Thesis for the Degree of Master of Engineering

Characterization of Wheat Germ Oil Converted by Immobilized Lipase Ethanolysis

by

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Department of Food Science & Technology

The Graduate School

Pukyong National University

August 2011

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고정화 리파아제를 이용한 에탄올화 반응으로 전환된 밀배아유의 특성

Advisor: Prof. Byung-Soo Chun

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Jeong-Eun Sim

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Contents

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심정은

부 경 대 학 교 대 학 원 식 품 공 학 과

요 약

현재 밀배아는 제분 과정에서 얻어지는 부산물로 대부분의 밀배아는 저렴한 사료로 이용되고 있어 밀배아의 활용도는 극히 낮은 실정이다. 그러나 밀배아에는 천연항산화제의 역할을 하는 비타민 E 등이 많이 함유되어 있고, tocopherol과 phenolic류 등의 천연기능성 물질들이 많이 함유되어 있어, 식물유의 산패를 억제하기 위해 이용되기도 한다.

이산화탄소는 낮은 임계점과 무독성, 그리고 용매의 사용량이 적고 부식성이 없이 친환경적이기 때문에 가장 널리 이용되는 초임계 유체이며 기존의 추출법이 가지는 어려움을 해결할 수 있는 새로운 혁신기술로서 주목받고 있다.

본 논문에서는 초임계 이산화탄소를 이용하여 밀배아를 추출하였고, 추출된 밀배아유에서 효소적 에탄올화 반응을 실시하였고 그 기능성에 대하여 연구하였다.

고도불포화 지방산 함유 diglyceride, monoglyceride 및 fatty acid methyl ester를 생성하기 위해 고정화 효소인 lipozyme TL-IM과 lipozyme RM-IM을 사용하여 밀배아유의 효소적 에탄올화 반응을 수행하였다. 반응은 에탄올, 온도, 효소량, 반응시간에 따른 밀배아유의 전환율을 실험해 보았고, 초임계 이산화탄소를 이용하여 추출한 오일과 일반 유기용매를 이용하여 추출한 오일의 전환율을 비교하였다.

초임계 유체 추출은 200 bar 40℃에서 2시간 동안 추출한 오일을 이용하였다. 오일의 지방산 및 밀배아의 분자량을 계산하기 위하여 gas chromatography를 이용하여 분석하였으며 TLC나 HPLC를 이용하여 지질의 정성분석을 하였다.

상압에서 반응된 밀배아 오일의 전환율을 비교해보았을 때 60℃에서 최대 반응전환율을 보였으며 초임계 이산화탄소 상태에서 반응해 보았을 때는 10 MPa, 50℃에서 최대 반응 전환율을 보였다. 본 연구에서 가압 조건에서 반응하였을 때 비가압에서의 반응에 비해 더 높은 수율을 얻었음을 확인 할 수 있었다.

반응 후 생성된 소재의 안정성을 확인하기 위해 산가, 과산화물가, 유리지방산 함량,

산화안정성 (thiocynate method, DPPH 라디칼 소거능, rancimat test)을 통하여 확인한 결과 40℃ 반응조건에서 생성된 소재가 가장 안정성이 높았으며 이는 반응온도가 높을수록 소재의 산화가 빨리 진행됨을 추측 할 수 있다.

또한 반응 후 생성물에 함유되어 있는 리파아제의 활성을 측정한 결과 가압 상태에서 회수된 생성물과 비가압에서 회수된 생성물의 리파아제 활성은 큰 차이가 없음을 확인 할 수 있었다.

1. INTRODUCTION

1.1 Background

Wheat germ is a by-product of the wheat milling industry. It is known that wheat germ may adversely affect the keeping and reprocessing quality of the flour. The human consumption of wheat germ is very limited, since it is used for animal feeding and other purposes (Zhu et al., 2006). Wheat germ was separated by wheat milling process and it constitutes about 2-3% of the wheat grain and contains about 11% oil. Depending on the variety of the wheat kernel, wheat germ contains 8-11% moisture, 27-30% proteins, 8-11% lipids, 15-20% sugars, 4-5% ash, 8-10% cellulose and hemi-cellulose and 10-14% vitamin E. (Xie et al., 2008; Zhou et al., 2005). Wheat germ is highest nutritional significant sources of α-tocopherol, vitamins of group B, proteins, dietary fiber and minerals (Amadò et al., 1992; Alabaster et al., 1997, Zhou et al., 2006). Vitamin E which is natural vitamin E complex in wheat germ is contained in an ideal state. Wheat germ is also rich in unsaturated fatty acids, mainly oleic, linoleic and α-linoleic acids (Sjovall et al., 2000). Wheat germ containing as much as 10% oil is used in products such as foods, biological insect control agents, medicals and cosmetic industries (Kahlon et al., 1989; Dunford et al., 2003).

Recently, it was shown the potential of processed wheat germ in the prevention and therapy of carcinogenesis (Reddy et al., 2000; Zalatnai et al., 2001) and it has been used as a functional food. Wheat germ is demanded technology for functional food development due to its high content of polyunsaturated fatty acids and bioactive compounds. (Ping et al., 2008)

Currently, esterification reaction is a great interest because of its application to several branches of industry. Products obtained by esterification of long chain fatty acids are biodiesel, trans-free fats and medium chain-length triglyceride. Transesterification methods are alcoholysis, acidolysis and interesterfication.

The by-products recovered from food processing can be turned into valuable products or at least converted into useful products. Manufacturing lipid products from natural resources normally yields significant amounts of low-value fats and oils next to the focused high valued processes. With better refining technologies these resources can be turned into value added products. Fats are possible to be converted into glycerin and fatty acid esters by alcoholysis. Accordingly, Wheat germ oils which are by-product are subject to alcoholysis to the triglyceride into di-, mono-glycerides and fatty acid esters.

Monoglyceride and diglyceride are used as emulsifier in food industry and yield higher market prices than oils. And they help to prevent weight and fat accumulation (Murase et al., 2001; Maki et al., 2002).

The chemically catalyzed reaction of lipids with alcohols is simple to carry out, but can generate many side products like soaps and free fatty acids which can be tedious to remove from the reaction mixture. As an alternative the lipase catalyzed reaction is more selective and ideally leads only to monoglycerides and fatty acid esters (Koichi et al; 2004).

Wheat germ oils contain a high percentage of poly-unsaturated fatty acid at the sn-2 position. Lipases which use this experiments, lipozyme RM-IM and lipozyme TL-IM, derived from each Rhizo-mucor miehei and derived from lanuginosus. These are immobilized 1, 3-regioselective lipase (Takaaki

Watanabe et al., 2003). Fig.1 is esterification of triglyceride and ethanol.

Generally, lipids are extracted by organic solvents. Several methods have been reported for extracting various materials (plants, food-by-products, algae etc). The most extraction way of the lipids by conventional methods, such as liquid solvent extraction using toluene, hexane, petroleum ether, chloroform, acetone etc. The disadvantages of requiring high temperature processing that result in decomposition or degradation of the thermally labile compounds cannot be avoided in a conventional separation method (Sanders et al., 1993). Organic solvents are also harmful to human health as well as the environment.

Supercritical fluid extraction (SFE) uses low temperature and high pressure. And it has been widely employed as alternative of organic solvent extraction. In supercritical state, densities are liquid-like, viscosity is more nearly that of normal gases and diffusivity is about two orders of magnitude higher than in typical liquids (Brunner et al., 2005). In addition, the solubility of solutes in supercritical fluids (SCFs) in strongly influenced by the density of the supercritical fluid which can be easily and rapidly controlled by modest variations in temperature and pressure. Fig. 2 shows the pressure-temperature phase diagram of SFs. The vapor pressure starts in triple point and ends at the critical point. The critical region arises at critical point. In critical region, there is only one phase and it possesses some of the properties of both gas and liquid (Taylor et al., 1996).

Several compounds have been examined as SFE solvents. For example, hydrocarbons such as hexane, pentane and butane, nitrous oxide, sulphur hexafluoride and fluorinated hydrocarbons (Smith et al., 1999). However, carbon dioxide is the most popular SFE solvent because it is safe, readily

available and has a low cost. It allows supercritical operations at relatively low pressures and at near-room temperatures (Reverchon et al., 1997). Supercritical carbon dioxide (SC-CO_2) also possesses excellent extractive properties such as high compressibility, liquid-like density, low viscosity, high diffusivity (Lim et al., 2002). Therefore, SFE is profitable when applied to high added value, to obtain ultra pure products or if imposed by regulatory restrictions on residues (Gomes *et al.*, 2007) and it can be useful when applied to food and pharmaceutical industry.

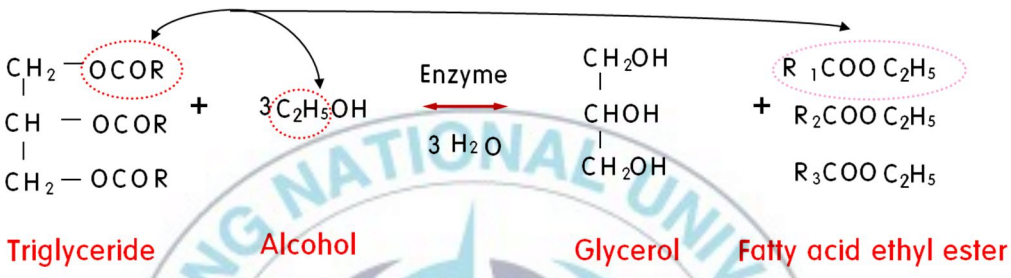

Fig. 1. Enzymatic ethanolysis of triglyceride by immobilized lipase

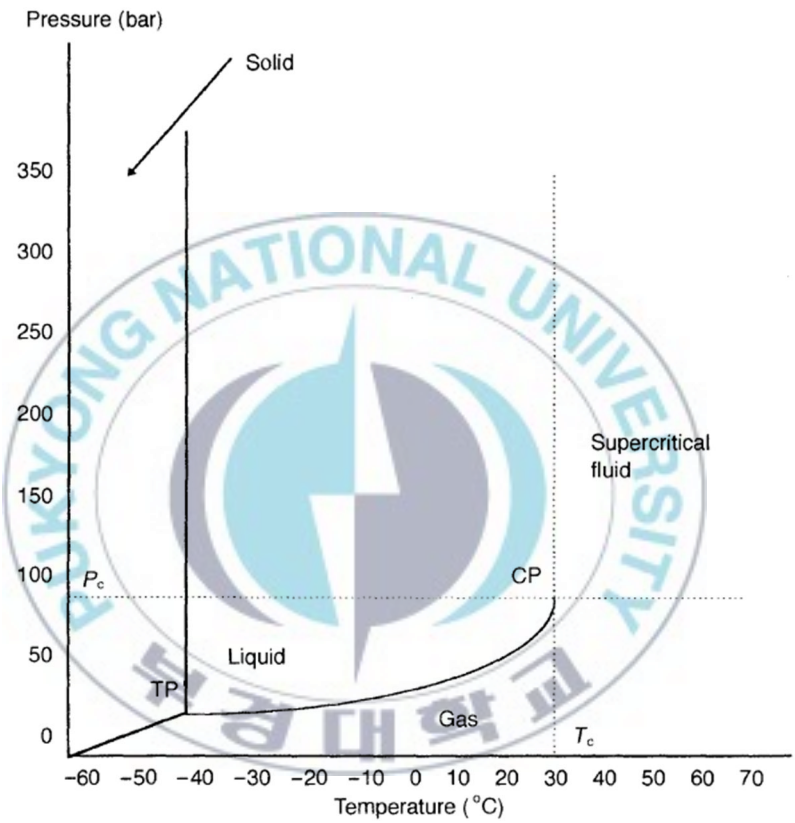

Fig. 2. Pressure-temperature phase diagram of a substance with critical temperature (Tc) and pressure (Pc).

1.2 Scope of this thesis

The objectives of this study are to compare the ethanolysis of SC-CO_2 extracted wheat germ oil and hexane extraction using immobilized lipase.

For this purpose, the compositions of fatty acid from extracted wheat germ oil were evaluated by GC analysis. TLC and HPLC was modified for analyzing mixture containing tri-, di-, mono-glycerides and fatty acid ester obtained by the enzymatic ethanolysis of wheat germ oil. In addition, the characterization of enzymatic ethanolysis reactant was compared using acid value, peroxide value, free fatty acid and oxidative stability and they were identified by comparing the feathers.

2. MATERIALS AND METHODS

2.1 Materials

The sample was kindly donated by Young-Nam Flour Mills Company (Busan, Republic of Korea). Carbon dioxide (99.99% pure) was supplied by KOSEM (Yangsan, Republic of Korea). Standard of wheat bran oil was purchased from Sigma Chemical Company (St. Louis, MO, USA). Immobilized commercial lipases specifically lipozyme TL-IM (thermomuces lanuginosa immobilized on silica gel) and lipozyme RM-IM (rhizomucor miehei immobilized on an ion exchange resin) were purchased by Novozymes (Bagsvaerd, Danmark). Lipid standard (Mono, Di, Triglyceride Mix., Supelco, USA) was used for identification. HPLC grade methanol, isoprpylalcohol, hexane and absolute ethanol (99.9%) were purchased Burdick & Jackson.

2.2 Sample preparation

After drying in an oven at 60℃, wheat germs were crushed in mechanical blender and sieved (500mesh) by a mesh. Then the samples were stored at 2℃ and used for SC-CO_2 extraction.

2.2.1 Supercritical carbon dioxide extraction

The Wheat germ oil was extracted by supercritical carbon dioxide for a laboratory scale. The flow diagram of the equipment is shown in Fig. 3. This apparatus can be operated at pressure up to 30 MPa. 50 g of wheat germ were loaded into the stainless steel extraction vessel which was 200 mL in volume. This vessel contains cotton at the bottom. Before plugging with a cap, another layer of cotton was used at the bottom of the extraction vessel. CO_2 was pumped to the extraction vessel by a high-pressure pump (pu-2-88, Jasco) up to the desired pressure which was regulated by a back-pressure regulator. The pressure of CO_2 was automatically maintained by the pump. The temperature of vessel was maintained heat by connecting with a water bath. The oil was extracted by supercritical carbon dioxide (SC-CO_2) at temperature of 40℃ and pressure of 25 MPa. The total extraction time was 2.5 hrs. The flow rate of CO_2 was kept constant at 26.81 g/min for all extraction conditions and CO_2 volume passing through the apparatus were measured using a dry gas meter. The extracted oil was collected on the glass separation vessels. The extracted oil was stored at -20℃ until further analysis.

2.2.2 Ethanolysis in atmospheric system using immobilized enzyme

In this experiment, wheat germ oil and ethanol was tested for ethanolysis reaction using immobilized enzyme (lipozyme TL-IM, lipozyme RM-IM) in atmospheric systems. Experiments which may affect the reaction by varying the conditions (wheat germ oil and ethanol ratio; 1.0 to 4.0 mole ration, temperature; 30℃ to 70℃, enzyme load; 1-5 w% of wheat germ oil, reaction time; 1 to 24hrs) were carried out ethanolysis. The molecular weight of wheat germ based on the triglyceride molecular weight was 863.3g/mole. And ethanol molecular weight was 46 g/mole and density of wheat germ oil was 0.922 g/mL and ethanol was 0.733 g/mL. The reactions were carried out in 25 mL erlenmeyer flasks containing mixtures of 5 g wheat germ oil and ethanol (99.9%) ranging from 1.0 to 4.0 mole ratio based on wheat germ oil using lipozyme TL-IM and lipozyme RM-IM.

Wheat germ oil was mixed with ethanol for emulsification of the reaction mixture, after which immobilized lipases was added. The reaction mixture was incubated at 40 to 70℃, 120 rpm in shaking incubator (HB-201SF: hanbaek Scientific Co). The reaction products were obtained by removing the debris and the enzyme and dissolved in the mixture of methanol, were analyzed by HPLC-UV. The reaction products were analyzed during storage in the freezer at -20℃.

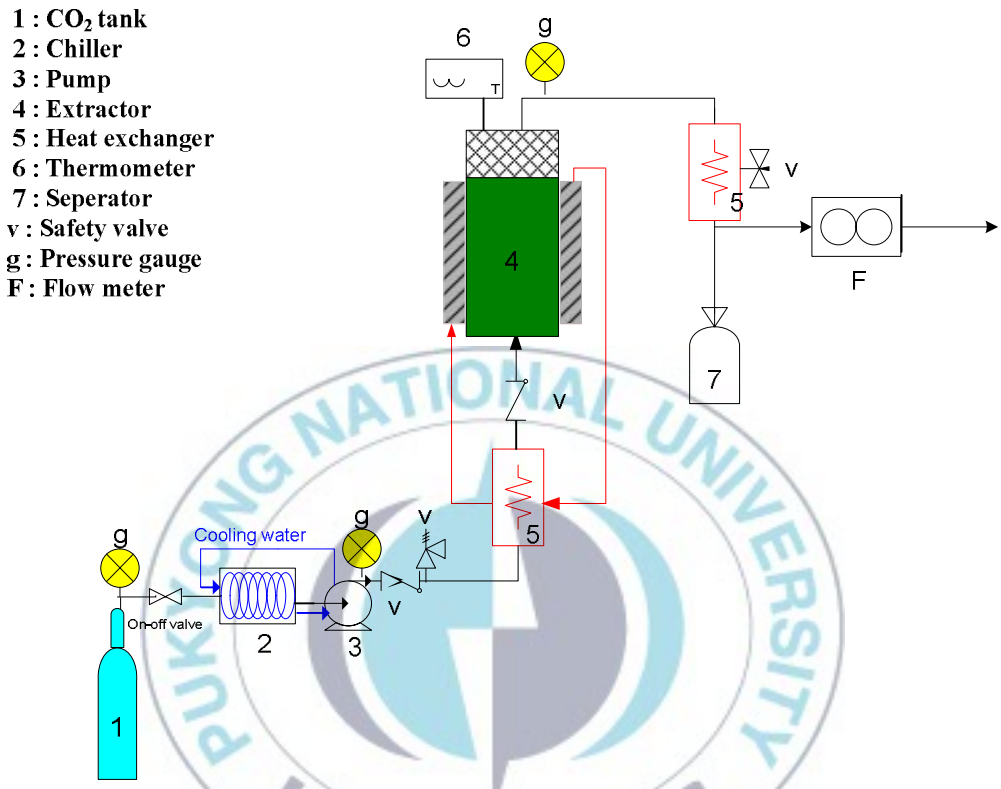

Fig. 3. Schematic diagram of SC-CO_2 extraction process.

2.2.3 Ethanolysis in SC-CO_2 System using Immobilized Enzyme

The set up of a laboratory scale of SC-CO_2 system is shown in Fig. 4. The stainless steel reaction vessel which was 55 mL in internal volume (112 mm x 25 mm i.d) consists of a stainless (SS-316), sight glass, spin bar stirrer and water jacket. Samples (extracted by SC-CO_2) were loaded in to the stainless steel reaction vessel with ethanol and enzyme. After injection, reactor was closed with reactor's head pressure was increased. The reaction temperature was maintained by connecting the reaction vessel with water bath and temperature was measured using FLUKE 52(FLUKE, USA) continuously. Samples of the effluent were taken in to sealed vials at periodic intervals (at 10, 20, 30, 60, 120 min, respectively). Finally, the reactor was depressurized to atmospheric and hexane added to the residue for washing. The effect of temperature and pressure studied at 40-60℃ and 7-10 MPa at constant reaction time of 2 hrs.

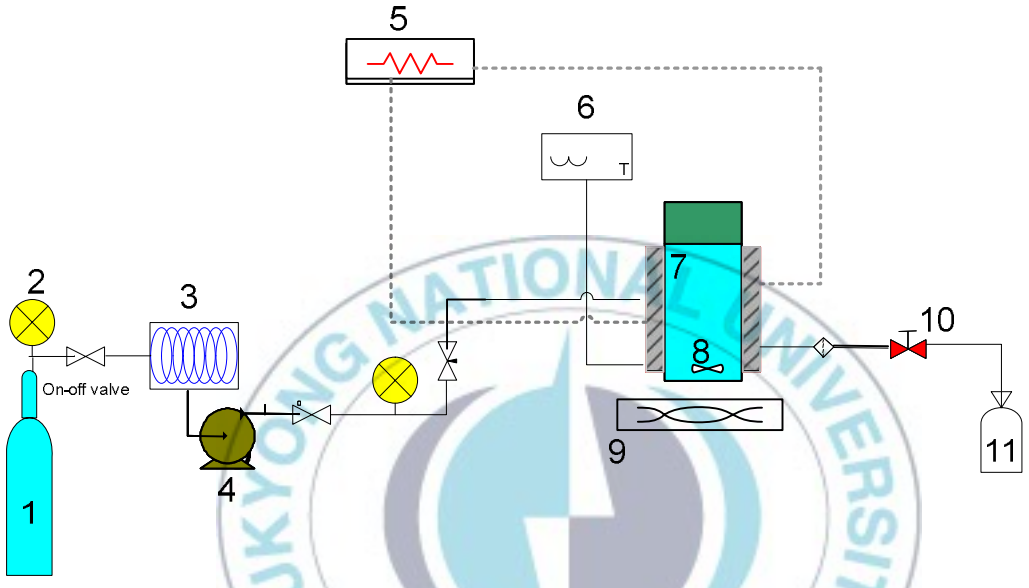

Fig. 4. Schematic diagram of the apparatus for enzymatic ethanolysis in supercritical carbon dioxide: 1. CO_2 cylinder, 2. Pressure gauge, 3. Cooling bath, 4. High pressure pump, 5. Heat exchanger, 6. Thermometer, 7. Reactor, 8. Magentic bar, 9. Stirrer, 10. Mitering valve, 11. Separator

2.3 Analytical methods

2.3.1 Gas chromatography (GC)

Prior to the experiment, wheat germ oil was performed by GC (HP5890 Series II, USA) to investigate the fatty acid composition. A Hewlett Packard gas chromatography with an Agilent DB-Wax capillary column (30m length × 0.250 mm internal diameter, 0.25 ㎛, Agilent) was used. The fatty acid methyl esters were prepared firstly according to AOCS official method of Ce 2-66 (AOCS, 1998b). Nitrogen at flow rate of 1mL/min was used as a carrier gas. The oven temperature was programmed at a constant temperature of 130℃ for 3 min, and then increased to 240℃ at a rate of 4℃/min and at 240℃ for 10 min. Injector and detector were used at a temperature of 250℃. It was assumed that the ratio of single component peak area to total peak area is the mass fraction of the component. Wheat germ oil methyl esters were identified by comparison of retention time with lipid standard (fatty acid methyl esters mixture; Supelco, USA). Heptadecanoic methyl ester served as internal standard.

2.3.2 Thin layer chromatography (TLC)

Wheat germ oil was separated by TLC to know fatty acid profile of mono-, di-, and triglyceride. Separation of individual lipid was performed by thin layer chromatography using 20×20 cm aluminum foil-backed plates pre-coated with 0.2 mm layer of a silica gel 60 (ALUGRAM®SIL G/UV254; Macherey-Nagel, Duren, Germany). The reaction lipid solutions were prepared in isopropylalcohol/hexane (5:4, v/v; 4000ppm). Solutions of the reactant were spotted onto a chromatographic plate in 1μL quantities. The mobile phase was composed cyclohexane: athylacetate (3:2 v/v). The development tank was saturated with solvent for 20min. The chromatogram was developed at the room temperature. After the solvent had risen to near the top of the plate (about 1cm from the top), the plate was removed. The plate was kept in the hood until the majority of the eluting solvent had evaporated from the plate about 10 min. Spots were visualized by iodine vapor.

2.3.3 High-performance liquid chromatography (HPLC)

HPLC analysis was carried out on a Waters HPLC equipped with a model 600 E system controller, a model 484 UV/VIS detector and a Atlantis®dC18 column (5 μ, 4.6 × 150 mm, Waters, USA). The different species of lipids as tri-, di-, mono-glycerides and ethyl esters were analyzed by the method of Holcapek et al. (Table 1). The mobile phase consisted of (A) methanol and (B) isopropylalcohol-hexane (5:4, v/v). A linear solvent gradient program was employed: at 0 min, 100/0; at 15min, 50/50; at 25min, 50/50; at 30min, 0/100 (%A/%B, respectively).

Samples (30μL) were dissolved in isopropanol-hexane (5:4, v/v) (1mL). The injection volume was 20μL and the flow-rate 1 mL/min. Mono-, di, triglyceride was detected at wavelength of 205 nm. The amount of MG, DG and TG in the extract was measured based on peak area of standard mono-, di-, triglyceride mix (Supleco, USA).

Table 1. HPLC conditions for the detection of tri, di, monoglyceride

Instrument	:	Waters 600E
Column	:	Atlantis® dC18 5 μ(4.6×150 mm, Waters, USA)
Column temp.	:	Room temp
Detector	:	Waters 484 UV Dectector
Wavelength	:	205 nm
Injection volumn	:	20 μL
Flow rate	:	1.0 ml/min
Moble phase	:	Methanol Hexane : Isopropanol (4:5)(v/v)

2.4 Measurement of oil stability

2.4.1 Free fatty acid content of ethanolysis reactants

Free fatty acid (FFA) of extracted oil from ethanolysis reactants were analyzed as describe by Bernardez et al. Accurately, 50 mg of oil was placed into pyrex tubes with the addition of 3 mL of cyclohexane and then 1 mL of cupric acetate-pyridine reagent was added. Tubes were vortexed for 30 sec. After centrifugation at 2000 g for 10 min, the upper layer was read 710 nm. The FFA content of oil was measured on a calibration curve constructed from oleic acid standard (Fig. 5).

Copper reagent was prepared according to Lowry and Tinsley. Briefly, 5% (w/v) aqueous solution of cupric acetate was prepared and filtered. Then the pH of cupric acetate solution was adjusted to 6.1 using pyridine.

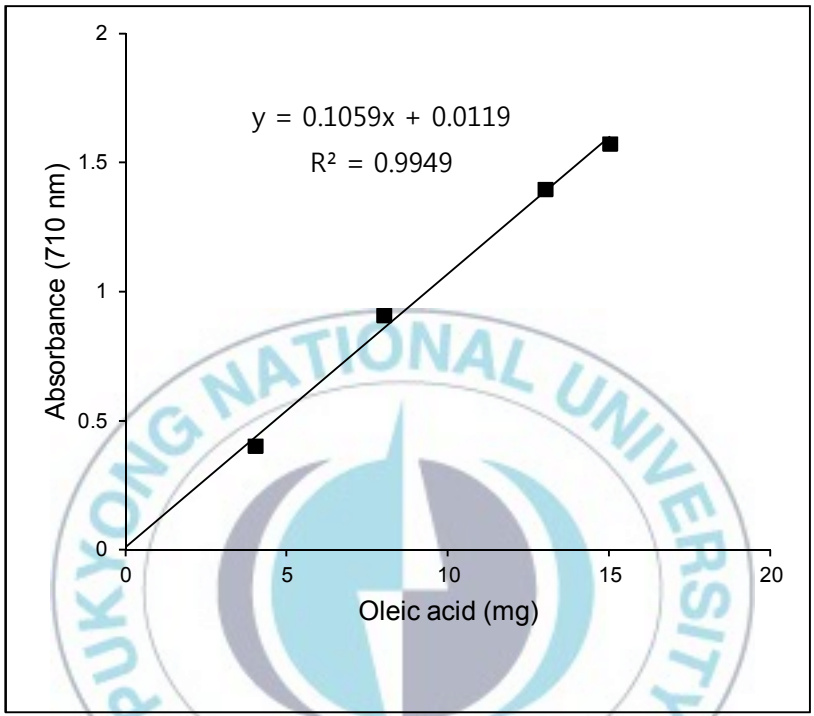

Fig. 5. Calibration curve of oleic acid for estimation of free fatty acids in ethanolysis reactants.

2.4.2 Acid Value

The acid value was assessed according to the method described previously by P. Sun et al. (2008); one g of sample was dissolved in 100 mL of ether: ethanol (1:1) and shaking. Then phenolphthalein as an indicator was added drop wise addition. Acid value of oil was analyzed by titration with 0.1N KOH-ethanol solution until the pink colour persists for at least 30s. The acid value calculated using following equation.

$$\text{Acid value (AV)} = 5.611 * A * F/S$$

A : Volume of the KOH-ethanol solution of the titration (mL)

F : Concentration of the KOH-ethanol factor

S : Mass of Oil (g)

5.611 : Molecular weight of KOH in mg

2.4.3. Peroxide Value

Peroxide value was determined by the AOCS method Cd 8-53 (AOCS, 1998) with modified amount of sample taken. 1g sample was dissolved in 30mL of chloroform: acetic acid (2:3). Then the 1mL of saturated KI solution was added to the mixtures and allowed the solution to stand with occasional shaking for 1min. 30mL of distilled water was immediately added to the solution and then shakes. Then the 1% starch indicator solution was added drop wise addition. The peroxide value of oil was analyzed by titration with 0.1N sodium thiosulfate ($Na_2S_2O_3$) until the yellow iodine color had almost disappeared. A blank determination was conducted with same procedure. The peroxide value calculated using following equation. Peroxide value was expressed as milliequivalents peroxide/1000 g sample.

$$\text{Peroxide value (POV)} = (S - B) \times N \times 1000 / M$$

S : Volume of titrant, mL of sample

B : Volume of titrant, mL of blank

N : Normality of sodium thiosulfate solution

M : Mass of sample, g

2.4.4 Thiocyanate method

To measure the oxidative stability, the oxidation of emulsion of ethanolysis reactant was in water (deionized and degassed water) at 37°C. Three emulsions of ethanolysis reactant in water (w/w) (ethanolysis reactant 5%, water 95%; astaxanthin 2%, ethanolysis reactant 4%, water 94%; linoleic acid 10%, water 90% (control)) were prepared. The mixtures were properly homogenized by a homogenizer. Oxidative stabilities were checked by thiocyanate (Mistuda et al., 1996) which was used to measure the antioxidant activity.

The peroxide formed by lipid peroxidation reacts with ferrous chloride and form ferric ions. Ferric ions then unite with ammonium thiocyanate and produce ferric thiocyanate. Briefly, 0.1 mL of emulsion solution was added to 4.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 0.02 M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance of red color was measured at 500 nm (UVIKON 933, Kontron Instrument). A blank determination was conducted with same procedure. Every 24 hr interval during incubation, the absorbance was recorded.

2.4.5 DPPH radical scavenging activity

The scavenging of DPPH radical was carried out according to the method described by Hsu et al. (2003). Aliquots of 1 mL methanolic samples and 5 mL of freshly prepared 0.1mM DPPH methanolic solutions were thoroughly mixed, and kept for 50 min in the dark. The absorbance of the reaction mixture at 517 nm was read with a spectrophotometer (UVIKON 933, Kontron Instrument). Methanol (1 mL), replacing the extract, was used as the blank. The percentage of free radical scavenging effect was calculated as follows:

$$\text{Scavenging effect (\%)} = [1-(A_{517\ nm,\ sample}/\ A_{517\ nm,\ blank})] \times 100$$

2.4.6. Rancimat test for antioxidant activity

A Metrohm Rancimat model 743 (Methrom Instruments, Herusau, Swizerland) was utilized. A steam of filtered, cleaned and dried air at flow rate 20 L/hr is bubbled into samples (3 g) contained in reaction vessel. These vessels are placed in an electric heating block which is set at 110℃. Effluent air containing volatile organic acids from the oil samples are collected in a measuring vessel with 60 mL of distilled water. The conductivity of the water is continuously recorded and the OSIs of the oil samples were automatically recorded at 120℃. In each time, oil samples were accommodated in the equipment and analyzed simultaneously. The oil samples for all determinations were randomized to determine their position in the heating block.

2.5 Enzyme activity

2.5.1. Lipases assay

Lipase activity was assayed using the modified method of Vorderwülbecke et al. (1992) described by Hatzinikolaou et al. (1999). The substrate emulsions were prepared by drop wise addition of 0.2 mL solution A (40 mg of *p*-nitrophenyl-laurate was dissolved in 12.0 mL of isopropanol) into 3.0 mL solution B (0.4 g Triton X-100 and 0.1 g gum arabic were dissolved in 90 mL of 0.1 M potassium phosphate buffer, pH 7.0) under intense vortexing. These emulsions were stable for 1 hr at room temperature. 0.1 mL of the crude extract was added to 3.2 mL of the substrate emulsion and the mixture was incubated for 20 min in a shaking water bath at 35°C. The reaction was terminated by boiling for 5 min. Following centrifugation (6000 rpm, 10 min) the absorbance of the clear supernatant was measured at 410 nm.

2.6 Statistical analysis

All determinations were carried out in triplicate, and statistical analyses were performed according to the Microsoft office excel 2007 (Microsoft Corporation, USA). Significant differences between means were determined by Duncan's multiple range tests. *P* values less than 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Fatty acid Composition

Fatty acids are merely carboxylic acids with long hydrocarbon chains which are either saturated or unsaturated. It can be an indicator of its stability, physical properties, and nutritional value. The fatty acid compositions of the oil obtained by SC-CO_2 extraction are shown in Table 2. As shown in Table 2 wheat germ extracted oil distributed 9 different fatty acids. The components are palmitic acid, palmitoleic acid, stearic acid, oleic acid, elaidic acid, linoleic aicd, arachidic acid, ɣ-linolenic acid and heneicosanoic acid. The most predominant fatty acid was oleic acid.

The average molecular weight of wheat germ oil was calculated and it was found 863.3 g/mol from fatty acid composition.

Table 2. Fatty acid composition and molecular weight of wheat germ oil extracted organic and supercritical fluids

Fatty acid (%)	Wheat germ oil	FAME M.W
Palmitic Acid	18	256.42
Palmitoleic Acid	0.17	254.41
Stearic Acid	0.7	284.48
Oleic Acid	16.5	282.46
Elaidic Acid	1.35	282.46
Linoleic Acid	57.69	280.45
Arachidic Acid	0.28	312.53
ɣ-Linolenic Acid	0.19	278.43
Heneicosanoic Acid	5.12	326.56
	100	

3.2. Ethanolysis in atmospheric system using immobilized enzyme

3.2.1. Characteristic of enzymatic ethanolysis

For fatty acid analysis, the individual mono-, di-, tri-glyceride and ester were separated by TLC (Fig. 6). TLC is a relatively simple experiment and able to find the composition of the lipids rapidly. So it was performance before HPLC analysis.

Before and after the reaction chromatogram of wheat germ oil is shown in the Fig. 7 and Fig. 8. After reaction, triglyceride contents significantly decrease and mono-, di-glyceride and ester contents increase. The results in all the Reaction were measured by conversion to total%.

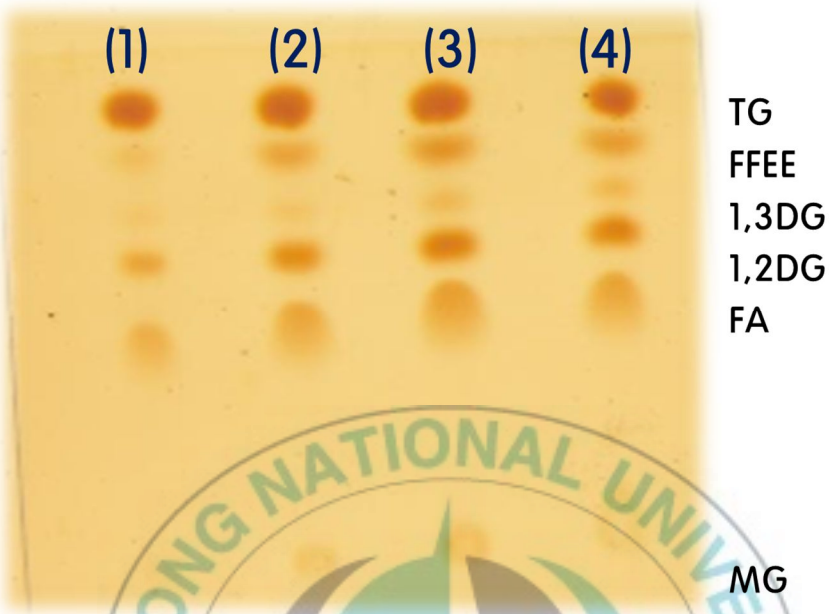

Fig. 6. Enaymayic ethanolysis of wheat germ oil using ethanol 99% at T=60 ℃, 120 rpm, 2w% of Lipozyme TL-IM: (1) 1hr, (2) 2hr, (3) 3hr, (4) 4hr.

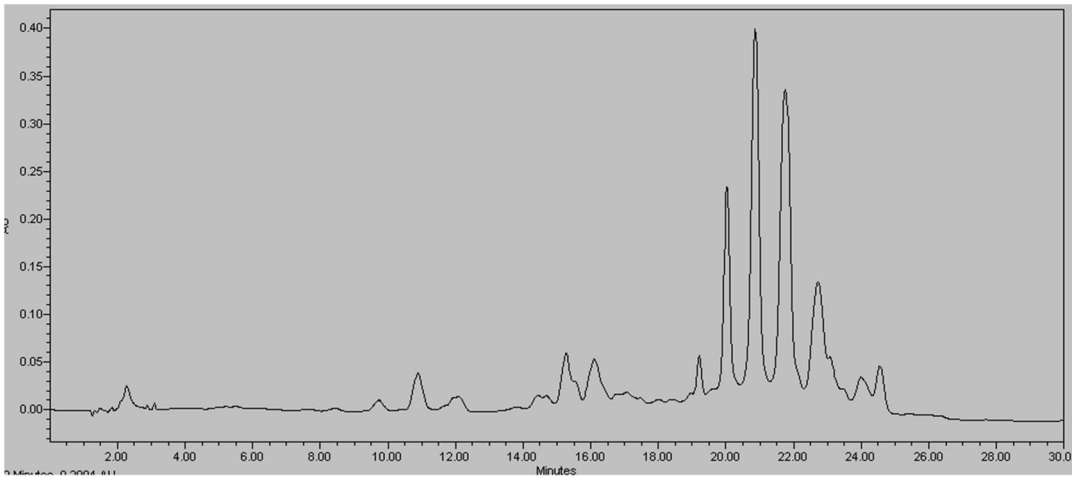

Fig. 7. HPLC-analysis of lipid compounds of wheat germ oil

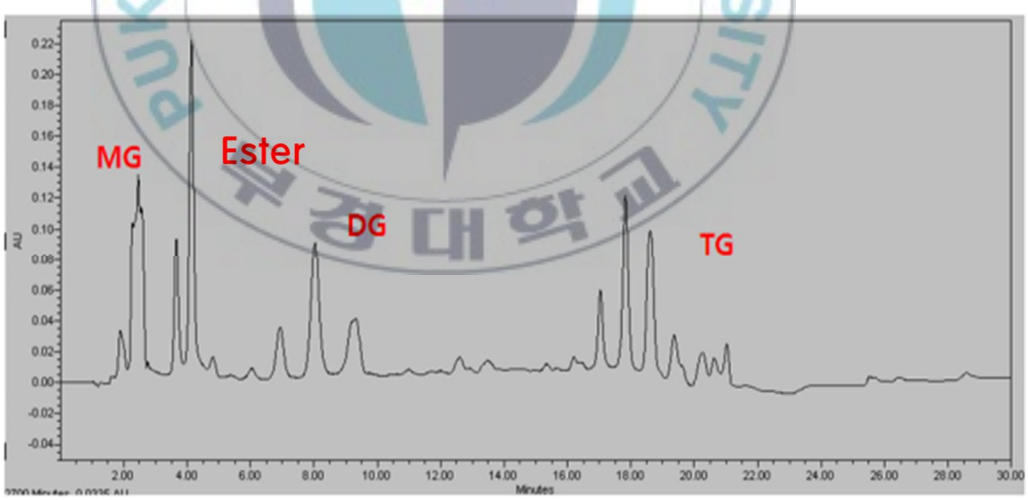

Fig. 8. HPLC-analysis of lipid compounds of wheat germ oil after ethanolysis

3.2.3. Effect of mole ratio on enzymatic ethanolysis

Generally, the high substrate mole ratio favors formation of the products… However, for enzymatic ethanolysis, the highest ethanolysis of wheat germ oil by immobilized lipases occurred at near a mole ratio of 1 to 1.5 ethanol to wheat germ oil (Re =1 or 1.5). From this Fig. 9 and Fig. 10, the optimized ethanol ratio for obtaining highest conversion is mole ratio 1 to 2 using lipozyme TL-IM, lipozyme RM-IM. When the mole ratio increased to 3, the enzyme activity decreased significantly. The reason, it suggested that ethanol inhibit enzyme activity (Simada et al., 2002).

3.2.4. Effect of temperature on enzymatic ethanolysis

The influence of temperature on the enzymatic ethanolysis was studied in a fixed condition at mole ratio (Re) of 1.0, 5w % lipase, 120 rpm shaking speed and 8hrs of reaction time. Wheat germ oil used supercritical fluid extraction at 200 bar and 40℃.

Experiment temperatures ranging from 40 to 70℃ were set up. The effect of temperatures on enzymatic ethanolysis of wheat germ oil is shown in Fig. 11. The enzyme activity was estimated as the amounts of free fatty acid ethyl ester (FFEE) formed. From Fig. 11, the optimized temperature for obtaining the highest conversion is 60℃. The reason would be that immobilization reduces the susceptibility to heat. And the most immobilized lipases are included between 30℃ and 62℃, where as free lipases tend to be slightly lower (Akoh et al., 1998). Temperatures higher than 60℃ lessen the productivity due to enzyme denaturation and most proteins become nastier beyond 60℃.

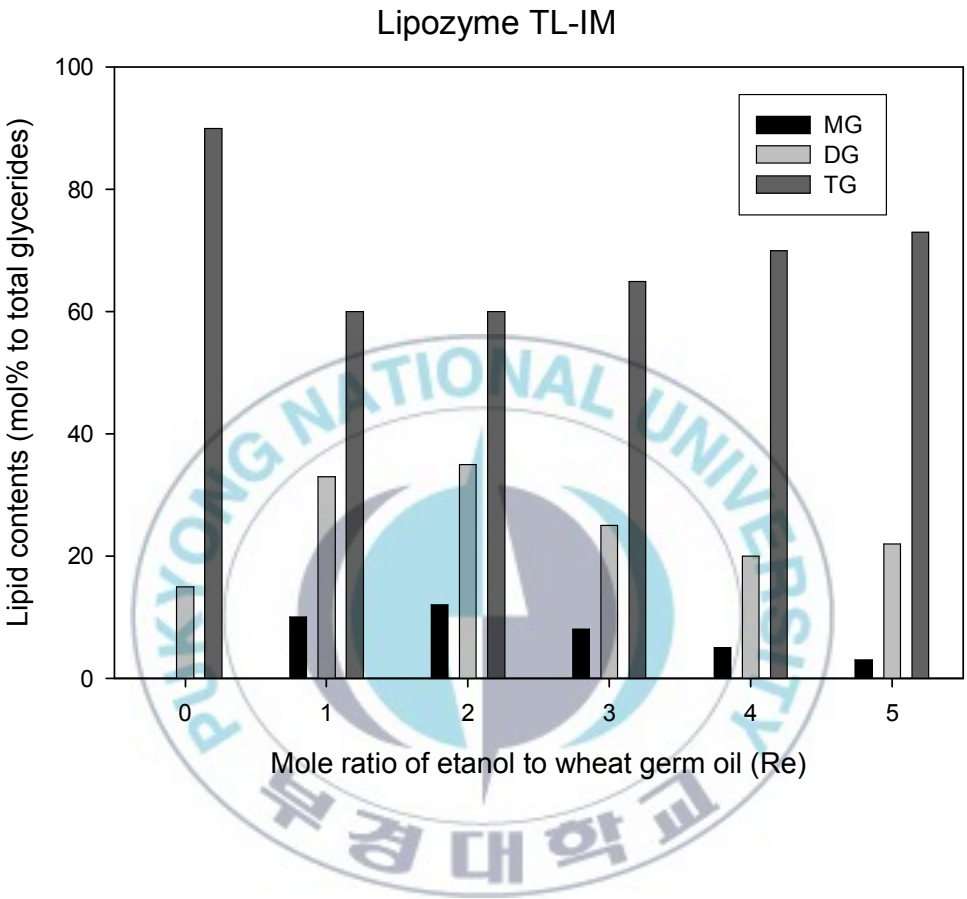

Fig. 9. Effect of ethanol mole ratio to wheat germ oil at 60℃, 3% lipozyme TL-IM, 120 rpm, 8hr.

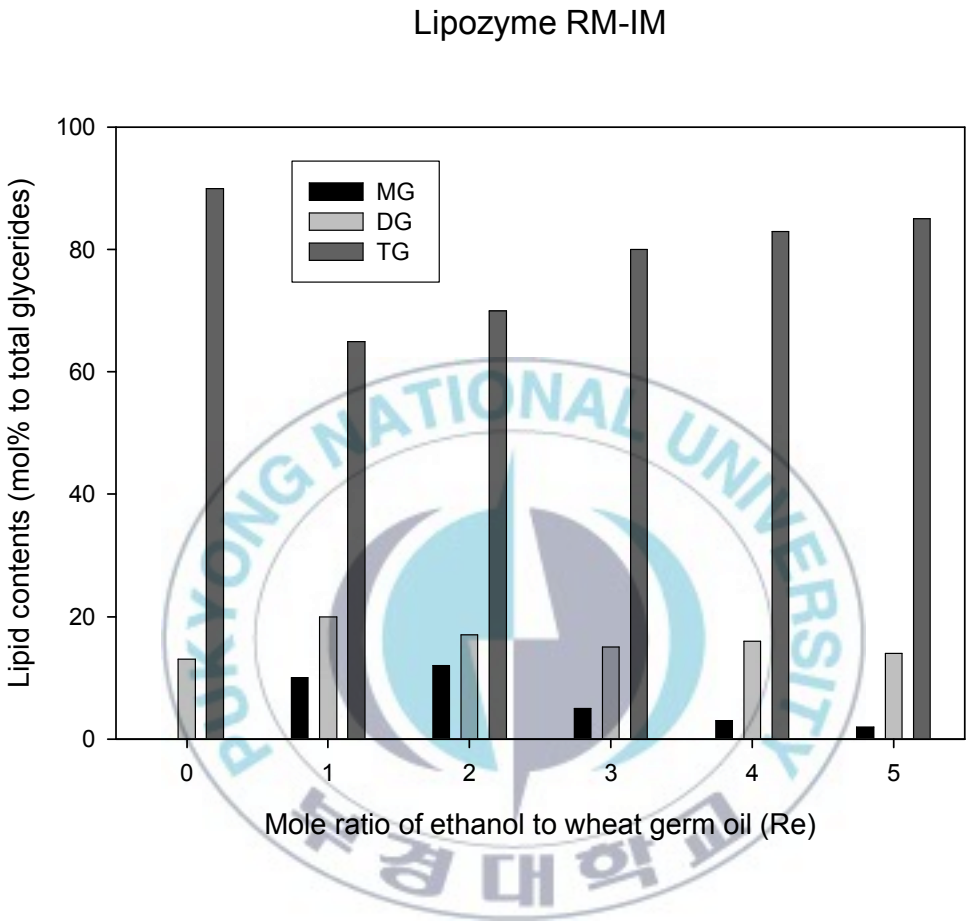

Fig. 10. Effect of ethanol mole ratio to wheat germ oil (Re) at 60℃, 3% lipozyme RM-IM, 120 rpm, 8hr

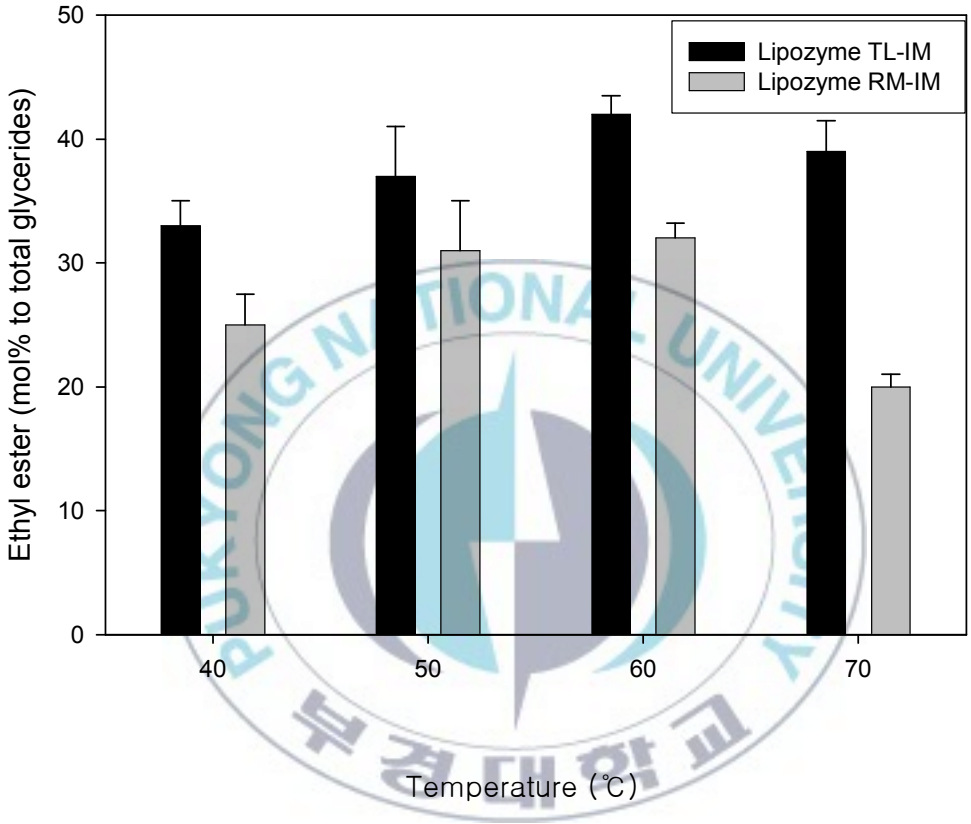

Fig. 11. Effect of temperature to wheat germ oil at 1.0 ethanol mole ratio, 5% lipozyme TL-IM, 120 rpm, 8hrs

3.2.5. Effect of enzyme load on enzymatic ethanolysis

The influence of enzyme load on the enzymatic ethanolysis was studied in a fixed condition at mole ratio (Re) of 1.0, 40℃, 120 rpm shaking speed and 8hrs of reaction time. Wheat germ oil used supercritical fluid extraction at 200 bar 40℃

Experiment enzyme load ranging from 2 to 4wt% were set up. The effect of enzyme load on enzymatic ethanolysis of wheat germ oil is shown Fig. 12. From this Fig. 12, the optimized temperature for obtaining the highest conversion is 4wt%. All of the lipozyme TL-IM and lipozyme RM-IM increase to 4wt% and then there is almost never change. It means that even adding more than 4wt% of the enzyme usage does not affect the yield of MG and DG.

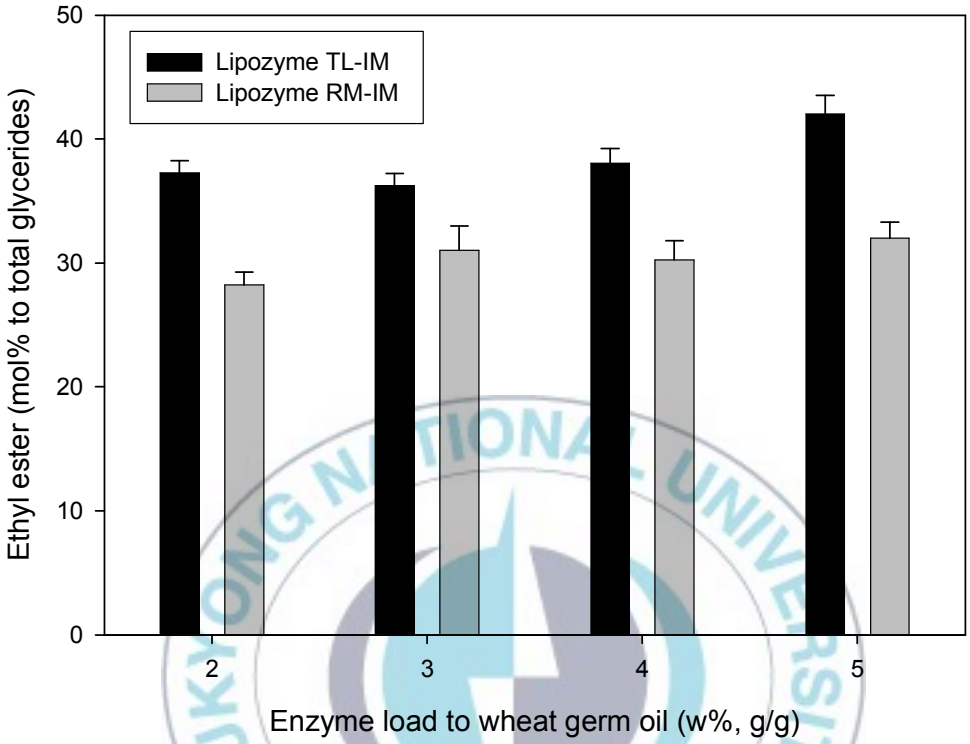

Fig. 12. Effect of enzyme load to wheat germ oil at 1.0 ethanol mole ratio, 60℃, 120 rpm, 8hr

3.2.6. Effect of reaction time on enzymatic ethanolysis

3.2.6.1. Initial reaction tendency

The influence of initial reaction tendency on the enzymatic ethanolysis was studied in a fixed condition at mole ratio (Re) of 1.0, 40℃, 4 w% lipase and 120 rpm shaking speed. Wheat germ oil obtained by supercritical fluid extraction at 200 bar and 40℃ was used.

Experiment reaction times ranging from 1 to 8 hrs were set up. The effect of reaction time on enzymatic ethanolysis of wheat germ oil is shown Fig. 13 and Fig. 14. Initial reaction, the conversions increase rapidly at 2 hr. After, the conversion rate was almost no change. And initial reaction tendency, lipozyme RM-IM is faster than lipozyme TL-IM.

3.2.6.2. Ethanol graded addition

The influence of reaction time (ethanol graded addition) tendency on the enzymatic ethanolysis was studied in a fixed condition at mole ratio of 1.0, 40℃, 4w% lipase and 120 rpm shaking speed.

And it added ethanol of mole ratio 0f 1.0 per 8 hours for 24 hrs. From this Fig. 15, the conversions increase for 2^{nd} ethanol addition. But 3^{rd} ethanol addition, the conversions decrease. Because it assumed that reverse reaction progress from 3^{rd} ethanol addition.

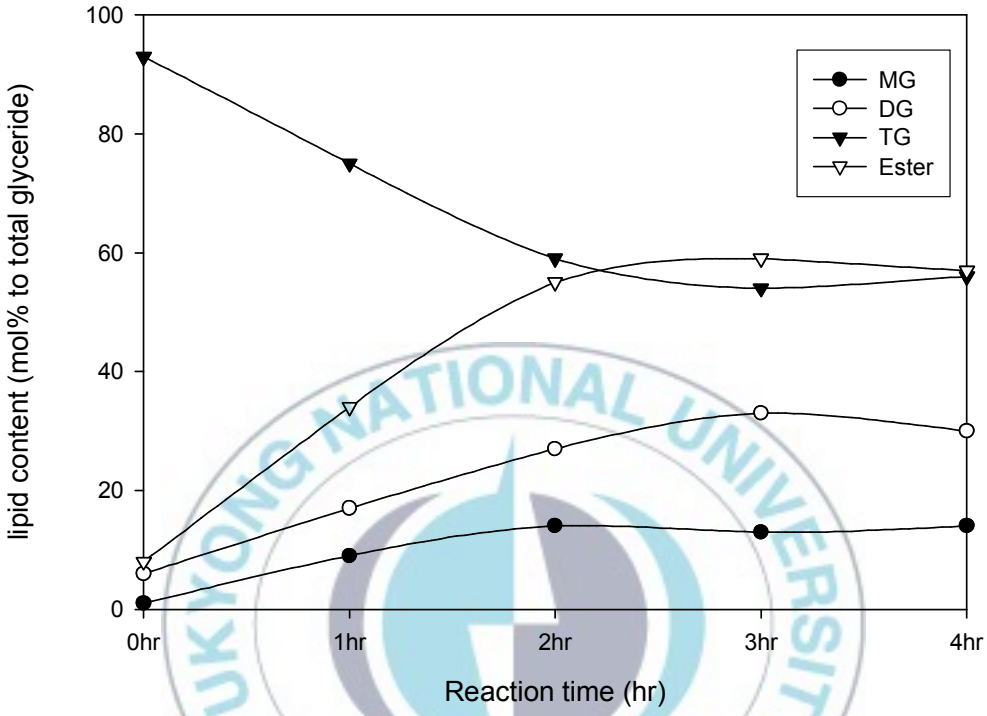

Fig. 13. Effect of initial time to wheat germ oil at 1.0 ethanol mole ratio, 60℃, 4% lipozyme RM-IM, 120 rpm, 4hr

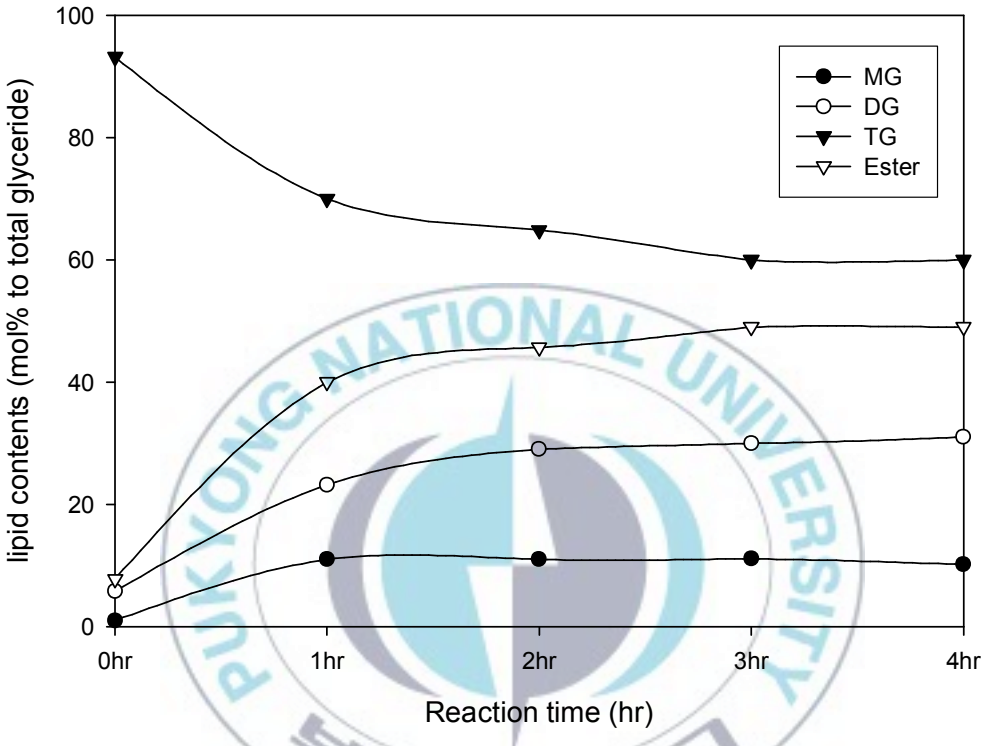

Fig. 14. Effect of initial time to wheat germ oil at 1.0 ethanol mole ratio, 60℃, 4% lipozyme RM-IM, 120 rpm, 4hr

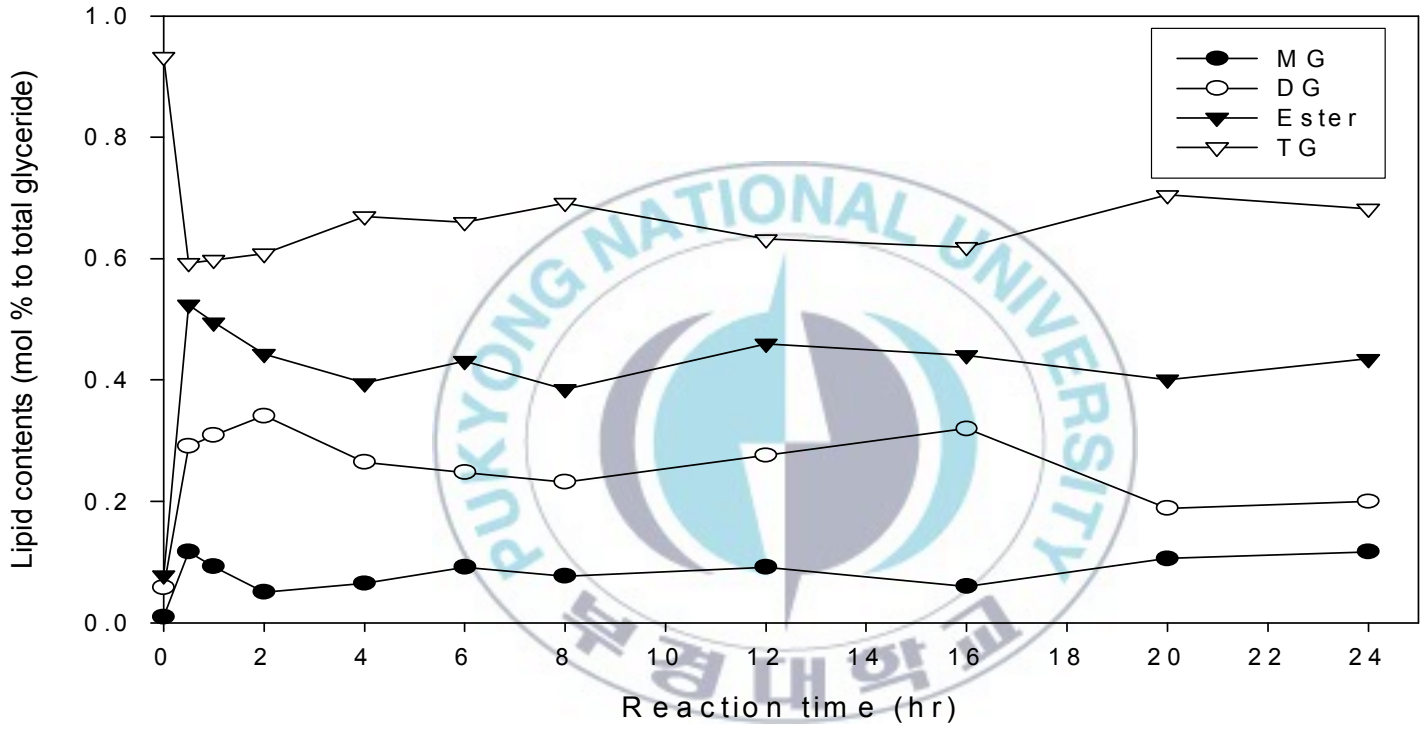

Fig. 15. Effect of time to wheat germ oil at 1.0 ethanol mole ratio (ethanol graded addtion, 60℃, 4% lipozyme RM-IM, 120 rpm, 24hrs

3.2.7. Comparison of conversion in organic extraction and SC-CO_2 extraction

In order to compare with organic solvent extraction and SC-CO_2 extraction was performed enzymatic ethanolysis at mole ratio of 1.0, 60℃, 4w% lipase and 120 rpm shaking speed (Fig. 16).

From this Fig. 16, conversion of SC-CO_2 extraction was higher than organic solvent extraction.

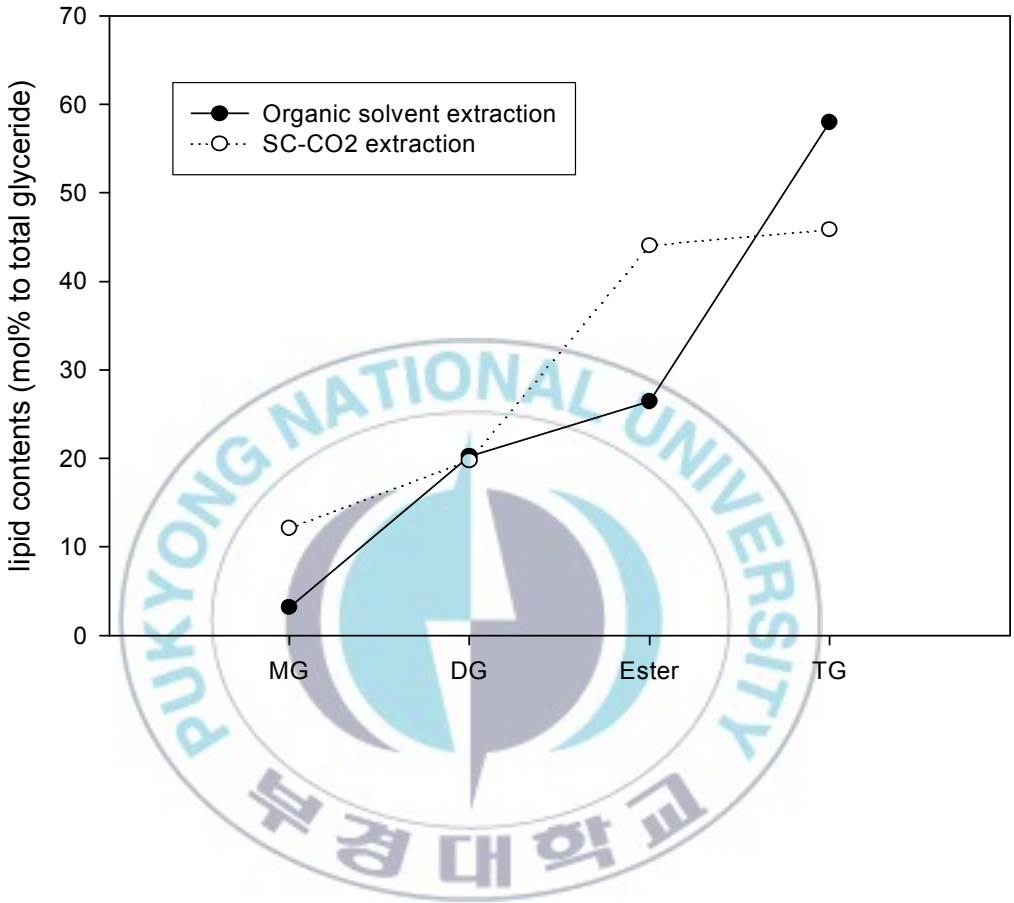

Fig. 16. Compare of lipid contents to organic solvent extraction and SC-CO2 extraction

3.3. Ethanolysis in pressured system using immobilized lipase

3.3.1. Effect of temperature in pressured system

The influence of temperature in pressured system was studied in a fixed condition at mole ratio (Re) of 1.0, 4w% lipase and 2hrs of reaction time. Wheat germ oil used supercritical fluid extraction at 200bar 40℃.

Experiment temperatures ranging from 40 to 60℃ were set up, at constant pressure of 8 MPa. The effect of temperatures on enzymatic ethanolysis of wheat germ oil is shown Fig. 17. The enzyme activity was estimated as the amounts of free fatty acid ethyl ester (FFAEE) formed. From this Fig. 17, the optimized temperature for obtaining the highest conversion is 50℃.

As increasing temperature increases has a positive effect on the kinetic constant, as defined by the transition state theory. However, high temperatures may promote side reactions, such as dehydration and denaturation if their activation energy is higher than that of esterification, reducing the ester yield (Hassan et al., 2006).

3.3.2. Effect of pressure in pressured system

Generally, pressure affects reaction rate in supercritical fluids can vary some physical parameters, such as the partition coefficient, dielectric constant, and fluid Hildebrand solubility parameter (Hassan et al., 2006).

The influence on enzymatic ethanolysis in pressured system was performed in a fixed condition at mole ratio (Re) of 1.0, 4w% lipase and 2hrs of reaction time. Wheat germ oil used supercritical fluid extraction at 200bar 40℃. Experiments pressure ranging from 6 to 10 MPa were set up. Temperature was fixed at 50℃ because it is related with enzyme activity and stability.

As it is shown in Fig. 18, the conversion yield was increased as the pressure increased. However, Kwon et al. (2009) reported that the effect of increasing pressure diminished above 12.5 MPa. Drescher et al. (2002) also reported that the concentration of ethanol in liquid phase slowly increased when pressure increased from 7 to 10 MPa at constant temperature of 50ºC.

3.3.3. Comparison of conversion in non-pressured system and pressured system

In order to compare with non-pressured system and pressured system was performed enzymatic ethanolysis at mole ratio of 1.0, 50℃, 4w% lipase (Fig. 19).

Fig. 19 shows that the effect of pressure was beneficial on the condition. Generally, change in pressure of CO_2 influences the density and transport properties such as viscosity, thermal conductivity, and diffusivity (Nagesha et al., 2004). Also, pressure can directly affect the reaction rate by changing the concentrations of reactants and products in solution because the partitioning of reaction components between the two phases depends on pressure (Erickson et al., 1990).

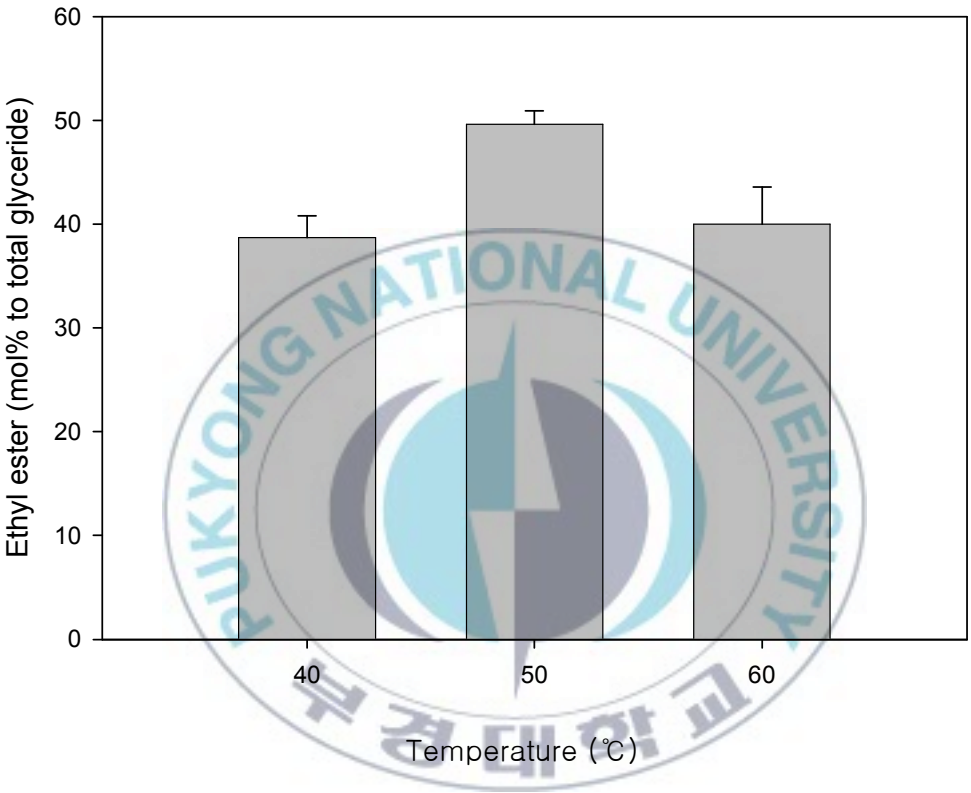

Fig. 17. Effect of temperature to wheat germ oil at 1.0 ethanol mole ratio, 4% lipozyme, 8MPa, 2hrs

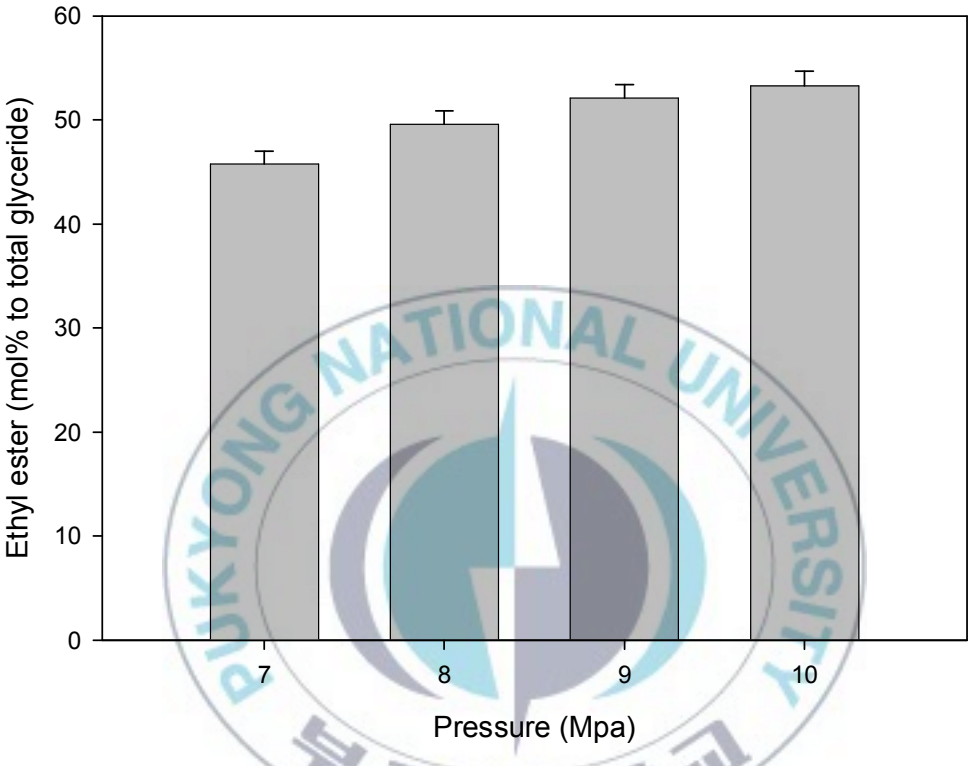

Fig. 18. Effect of pressure to wheat germ oil at 1.0 ethanol mole ratio, 4% lipozyme, 50ºC, 2hrs.

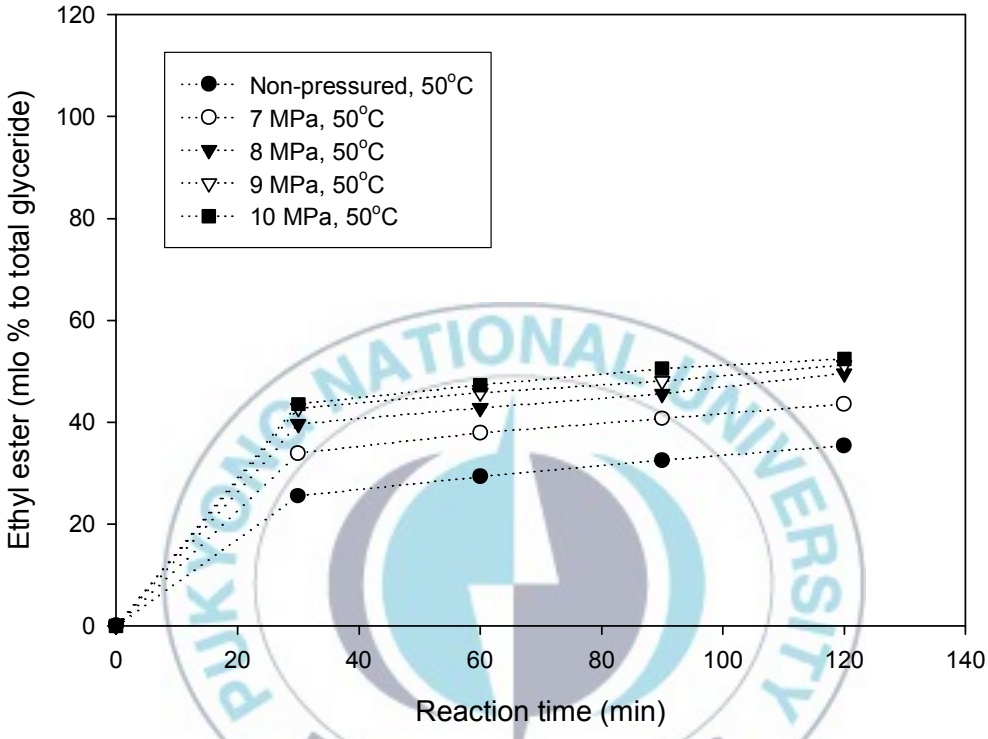

Fig. 19. Compare of non-pressured system and pressured system to wheat germ oil at 1.0 ethanol mole ratio, 4% lipozyme TL-IM, 50ºC, and 2hrs.

3.4 Characterization of ethanolysis reactants

3.4.1 Fatty acid composition of ethanolysis reactants

Fatty acids compospiton of the oil obtained by enaymatic ethanolysis are shown in Table 3. Fatty acids were identified in different reaction. Fatty acids were identified in different reaction temperature. There were no remarkable changes in the fatty acid composition wheat germ oil obtained by enzymatic ethanolysis, because esterification is converted lipid contractual.

3.4.2 Oil Stability

3.4.2.1 Comparison of free fatty acid of enzymatic ethanolysis reactant

Oxidation of oil is related with free fatty acid (FFA) contents. The quality of oil is deteriorated at production and storage conditions. In this study, FFAs of enzymatic ethanolysis reactants were compared and are given in Fig. 20. It was found that as the temperature increase, the amounts of FFA significantly increase. This result agreed with higher temperature caused a significant increment of the FFA in the byproduct oil (Rubio-Rodriquez et al., 2008).

3.4.2.2 Comparison of acid value and peroxide value of enzymatic ethanolysis reactants

In the present study, storage properties of obtained by enzymatic ethanolysis reactant were compared. Acid value (AV) and peroxide value (POV) was carried out to measure the quality of the oil and oxidation state of lipid. It is used to mesasure the rancidity which occurs by auto oxidation state of lipid.

It was also found that as the temperature increase, the amount of AV and POV increase (Fig. 21 and Fig. 22). This result associated with FFAs contents. It was also found that higher temperature caused a significant increment of the AV and POV.

Table 3. Major fatty acid composition of wheat germ oil obtained by enzymatic ethanolysis reaction

Fatty acid (%)	Before reaction	After reaction			
	Wheat germ oil	40℃	50℃	60℃	70℃
Palmitic Acid	18	16.43	16.58	16.54	16.52
Palmitoleic Acid	0.17	0.16	0.16	0.16	0
Stearic Acid	0.7	1.19	1.21	1.21	1.21
Oleic Acid	16.5	16.61	16.59	16.59	16.6
Elaidic Acid	1.35	1.36	1.36	1.36	1.37
Linoleic Acid	57.69	57.67	57.51	57.55	57.71
Arachidic Acid	0.28	0.27	0.27	0.27	0.25
ɣ-Linolenic Acid	0.19	0.19	0.18	0.19	0.19
Heneicosanoic Acid	5.12	6.12	6.13	6.14	6.16
	100	100	100	100	100

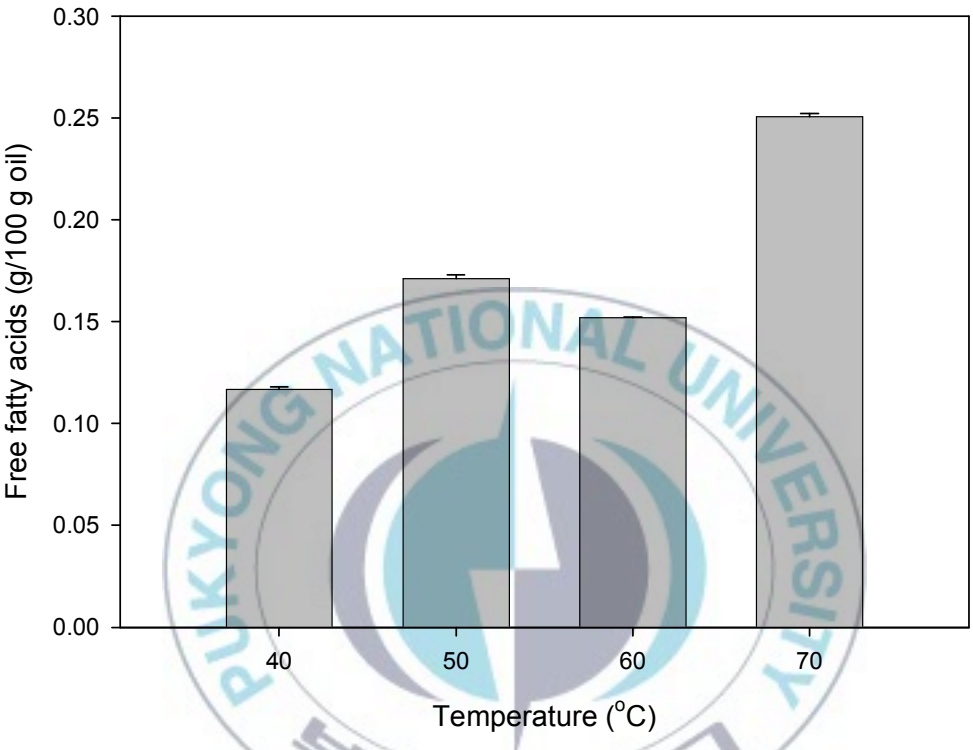

Fig. 20. Free fatty acid of wheat germ oil by ethanolysis reactants.

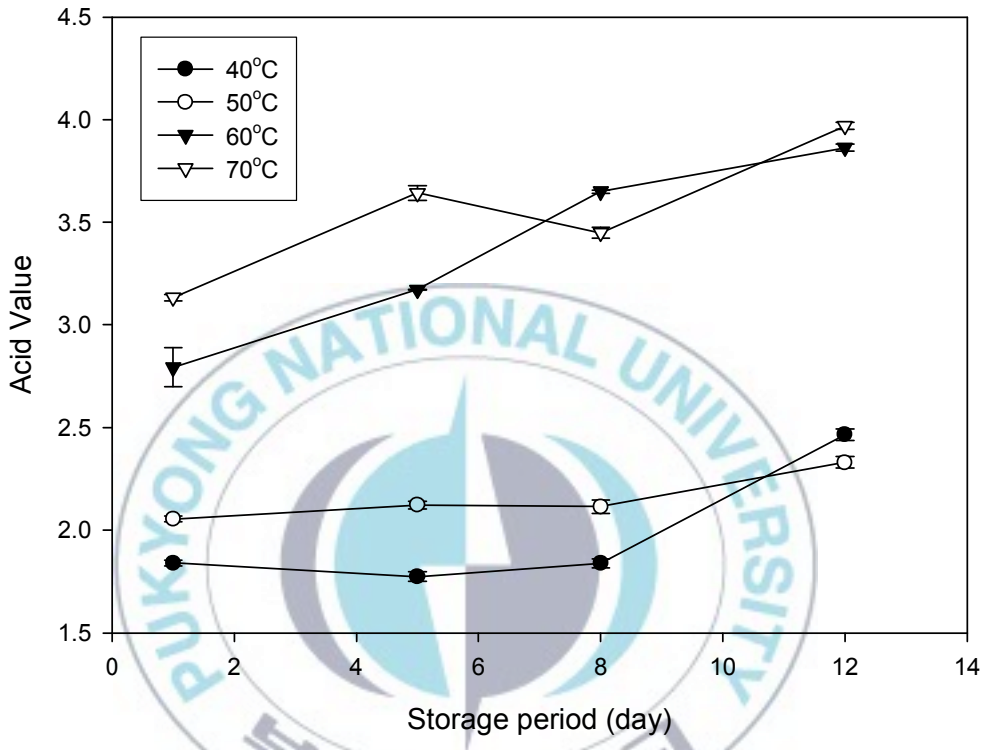

Fig. 21. Comparison of the acid value of enzymatic ethanolysis of reactants.

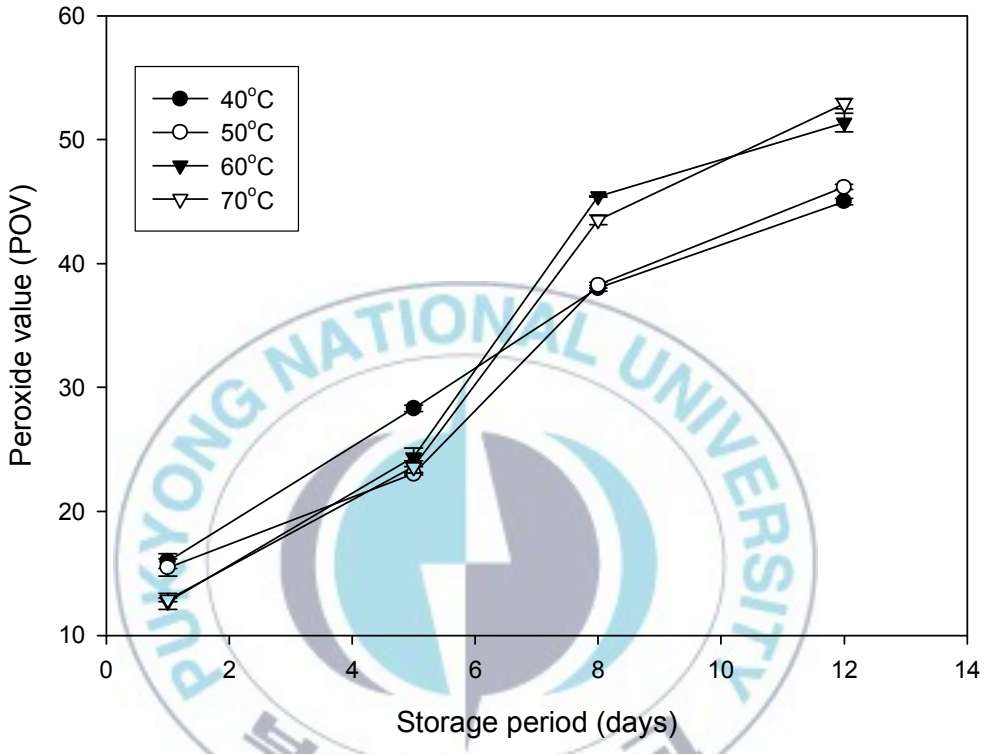

Fig. 22. Comparison of the peroxide value of enzymatic ethanolysis of reactants.

3.4.3 Oxidative Stability

For determination of oxidative stability, wheat germ oil obtained by ethanolysis reactants was characterized by measuring the thiocyanate method, DPPH radical scavenging effect and rancimat test.

3.4.3.1 Thiocyanate method

The oxidative stabilities of ethanolysis reactants are shown in Fig. 23 A and B. In this study oxidation trend evaluated determining the state of oxidation of incubated sample. The increase in absorbance value was an indicator of auto oxidation by formation of peroxides during incubation. Astaxanthin as antioxidant inhibited the peroxide formation from lipid. And linoleic acid emulsion indicated a low oxidative stability. Fig. 23A indicated that the ethanolysis reactants increase during storage period. The ethanolysis reactants showed significant increased oxidation after 10 days. Initially, ethanolysis reactant emulsion showed slightly high absorbance comparing to lionoleic acid emulsion. And Fig. 23 B shows that the higher reaction temperature increased oxidation. This result also agreed with higher temperature caused a significant increment of the oxidation stability.

3.4.3.2 DPPH radical scavenging effect

The DPPH radical scavenging effect of reaction temperature to ethanolysis reactants are shown in Fig. 24. According to Fig. 24, the condition of 40℃ shows the biggest scavenging activity among the various conditions. But no significant difference of the DPPH radical scavenging activities of wheat germ oil obtained ethanolysis reactants.

3.4.3.3 Rancimat test

The Rancimat is used to determine the oxidative stability of fatty acid methyl ester. Tests of the induction period were performed. The instrument measures the ability of a sample to resist oxidation under conditions of heat and continuous air flow. The instrument is used extensively in determining the detrimental effects of metallic contaminants and the ameliorating effects of chelators and antioxidants.

The Fig. 25 shows the induction time of the ehtanolysis reactants to reaction temperature. When comparing reactants, reaction temperature is lower, the induction period is higher. As a result, at the process of ethanolysis from the high temperature, the oxidation was already in progress.

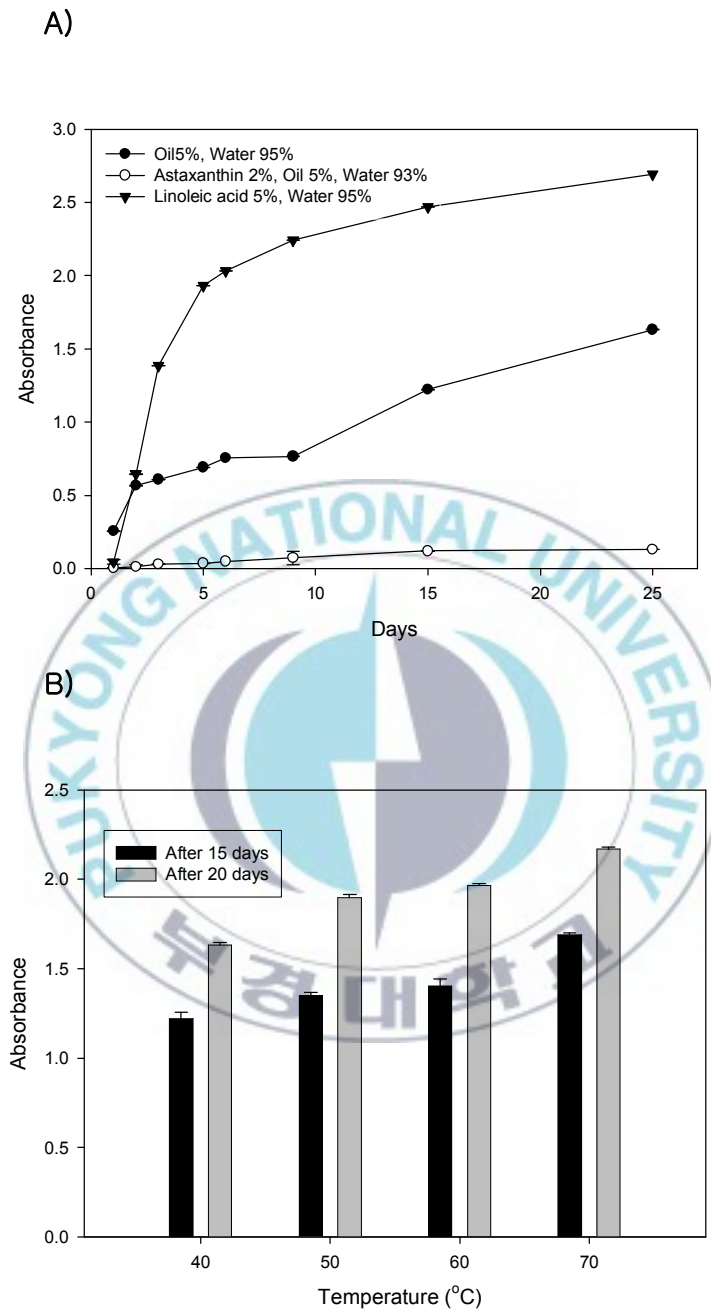

Fig. 23 A-B. Oxidative stability of ethanolysis reactants according to A) storage period and B) reactant temperature.

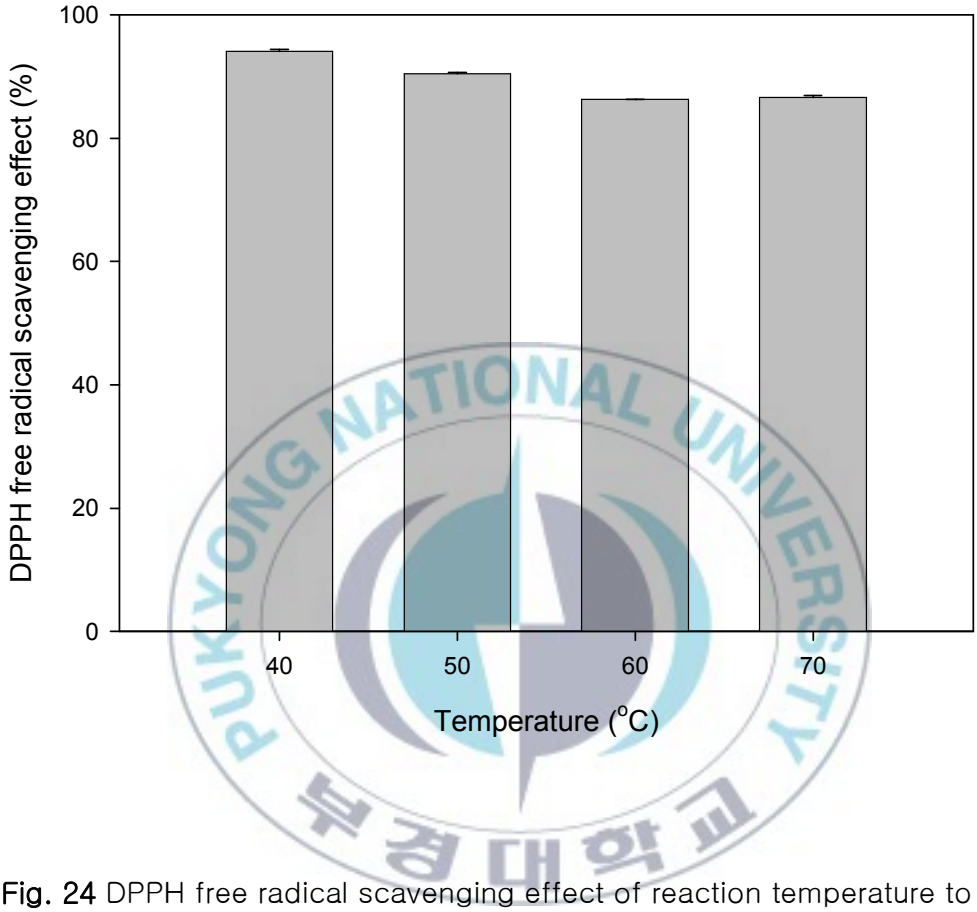

Fig. 24 DPPH free radical scavenging effect of reaction temperature to ethanolysis reactants

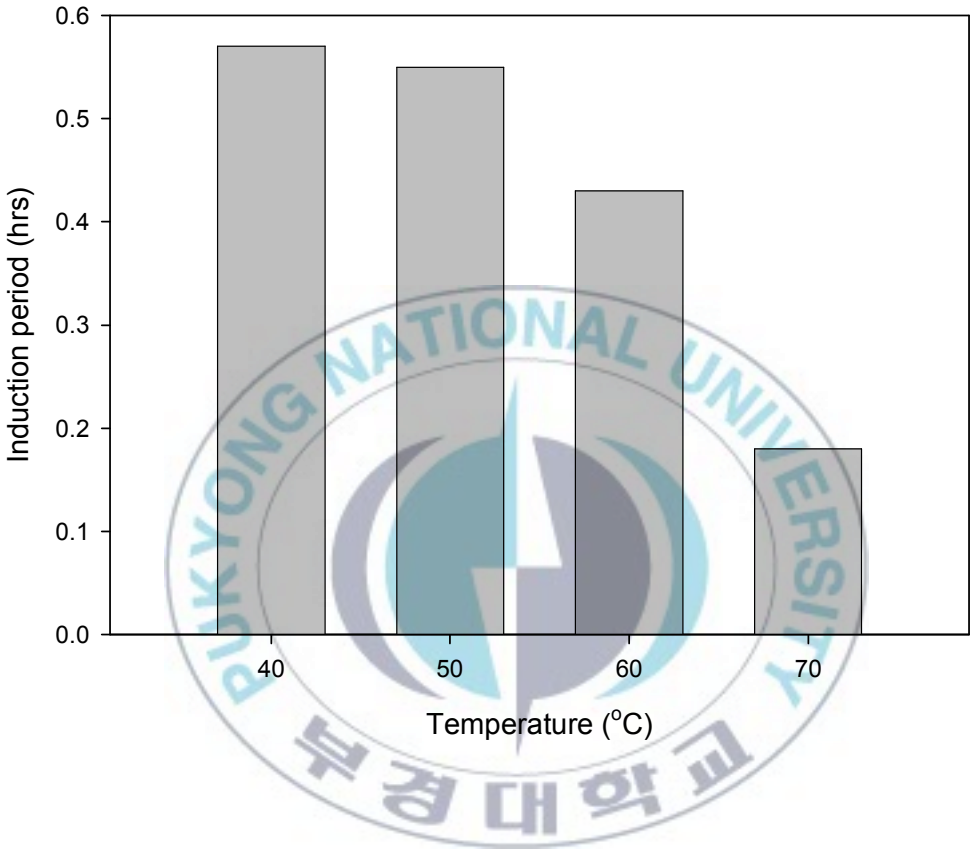

Fig. 25 Induction period of ethanolysis reactants at different reaction temperature.

3.5 Lipase activity

Fig. 26 A-B shows the lipase activity of ethanolysis reactants of wheat germ oil. Fig.26-A indicates the lipase activity of reactants at different reaction temperature and Fig. 26 B indicates the lipase activity of reactants at different reaction pressure. Among the different condition, the lipases activity is no significant difference. This may have resulted from a no difference of enzyme activity after ethnolysis at SC-CO_2 system. The loss of enzyme activity occurs when the reactions were finished. Because the reaction was finished, the enzyme activity decreased significantly.

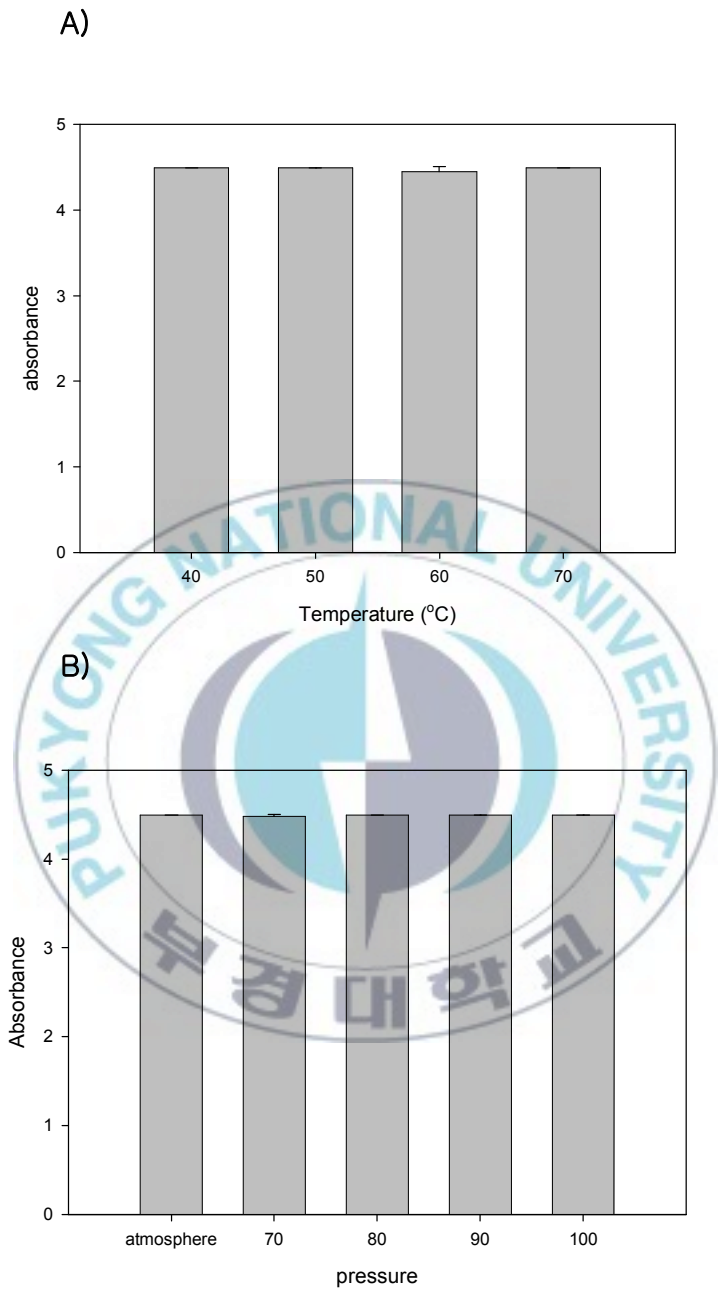

Fig. 26 A-B. Lipase activity of ethanolysis reactants according to A) reaction temperature and B) reaction pressure

4. CONCLUSION

Production of rich monoglyceride, diglyceride, and ethyl ester from wheat germ oil and ethanol with immobilized 1, 3-regiospecific lipases was performed. Changing main experimental parameters affecting reaction, the temperature, enzyme amount, reaction time and enzyme, the optimal reaction conditions were established. And oxidation stability compared enzymatic ethanolysis reactant with wheat germ oil.

When the ethanol mole ratio was increased from 1 to 4, the conversion efficiency was high at mole ratio 1.0. For non-pressured system, the optimal temperature and enzyme load are 60℃, 4% lipase. And reaction time, conversion increase 2^{nd} ethanol addition and then decrease. However, in pressured system, the optimal temperature was found 50℃. The increasing temperature has a positive effect. However, high temperatures may promote dehydration and denaturation (Hassan et al., 2006).

Compared with non-pressurized system and pressure system, the effect of pressure was beneficial. Generally, change in pressure of CO_2 influences the density, activity and stability of enzymes. Also, pressure can directly affect the reaction rate by changing the concentrations of reactants and products in solution because the partitioning of reaction components between the two phases depends on pressure (Erickson et al., 1990).

And wheat germ oils obtained by enzymatic ethanolysis characterized by measuring the composition of fatty acid, oil stability (FFA content, acid value, peroxide value) and oxidative stability (thiocynate method, DPPH scavenging effect and rancimat test). There were no remarkable changes in the fatty acid composition of wheat germ oil obtained by enzymatic ethanolysis. It caused that esterification is converted lipid construction.

The condition of 40℃ shows the highest oil stability and oixdatiive stability among the various conditions. But there is no significant difference of the DPPH radical scavenging activities. The higher reaction temperature increased oxidation. This result agreed with higher temperature caused a significant increment of the oxidation stability.

5. ACKNOWLEDGEMENT

본 논문이 완성되기까지 도움을 주신 많은 분들께 감사의 인사를 드립니다. 먼저 석사과정 동안 학문의 길로 인도해주시고 부족한 저에게 아낌없는 격려와 지도를 해주신 전병수 교수님께 깊은 감사의 인사를 드립니다. 그리고 바쁘신 시간 와중에 저의 논문 심사를 맡아주시고, 소중한 충고와 조언을 해주신 김선봉 교수님, 안동현 교수님께도 깊은 감사의 마음을 전하고 싶습니다. 그리고 많은 가르침을 주신 조영제 교수님, 이양봉 교수님, 양지영 교수님, 김영목 교수님께도 진심으로 감사의 말씀 전합니다.

그리고 식품공학실험실 이름 아래 많은 도움을 주신 MD. Salim Uddin, Ali Nehari Abdelkader 박사님과 신상규 선배님, 안향민 선배님, 권경태 선배님, 정고운 선배님께 감사의 인사 드리며 실험실 생활동안 많은 도움을 준 승미, 정남이 오빠, 혜연이언니, 준호, 주희와 실험실 학부생으로 많은 도움을 준 박현민, 오준구, 정미림, 이인정, 김선준, 백창주, 정다혜, 이경진에게도 너무 고맙다는 말을 전하고 싶습니다. 또한 학부생활때부터 많은 힘을 주었던 선경이, 은주, 지은, 아롱이에게도 고마움을 전하며 항상 힘들 때마다 힘이 되어준 주희, 연정이, 주혜, 현화, 선정이에게 고맙다는 말을 전하고 싶습니다. 이들이 있기에 항상 힘을 얻을 수 있었으며 이들에게 항상 좋은 일만 있기를 바랍니다.

마지막으로 넘치는 사랑으로 힘이 되어주시고 믿어주신 부모님께 이 논문을 바칩니다. 부모님 너무너무 사랑합니다. 그리고 항상 철없는 누나를 항상 이해해주고 잘 챙겨준 동생 경태에게도 고맙다고 전하고 싶습니다. 이제는 이러한 가족들의 은혜에 조금이나마 보답할 수 있도록 노력하겠습니다.

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