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# Enzymatic fractionation of conjugated linoleic acid isomers by selective esterification

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**Abstract**

Attempt was done to prepare food supplements with high content of *c*9, *t*11-CLA or *t*10, *c*12-CLA. A free acid mixture containing CLA isomers was esterified with ethanol by enzyme catalysis. Novozyme 435 and Lipase AY30 were screened, and Lipase AY30 was employed to catalyze esterification reaction because of its high fractionation efficiency. Effect of reaction conditions on total esterification was investigated, and the optimal reaction conditions were: 140 U of lipase amount, reaction temperature at 50 °C, a pH of 6.5, and molar ratio of FFA–CLA to ethanol at 1:1. Based on the studies above, experiments of esterification and purification were done, and the best fractionation efficiency was obtained when the total esterification was 37%, and the corresponding purity and recovery of *c*9, *t*11-CLA were 75.50 and 49.85%, and that of *t*10, *c*12-CLA were 72.02 and 62.32%.


*Keywords:* Fractionation; Lipase; CLA isomers

## 1. Introduction

Conjugated Linoleic acid (CLA) is a collective term which refers to a mixture of positional and geometric isomers of linoleic acid with conjugated double bonds. The natural CLA resources are mainly from meat and milk products of ruminants where *c*9, *t*11-CLA is the predominant isomer. However, commercially available CLA products, which are usually manufactured by alkali conjugation of safflower oil in propylene glycol, are complex mixtures including more than seven isomers of CLA, of which *c*9, *t*11-CLA and *t*10, *c*12-CLA are two major components in almost equal amount. CLA has attracted considerable attention due to its physiological activities, such as the reduction of cancer incidence [1,2], a decrease in body fat content [3,4], profitable effects on atherosclerosis [5,6], and improvement of immune system [7]. Recently, it has been reported that each isomer has different bioactivities: *c*9, *t*11-CLA has anticancer activity [8]; *t*10, *c*12-CLA has activities to decrease body fat content [9,10], to increase energy expenditure [11], and to suppress the development of hypertension [12]. To further study the biological effects of CLA, and to develop nutraceuticals with different ratio of CLA isomers, it is strongly desirable to explore simple and economic methods to fractionate of CLA isomers.

More interests have been increased in the use of enzymatic methods to separate the CLA isomers because of difficulties by current methods [13,14]. It is well known that some lipases have substrate selectivity towards positional isomers of unsaturated fatty acids. Borgdorf and Warwel [15,16] screened lipases with substrate selectivity to the *cis*/*trans* configuration of the C=C double bond by the esterification of oleic and elaidic acid with butanol in hexane, and found that *Candida antarctica* lipase A had extraordinary substrate selectivity on *trans*-9-isomers, such as linolelaidic (9*t*, 12*t*-18:2) acid, and lipases from *Candida cylindracea* and *Mucor miehei* have a high preference for fatty acids containing a (first) *cis*-double bond in ≥9-position. Researches on fractionation of CLA isomers were reported by several labs. *Candida rugosa* lipase and *Geotrichum candidum* lipase (a commercial *G. candidum* Amano GC-4, a cloned *G. candidum* lipase B) are found to recognize *c*9, *t*11-CLA more readily than *t*10, *c*12 -CLA, and to be effective to fractionate CLA isomers. Fractionation efficiency of each lipase and the recovery of isomers by different process were shown in Tables 1 and 2 [17–22], and the results were compared: 1(Purity of *c*9, *t*11-CLA and *t*10, *c*12 -CLA are higher in the esterifi-

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Table 1
Fractionation of CLA isomers by *Candida rugosa* lipase

| References | Process | Reaction substrate | *Candida rugosa* lipase | | | |
|---|---|---|---|---|---|---|
| | | | c9, t11-CLA | | t10, c12-CLA | |
| | | | Purity (%) | Recovery (%) | Purity (%) | Recovery (%) |
| [17] | A | CLA–FFA[a] Lauryl alcohol | 95.6 | 34.5 | 94.9 | 52.3 |
| [18] | B | CLA–FFA[b] Menthol | 94.0 | 42.0 | 91.0 | 40.0 |
| [19] | C | CLA–FFA[c] Lauryl alcohol | 93.1 | 34.0 | 95.3 | 31.0 |
| [20] | D | Acylglycerol–CLA | 72.9 | 26.9 | 81.3 | 23.1 |

Purity of *c*9, *t*11-CLA and *t*10, *c*12-CLA was based on the total CLA content. Recovery of *c*9, *t*11-CLA and *t*10, *c*12-CLA was expressed relative to the initial amount of each isomer. (A) Two-step selective esterification; hexane extraction; molecular distillation; urea adduct fractionation, (B) two-step selective esterification; hexane extraction; molecular distillation, (C) selective esterification, molecular distillation, urea adduct fractionation and (D) selective hydrolysis, hexane extraction.
[a] 33.1% *c*9, *t*11-CLA; 33.9% *t*10, *c*12-CLA.
[b] 33.3% *c*9, *t*11-CLA; 34.2% *t*10, *c*12-CLA.
[c] 45.1% *c*9, *t*11-CLA; 46.8% *t*10, *c*12-CLA.

Table 2
Fractionation of CLA isomers by *Geotrichum candidum* lipase

| References | Process | Reaction substrate | Purity of c9, t11-CLA | | Purity of t10, c12-CLA |
|---|---|---|---|---|---|
| | | | Amano GC-4 | *G. candidum* lipase B | Amano GC-4 |
| [21] | Hydrolysis | CLA–ME[a] | 77% | 94% | – |
| [21] | Esterification in organic solvent | CLA–FFA[a] Methanol/ethanol | 80% | 96% | – |
| [22] | Esterification | CLA–FFA[b] Lauryl alcohol | 91% | – | 82% |

[a] 49.7% *c*9, *t*11-CLA and 50.3% *t*10, *c*12-CLA.
[b] 34.2% *c*9, *t*11-CLA and 34.6% *t*10, *c*12-CLA.

cation process by *Candida rugosa* lipase than by *Geotrichum candidum* lipase; 2) some drawbacks exits such that lauyl alcohol and methanol were toxic, and reaction in organic solvent was unsuitable in the food processing; 3) The enzymatic esterification process is more practical than the hydrolysis reaction because CLA is produced industrially in the form of fatty acid by alkali isomerization of linoleic acid.

The aim of our research is to develop an efficient process to produce a safe food supplements with high content of different CLA isomer. Enzymatic esterification of CLA–FFA and ethanol was adopted because ethanol can be used in food processing. Novozyme 435 (from *Candida Antarctica* lipase B), that is known to have high esterification activity, was also screened in addition to *Candida rugosa* lipase. Different reaction conditions, such as substrate mole ratio, temperature, pH, enzyme load, and reaction time, were investigated.

## 2. Materials and methods

### 2.1. Materials

Immobilized lipase from *Candida antarctica* lipase B (Novozyme 435) was kindly provided by Novo Nordisk (Bagsvaerd, Denmark). Lipase from *Candida rugosa* (Lipase AY 30) was a gift from Amano Pharmaceutical Co. Ltd. (Nagoya, Japan), and the lipase was dissolved in the phosphate buffer at different concentration of 5.6–16.8 U/ml, and 10 ml of the lipase solution was added to a reaction mixture.

The mixture of CLA isomers (FFA–CLA) was prepared by our lab and it was produced by alkali isomerization of sunflower oil with 50% KOH in propylene glycol at 170 °C for 1 h. The FFA–CLA was composed of 30.17% *c*9, *t*11-CLA, 30.75% *t*10, *c*12-CLA and 0.20% other CLA isomers; 7.83% palmitic acid, 3.37% stearic acid, 15.21% oleic acid, 3.02% linoleic acid. Molecular weight of FFA–CLA was calculated based on the composition of fatty acids. Heptadecanoic acid (C17:0) and ethyl ester of heptadecanoic acid was purchased from Sigma–Aldrich Chemie GmbH, Guangzhou, China. Sunflower oil was purchased from a local supermarket. Other chemicals were of analytical grade.

### 2.2. Reactions

The esterification reaction was carried out in a 50 ml conical flask with stirring at 180 rpm. The reaction mixture was composed of FFA–CLA, absolute ethanol, and lipase or lipase solution. Reaction temperature, pH, molar ratio of FFA–CLA/ethanol, and lipase amount were changed to study

their effects on the degree of esterification of total fatty acids and each CLA isomers.

### 2.3. Separation of ethyl ester and FFA in reaction mixture

Separation of ethyl ester was conducted by hexane extraction [18]. Ethyl ester was extracted twice with 200 ml hexane after adding 70 ml 0.5 N KOH solution in 20% ethanol into 10 g reaction mixture. The fatty acids in the water phase were extracted twice with 150 ml hexane after acidification with HCl. Hexane was recovered by rotatory evaporator.

### 2.4. Analyses

#### 2.4.1. Determination of lipase activity

Lipase activity assay was performed according to an olive oil emulsion method [23]. Four millilitre of olive oil emulsion, 5 ml of 0.05 M citric acid buffer and 1 ml of enzyme solution were mixed and incubated at 40 °C for 15 min. The reaction was terminated with the addition of 95% ethanol (15 ml) after incubation, and the liberated fatty acids were titrated with 0.05 M NaOH. Blanks were measured with a heat-inactivated enzyme sample. Each sample was assayed three times and the average value was taken. One unit of lipase (U) is defined as the amount of enzyme which releases 1 μmol titratable free fatty acids per minute under the described conditions. The hydrolysis activity of Novozyme 435 is 138 U/g (its esterification activity is given as 10,000 PLU/g by provider). The hydrolysis of *Candida rugosa* lipase is 14,800 U/g.

#### 2.4.2. Determination of fatty acid composition of free fatty acids and ethyl esters in the reaction mixture

About 1 g of the reaction mixture was separated by centrifugation at 6000 × *g* for 5 min. The upper mixture of ethyl esters and fatty acids was withdrawn into another tube with anhydrous sodium sulfate, and then 1 ml hexane was added and mixed by swirling. After centrifugation at 10,000 × *g* for 1 min, the supernatant liquid was drawn for GC analyses.

A Hewlett-Packard 5890 gas chromatograph (GC) was used to analyze the fatty acid composition of free fatty acids and ethyl esters produced in esterification. The products were separated on a OV-351 capillary GC column (60 m × 0.32 mm) using helium as carrier gas. A temperature program was used as the column oven holding at 150 °C for 3 min, then rising to 220 °C at 5 °C/min and running for 32 min, giving a total run time of 49 min. The split ratio was 1:50. The injector and the flame ionization detector temperature were set as 250 and 300 °C, respectively. Heptadecanoic acid (C17:0) and ethyl ester of heptadecanoic acid was used as the internal standard for calculation.

Peaks in GC were appointed by comparison of their retention times with those of known standards. Peak percentages and areas were calculated using Hewlett-Packard PC integration software (HP 3398A ChemStation Version A.01.01). The degree of esterification of the total fatty acids was determined as the content of ethyl esters by GC. The degree of esterification of each CLA isomer was determined as the ratio of isomer content in the ethyl esters to total corresponding isomer in reaction mixture.

## 3. Results and discussion

### 3.1. Enzyme screening

Two commercially available lipases from different sources were used to fractionate CLA isomers by selective esterification with ethanol. Lipase AY30 was non-immobilized, and Novozyme 435 was an immobilized lipase. They were screened by incubating lipase with a 1:1 molar ratio of FFA–CLA and ethanol at 50 °C. Table 3 shows the effect of different enzyme on the degree of esterification of total fatty acids and each CLA isomer. Novozyme 435 had stronger activity in the esterification than Lipase AY 30 did, and the total esterification was 76.71% with Novozyme 435, 24.44% with lipase AY30 when the reaction was done for 4 h. Moreover, Lipase AY 30 and Novozyme 435 had different selectivity on the *c*9, *t*11-CLA and *t*10, *c*12-CLA, the former with little preference on *t*10, *c*12-CLA, whereas the latter with high selectivity on *c*9, *t*11-CLA. Lipase AY30 catalyzed the esterification of *c*9, *t*11-CLA 3.1–4.1 times faster than the corresponding reaction of *t*10, *c*12-CLA, while Novozyme 435 esterified *t*10, *c*12-CLA about 1.1–1.8 times faster than *c*9, *t*11-CLA. It can be clear that Novozyme 435 had an extraordinary activity in esterification, but Lipase AY30 had higher fractionation efficiency. In order to fractionate *c*9, *t*11-CLA and *t*10, *c*12-CLA efficiently, Lipase AY 30 was selected for the rest of study.

Table 3
Effect of lipase on the degree of esterification of total fatty acids and each CLA isomer

| Time (min) | Lipase AY30 | | | Novozyme 435 | | |
|---|---|---|---|---|---|---|
| | TE (%) | *c*9, *t*11-CLA (%) | *t*10, *c*12-CLA (%) | TE (%) | *c*9, *t*11-CLA (%) | *t*10, *c*12-CLA (%) |
| 20 | – | – | – | 9.62 | 8.79 | 15.82 |
| 30 | – | – | – | 18.44 | 19.12 | 23.10 |
| 60 | 8.31 | 19.74 | 4.77 | 32.08 | 34.92 | 39.08 |
| 120 | 16.33 | 34.78 | 10.32 | 48.70 | 50.29 | 56.88 |
| 240 | 24.44 | 44.56 | 14.33 | 76.71 | 79.02 | 87.28 |

TE (%): total esterification (the degree of esterification of total fatty acids); *c*9, *t*11-CLA (%): degree of esterification of *c*9, *t*11-CLA; *t*10, *c*12-CLA (%): degree of esterification of *t*10, *c*12-CLA. A reaction mixture includes: 10 g FFA–CLA, 1.6 g ethanol, 10 ml Lipase AY30 solution (140 U, pH 7.0) or 6.9 U of Novozyme 435. The reaction mixture was stirred at 50 °C.

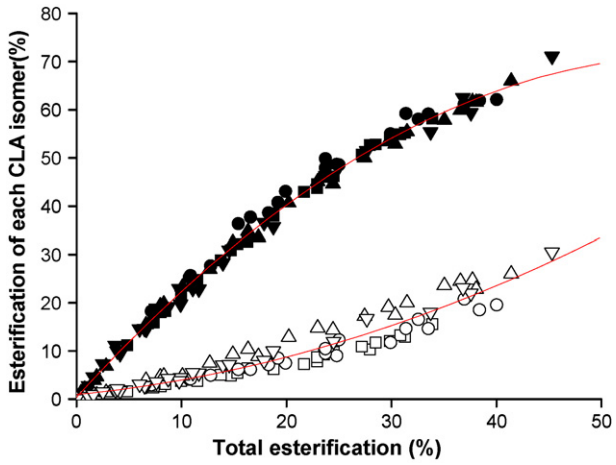


Fig. 1. Relationship between the degree of esterification of each CLA isomer and total esterification in selective esterification with ethanol under different reaction conditions. A reaction mixture of 10 g FFA–CLA, different ratio of FFA–CLA/ethanol (4:1–1:2, mol/mol), 10 ml phosphate buffer with different pH, and 56–168 U Lipase AY 30 was stirred at different temperature (45–60 °C). (■) Reaction for 1–8 h with FFA–CLA/ethanol (2:1, mol/mol) and different lipase amount at 50 °C and pH 6.5. (●) Reaction for 1–8 h with FFA–CLA/ethanol (2:1, mol/mol) and 140 U Lipase AY30 at pH 6.5 and different temperature. (▲) Reaction for 1–8 h with FFA–CLA/ethanol (2:1, mol/mol) and 140 U Lipase AY30 at 50 °C and different pH. (▼) Reaction for 1–8 h with 140 U Lipase AY30 and different ratio of FFA–CLA/ethanol at 50 °C and pH 6.5. The open and closed symbols show *t*10, *c*12-CLA and *c*9, *t*11-CLA, respectively.

### 3.2. Relationship between the degree of esterification of total fatty acids and each CLA isomer

The FFA–CLA was esterified with ethanol at various conditions: lipase amount from 56 to 168 U in the reaction mixture; reaction temperature from 45 to 60 °C; pH from 6.0 to 8.0; molar ratio of FFA–CLA to ethanol from 4:1 to 1:2; reaction time from 1 to 10 h. Fig. 1 shows the relationship between the degree of esterification of each CLA isomer and total fatty acids (total esterification) in the selective esterification with ethanol. By analysis on the experimental data, the values of regression coefficients were calculated and equation model was given as follows:

$$Y_1 = 0.61 + 2.42X - 0.02X^2 \quad (1)$$

$$Y_2 = 0.99 + 0.21X + 0.01X^2 \quad (2)$$

where $Y_1$ and $Y_2$ represent the degree of esterification of *c*9, *t*11-CLA and *t*10, *c*12-CLA, respectively. $X$ is the degree of esterification of total fatty acids. The coefficient of determination $R^2$ of the Eqs. (1) and (2) were calculated to be 0.994 and 0.918, respectively. This indicates that the model explains 99.4 and 91.8% of the variability in the data.

Lipase AY30 had a higher selectivity on *c*9, *t*11-CLA and a less preference on *t*10, *c*12-CLA in the esterification, and the enzymatic fractionation efficiency of *c*9, *t*11-CLA and *t*10, *c*12-CLA was determined mainly by total esterification, while the relationship was not significantly influenced by reaction conditions such as the amount of lipase, pH, temperature, molar ratio of FFA–CLA/ethanol, and reaction period. As shown in Fig. 1, the optimal fraction efficiency was observed when the total esterification was between 30 and 45%. From Eqs. (1) and (2), it could be calculated that the maximal distance between two equations was attained when the total esterification was 36.8%. Furthermore, it disclosed that 36.8% total esterification most efficiently fractionated *c*9, *t*11-CLA into the ethyl ester fraction and *t*10, *c*12-CLA into the fatty acid fraction, and the corresponding degree of esterification of *c*9, *t*11-CLA and *t*10, *c*12-CLA was 59.7 and 21.5%, respectively.

### 3.3. Effect of reaction conditions on total esterification

Total esterification played a critical role in the enzymatic fractionation efficiencies of *c*9, *t*11-CLA and *t*10, *c*12-CLA. So it is very important to investigate the catalytic effect of different reaction conditions on total esterification. The factors to be discussed include lipase amount, reaction temperature, pH, molar ratio of FFA–CLA/ethanol, and reaction time.

#### 3.3.1. Effect of lipase amount

The esterification was performed at 50 °C using 56–168 U Lipase AY30.The ratio of FFA–CLA and ethanol was 2:1. The results were shown in Fig. 2. Reaction velocity depended on the amount of lipase, and it increased with the increment of lipase used. When the lipase was over 140 U/g mixture, the degree of total esterification was more than 30% at 8 h. The degree of total esterification increased less when more lipase was added, and the amount was, therefore, fixed at 140 U in the latter reaction mixture.

#### 3.3.2. Effect of reaction temperature

Effect of reaction temperature on esterification of FFA–CLA with ethanol was investigated. The temperature was set as 45, 50, 55 and 60 °C, respectively. The molar ratio of FFA–CLA and ethanol was 2:1, and 140 U lipase was used in the esterification. Total esterification increased with the temperature rising, and it reached 40.01% at 8 h when the temperature was 50 °C, but it degraded with higher reaction temperature (Fig. 3). It may

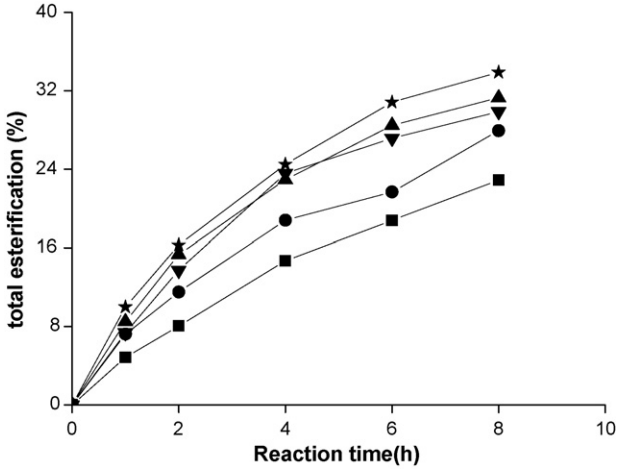


Fig. 2. Effect of lipase amount on total esterification of FFA–CLA with ethanol during the reaction period. A reaction mixture of 10 g FFA–CLA and 1.6 g ethanol and 10 ml (56–168 U) lipase solution was stirred at 50 °C and pH 6.5. (■) 56 U; (●) 84 U; (▼) 112 U; (▲) 140 U; (⋆) 168 U.

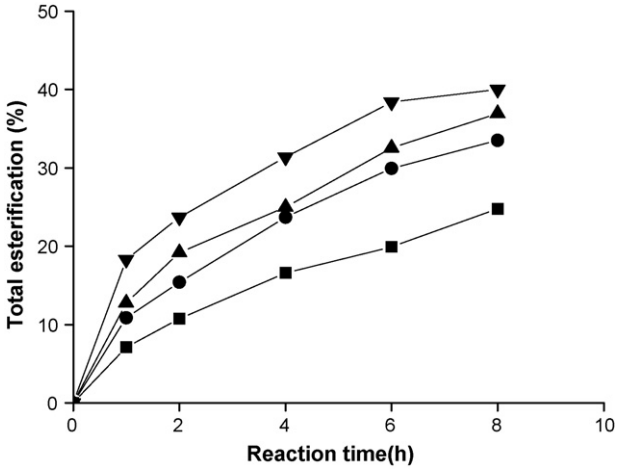


Fig. 3. Effect of temperature on total esterification of FFA–CLA with ethanol during the reaction period. A reaction mixture of 10 g FFA–CLA and 1.6 g ethanol and 10 ml (140 U) lipase solution was stirred at pH 6.5 at different reaction temperature. (■) 60 °C; (●) 55 °C; (▼) 50 °C; (▲) 45 °C.

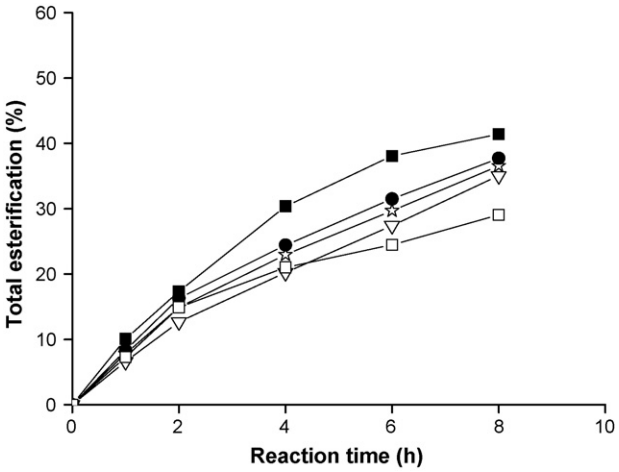


Fig. 4. Effect of pH on total esterification of FFA–CLA with ethanol during the reaction period. A reaction mixture of 10 g FFA–CLA and 1.6 g ethanol and 10 ml (140 U) lipase solution was stirred at 50 °C at different reaction pH: (▽) 8.0; (☆) 7.5; (●) 7.0; (■) 6.5; (□) 6.0.

be probably because lipase protein was denatured and lost its catalytic function at such a high reaction temperature as 55 °C or higher. Based on results above, the reaction temperature was fixed at 50 °C in the following reactions.

### 3.3.3. Effect of pH

The esterification was carried out at 50 °C at pH of 8.0, 7.5, 7.0, 6.5 and 6.0, respectively, and molar ratio of FFA–CLA/ethanol was 2:1, and 140 U lipase was used in the reaction. As shown in Fig. 4, Lipase AY 30 displayed catalytic activity between 6.5 and 8.0, and the optimal activity of the lipase was observed at pH of 6.5, and the corresponding total esterification was 41.4%.

### 3.3.4. Effect of substrate mole ratio

The molar ratio of reaction substrate is a crucial factor which affects the total esterificatoin. Molar ratio of FFA–CLA/ethanol was selected as 4:1, 3:1, 2:1, 1:1 and 1:2, respectively, and its effect on total esterification was explored. The esterification was performed at pH of 6.5 at 50 °C with 140 U lipase amount. In the reaction mixture, the content of FFA–CLA was kept at constant, and the amount of ethanol was changed. The results were shown in Fig. 5. The total esterification rose with the molar ratio of FFA–CLA/ethanol reducing from 4:1 to 1:1 and it is probably because larger amounts of ethanol enhanced the interacting opportunity of FFA–CLA and ethanol. But due to the toxicity of ethanol towards enzyme the total esterification degraded greatly when more ethanol was added. When the reaction was proceeded

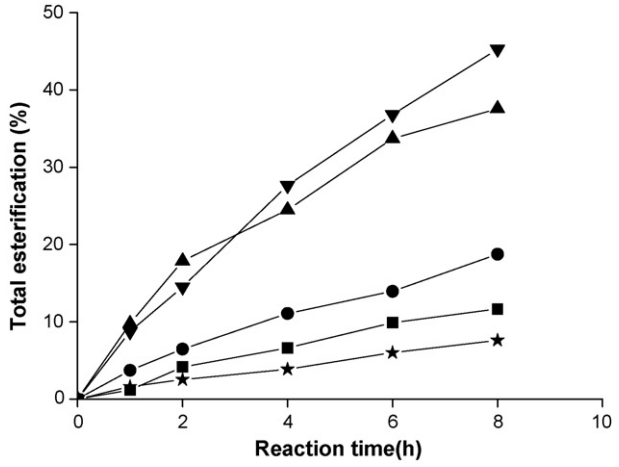


Fig. 5. Effect of the molar ratio of FFA–CLA/ethanol on total esterification of FFA–CLA with ethanol during the reaction period. A reaction mixture of 10 g FFA–CLA and different amount of ethanol and 10 ml (140 U) lipase solution was stirred at 50 °C at pH 6.5. (■) 4:1; (●) 3:1; (▲) 2:1; (▼) 1:1; (⋆) 1:2.

Table 4
Fatty acid compositions of fractionated products and purity and recovery of CLA isomers

| Preparation | FA composition (wt%) of CLA | | | | | | Purity (%) | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| | 16:0 | 18:0 | 18:1 | 18:2 | *c*9,*t*11-CLA | *t*10,*c*12-CLA | | |
| Original CLA–FFA | 7.83 | 3.37 | 15.21 | 3.02 | 30.17 | 30.75 | | |
| Ethyl ester product | 6.21 | 0.96 | 11.37 | 3.46 | 57.03 | 18.51 | 75.50[a] | 49.85[b] |
| Fatty acids product | 6.47 | 3.65 | 11.81 | 2.71 | 18.76 | 48.28 | 72.02[c] | 62.32[d] |

[a] Purity of *c*9, *t*11-CLA = *c*9, *t*11-CLA / (*c*9, *t*11-CLA + *t*10, *c*12-CLA).
[b] Recovery of *c*9, *t*11-CLA = *c*9, *t*11-CLA in ethyl ester/*c*9, *t*11-CLA in original CLA–FFA.
[c] Purtiy of *t*10, *c*12-CLA = *t*10, *c*12-CLA/(*c*9, *t*11-CLA + *t*10, *c*12-CLA).
[d] Recovery of *t*10, *c*12-CLA = *t*10, *c*12-CLA in fatty acids/*t*10, *c*12-CLA in original CLA–FFA.

at 1:1 molar ratio of FFA–CLA/ethanol, the total esterification was up to be 45.30% at 8 h, which was the best result among the five different susbstrate molar ratio conditions.

Under the optimal conditions described above, the esterification of FFA–CLA and ethanol was carried out by Lipase AY 30. The reaction was stopped when the total esterification was up to 37%, and then the reaction mixture of fatty acids and ethyl ester was separated. The results were shown in Table 4. The purity and recovery of *c*9, *t*11-CLA and *t*10, *c*12-CLA would be improved if multi-step enzymatic esterification was performed [17,18].

## 4. Conclusions

A developed process for fractionating CLA isomers was established. Ethanol was used as substrate because of its safe application in the food processing, and ethyl ester is also a permitted food supplements. Although enzymatic fractionation of CLA isomers has been reported by some lab, there is no detailed paper published on the fractionation efficiency in the esterification of CLA–FFA and ethanol in solvent-free system by lipase. From the results and analysis, the final conclusions were drawn:

(1) Novozyme 435 had an extraordinary activity in esterification, but Lipase AY30 had higher fractionation efficiency. The maximal fraction efficiency was observed when the total esterification was 36.8% catalyzed by Lipase AY30.
(2) The optimal reaction conditions for total esterification were: 140 U of lipase amount, reaction temperature at 50 °C, a pH of 6.5, and molar ratio of FFA–CLA to ethanol at 1:1.
(3) When the total esterification was 37%, the purity and recovery of *c*9, *t*11-CLA were 75.50 and 49.85%, and that of *t*10, *c*12-CLA were 72.02 and 62.32%.

Comparing the results with the published data on fractionation of CLA isomers in different system, the purity and recovery of *c*9, *t*11-CLA and *t*10, *c*12-CLA was relatively lower, but it would be improved by the multi-step esterification process [17,18]. Nevertheless, this is a new process there is enough room for further research to develop industrial production techniques for food supplements.

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