lipase), a Sn-1,3 specific lipase, was used to catalyze the hydrolysis of seabuckthorn fruit oil and produce monoglycates rich in palmitoleic acid. The fruit oil of seabuckthorn was used as the research object, the dosage of enzyme taking 10% wt.% oil, the influence of water/oil ratio, pH value and reaction temperature to hydrolysis rate were investigated, and then the response surface methodology was applied for optimizing the process conditions. Further, the relationship between the hydrolysis rate and the content of monoacylglycerol was studied under the optimal hydrolysis conditions. The result indicated that the optimal combined condition is as follows: the reaction temperature is 55°C, the pH is 7, the water-oil quality ratio is 1:1. The hydrolysis rate achieved 79.59% within 12 h in the condition above. Under the optimal conditions, the hydrolysis rate was positively correlated with the content of monoester, and the correlation coefficient was 0.999 within the first 10 h of the reaction. The hydrolysis rate was 66.76% at 10 h, and the content of monoglyceride reached the highest of 61.11%. Hereafter, the hydrolysis of monoglyceride began if the reaction continued. The content of palmitoleic acid increased from 27.11% to 40.84%, and that of linoleic acid increased from 4.51% to 33.34%, so the total amount of the two functional fatty acids reached 74.18%. Enzymatic hydrolysis can be an effective way to improve the function of seabuckthorn fruit oil.

Keywords: palmitoleic acid; sn-1,3 specific lipase; seabuckthorn fruit oil; sn-2 monoglyceryl ester.

Practical Application: Palmitoleic acid can be used to treat chronic diseases such as metabolic syndrome, diabetes and inflammation

1 Introduction

Palmitoleic acid (16:1n7, POA), otherwise called (9Z)-hexadecenoic acid, is an omega-7 monounsaturated fatty acid (Oh et al., 2022), which has been demonstrated a variety of beneficial properties to human health and a wide range of applications in nutrition in previous studies, medical and chemical industries. Palmitoleic acid supplementation can decrease systolic blood pressure and improve aortic remodeling (Tang et al., 2021; Astudillo et al., 2020; Souza et al., 2020), increase insulin signaling in muscles and the intake and utilization of glucose in fat cells (Yang et al., 2021). At the same time, it has been thought to exert multiple beneficial effects, such as anti-hypertrophic (Simão et al., 2022), function/dysfunction of white adipose tissue (Cruz et al., 2020), decrease the catecholamine-induced cardiac damage (Betz et al., 2021), and suppression myoblast differentiation (Tokunaga et al., 2021), etc. In view of its excellent pharmacological function, the development of palmitoleic acid products has great market potential.

As a functional monounsaturated fatty acids, POA is very expensive in the market, and the market price of high-purity POA can reach more than 200 RMB per gram, which is comparable to gold (Hanum et al., 2019; Ding et al., 2022). Queensland fruit oil, sea buckthorn, and fish oil are the major sources of POA (Hu et al., 2019). Due to the lack of fishery resources, the source of POA is limited, which is not enough to meet the market demand. Among these, the content of palmitoleic acid in seabuckthorn fruit oil is as high as 30% (Ciesarová et al., 2020), which has great development potential to separate palmitoleic acid from seabuckthorn fruit oil.

At present, the common method of separation of palmitoleic acid is to separate free fatty acids from seabuckthorn fruit oil after saponification, and then methods for further purification have been used to enrich palmitoleic acid from natural oils, including low-temperature crystallization, supercritical CO_2 extraction, urea complexation (Wang et al., 2020) and molecular distillation. Traditional saponification is obtained by chemical method, and the Free fatty acid (FFA) after saponification is easily lost after subsequent separation. Compared with chemical method, Lipase hydrolysis is gradually attracting more and more attention because the product hydrolyzed by lipase has good flavor, mild reaction conditions and little environmental pollution (Abdulmalek & Yan, 2022). Ciesarová et al. (2020) found that the content of palmitoleic acid in the Sn-2 triglyceride of seabuckthorn fruit oil was as high as 50%. Hydrolyzing the 1 and 3 positions of triglycerides in sea-buckthorn fruit oil can obtain POA-rich MAG. At present, the research on enzymatic preparation of monoacylglycerols has made some progress. The long-chain polyunsaturated fatty acids (such as EPA, DHA and linolenic acid) of monoacylglycerols (MAG) mostly have similar

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physiological activities. Compared with 1-monoacylglycerol, 2- monoacylglycerol can be directly absorbed by human body, thus exerting its physiological activities. Palacios Santamaría et al. (2022) concentrated monoacylglycerols rich in polyunsaturated fatty acids by enzymatic glycerolysis. Enzymatic hydrolysis has the advantages of stable properties and mild conditions, while the disadvantages of free enzyme such as poor stability and difficulty in recycling increase its use cost, thus limiting its application in industrial production (Weng et al., 2022). Immobilization of enzyme is one of the important technologies to solve the above problems (Ribeiro et al., 2021). At present, there is no report on the enrichment of palmitoleic acid in seabuckthorn fruit oil by using Sn-1,3 specific immobilized lipase hydrolysis.

In this paper, seabuckthorn fruit oil was used as the research object, and Sn-1,3 specific lipase reaction was used to enrich palmitoleic acid. The effects of pH value, reaction temperature, water-oil ratio, reaction time and other factors on the hydrolysis effect were analyzed. Based on the single factor study, the response surface methodology was used to optimize the hydrolysis process conditions, and the corresponding relationship between hydrolysis rate and monoacylglycerol content and the effect of hydrolysis to monoacylglycerol on the enrichment of palmitoleic acid were studied, so as to provide reference for the follow-up research and industrial production of enzymatic enrichment of palmitoleic acid.

2 Materials and methods

Lipozyme® RM IM, an immobilized lipases, was purchased from Novozymes, and it's a Sn-1,3 specific lipase with activity of 275 IUN/g. Seabuckthorn fruit oil (SFO) was provided by Xinjiang Zhongke Seabuckthorn Technology Co., LTD. Standard monoacylglycerol palmitate (≥98%), diglyceride oleate (≥98%), triglyceride palmitate (≥98%) and palmitoleic acid free fatty acid (≥99%), Span 80 (analytical grade), Tween 80 (analytical grade), were all purchased from Aladdin Reagent Co., LTD. Methanol (chromatographic purity), n-hexane, (chromatographic purity) were purchased from Sinopharm Chemical Reagents Co., LTD. Formic acid, 30 °C~60 °C boiling range petroleum ether, diethyl ether, isopropyl alcohol, anhydrous ethanol, anhydrous sodium sulfate, potassium hydroxide, dichloromethane, etc. were all analytically pure and were purchased from Sinopharm Chemical Reagents Co., LTD.(Shanghai, China).

2.1 Lipase catalyzed hydrolysis of seabuckthorn fruit oil

Seabuckthorn fruit oil (2 g) was mixed with 0.1mol/L phosphate buffer at different pH value from 6 to 10 and different substrate ratios (water/seabuckthorn fruit oil) ranging from 0.5:1 to 2.5:1 (m/m) in a conical flask with a stopper. Then the lipases (10 wt.%, per weight of Seabuckthorn fruit oil) and the mixture of Span80 (1.4 g) and Tween 80 (0.26 g) with HLB=6.0 were added. Samples were flushed with nitrogen and incubated in water bath with magnetic agitation at 300 rpm at temperatures of 20~60 °C. When sampling, an appropriate amount of anhydrous alcohol was added to inactivate the enzyme and stop the reaction. The samples were regularly taken and the hydrolysis rate were analyzed.

2.2 Hydrolysis rate determination

The acid value was analysed according to the cold solvent automatic potentiometric titration method in GB 5009.229-2016. The saponification value was analyzed according to GB/T 5534- 2008.The hydrolysis rate (HR) was calculated based on the following Formula 1.

$$\mathrm{HR} = \frac{AV_t - AV_0}{SV - AV_0} \times 100\% \quad (1)$$

where HR is the hydrolysis rate, %; AV_0, AV_t are the oleic acid values of the sample at time 0 and t h respectively, mg KOH/g; SV is the saponification value of seabuckthorn fruit oil, mg KOH/g.

2.3 Monoacylglycerol content determination

The content of monoacylglycerol was determined according to GB/T 22328-2008. The content of monoacylglycerol content (X) was calculated based on the following Formula 2.

$$\mathrm{X} = \frac{(V_0 - V_1) \times C \times r \times M_r}{20 \times m} \quad (2)$$

X is the content of monoacylglycerol,%;V0 are the milliliters of sodium thiosulfate standard solution consumed by titration of the blank solution, mL; V1 is the number of milliliters of sodium thiosulfate standard solution consumed in titration of sample solution, mL; C is the concentration of sodium thiosulfate standard solution, mol/L; Mr is the specific relative molecular weight of monoacylglycerol; m is the mass of the sample, g; r is the ratio of chloroform phase volume (100 mL) to the volume used for measurement (50 mL or less), in milliliters per milliliter (mL/mL).

2.4 TLC analysis of glycerides

The samples were analyzed by thin layer chromatography (TLC) with silica gel G plate (Qingdao Ocean Chemical Co., LTD.). The developing agent was petroleum ether: anhydrous ether: formic acid of 70:30:1 (V/V/V). After unfolding, iodine vapor was placed in a closed container for color development. Taking monoacylglycerol, diglyceride, triglyceride and free fatty acid standard as control, the composition of hydrolysis sample was qualitatively analyzed.

2.5 Fatty acid composition analysis

The monoacylglycerol (MG) fraction obtained by TLC was extracted with n-hexane, dried with N_2, methylated by alkaline methylation (Zaouay et al., 2020), and then fatty acid composition was analyzed by GC-MS, (Shimadzu GCMS-QP2010 Ultra System GMS, Shimadzu (Shanghai) Laboratory Equipment Co., LTD.) according to Zou et al. (2020).

2.6 Experimental design and statistical analysis

All experiments and sample analyses were conducted in triplicate. The data were subjected to ANOVA using the Microsoft Excel 2010 and Origin 2021 software, and presented as mean value with the standard deviation. The response surface

experimental design and variance analysis were carried out with the software Design-Expert.V8.0.6.1, and the significance level was analyzed under the condition of $P<0.05$. Software Origin 2021 was used to draw the graph.

3 Results and discussion

3.1 Influence of water-oil ratio (m/m)

Under the conditions of temperature 50 °C, pH 7, enzyme dosage 10 wt.% and rotation speed 300 r/min, the reaction was carried out for 6 h and 12 h. Water-oil ratios (m/m)of 0.5:1, 1:1, 1.5:1, 2:1 and 2.5:1 were selected to investigate the effect of water-oil ratio (m/m) on hydrolysis rate, as shown in Figure 1. With the increase of water addition, the hydrolysis rate increased greatly. When the water-oil ratios (m/m) was 1:1, the hydrolysis rate was the highest, and then decreased gradually. The reason might be that as the water content increase to a certain amount, the oil-water interface area increase and more enzyme with catalytically active conformation dominate. However, when water is excessive, a layer of water will be formed around the enzyme, making it more difficult for products with poor water solubility to contact with the active sites of the enzyme, and reducing the contact area with oil droplets, which will lead to enzyme inhibition, loss of enzyme activity and a decrease in hydrolysis rate.

3.2 Influence of temperature

Under the conditions of pH 7, enzyme dosage of 10 wt.%, water-oil ratio (m/m) of 1:1 and rotating speed of 300 r/min, the effects of pH value on hydrolysis rate were investigated at 20 °C, 30 °C, 40 °C, 50 °C and 60 °C. The effects of hydrolysis temperature on hydrolysis reaction are shown in Figure 2. When the temperature of lipase reaction system below 50 °C, the hydrolysis rate increased with the increase of temperature. When the reaction system temperature reached 60 °C, the hydrolysis rate decreased significantly. It may be related to the inhibition of lipase activity by high temperature. Therefore, the optimum hydrolysis temperature of seabuckthorn fruit oil by lipase was determined to be 50 °C.

3.3 Influence of pH value

Under the conditions of 50 °C, 10 wt.% enzyme dosage, 1:1 water-oil mass ratio and 300 r /min rotation speed for 2 h, the pH values of 6, 7, 8, 9 and 10 were selected to investigate the effect of pH value on the hydrolysis rate. As shown in Figure 3,

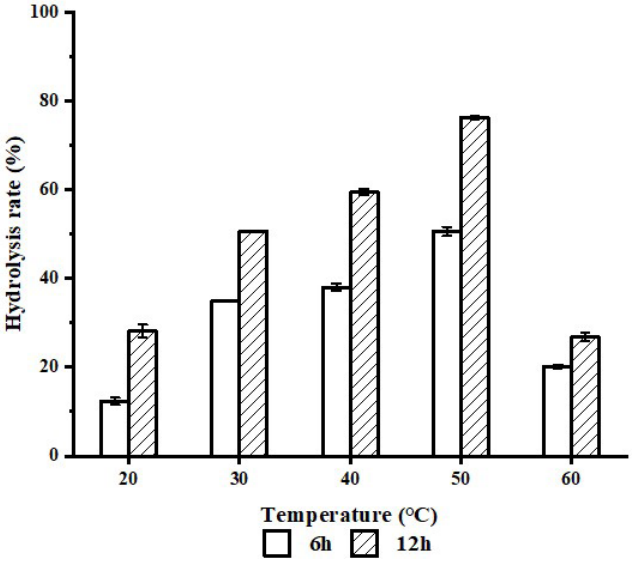

Figure 2. Influence of temperature to hydrolysis rate.

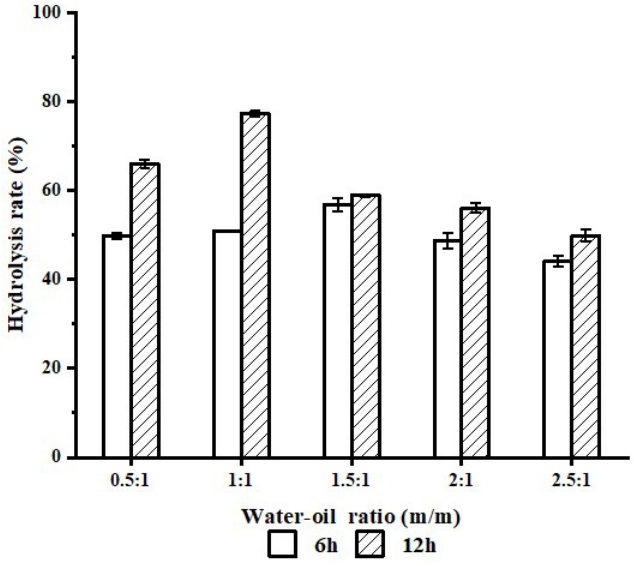

Figure 1. Influence of water-oil rate to hydrolysis rate.

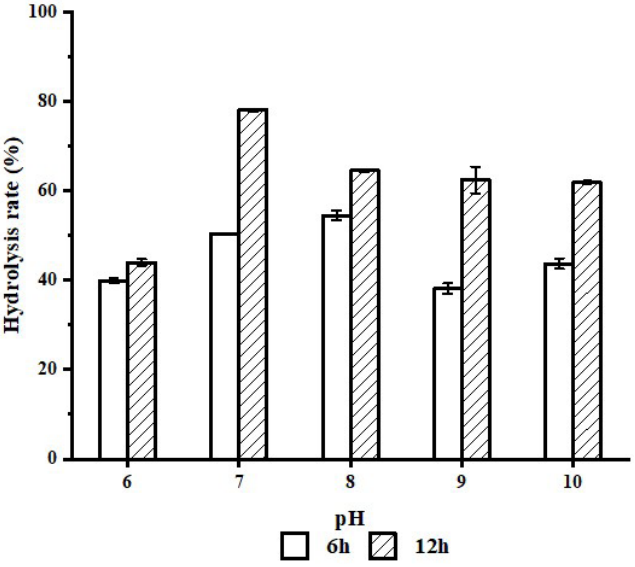

Figure 3. Influence of pH value to hydrolysis rate.

when the pH value of the system was 7, the hydrolysis rate of lipase hydrolyzed seabuckthorn fruit oil was higher. However, when the pH of the system was higher than 7, the hydrolysis rate decreased, which may be due to the inhibition of lipase activity by alkaline environment. Therefore, the optimum pH value of lipase catalyzing the hydrolysis of seabuckthorn fruit oil was determined to be 7.

3.4 Response surface optimization

In order to explore the nonlinear effects of water-oil ratio, reaction temperature and pH value on hydrolysis rate and the interaction between different factors, and to optimize the process conditions, response surface method was used to optimize the above three factors on the basis of single factor experiment.

3.5 Design and results of response surface experiment

On the basis of single factor experiment, the water-oil ratio (m/m), temperature and pH value were selected as the investigation factors, and the hydrolysis rate was the response value. Box-Benhnken experimental design (Table 1) was used to optimize the hydrolysis process of seabuckthorn fruit oil. The results were shown in Table 2.

The results in Table 2 are fitted by quadratic multinomial regression using Expert-Design X8 software. The regression equation model is: $HR=77.18+0.69A+9.38B+0.37C+3.76AB-1.57AC+1.48AD+0.98BC-17.70A^2-9.51B^2-12.19C^2$.

3.6 Analysis of variance and significance test of factors

The regression equation of hydrolysis rate of seabuckthorn fruit oil was analyzed by variance analysis and significance test, and the results are shown in Table 3.

According to Table 3, the P of the model is < 0.0001, indicating that the model is highly significant; P=0.1486>0.05, indicating that the missing item is not significant; The regression model is reliable. The determination coefficient R2 of the model is 0.9848, and the adjustment coefficient (R^2 adj) is 0.9654, which indicates that the model has good fitting degree, and the predicted value is highly correlated with the experimental value. The variation coefficient of the model is 4.63%, which indicates that the output data of the model has high accuracy and small error, and can predict the hydrolysis rate well. It can be used to optimize the technological conditions of Lipozyme® RM IM catalytic hydrolysis of seabuckthorn fruit oil.The coefficient significance test of regression equation model shows that the interaction term AB and quadratic term B^2 have significant effects on the hydrolysis rate, while the first term B, quadratic term A^2 and C^2 have extremely significant effects on the hydrolysis rate. The order of influence of three factors on hydrolysis rate is reaction temperature > water-oil ratio > pH value.

The response surface model analysis showed that Lipozyme® RM IM, with 10 wt.% enzyme (seabuckthorn fruit oil), catalyzed the hydrolysis of seabuckthorn fruit oil. The optimum technological conditions were as follows: the reaction temperature was 55.10 °C, the pH value was 7.03, and the water-oil ratio (m/m) was 1.03:1. In practical application, considering the precision of equipment control, the optimum conditions are determined as follows: reaction temperature 55 °C, pH value 7 and water-oil ratio (m/m) 1:1. Under this condition, the predicted hydrolysis rate is 79.59% after reaction for 12 hours. After many validation experiments, the actual hydrolysis rate is 79.49%, and the error with the predicted value is less than 5%, which is close to the predicted value. The model can be used to optimize the hydrolysis process of seabuckthorn fruit oil catalyzed by Lipozyme® RM IM.

Table 1. Response surface analysis factor level design.

Level	Factor A	Factor B	Factor C
	Water to oil ratio(m/m)	T/°C	pH
-1	0.5:1	40	6
0	1	50	7
1	1.5:1	60	8

3.7 Kinetics of enzymatic hydrolysis reaction

Under the optimum reaction conditions, the reaction time was 2 h, 4 h, 6 h, 8 h, 10 h, 12 h and 18 h. And the effect of reaction time on the hydrolysis rate was investigated. As shown in Figure 4, with the extension of the reaction time, the hydrolysis increased rapidly at first, then slowed down after two hours, and the hydrolysis rate stopped increasing after 12 hours. The reason is that, with the progress of the reaction, the concentration of substrate glyceride gradually decreases, and the concentration of product fatty acid in the oil phase and oil-water interface gradually increases, which leads to an increase in the collision frequency between fatty acid and enzyme, and a decrease in the collision frequency between glyceride and enzyme, which is not conducive to the progress of the reaction and reduces the reaction rate.

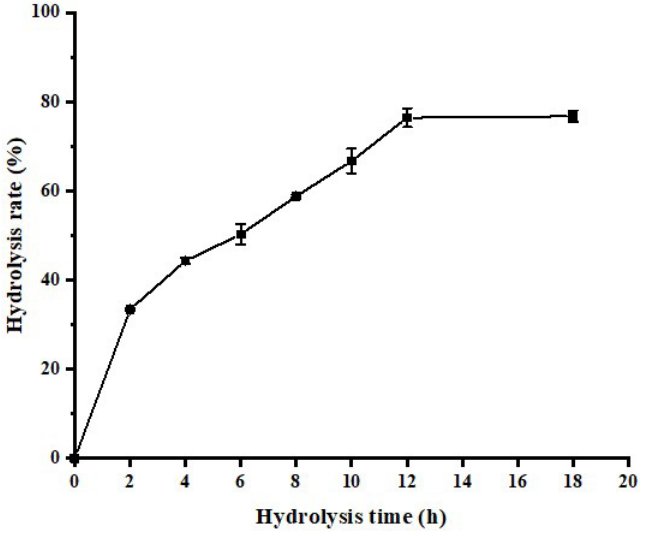

Figure 4. Kinetic curves of lipase hydrolysis reaction.

Table 2. Response surface analysis experimental design results.

Test number	Factor A	Factor B	Factor C	Hydrolysis rate/%
	Water to oil ratio(m/m)	T/°C	pH	
1	1	0	-1	50.32
2	-1	0	-1	44.61
3	1	1	0	61.22
4	0	1	1	68.71
5	0	0	0	78.58
6	1	-1	0	38.89
7	-1	1	0	53.53
8	0	-1	1	44.06
9	1	0	1	46.82
10	0	-1	-1	44.2
11	0	1	-1	64.93
12	-1	0	1	47.4
13	0	0	0	78.23
14	0	0	0	75.57
15	-1	-1	0	46.23
16	0	0	0	74.59
17	0	0	0	78.91

Table 3. Significant analysis of differences in regression models.

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob > F	Significance
Model	3352.70	9	372.52	50.55	< 0.0001	**
A-water to oil ratio	3.75	1	3.75	0.51	0.4985	
B-temperature	703.31	1	703.31	95.44	< 0.0001	**
C-pH	1.07	1	1.07	0.15	0.7141	
AB	56.48	1	56.48	7.66	0.0278	*
AC	9.89	1	9.89	1.34	0.2846	
BC	3.84	1	3.84	0.52	0.4937	
A^2	1318.82	1	1318.82	178.96	<0.0001	**
B^2	380.84	1	380.84	51.68	0.0002	*
C^2	625.72	1	625.72	84.91	< 0.0001	**
Lack of Fit	36.23	3	12.08	3.15	0.1486	
R-Squared	0.9848					
Adj R-Squared	0.9654					

*Significant (P<0.05); **Extremely significant (P<0.01).

3.8 TLC analysis of hydrolysis sample

The main component of natural oil is Triglyceride (TAG), the content of which is over 95%, and it also contains a small amount of free fatty acids. Figure 5 Thin layer chromatography with reaction time of 6 h and 10 h at temperature of 55.10 °C, pH value of 7.03, water-oil ratio (m/m) of 1.03:1, enzyme dosage of 10 wt.% (seabuckthorn fruit oil) and rotation speed of 300 r/min. It can be seen from Figure 5 that seabuckthorn fruit oil is hydrolyzed by Lipozyme® RM IM, and compared with the standard substance, the main components of triglyceride after hydrolysis are as follows Lipozyme® RM IM is a highly selective lipase at 1,3 positions, and the monoacylglycerols of its hydrolysis products are mainly Sn-2 monoacylglycerols and free fatty acids.

3.9 Correlation analysis between hydrolysis rate and monoacylglycerol content

66.67% is the theoretical maximum hydrolysis rate of triglyceride to monoacylglycerol. The effect of hydrolysis time on monoacylglycerol content and the relationship between hydrolysis rate and monoacylglycerol content were investigated under the optimal hydrolysis conditions (Figure 6). It can be seen from Figure 6 that within the first 10 hours of the reaction, with the gradual increase of hydrolysis rate, the content of monoacylglycerol gradually increased, and the hydrolysis rate reached 66.76% at 10 hours, at which time the content of monoacylglycerol was the highest, reaching 61.11%. After the hydrolysis rate exceeded 66.67%, the content of monoacylglycerol began to decrease. The correlation between hydrolysis rate and monoacylglycerol

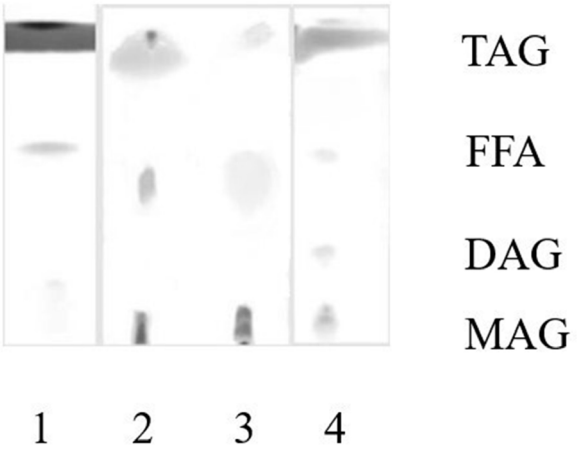

Figure 5. TLC of hydrolysis sample. Note: 1. Seabuckthorn fruit oil substrate; 2. Hydrolyzed sample of seabuckthorn fruit oil (6h); 3. Hydrolyzed sample of seabuckthorn fruit oil(10h); 4. Mixed standards.

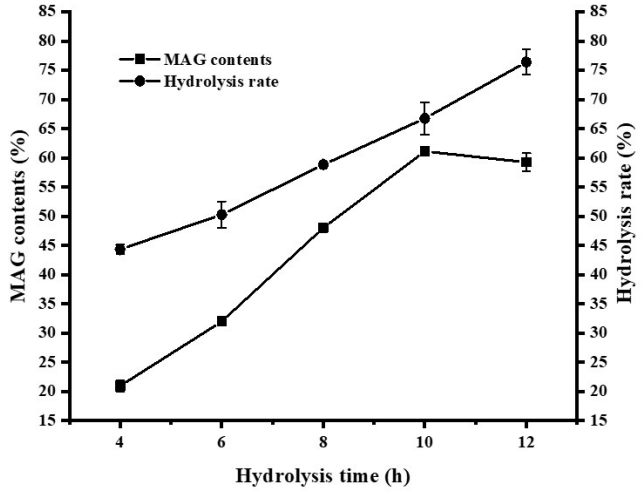

Figure 6. Effects of hydrolysis time on hydrolysis rate and monoacylglycerol content.

content within 0-10 h was investigated, and the correlation coefficient was 0.999, and the correlation coefficient between hydrolysis rate and monoacylglycerol content within 0-12 h was 0.942, indicating that when the hydrolysis rate exceeded 66.67%, monoacylglycerol began to degrade further, and the correspondence between hydrolysis rate and monoacylglycerol content decreased at this time. Combined with the hydrolysis specificity of the enzyme used in this study, it was shown that when the hydrolysis rate was 66.76%, the hydrolysis of the ester bond of Sn-1,3 in triglyceride was completed, and the monoacylglycerol obtained was Sn-2 monoacylglycerol.

3.10 Analysis of fatty acid composition in monoacylglycerol

The fatty acid types and contents of monoacylglycerol after hydrolysis of seabuckthorn oil are shown in Table 4. The content of palmitoleic acid in monoacylglycerol is 40.84%, which is higher than that in raw materials of 27.11%, so the enrichment of palmitoleic and was realized to a certain extent. In addition, the content of essential fatty acid linoleic acid increased from 4.51% to 33.34%. After lipase-catalyzed hydrolysis, the total content of the two functional fatty acids palmitoleic and and linoleic acid in monoacylglycerol was 74.18%, and the content of saturated fatty acid palmitic acid decreased from 29.86% to 8.66%.

Table 4. Fatty acids composition of monoglycerol from lipase-catalyzed hydrolysis of seabuckthorn fruit oil.

Fatty Acid	Seabuckthorn fruit oil /%	Monoacylglycerol /%
Myristic acid	0.46	3.98
Palmitic acid	29.86	8.66
Palmitoleic acid	27.11	40.84
Stearic acid	1.29	13.18
Linoleic acid	4.51	33.34

4 Conclusion

In this study, palmitoleic acid was enriched by lipase hydrolysis from seabuckthorn fruit oil. The influence of water/oil ratio, pH value and reaction temperature to hydrolysis rate was investigated, and then the response surface methodology was applied for optimizing the process conditions. The results showed that temperature was the most important influence factor for the hydrolysis rate, and the water/oil ratio was the next. The optimal hydrolysis conditions were as follows: Novozym® RM IM as biocatalyst at 8 wt%, the water-oil quality ratio of 1.03:1, reaction temperature of 55.10 °C, and pH of 7.03 and the hydrolysis rate can achieve 79.59% within 12 h. With hydrolysis progress, the content of monoacylglycerol increased and the correlation coefficient of hydrolysis rate and the content of monoacylglycerol was 0.999 within the first 10 h of the reaction. The content of monoacylglycerol reached the highest point of 61.11% at 10 h when the hydrolysis rate was 66.76% . Hereafter, the hydrolysis of monoacylglycerol began if the reaction continued. The monoacylglycerol was isolated and the compositions were analyzed using GC-MS. The result showed that the contents of palmitoleic acid and linoleic acid in the monoacylglycerol were 27.11% 33.34% respectively, and the total amount of the two functional fatty acids reached 74.18%. Compared with the free fatty acids formation from saponification reaction, the monoacylglycerol formation of palmitoleic acid and linoleic acid is more stable, also which is more conducive to the digestion and absorption of the body. The monoacylglycerol obtained from seabuckthorn fruit oil by controlled Sn1,3 specific lipase catalyzed hydrolysis was rich in palmitoleic acid and linoleic acid, and its functionality deserves further study.

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