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Lipase Catalyzed Incorporation of Conjugated Linoleic Acid by Transesterification into Sunflower Oil Applying Immobilized Lipase (Lipozyme *Thermomyces lanuginosus* and *Rhizomucor mehei*)

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A B S T R A C T

Conjugated Linoleic Acid (CLA), Glycerol (G) and sunflower oil blends with varying concentration were subjected to enzymatic esterification using a 1, 3- specific immobilized lipase. CLA was used as acyl due to its purported health benefits. The transesterified lipids were evaluated for free fatty acids (FFA) and composition of fatty acids by gas chromatography. Lipozyme *Rhizomucor mehei* immoblized (RM IM) is preferred for enzymatic esterification because of more CLA incorporation and less free fatty acids at the end of reaction than Lipozyme *Thermomyces lanuginosus* (TL) IM. Response surface methodology (RSM) was used to determine the effects of three variables Glycerol concentration, reaction temperature, and amount of enzyme on the lipase catalyzed incorporation of CLA into Sunflower oil. The optimum condition for maximum CLA incorporation (96.7% Incorporation Yield), maximum reaction rate (0.0271 M/h) and minimum free fatty acids production (Acid No.2.73) was obtained, at 75℃ by using 3 % enzyme and 0.27 M glycerol.

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NOMENCLATURE

CLA	Conjugated Linoleic Acid	FFA	Free fatty acids
G	Glycerol	RSM	Response surface methodology

1. INTRODUCTION

Functional foods are foods that have potentially positive effects on health, beyond basic nutrition. Increased understanding of the nutritional values of lipids, in particular, the metabolic effects associated with consumption of triacylglycerols containing substantial levels of specific fatty acid residues, has led to the development of novel technologies for modifying fats and oils to enhance the health benefits resulting from ingestion of these substances [1].

Conjugated linoleic acid (CLA), a natural derivative of the essential fatty acid -linoleic acid, has received increasing attention in recent years due to its health effects potential [2] such as having anticarcinogenic activity [3], enhancing the immune system or decreasing body fat content [4, 5]. Conjugated linoleic acid is a term used to describe a heterogeneous group of positional and geometric isomers of linoleic acid (cis-9, cis-12 C18:2) with conjugated double bonds [6]. Theoretically, 54 CLA isomers are possible, but only about 28 of them have been identified so far, including cis, cis; trans, trans; cis, trans and trans, cis isomers of 7,9; 8,10; 9,11; 10,12; and 11,13 C18 diene acids [7]. The most abundant isomer is cis-9, trans-11 (C9, T11 CLA, also denominated rumenic acid) which may represent up to 80% of total CLAs in food [8]. Other isomers namely trans-10, cis-12 (T10, C12 CLA) are found in much smaller quantities. Rumenic acid and T10, C12 CLA are considered to be the most bioactive isomers [2, 9]. The current CLA dietary intake by humans seems to be insufficient to exert its beneficial effects.

Sunflower oil contains predominantly linoleic acid in triglyceride (TG) form. It is a combination of monounsaturated and polyunsaturated fats with low saturated fat levels. One logical approach to facilitating

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ingestion of both CLA and poly unsaturated fatty acids is to employ enzymatic processes to produce fats and oils triacylglycerols in which the residues are substantially enriched in these substances. Transesterification is a reaction which can be catalyzed by enzymes or chemical catalysts to produce modified lipids by changing the fatty acid composition to improve the physical and nutritional properties of triacylglycerols molecule.

Chemical transesterification is less expensive and leads to a random distribution of fatty acids on the triacylglycerols. Enzymatic transesterification has certain advantages over Chemical Transesterification such as milder reaction conditions and region specificity, which may be less harmful to the flavor of the product [10]. Positional specificity allows lipases to distinguish between the sn-1 and⁄or sn-3 position on the triacylglycerol molecules.

Several reports of enzymatic esterification reactions of CLA are available in the literature. Enzymatic transesterification of fractionated rice bran oil has been carried out with CLA [11], butterfat with CLA in a batch reactor [12], tricaprylin with CLA [13], safflower oil with CLA [14], menhaden oil with CLA [15], triglycerides with CLA [16], corn oil with CLA [17], canola oil with CLA [18].

In this investigation, sunflower oil was transesterified with CLA isomers, mainly due to its beneficial health effect, via lipase-catalysis, to produce transesterified lipid. RSM was used to evaluate the effect of three variables such as Glycerol concentration, reaction temperature and the quantity of enzyme, on the rate of CLA incorporation into sunflower oil. The estimated combination (three variables) for maximizing CLA incorporation was obtained by RSM.

2. MATERIALS AND METHODS

2. 1. Materials Refined, deodorized and bleached sunflower oil were purchased from Behshahr Co.(Tehran, Iran) and CLA was obtained from Webber naturals (Mississauga, Canada). Analytical grade glycerol (free of water) was obtained from Merck (Darmstadt, Germany). Lipozyme TL IM and IM RM, 1,3 specific as immobilized lipase from the fungus *Thermomyces lanuginosus* and *Rhizomucor mehei* kindly supplied by Novozyme (Denmark) which was utilized for esterification process. Standards of fatty acids methyl esters (C16:0, C18:0, C18:1) were purchased from Wako (Osaka, Japan) and Standard of CLA fatty acids methyl ester (cis-9, trans-11-octadecadienoic acid 42%, trans-10, cis-12 octadecadienoic acid 44%, cis-10 cis-12-octadecadienoic acid 10%, and others 5%) and linoleic acid(cis-9, cis-12-octadecadienoic acid) were purchased from Sigma (St. Louis, MO). Methanolic boron trifluoride were purchased from Fluka (Switzerland). All other chemicals were analytical grade and from Merck (Darmstadt, Germany).

2. 2. Enzymatic Esterification Prior to the esterification experiments, blends of CLA, G and sunflower oil were designed according to Table 1. Before esterification, enzyme granules were leaded in two separate steps for removing air and water as described in our previous research [19].

Esterification reactions were performed in heated double wall reactor. 100 g of crude sunflower oil exhibiting different free fatty acid contents were mixed with various amounts of glycerol (Table 1) by a magnetic stirrer at 400 rpm in order to obtain a homogenous mixture of the components. The reaction mixture was heated to 65 ℃. To start the esterification reaction 1.5% (w/w) of immobilized Lipase was added. Water was eliminated from the reaction system by pure nitrogen stripping in order to suppress reverse reaction and to inhibit oil oxidation. Before sampling, stirring was stopped and the enzyme was allowed to fully settle at the bottom while, products were withdrawn from the top. Samples were taken after a reaction time of 1, 2, 4, 5, 6, 7 and 8 h. After sampling, the acid value was determined according to the DGF-Einheits method C-V2 described by Gofferje et al. [20]. All the experiments were carried out in duplicates.

Esterified oil was immediately vacuum filtered through Whatman No. 4 filter paper to eliminate remained fine enzyme particles [21]. Removal of FFA and partial acylglycerols were performed according to Rousseau and Marangoni with some modifications [19, 22]. Esterified oil was subsequently blended with an equal volume of 96% ethanol (40–50 ℃) in a separatory funnel. The ethanol phase was then extracted and the procedure was repeated five times. Traces of ethanol were eliminated by passing the stream of pure nitrogen through fats at 90 ℃ for 40 min. Dried fats were then filtered under vacuum through Whatman No. 4 filter paper to remove any fine particles remaining in the fat [19, 21] .Then, samples were taken for fatty acids analysis with gas chromatography mass selective detector (GC MSD). For fatty acids analysis, fatty acids methyl esters was prepared by esterifying with methanolic boron trifluoride reagent according to the AOCS Ce 2-66 procedure [23]. The fatty acids methyl esters was analyzed by an Agilant 5975c GC MSD (Agilent Technologies, Santa Clara, CA, USA) equipped with Hp-5 MS fused silica capillary column (30 m, 0.32 mm i.d., 0.25μm film thickness).

TABLE 1. Compositional design of the blends CLA, G and sunflower oil

Parameter	CLA	Glycerol	Sunflower oil
Levels investigated	0.05,0.1,0.15.0.2,0.25,0.3(M)	0.1,0.2,0.3, 0.4,0.5(M)	100(g)

The injection volume was 1.0µl. The temperature of the GC oven was programmed at 110 ℃ for 2 min and increased from 110 ℃ to 230 ℃ at a rate of 5 ℃ /min where it was held for 5min and increased again from 230 ℃ to 270 ℃ at a rate of 10 ℃ /min and held again for 5min. The injector and detector temperatures were 250 ℃, 270 ℃. Helium was used as the carrier and make-up gas. The flow rate of the make-up gas was 30 ml/min. The pressure was maintained at 10 psig on the column in order to obtain the flow rate of the carrier gas at 1.2 ml/min. The split ratio was set at 1:100. The C-9, T-11 CLA methyl ester eluted at 23.14 min was identified by comparing the retention time with the standard methylated CLA (Sigma Chemical Co., St. Louis, MO, USA). To analyze the quantity of fatty acids methyl esters, the calibration of standard methyl esters of CLA in linear correlation was illustrated with good accuracy (R^2 = 0.996). To verify the accurate location of each fatty acid, the amount of one micro liter of standard liquids was injected into the system and retention time was set to recognize each peak [19]. Fatty acid levels were reported as relative proportions of the total composition. Incorporation Yield were calculated according to the following formula (Equation (1)):

Incorporation Yield= (CLA incorporated/CLA reacted)*100 (1)

2. 3. Optimization of Processing for Maximum CLA Incorporation, Reaction Rate Maximization And FFA Minimization Optimization of CLA incorporation, reaction rate and FFA content are the most important subjects in the esterification process of oil and fats. So, optimization of the process for maximum CLA incorporation, maximum reaction rate and minimum production of FFA to minimize the falling of oil was studied. The surface response method was used for optimization of the process. Also, the planning of tests were done based on central combinational plan with three factor and each factor in three levels with three repetition in central point containing seventeen tests. The values of independent variables consisting of reaction temperature (℃), Glycerol concentration (M) and the quantity of enzyme (%) converted to the encrypted codes +1, 0, –1 that represent high, medium and low levels accordingly, are presented in Table 2. Design expert version eight software were utilized for the purpose of statistical analysis include of variance analysis, regression, optimization and the graphical shapes [24].

3. RESULTS AND DISCUSSION

3. 1. Quality of Sunflower Oil Table 3 shows the properties and FFAs composition of sunflower oil.

TABLE 2. Independent variables and their levels for the combinational central plan in enzymatic estrification process

Independent variables	Symbol	Variable levels Encrypted		
		-1	0	+1
Glycerol concentration (M)	X1	0.1	0.3	0.5
Reaction temperature (℃)	X2	55	65	75
Quantity of enzyme (%)	X3	1	2	3

The following formula were used for converting of real Independent variables to Encrypted variable levels

X_1= (Glycerol concentration – 0.3)*5 and X_2 = (temperature –6.5)/10 and X_3 = the quantity of enzyme – 2

TABLE 3. Properties of sunflower oil

Properties		Allowable limit	Fatty acids (%)	
Free fatty acids	0.07	Max 0.2	$C_{16:0}$	7.44
Iodine Value	127.5	122-140	$C_{18:0}$	4.01
Peroxide Value	2.6	Max 7	$C_{18:1}$	29.08
Slip Melting Point	-5℃	-	$C_{18:2}$	57.75
Refractive Index	1.4715	-	**Others**	1.5

The properties of sunflower oil were compared to Iranian National Standard and showed to be approximately within the range specified by the standard.

3. 2. Influence of Lipase on Enzymatic Esterification The use of lipases as biocatalysts for the production of specific structured triacylglycerols has more potential benefits for future developments, besides the specificity of lipases [5]. Figure 1 shows the impression of Lipase on transesterified lipid and enzymatic esterification. CLA incorporation is greater for Lipozyme RM IM (6.13%) than Lipozyme TL IM (4.91%) and free fatty acids at the end of reaction is greater for Lipozyme TL IM (15.68) than Lipozyme RM IM (7.28). Therefore, use of Lipozyme RM IM is preferred. Goli et al. have esterified CLA with canola oil by *Thermomyces lanuginosus* lipase (Lipozyme TL IM). Their reaction mixture was flushed with nitrogen, stoppered and incubated for 48 h in an orbital shaker at 200 rpm and 55℃ [18]. Thus, the time of their esterification reaction was too long.

3. 3. Esterification Analysis The reaction time of glycerol, CLA and sunflower oil esterification by lipozyme RM IM was investigated. Figure 2 shows the degree of conversion of CLA to sunflower oil as a function of reaction time. At the beginning, the reaction was shifted very fast to the esterification equilibrium and nearly about 89% of the free fatty acids were esterified after approximately 8 h.

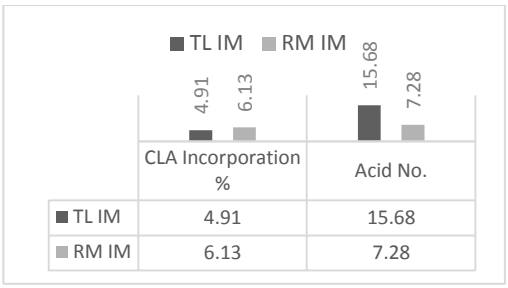

Figure 1. Impression of Lipozyme TL IM and RM IM on transesterified lipid. Reaction conditions: 100 g sunflower oil, 2% (w/w) Lipozyme, 0.3M glycerol, 0.2M CLA, temperature 65℃.

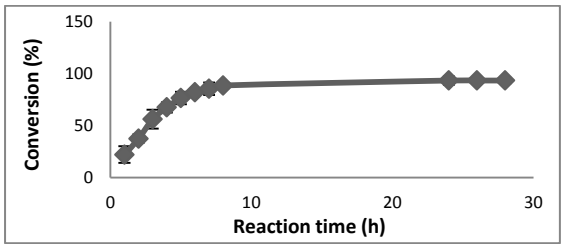

Figure 2. Conversion of CLA versus reaction time in enzymatic esterification of sunflower oil and glycerol. Reaction conditions: 100 g sunflower oil, 1.5% (w/w) Lipozyme RM IM, 0.3M glycerol, 0.3M CLA, temperature 65 ℃.

Esterification was studied according to the method described in the materials and methods, and free fatty acids and incorporated CLA were determined. Figure 3, shows the percentage of fatty acids in the samples of oils obtained by Gas Chromatography.

Considering higher glycerol concentrations of 0.1and 0.2M, the CLA incorporation increases up to glycerol concentration of 0.3 M. With higher glycerol contents, the CLA incorporation also decreases, probably indicating increase in the amount of acyl acceptors (mono and diacylglycerides) present in the Transesterified lipid and removing them at the end of reaction according to the Rousseau & Marangoni method described [22].

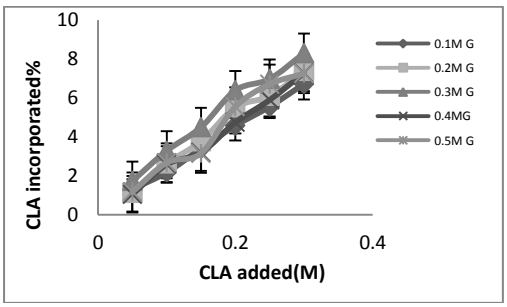

Figure 3. CLA Incorporation of esterified blends as a function of Glycerol (M). Reaction conditions: 100 g sunflower oil, 1.5% (w/w) Lipozyme RM IM, temperature 65 ℃, Reaction time 8 h.

3. 4. Impression of Substrate Concentrations on Reaction Rates Figure 4 shows the reaction rates as a function of glycerol concentration (0.1–0.5 M) for different CLA concentrations. At high acid level (0.15–0.3 M), reaction rate increased at low glycerol concentration (0.1-0.2 M) and decreased with higher glycerol concentration. These results represent lipase inhibition by the alcohols (glycerol, mono and diacylglycerols). The inhibition can probably be explained by the high polarity of the acyl acceptors promoting and blocking of the nucleophilic site of the enzyme in the catalytic center [25]. At low acid concentrations, the reaction rate decreased slightly with increasing glycerol contents. These results tend to confirm the enzyme inhibition by glycerol or other acyl acceptors present in the reaction system. Therefore, glycerol has an optimal level which depended on FFAs concentration. The optimal level of glycerol increases with increase of FFAs concentration (Figure 4).

Figure 5 shows the reaction rates as a function of CLA concentration (0.05–0.3 M) for different glycerol concentrations. Reaction rate was increased with increasing free fatty acid concentration and this indicates that FFAs concentration has no influence on the lipase activity. [20, 26] The results of the present study indicate lipase inhibition by the acylacceptors (Glycerol, mono-, diacylglycerols).

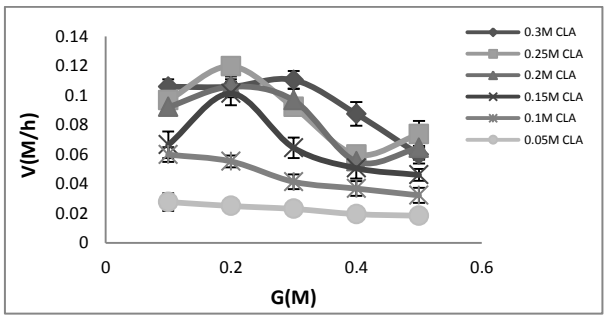

Figure 4. Reaction rate of esterified blends as a function of CLA (M). Reaction conditions: 100 g sunflower oil, 1.5% (w/w) Lipozyme RM IM, temperature 65℃, Reaction time 8h.

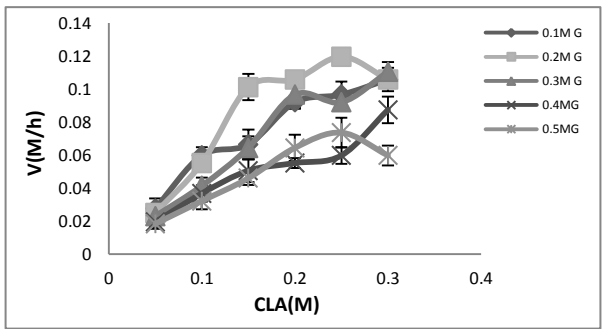

Figure 5. Reaction rate of esterified blends as a function of Glycerol (M). Reaction conditions: 100 g sunflower oil, 1.5% (w/w) Lipozyme RM IM, temperature 65℃, Reaction time 8 h.

3. 5. Optimization of Processing Response surface methodology (RSM) has been an affective and a strong statistical method for optimizing experimental conditions and survey of critical processes by reducing the number of experimental trials [27]. The optimization of a process by RSM is a more economical and faster method for collecting research results than classical one variable at a time or full factorial experimentation. RSM has been successfully applied for enzymatic transesterification optimization in organic solvent reactions [28].

Using RSM, the effects of three variables, namely Glycerol concentration, reaction temperature, and amount of enzyme on the CLA incorporation, reaction rate and free fatty acids were studied. In RSM, uncoded variables were converted to coded variables (Table 4) with a zero mean and standard deviation that had been determined dimensionless [19]. Table 4 shows experimental settings of the reaction factors for the faced centered cube design scheme and responses for the reaction rate, CLA incorporation and free fatty acids.

In process optimization for maximum CLA incorporation, maximum reaction rate and production of minimum free fatty acids were obtained. In order to minimize the falling of oil, the optimum terms at temperature of 75 ℃ by using 3 % enzyme and 0.27 M G were 6.02% CLA incorporation, 96.7% Incorporation yield, reaction rate 0.0271 M/h, acid No.2.73. The high cost of enzymes is one of the most important issues in the enzymatic esterification process of oil and fats. So, optimization of the process for minimum enzyme consumption was determined. the optimum terms at 75 ℃ by using 2 % enzyme and 0.29 M G were 5.83% CLA incorporation, 93.7% incorporation yield, reaction rate 0.0219 M/h, acid No. 4.66 (Table 5).

Figure 6 shows the all optimized conditions and finds the ideal process settings to achieve optimal performance.

The hydrolysis of triacylglycerols causes formation of free fatty acids in the oil. Existence of these compounds accelerates the subsequent hydrolyze of triacylglycerols [19]. Also, it seems that these compounds accelerate the oxidation of oil by increasing the oxygen in oil due to the peroxide properties of their carboxyl groups. That is why the free fatty acids in oils should be minimized. For all models values of "Prob > F" less than 0.0500 indicate that model terms are significant.

Also, the relation between independent variables and the responses were obtained as following Equations (2-4):

$$CLA = -3.25606 + 0.093705 * Temp + 1.85026 * E - 0.12125 * G * - 14.05634 * G^2 - \quad (2)$$

$$Vi = +0.088690 + 0.16805 * G - 3.22836E-003 * Temp - 5.67732E-003 * - 0.16136 * G2 \quad (3)$$

$$Acid\ No = +19.47762 - 71.06011 * G + 0.027591 * Temp + 2.58497 * E + 0.68250 * G * Temp + 44.61268 * G2 - \quad (4)$$

TABLE 4. Experimental settings of the reaction factors for the faced-centered cube Design arrangements and responses for the CLA incorporation, reaction rate and free fatty acids

Std	G	Temp	E	CLA%	Vi	Acid no.	Incorporation Yield
1	0.1(-1)	55(-1)	1(-1)	3.52	0.0040	11.76	74.27653
2	0.5(1)	55(-1)	1(-1)	4.6	0.0115	8.4	80.06431
3	0.1(-1)	75(1)	1(-1)	5.02	0.0126	7.84	86.81672
4	0.5(1)	75(1)	1(-1)	4.62	0.0080	10.36	88.26367
5	0.1(-1)	55(-1)	3(1)	4.98	0.0097	10.64	87.62058
6	0.5(1)	55(-1)	3(1)	5.4	0.0184	5.6	77.97428
7	0.1(-1)	75(1)	3(1)	5.49	0.0189	4.76	81.8328
8	0.5(1)	75(1)	3(1)	5.45	0.0224	4.76	86.33441
9	0.1(-1)	65(0)	2(0)	4.85	0.0057	9.52	91.31833
10	0.5(1)	65(0)	2(0)	5.09	0.0161	5.04	77.97428
11	0.3(0)	55(-1)	2(0)	5.37	0.0184	6.72	95.49839
12	0.3(0)	75(1)	2(0)	5.68	0.0218	3.92	75.08039
13	0.3(0)	65(0)	1(-1)	4.85	0.0126	7.56	88.42444
14	0.3(0)	65(0)	3(1)	5.94	0.023	3.64	99.35691
15	0.3(0)	65(0)	2(0)	5.3	0.0138	7.28	74.27653
16	0.3(0)	65(0)	2(0)	5.5	0.0138	6.44	80.06431
17	0.3(0)	65(0)	2(0)	6.13	0.01955	7.28	86.81672

Reaction conditions: 100g sunflower oil, 0.2M CLA, Reaction time 8h.

TABLE 5. Optimal condition for CLA incorporation in to sunflower oil, reaction rate and free fatty acids production

Factor	Name	Level(A)	Level(B)
A	G	0.27	0.29
B	Temp	75	75
C	Enzyme	3	2.06
Response		**Prediction**	**Prediction**
CLA		6.02935	5.83716
V		0.0271	0.0219
Acid No		2.73287	4.6646

Level (A) optimization of the process for maximum CLA incorporation, maximum reaction rate and production of minimum free fatty acids, Level (B) optimization of the process for maximum CLA incorporation, maximum reaction rate, production of minimum free fatty acids and minimum enzyme consumption. Reaction conditions: 100 g sunflower oil, 0.2 M CLA, Reaction time 8 h.

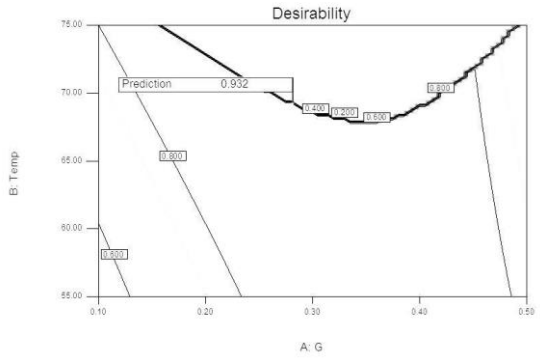

Figure 6. Desirability plot for all optimized conditions.

According to Equations (2-4), Glycerol concentration showed parabolic effects on CLA incorporation, reaction rate and FFA content.

4. CONCLUSION

In the present study CLA, a natural derivative of linoleic acid, was esterified with sunflower oil due to its health effects potential. The results showed that glycerol has more effect on enzyme affinity than CLA and indicated lipase inhibition by alcohols (Glycerol, mono-, diacylglycerides). Lipozyme RM IM is preferred for enzymatic esterification and neutralization because of more CLA incorporation and less free fatty acids production at the end of reaction compared with Lipozyme TL IM. In optimization of the process for maximum CLA incorporation, maximum reaction rate and production of minimum free fatty acids to minimize the falling of oil, the optimum terms (6.02% CLA incorporation, 96.7% Incorporation yield, reaction rate of 0.0271 M/h, acid No.2.73) were obtained at 75 ℃ by using 3 % enzyme (Lipozyme RM IM) and 0.27 M G.

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Lipase Catalyzed Incorporation of Conjugated Linoleic Acid by Transesterification into Sunflower Oil Applying Immobilized Lipase (Lipozyme *Thermomyces lanuginosus* and *Rhizomucor mehei*)

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چکید

مخلوط اسید لینولئیک مزدوج CLA، گلیسرول و روغن آفتابگردان با ترکیب درصدهای مختلف جهت انجام واکنش استریفیکاسیون آنزیمی به وسیله لیپاز تثبیت شده با انتخاب پذیری جایگاه ۱و۳ استفاده شد. اسید لینولئیک مزدوج به سبب خواص بیولوژیکی بسیار مفید استفاده شد. ترکیب اسیدهای چرب نمونه‌های نهایی پس از فرآیند استری شدن توسط کروماتوگرافی گازی به دست آمد. با توجه به نتایج آنزیم لیپوزیم RM IM و جذب بیشتر CLA به بدنه تریگلیسرید و کاهش قابل توجه اسیدیته مخلوط واکنش در طول انجام واکنش جهت انجام این واکنش ارجحیت دارد. روش پاسخ سطحی جهت بررسی اثرات متغییرهای غلظت گلیسرول، دمای واکنش و مقدار آنزیم به کار گرفته شد. در بهینه سازی فرآیند برای تولید روغنی با حداکثر CLA جذب شده، حد اکثر سرعت واکنش، حداقل اسیدهای چرب آزاد و به حداقل رساندن افت روغن، شرایط بهینه در دمای۷۵ درجه سانتی‌گراد و با استفاده از ۳ درصد آنزیم و ۰.۲۷(M) گلیسرول با ۹۶.۷٪ بازده جذب CLA به دست آمد.

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