BABES-BOLYAI UNIVERSITY CLUJ-NAPOCA
FACULTY OF CHEMISTY AND CHEMICAL ENGINEERING

PhD THESIS

STUDY OF BIODIESEL FUEL PRODUCTION THROUGH ENZYMATIC METHODS

ABSTRACT

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Cluj-Napoca
2011

Content

Chapter I
Introduction

Biodiesel is a mixture of fatty acid alkyl esters and due to its similar properties to diesel fuel it can be used as a natural substitute for petroleum diesel. Biodiesel is a fuel derived from renewable sources, mainly vegetable oils and animal fats.

Biodiesel production using enzymatic methods has gained a special interest because it eliminates the disadvantages of alkaline process, currently applied at industrial level for biodiesel production.

The PhD thesis addresses a topical theme, namely biodiesel fuels production using enzymatic methods.

The theoretical and experimental research activities realized within the current PhD thesis can be highlighted with the following original contributions:

- Characterization of locally obtained sunflower oil used as feedstock for biodiesel production through enzymatic methods. This characterization is necessary because the raw material significantly influence the fuel characteristics of resulted biodiesel. The following characteristics were determined: fatty acid composition, acid value, iodine value, water content, sulfur content.
- Lipase screening for sunflower oil methanolysis: lipase from *Candida rugosa* in free form (CRL), lipase B from *Candida antarctica* immobilized on acrylic resin (Novozym 435), porcine pancreatic lipase in free form (PPL), lipase from *Mucor miehei* immobilized on macroporous ion exchange resin (Lipozyme MM IM), lipase AK from *Pseudomonas fluorescens* in free form and lipase A from *Candida antarctica* in immobilized form (Cal A).
- The optimum conditions determined for the methanolysis of sunflower oil catalyzed by Novozym 435, were the following: the presence of *tert*-butanol used as reaction medium to avoid lipase inactivation caused by methanol excess, optimum reaction temperature 40°C, *tert*-butanol:oil ratio 6:1 (v/v), methanol:oil molar ratio 6:1, Novozym 435 amount10 % (m/m) by oil weight.
- Time-course methanolysis of sunflower oil catalyzed by Novozym 435 using two reaction systems, namely: a continuous stirring reaction system (batch reactor) and a plug flow reaction system with recirculation (packed-bed column);
- Characterization of biodiesel obtained through enzymatic methanolysis of sunflower oil and the evaluation of its quality specifications according to European biodiesel quality standard, SR EN 14214:2010. Several physico-chemical characteristics have been determined: ester content, density at 15°C, viscosity at 40°C, flash point, sulfur content, water content, acid value, iodine value, methanol content, mono-, di-, triglycerides, free and total glycerol content, Na, K content, calorific power.

Chapter IV
Physico-chemical characterization of sunflower oil – the feedstock used for biodiesel production using enzymatic methods

3.1 Fatty acid composition of sunflower oil

Fatty acid composition of sunflower oil was determined using gas-chromatography. Thus fatty acids are converted into more volatile methyl esters using alkaline methanolysis. The chromatogram obtained from the gas chromatographic analysis is shown in Figure 4.1.

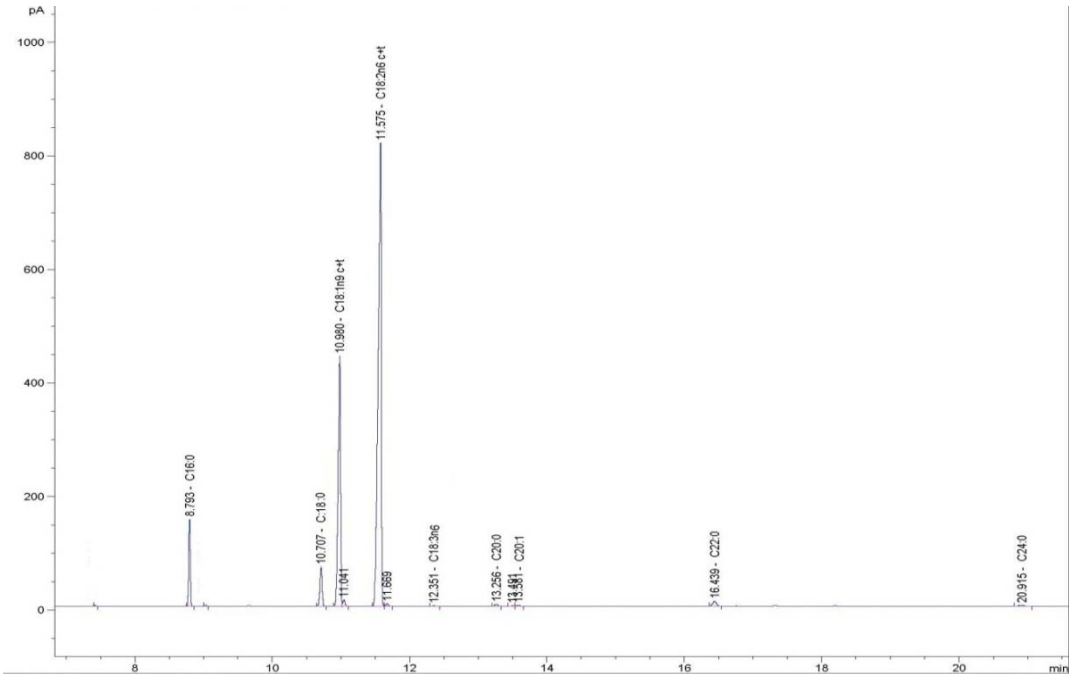

Figure 4.1. *The chromatogram obtained for the fatty acid analysis of sunflower oil*

The fatty acid composition of sunflower oil determined using gas chromatography with flame ionization detector is shown in Table 4.1.

Table 4.1. *Fatty acid composition of sunflower oil used as feedstock for biodiesel production*

Systematic name	**Common name**	**Abbreviation**	**RT (min)**	**Content [%] (m/m)**
Hexadecanoic acid	Palmitic acid	C16:0	8,793	6,16
Octadecanoic acid	Stearic acid	C18:0	10,707	3,88
cis-9-Octadecenoic acid	Oleic acid	C18:1n9	10,980	26,3
cis,cis-9,12-Octadecadienoic acid	Linoleic acid	C18:2n6	11,575	62,65
6,9,12-octadecatrienoic acid	γ-Linolenic acid	C18:3n6	12,351	0,10
Eicosanoic acid	Arahidic acid	C20:0	13,256	0,16
cis-11-Eicosenoic acid	Gadoleic acid	C20:1 c	13,561	0,02
Docosanoic acid	Behenic acid	C22:0	16,439	0,63
Tetracosanoic acid	Lignoceric acid	C24:0	20,915	0,10

Fig. 4.1 and table 4.1 show that the major fatty acid is linoleic acid with a mass percentage of 62,6%, followed by oleic acid with a mass percentage of 26,3%. The results obtained were used to determine the fatty acid composition according to degree of unsaturation, given in Table 4.2.

Table 4.2. *Fatty acid composition of sunflower oil according to degree of unsaturation*

Fatty acid composition	**Content %(m/m)**
Saturated fatty acids	11.0
Monounsaturated fatty acids	26.3
Polyunsaturated fatty acids	62.7

The obtained results show that the sunflower oil has some disadvantages due to its fatty acid composition in terms of several motor characteristics, namely: oxidation stability, storage stability, engine performance, although for the mixtures with diesel in the proportions used at present (5-20%), their influence is less significant. Instead, the main advantage is the decrease of CFPP (cold filter plugging point) making the resulted biodiesel more suitable for winter use.

The results obtained for the other characteristics studied for sunflower oil: iodine value, acid value, sulphur content, water content, are given in Table 4.5, which are discussed next.

Table 4.5 *Physico-chemical characteristics of sunflower oil used as feedstock for biodiesel production using enzymatic methods*

Parameter	**UM**	**Determined value**
Sulphur content	mg/kg	0.27
Water content	mg/kg	360.6
Acid value	mg KOH/g	0.06
Iodine value	g I_2/100g	128

3.2 Iodine value

Iodine value is expressed in grams of iodine which react with 100 grams of fat or oil under certain conditions, being a parameter that quantifies the degree of unsaturation of the fat/oil. The iodine value obtained for the sunflower oil was 128 g iodine I_2/100 g oil, which is its specific domain, mentioned in the literature, namely 125-135 g I_2/100 g oil [5]. Concerns about possible problems caused by biodiesel on engines were often assigned to high iodine values although studies have shown that biodiesel stability depends, besides the degree of unsaturation, on the content of antioxidants, and the production technology used.

3.3 Acid value

Acid value is pointing out the presence of free fatty acids or acids formed as a result of the oil degradation and burning (during or after processing). The acid value represents the amount of base required to neutralize the oil sample and is expressed in mg KOH/g sample. This parameter is of great interest, especially for alkaline methanolysis where the presence of free fatty acids must be limited due to soap formation which lead to the formation of emulsions. In case of enzymatic biodiesel, the presence of free fatty acids in the raw material is not a problem, because they are converted into esters along with triglycerides. The acid value of sunflower oil used as feedstock for biodiesel production is 0,06 mg KOH/g oil, a value that indicates a low content of free fatty acids.

3.4 Water content

Water is a minor component found in most raw materials for biodiesel production. In general, high water content causes a decrease in ester yields as undesirable reaction occurs by hydrolysis of triglycerides. For the enzymatic processes, a certain amount of water is required to "lubricate" the polypeptide chains, thus maintaining the enzyme in its active conformation. The water content determined for the sunflower oil used as raw material was 360 mg/kg, a value below the maximum value allowed for biodiesel by the European quality standard EN 14214:2010, namely 500 mg/kg [1].

3.5 Sulphur content

Sulfur content of biodiesel is limited to 10 mg/kg by the SR EN 14214:2010 quality standard. Therefore it is important to know the sulfur content of raw material because it can contribute to the sulfur content of resulted biodiesel. The value obtained for the sulphur

content of the sunflower oil was 0,27 mg/kg, far below the value required by the European quality standard, EN 14214:2010 [1].

Chapter V
Original contributions regarding biodiesel fuels production using enzymatic methods

2. 2. Lipase screening for biodiesel production from sunflower oil

In this experimental research activity a screening process was conducted to determine the suitable enzyme for the sunflower oil methanolysis. The lipases tested were the following: *Candida rugosa* lipase in free form (CRL), *Candida antarctica* lipase B immobilized on acrylic resin (Novozym 435), porcine pancreatic lipase in free form (PPL), *Mucor miehei* lipase immobilized on macroporous ion exchanger resin (Lipozyme IM MM), lipase AK from *Pseudomonas fluorescens* in free form (AK) and *Candida antarctica* lipase A in immobilized form.

For each of these lipases the sunflower oil methanolysis reaction was carried in *tert*-butanol, for four different reaction temperature: 25°C, 40°C, 50°C and 60°C. The experiments were conducted in the presence of *tert*-butanol to avoid lipase inactivation by methanol and by resulted glycerol. *tert*-Butanol dissolves both methanol and glycerol and is not a substrate for lipases because they do not act on tertiary alcohols.

Reactions were monitored for 24 h, and samples from the reaction mixture were taken at specific periods of time and were analyzed. The analyses performed have aimed the determination of methyl esters and were carried out using gas chromatography with flame ionization detector.

The obtained data showed the influence of lipase type and reaction temperature on transesterification reaction of sunflower oil with methanol.

Figures 5.2-5.3 show the results obtained for the methanolysis of sunflower at 25°C for the lipases tested. The best results after 24 h reaction time have been obtained for Novozym 435 with an ester yield of 70,2%, followed by Lipozyme MM and AK lipases with yields of 46,5%, and 41,5%, respectively. PPL gave a yield of 11,7% while CRL showed a low enzymatic activity, with a yield of only 1,8 %. *Candida antarctica* lipase A didn't show any enzymatic activity towards sunflower oil triglycerides.

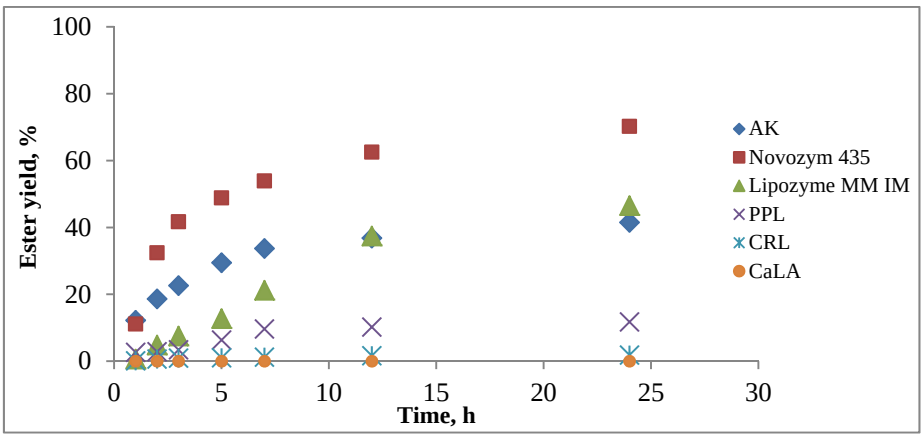

Figure 5.2 *Time-course methanolysis for the lipases tested at 25 ℃; Reaction conditions: methanol/oil 6:1(mol/mol); tert-butanol:oil 4:1 (v/v), 2% enzyme (m/m) by oil weight; 24 h reaction time, 24 h, stirring rate 200 rpm*

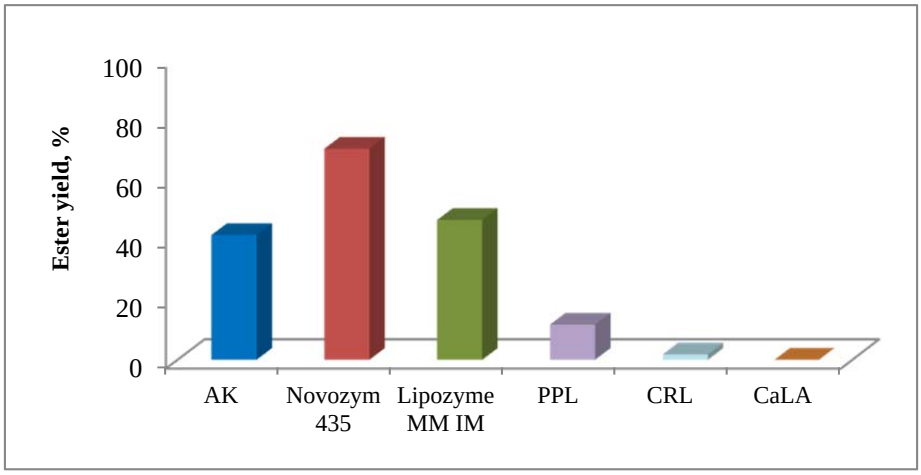

Figure 5.3 *Lipase screening for sunflower oil methanolysis in the presence of tert-butanol at 25℃. Reaction conditions: methanol/oil 6:1(mol/mol); tert-butanol/oil 4:1 (v/v), 2% enzyme (m/m) by oil weight; reaction time, 24 h, stirring rate 200 rpm*

The results obtained for the methanolysis of sunflower oil at 40°C, are graphically shown in Figures 5.4-5.5. The best results obtained at 40°C were given by Novozym 435 with a yield of 88.7%, followed by lipase AK and Lipozyme MM IM with yields of 59.3% and 53.2%. PPL has led to a yield of 12.2% while the CRL showed a low enzymatic activity, with a yield of only 2.2%. Lipase A from *Candida antarctica* showed no enzymatic activity.

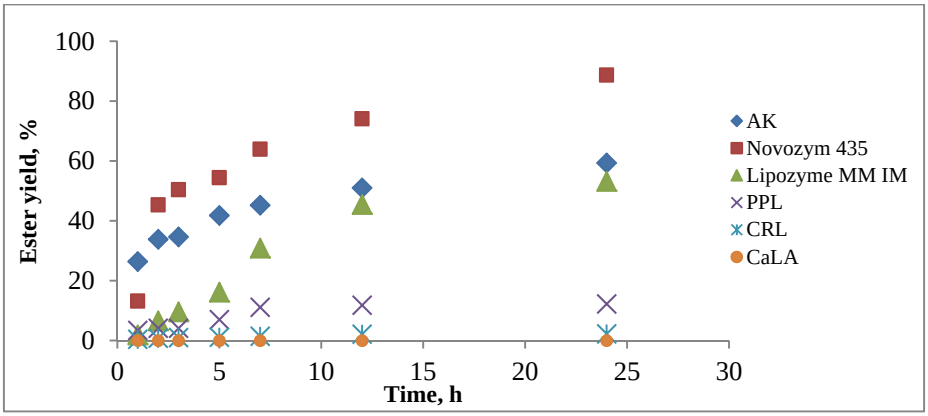

Figure 5.4 *Time-course methanolysis for the lipases tested at 40℃; Reaction conditions: methanol/oil 6:1(mol/mol); tert-butanol:oil 4:1 (v/v), 2% enzyme (m/m) by oil weight; 24 h reaction time, 24 h, stirring rate 200 rpm*

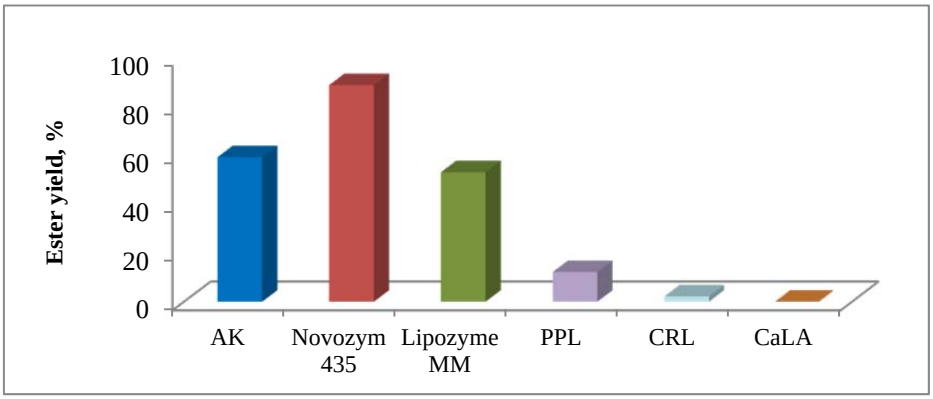

Figure 5.5 *Lipase screening for sunflower oil methanolysis in the presence of tert-butanol at 40℃. Reaction conditions: methanol/oil 6:1(mol/mol); tert-butanol/oil 4:1 (v/v), 2% enzyme (m/m) by oil weight; reaction time, 24 h, stirring rate 200 rpm*

The results obtained for the sunflower oil methanolysis at 50°C for the screened lipases, are graphically showed in Figures 5.6-5.7.

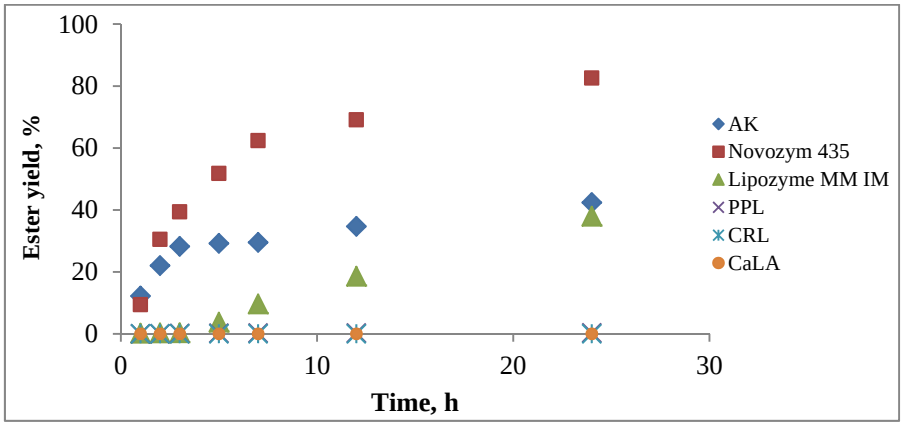

Figure 5.6 *Time-course methanolysis for the lipases tested at 50℃; Reaction conditions: methanol/oil 6:1(mol/mol); tert-butanol:oil 4:1 (v/v), 2% enzyme (m/m) by oil weight; 24 h reaction time, 24 h, stirring rate 200 rpm*

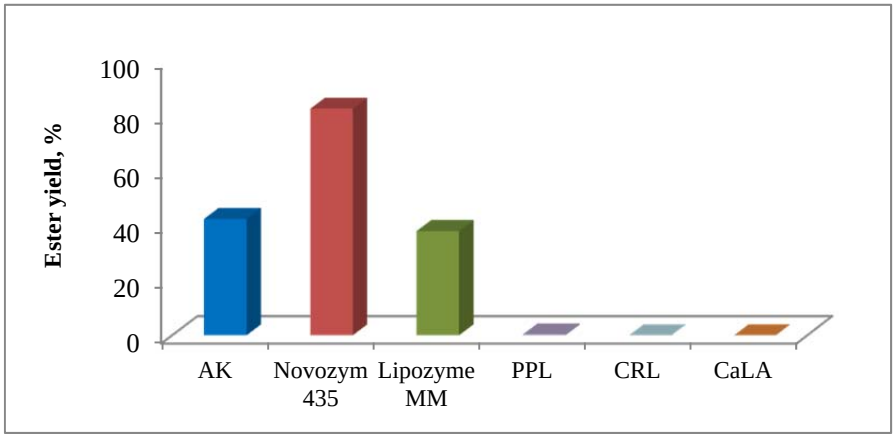

Figure 5.7 *Lipase screening for sunflower oil methanolysis in the presence of tert-butanol at 50℃. Reaction conditions: methanol/oil 6:1(mol/mol); tert-butanol/oil 4:1 (v/v), 2% enzyme (m/m) by oil weight; reaction time, 24 h, stirring rate 200 rpm*

After 24 h, Novozym 435 gave the best results at 50ºC with an ester yield of 82,7%, followed by AK lipase and Lipozyme MM with yields of 42,4%, and 38,0%, respectively. PPL has a very low enzymatic activity with a yield of only 0,3 %. CRL and CaL A showed no enzymatic activity.

The results obtained for the sunflower oil methanolysis at 60°C for the screened lipases, are graphically showed in Figures 5.8-5.9.

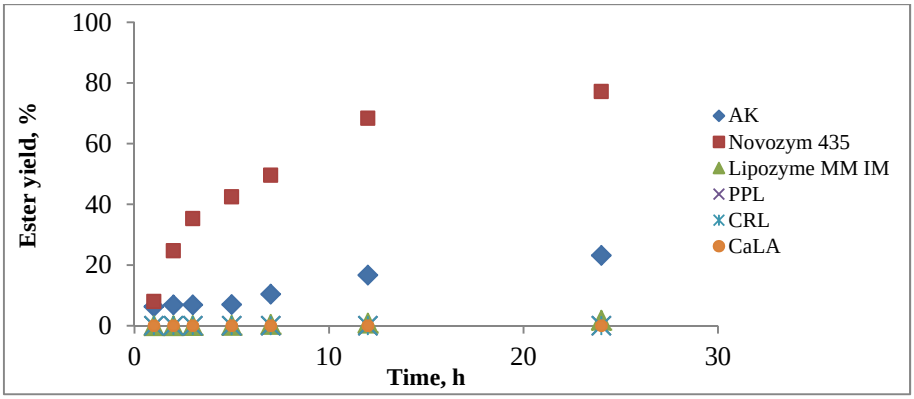

Figure 5.8 *Time-course methanolysis for the lipases tested at 60℃; Reaction conditions: methanol/oil 6:1(mol/mol); tert-butanol:oil 4:1 (v/v), 2% enzyme (m/m) by oil weight; 24 h reaction time, 24 h, stirring rate 200 rpm*

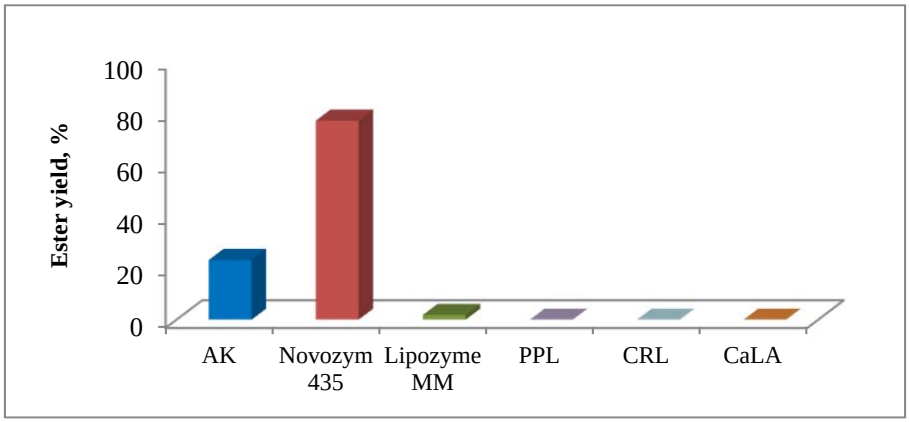

Figure 5.9 *Lipase screening for sunflower oil methanolysis in the presence of tert-butanol at 60℃. Reaction conditions: methanol/oil 6:1(mol/mol); tert-butanol/oil 4:1 (v/v), 2% enzyme (m/m) by oil weight; reaction time, 24 h, stirring rate 200 rpm*

The best results were obtained for Novozym 435, although the ester yield decreased to 77.2%. For AK lipase an ester yield of only 23,2% was obtained, while Lipozyme MM presented a very low enzymatic activity with an ester yield of only 1.84%. The other three lipases showed no enzymatic activity.

The effect of temperature on enzymatic activity of lipases used for the methanolysis of sunflower oil is showed in Figure 5.10. For all the screened lipases, when the temperature increased over 40°C the enzymatic activity decreased.

In conclusion all the experiments performed to find the most suitable enzyme for the methanolysis of sunflower oil, have showed that the best results were obtained for Novozym 435 which gave an ester yield of 88.7% yield for a reaction temperature of 40°C. AK lipase gave an ester yield of 59,3% and Lipozyme MM IM gave an ester yield of 53,2%. PPL showed a low enzymatic activity, with an ester yield of 12,2% while CRL showed almost no enzymatic activity (2,2% yield)

Candida antarctica lipase A showed no enzymatic activity which means that triglycerides are no substrate for this enzyme. In what concerns the reaction temperature, it was noted that, for all lipases, the temperature of 40°C gave the highest yields in methyl esters.

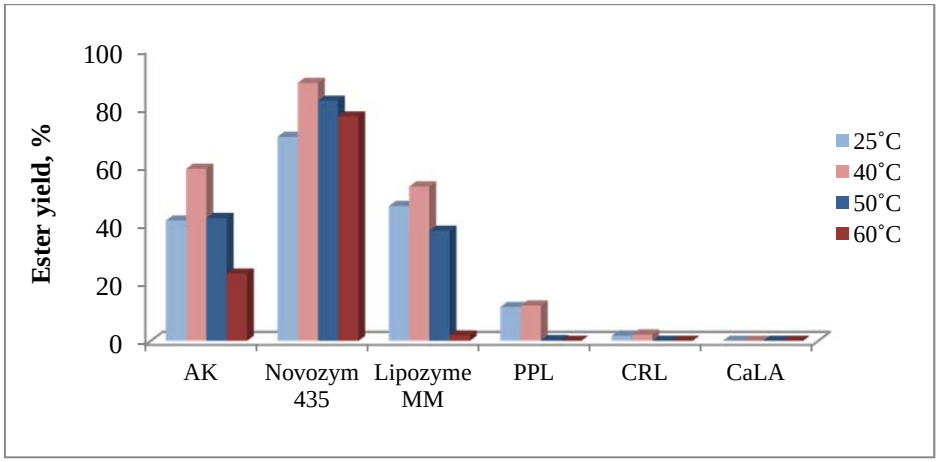

Figure 5.10 *The effect of temperature on enzymatic activity of lipases screened for the methanolysis of sunflower oil in the presence of tert-butanol; Reaction conditions: methanol/oil 6:1(mol/mol); tert-butanol:oil 4:1 (v/v), 2% enzyme (m/m) by oil weight; reaction time 24 h, stirring rate 200 rpm*

3. Optimization of enzymatic methanolysis reaction of sunflower oil for biodiesel production

The objective of this experimental research activity was to identify the optimal reaction conditions for the methanolysis of sunflower oil, catalyzed by Novozym 435, the lipase B from *Candida antarctica*. From the enzymes tested in the screening process, for the methanolysis of sunflower oil, Novozym 435 yielded the best results. The optimum temperature for the transesterification reaction of triglycerides was found to be 40°C. Good results were obtained also at room temperature. The following reaction parameters were studied: the presence of solvent and the solvent amount, the enzyme amount and the methanol/oil molar ratio.

3.2.1 The effect of *tert*-butanol as reaction medium for the methanolysis of sunflower oil

tert-Butanol was used as reaction medium for the methanolysis of sunflower oil catalyzed by Novozym 435, because it dissolves both methanol and glycerol and is not a substrate for lipases (lipases do not show enzymatic activity towards tertiary alcohols) [6]. Moreover, *tert*-butanol is a non-toxic solvent, and in terms of price it has a relatively low cost. In this work we studied the effect of *tert*-butanol on the enzymatic methanolysis of sunflower oil by determining the ester yield when the *tert*-butanol/oil volumetric ratio varied in the range 0:1- 8:1 (v/v). The results obtained are showed in Figure 5.11.

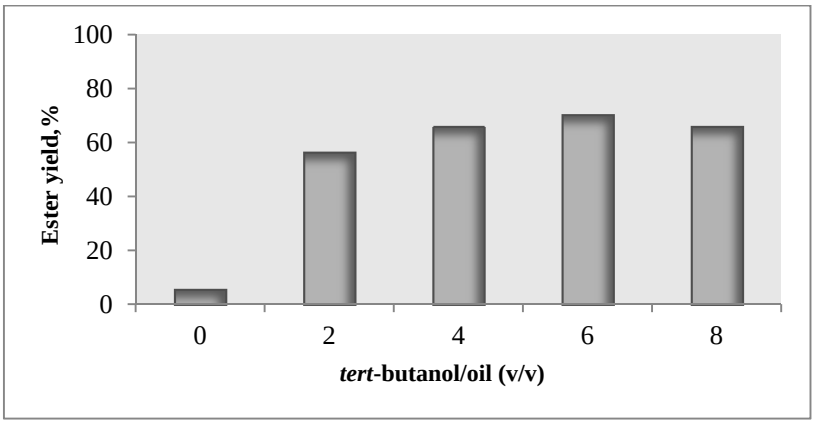

Figure 5.11 *The effect of tert-butanol on the enzymatic methanolysis of sunflower oil. Reaction conditions: methanol/oil 6:1 (mol/mol), 10% Novozym 435 by oil weight, reaction time 8 h*

In the absence of *tert*-butanol solvents, the ester yield was very low, of only 5,7% (w/w) due to methanol toxicity on lipase activity. When *tert*-butanol was introduced in the reaction mixture the efficiency significantly increased. The highest yield, namely 70% (w/w) was obtained for a volumetric ratio of 6:1 *tert*-butanol/oil, which was used in subsequent experiments.

3.2.2 The effect of methanol/sunflower oil molar ratio

Another studied parameter was the effect of methanol on esters yield. A set of experiments was performed in which the methanol/oil molar ratio was varied in the range 3:1-8:1 (mol/mol), the results being shown in Figure 5.12.

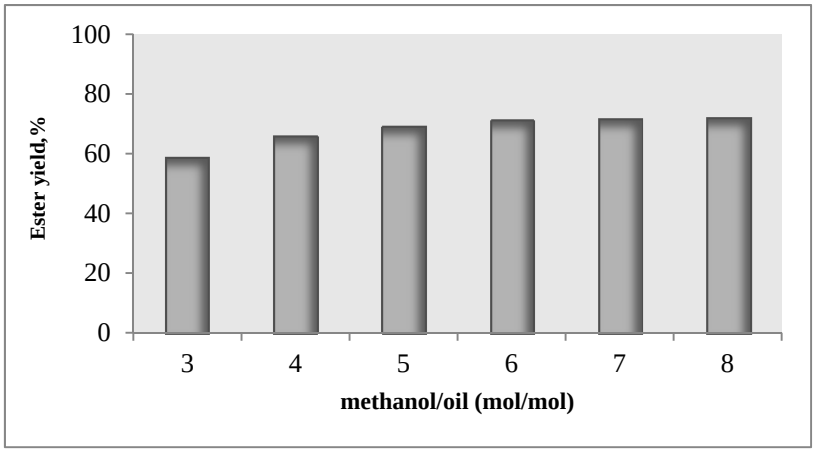

Figure 5.12 *The effect of methanol/oil molar ratio on the methanolysis of sunflower oil. Reaction conditions: tert-butanol/oil 6:1 (v/v), 10% Novozym 435 by oil weight, reaction time 8 h*

The results obtained (Fig. 5.12) showed that the methyl ester yield increased with increasing the methanol/oil molar ratio and the presence of *tert*-butanol allowed the use of methanol in large excess without cause lipase inactivation. Since the results for methanol/oil molar ratios above 6:1 did not vary substantially, methanol/oil molar ratio of 6:1 was chosen as optimal, further used.

3.2.3 The effect of enzyme amount

Another studied parameter was the amount of Novozym 435. The effect of enzyme amount on ester yield was determined by varying the enzyme amount between 1 and 15% (w/w), by oil weight. The obtained results are shown in Figure 5.13.

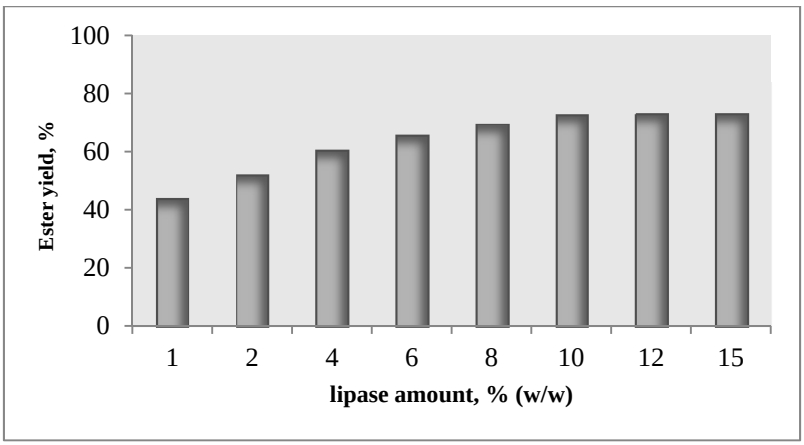

Figure 5.13 *The effect of lipase amount on the methanolysis of sunflower oil. Reaction conditions: methanol/oil molar ratio 6:1, terţ-butanol/oil 6:1 (v/v), reaction time 8 h*

The results obtained showed that the ester yield increased with increasing the enzyme amount and an amount of 10% (w/w) lipase, by oil weight, a yield of 72% was achieved after 8 h reaction time. Further increase of lipase amount did not result in significant changes in ester yield. In conclusion an enzyme amount of 10% (w/w) was further used as optimum.

The experiments performed for the methanolysis of sunflower oil catalyzed by Novozym 435 have showed that the optimum conditions are as follow:

- *tert*-butanol is required as reaction medium to avoid lipase inhibition caused by excess methanol;
- *terţ*-butanol/oil volumetric ratio 6:1;
- methanol/oil molar ratio 6:1;
- Novozym 435 amount - 10 % (w/w), by oil weight.

4. Biodiesel production by enzymatic methanolysis of sunflower oil: batch reactor vs. packed-bed reactor

In this experimental research activity the main objective was to compare two reaction systems for enzymatic biodiesel production, namely: methanolysis using continuous stirring system, and methanolysis using plug-flow reaction system.

In the first case the reaction was performed using a batch reactor in which the enzyme is subjected to continuous stirring together with the reaction mixture, while in the second case, the reaction mixture was introduced using a peristaltic pump over the packed-bed enzyme layer within a column reactor.

The reaction conditions used in both cases are those which have proved to be optimal for the methanolysis of sunflower oil catalyzed by Novozym 435. Reactions were monitored for 24 h, and samples from the reaction mixture were taken at regular periods of time and analyzed. The analyses consisted in the determination of methyl ester yield and were performed using gas chromatography

4.2 Results and discussions

Time-course reactions were monitored using gas chromatography with flame ionization detection and methyl heptadecanoate as internal standard.

For each reaction, in addition to global ester yield monitored, each individual ester was also quantified. The obtained data were graphically plotted in Figures 5.14-5.20.

Data presented in Fig. 5.14 - 5.20 showed that the global ester yield variation is given by the fatty acid methyl esters that are present in high content in the sunflower oil, namely linoleic acid (C18:2, 63.25% (w/w)) and oleic acid (C18:1, 25.23% (w/w)). For both systems the triglycerides conversion was almost complete after 24 h. For the continuous stirring reaction, after 24 h, the ester yield reached 98.6% while for the packed-bed column, the ester yield was slightly lower, namely 95.6%.

After 24 h reaction time, the batch reactor gave better results compared with the packed-bed column. The reason was due to the fact that, in this case, from the beginning of the reaction the enzyme was in contact with the entire amount of oil, caused by the stirring effect. The consequence is a higher reaction speed for the batch reactor at the beginning of reaction. Thus, after the first 15 min, the ester yield obtained for the batch reactor was 23.6%, while, for the packed-bed reactor the conversion of triglycerides has not started yet. This advance of the continuous stirring reaction persists throughout the linear variation of ester yield. After about 4 h, the ester yield for the continuous stirring reaction was 75.1% compared with only 57.7% for the paked-bed reaction system (Figure 5.14). Further, a deceleration of continuous stirring reaction was observed comparatively to that using packed-

bed enzyme and recirculation of reaction mixture. After 6 h reaction time, the ester yield for the batch reaction was 78,2% while for the packed-bed reaction was 70,2%.

This decrease of the reaction rate for the batch reactor was caused by the by-product glycerol that is also entrained in the reaction mixture due to the continuous stirring, and creates obstacles in terms of direct contact between the enzyme and oil. For the plug-flow system most of the glycerol was situateded at the bottom of the column. After 12 h reaction time, the yield for the batch reactor was 86.5% while for the packed-bed reactor the yield was 82.9%. This small difference was observed also after 24 h reaction time, when the ester yield reached 98.6% for the batch reactor, respectively 95.6% for the packed-bed column.

The results obtained showed that the variation mode for the global ester yield was retrieved for each fatty acid methyl ester individually quantified, namely: the yield varies linearly in the first period of time, after 4 h of reaction this variation becomes asymptotic, reaching a maximum after about 24 h.

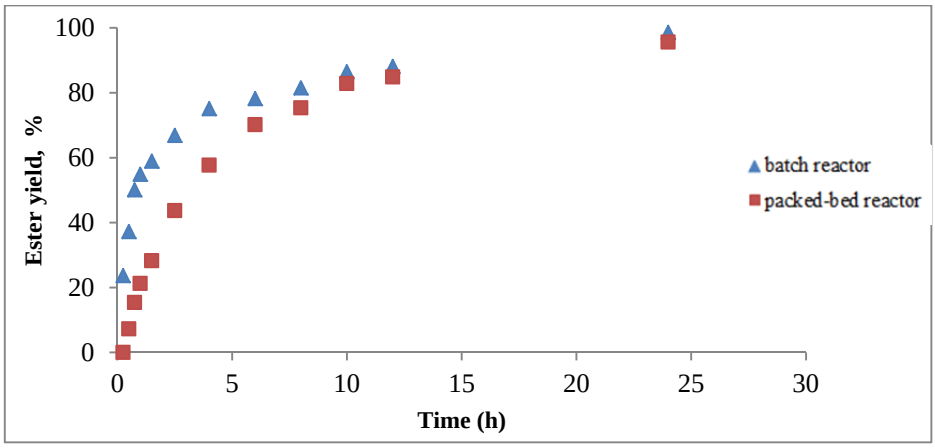

Figure 5.14 *Time-course methanolysis for biodiesel production from sunflower oil. Reaction conditions: methanol/oil molar ratio 6:1, tert-butanol/oil volumetric ratio 6:1, 10% Novozym 435, 24 h reaction time*

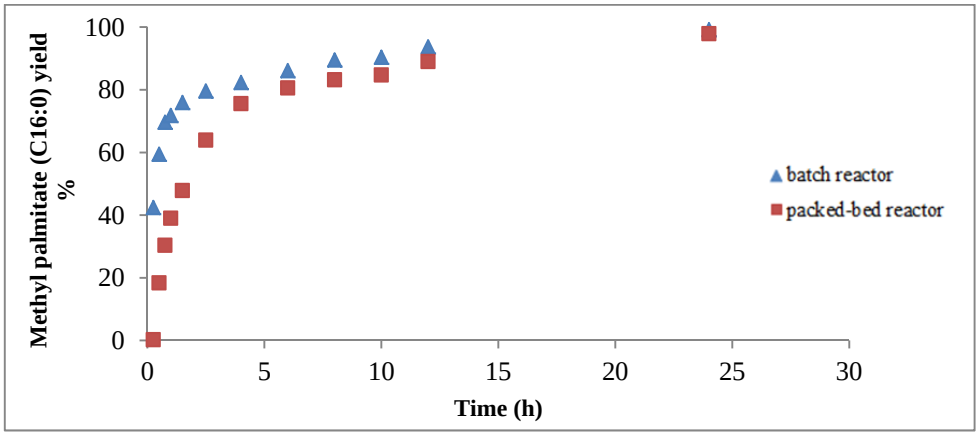

Figure 5.15 *Time-variation of methyl palmitate (C16:0) yield for the enzymatic methanolysis of sunflower oil. Reaction conditions: methanol/oil molar ratio 6:1, tert-butanol/oil volumetric ratio 6:1, 10% Novozym 435, 24 h reaction time*

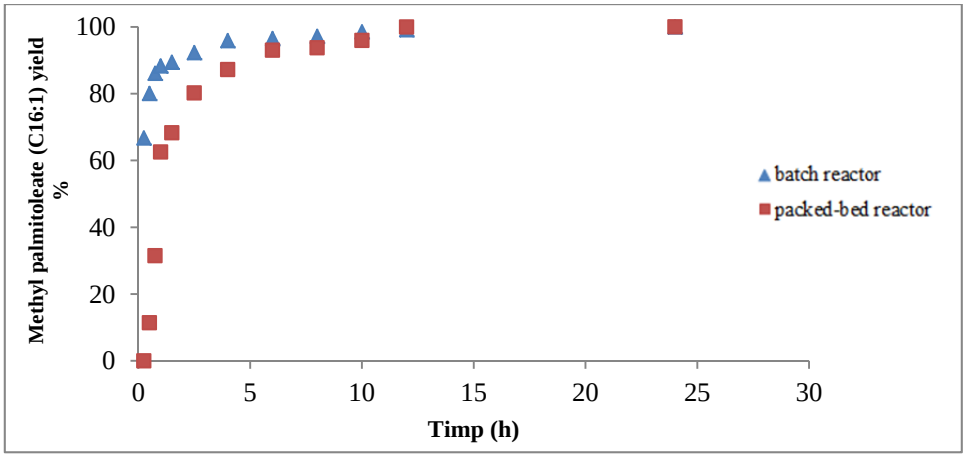

Figure 5.16 *Time-variation of methyl palmitoleate (C16:1) yield for the enzymatic methanolysis of sunflower oil. Reaction conditions: methanol/oil molar ratio 6:1, tert-butanol/oil volumetric ratio 6:1, 10% Novozym 435, 24 h reaction time*

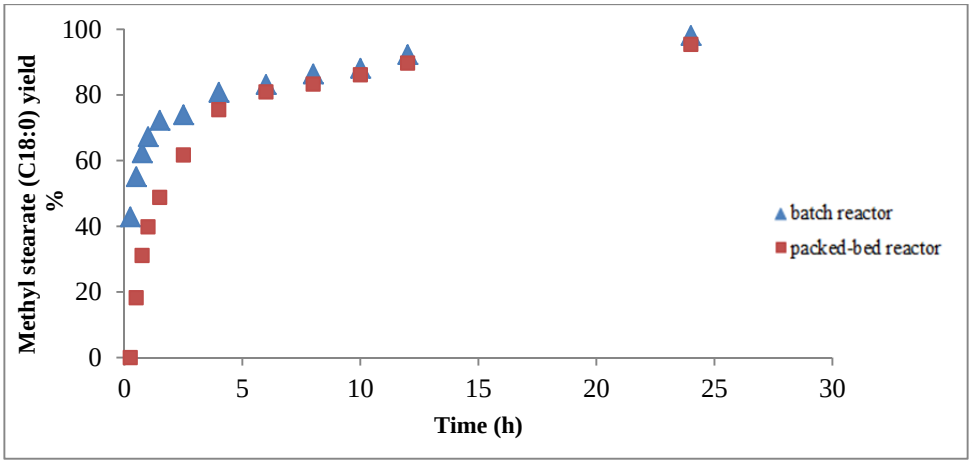

Figure 5.17 *Time-variation of methyl stearate (C18:0) yield for the enzymatic methanolysis of sunflower oil. Reaction conditions: methanol/oil molar ratio 6:1, tert-butanol/oil volumetric ratio 6:1, 10% Novozym 435, 24 h reaction time*

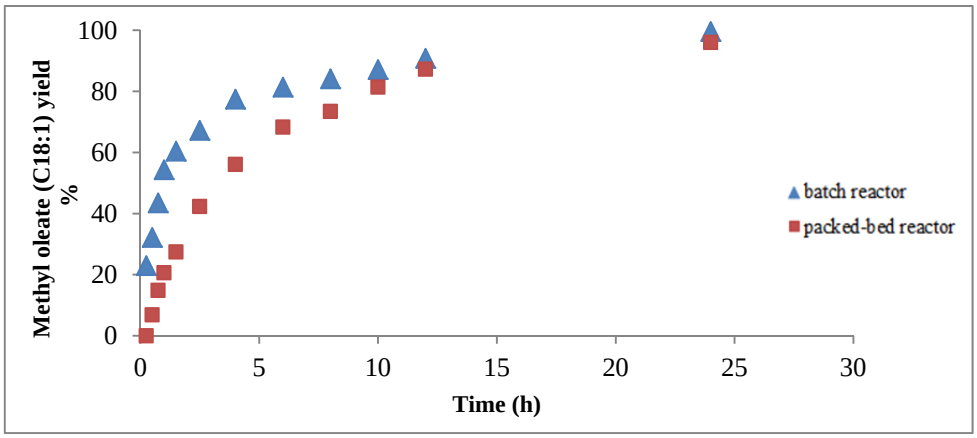

Figure 5.18 *Time-variation of methyl oleate (C18:1) yield for the enzymatic methanolysis of sunflower oil. Reaction conditions: methanol/oil molar ratio 6:1, tert-butanol/oil volumetric ratio 6:1, 10% Novozym 435, 24 h reaction time*

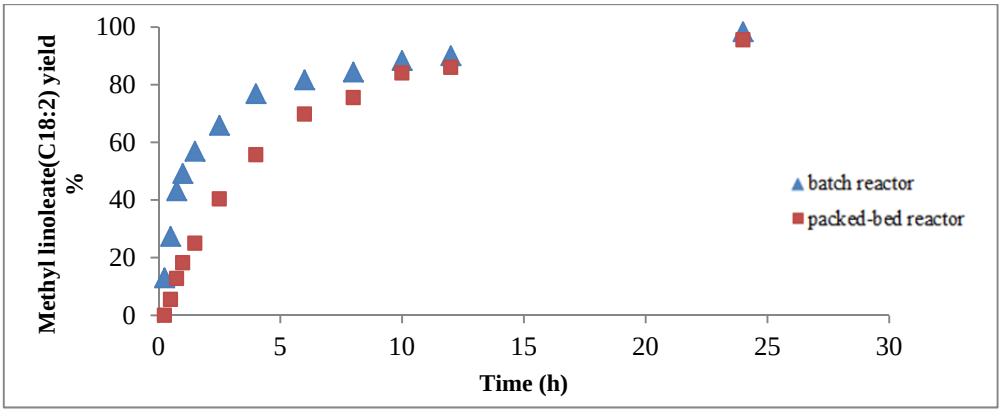

Figure 5.19 *Time-variation of methyl linoleate (C18:2) yield for the enzymatic methanolysis of sunflower oil. Reaction conditions: methanol/oil molar ratio 6:1, tert-butanol/oil volumetric ratio 6:1, 10% Novozym 435, 24 h reaction time*

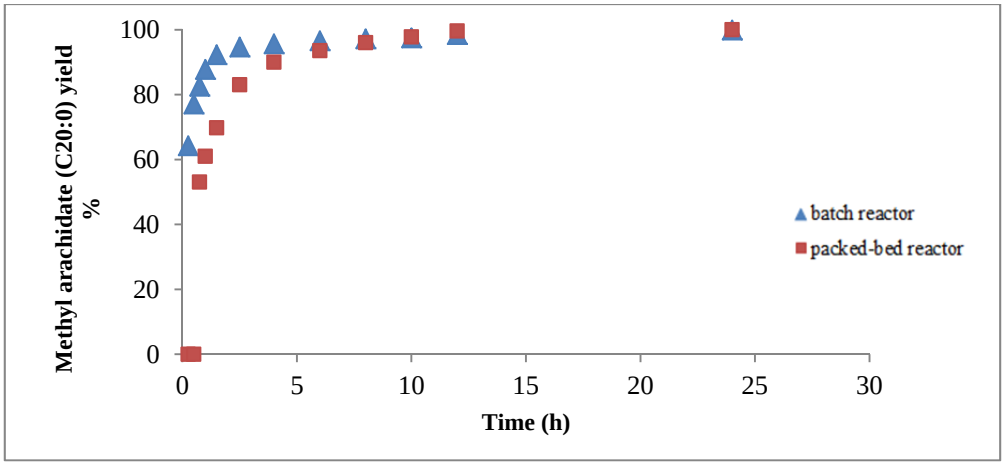

Figure 5.20 *Time-variation of methyl arachidate (C20:0) yield for the enzymatic methanolysis of sunflower oil. Reaction conditions: methanol/oil molar ratio 6:1, tert-butanol/oil volumetric ratio 6:1, 10% Novozym 435, 24 h reaction time*

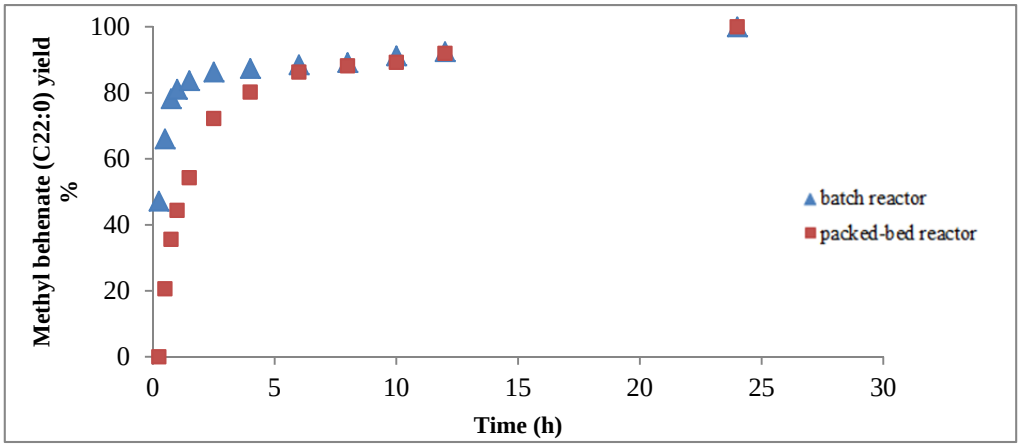

Figure 5.21 *Time-variation of methyl behenate (C22:0) yield for the enzymatic methanolysis of sunflower oil. Reaction conditions: methanol/oil molar ratio 6:1, tert-butanol/oil volumetric ratio 6:1, 10% Novozym 435, 24 h reaction time*

For methyl oleate (C18:1), one of the major components of biodiesel result, there is substantial difference for the two reaction systems at the beginning of the reaction, where the yield variation is linear. Thus, for the batch system, after the first 15 min the yield is 23%, while for the packed-bed reactor the oleic acid conversion has not started yet. This difference

was retrieved after the first 4 h, when the yield for the batch reactor reached 77.5% and for the packed-bed reactor was only 56.1%. After 24 h reactiontime, the yield reached 99.6% for the batch reaction, respectively 96% for the packed-bed reaction (Figure 5.18).

Methyl linoleate represents the major component of the biodiesel resulted from the methanolysis of sunflower oil. Thus, the variation mode of methyl linoleate yield has the most significant effect on the variation of global ester yield (Figure 5.19). After the first 4 h reaction time, when the variation mode is almost linear, the yield reached 76,9% for the batch reactor while for the packed-bed reactor the value reached was 55,7%. After this moment, the variation becomes asymptotic, reaching the maximmum after 24 h, of 98,4% for the batch reactor, and 95,6% for the packed-bed reactor, respectively. It can be seen that these values can be retrieved also for the global ester yield after 24 h reaction time.

Both reaction systems used for the methanolysis of sunflower oil, catalyzed by Novozym 435, resulted in high yields, above 95% after 24 h reaction time. However, the batch reactor gave the best results, an ester yield of 98.6%, while the yield for the packed-bed reactor was 95.6%. The major difference between the two reaction systems can be observed at the beginning of the reaction and in the first 4 hours when the variation is linear. The batch reactor for this period of time has a superior advantage because the reaction rate is higher in this case. Subsequently, this difference became lower due to problems caused by glycerol for the batch reaction, after 24 h ,a difference of only 3% being found between the two systems studied.

Chapter VI
Physico-chemical characterization of biodiesel obtained through enzymatic methanolysis of sunflower oil

The biodiesel obtained by enzymatic methanolysis of sunflower oil was analyzed by specific methods to determine physico-chemical characteristics that indicate its quality compared to quality specifications required by the European standard for biodiesel, EN 14214:2010. The following physico-chemical characteristics were determined: ester content, density at 15°C, viscosity at 40°C, flash point, sulphur content, water content, acid value, iodine value, methanol content, mono-, di-, triglycerides content, free and total glycerol content, Na, K content, calorific power. The obtained values are given in Table 6.5. These characteristics are detailed below, showing their direct influence on engine parameters.

3.1 Ester content

Ester content is a measure of transesterification reaction completion. A higher conversion of triglycerides into methyl esters leads to a better engine performance. Ester content of biodiesel can vary greatly depending on the different technologies used and the raw materials available. European biodiesel standard, EN 14214:2010, sets a limit for the ester content of at least 96.5% while the American standard for biodiesel, ASTM D 6751 does not specify a minimum for the ester content [7].

The ester content determined for the biodiesel obtained by enzymatic methanolysis of sunflower oil was determined by gas chromatography; with methyl heptadecanoate as internal standard, the chromatogram obtained being showed in Figure 6.1. The resulted data were processed, yielding a value of 98.9% ester content, indicating an almost complete conversion of triglycerides into methyl esters.

Table 6.5. *Caracteristici fizico-chimice ale biodieselului obţinut prin transesterificarea enzimatică a uleiului de floarea soarelui*

Caracteristica	Unit	Value	EN 14214:2010 [1]	
			min	max
Ester content	%	98.9	96.5	
Density at 15°C	kg/m^3	890	860	900
Viscosity at 40°C	mm^2/s	4.36	3.50	5.00
Flash point	°C	133	120	-
Sulphur content	mg/kg	0.10	-	10
Water content	mg/kg	479	-	500
Acid value	mg KOH/g	0.11		0.50
Iodine value	g I_2/100g	127		120
Methanol content	%	0.003		0.20
Monoglyceride content	% (m/m)	0.65		0.80
Diglyceride content		0.17		0.20
Triglyceride content	% (m/m)	0.01		0.20
Free glycerol	% (m/m)	0.015		0.02
Total glycerol	% (m/m)	0.21		0.25
Na content	mg/kg	<0.25		5
K content	mg/kg	0.11		5
Calorific power	MJ/Kg	32.7		

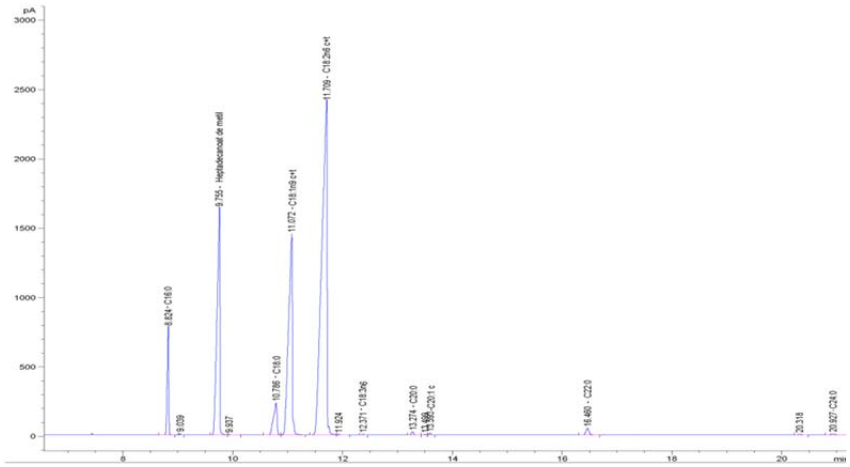

Figure 6.1 *The chromatogram of ester content for the biodiesel obtained from the enzymatic methanolysis of sunflower oil*

3.2 Density at 15 ºC

The European standard for biodiesel, EN 14214:2010, specifies an allowable domain for density at 15°C ranging from 860-900 kg/m3. The American standard for biodiesel, ASTM D 6751, does not set a limit on density, and stipulates that biodiesel density falls between 860 and 900 kg/m^3 (typical values between 880 and 890 kg/m^3) when other quality specifications are met.

Density value for the biodiesel produced by enzymatic methanolysis of sunflower oil is 890 kg/m^3, thus meeting the quality specified by EN 14214:2010 standard.

3.3 Viscosity at 40 ºC

Viscosity determines the fuel flow through pipes, nozzles and injection holes as well as the temperature range for the proper functioning of the burning fuel. A high viscosity can cause problems for the spray effect of the injector which can cause excessive coking and oil dilution. These problems are associated with reduced engine life.

The quality standards specify also a minimum limit for viscosity to prevent wear of the friction produced by the fuel injection system, which would reduce engine power. A value of viscosity between the limits imposed by the quality standards ensures proper lubrication and corresponding pumping characteristics [9]. The European biodiesel standard, EN 14214:2010, specifies a viscosity domain between 3.5-5 mm^2/s. The American standard for biodiesel, ASTM D 6751 specifies a viscosity domain between 1.9-6 mm^2/s.
The biodiesel produced by enzymatic methanolysis of sunflower oil has a viscosity of 4.36 mm^2, meeting the quality specification imposed by both EN 14214:2010 and ASTM 6751, respectively

3.4 Flash point

The flash point determines the flammability of the material. In general, the flash point value specified by the quality standards is relatively high, for safety reasons regarding storage and transport and also to ensure that the alcohol is removed from the finished product. Low flash points may indicate alcohol residue in biodiesel.
European biodiesel standard, EN 14214:2010, sets a minimum limit for the flash point of 120°C while the American standard for biodiesel, ASTM D 6751, sets a minimum limit for the flash point of 130°C.

The flash point for the biodiesel obtained by enzymatic methanolysis of sunflower oil was 133°C which corresponds to quality requirements imposed by the quality standard EN 14214:2010 and ASTM D 6751.

3.5 Sulphur content

The engine combustion of fuels that contain sulphur leads to the formation of sulphur dioxide emissions and particulate matter. Sulphur limits are generally imposed for environmental reasons.

European biodiesel standard, EN 14214:2010, sets a maximum limit of sulphur content of 10 mg/kg while the American standard for biodiesel, ASTM D 6751 sets a maximum limit on the sulphur content of 50 mg/kg.

For the biodiesel obtained from sunflower oil by enzymatic methanolysis, the sulphur content was 0.10 mg/kg, a very low value compared with the maximum value imposed by the quality standards. The immediate consequence is a decrease in sulphur dioxide emissions.

3.6 Water content

The European biodiesel standard, EN 14214:2010 together with the American standard for biodiesel, ASTM D 6751, set a maximum limit for water content of 500 mg/kg.
The water content determined for the biodiesel obtained by enzymatic methanolysis of sunflower oil is 379 mg/kg, a value that corresponds to the quality requirements imposed by the quality standards.

3.7 Acid value

Acid value is an pointer of the presence of free fatty acids or acids formed as a result of the degradation and burning of oil (during or after processing). European biodiesel standard, EN 14214:2010, sets a maximum limit for acid value of 0.5 mg KOH/g. The American standard for biodiesel, ASTM D 6751, sets a maximum limit for the acid value of 0.80 mg KOH/g.
For the biodiesel obtained by enzymatic methanolysis of sunflower oil the acid value was 0.11, below the maximum limit required by the quality standards.

3.8 Iodine value

For the biodiesel obtained by enzymatic methanolysis of sunflower oil a iodine value 127 was obtained, exceeding the maximum limit imposed by the standard EN 14214:2010. The literature specifies a range between 110-143 g I_2/100g for the biodiesel produced from sunflower oil [16]. If the European biodiesel standard, EN 14214:2010, sets a maximum limit for iodine value of 120 g I_2/100g, the American standard for biodiesel, ASTM D 6751, does not set a limit for iodine value. Iodine value required by the European standard for biodiesel quality limit the raw material that can be used for biodiesel production. The presence of this parameter in certain quality standards can actually be a political tactic to limit imports of certain raw materials for this purpose.

3.9 Methanol content

The value obtained for the methanol content of biodiesel produced by enzymatic methanolysis of sunflower oil is 0.003%, the chromatogram being illustrated in Figure 6.2. The value is far below the maximum limit allowed by the European biodiesel standard EN 14214:2010 which is 0.2%.

Studies in literature have shown that a methanol content of only 1% biodiesel can reduce the flash point of 170°C to less than 40°C. Thus, by introducing a quality specification of minimum flash point of 120°C, the European standard EN 14214:2010 implicitly limits the amount of alcohol at a very low value (<0.1%).

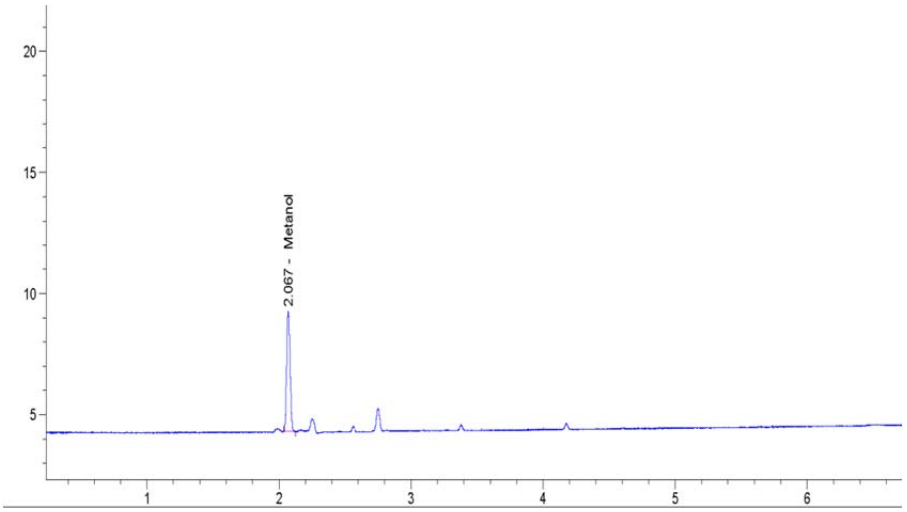

Figure 6.2 *The chromatogram obtained for the methanol content for the biodiesel obtained by the enzymatic methanolysis of sunflower oil*

3.10 Mono- di- and triglyceride content, free and total glycerol content

The content of mono-, di-, triglycerides, free and total glycerol was determined by gas chromatography using two internal standards: 1,2,4-butanetriol (internal standard used for measuring free glycerol) and tricaprin (internal standard used for the quantification of mono-, di-, triglycerides). The chromatogram obtained after the GC analysis is given in Figure 6.3.

For the biodiesel obtained by enzymatic methanolysis of sunflower oil the content of mono-, di-and triglycerides, is within the limits imposed by the quality standards, with values of 0.65%, 0.17% and 0.01%, respectively. The European biodiesel standard, EN 14214:2010, sets a limit for the maximum content of 0.8% for monoglycerides, a maximum content of 0.2% for diglycerides and triglyceride content up to 0.2%. The American standard for biodiesel, ASTM D 6751, does not set a limit for the content of mono-, di-, and triglycerides.

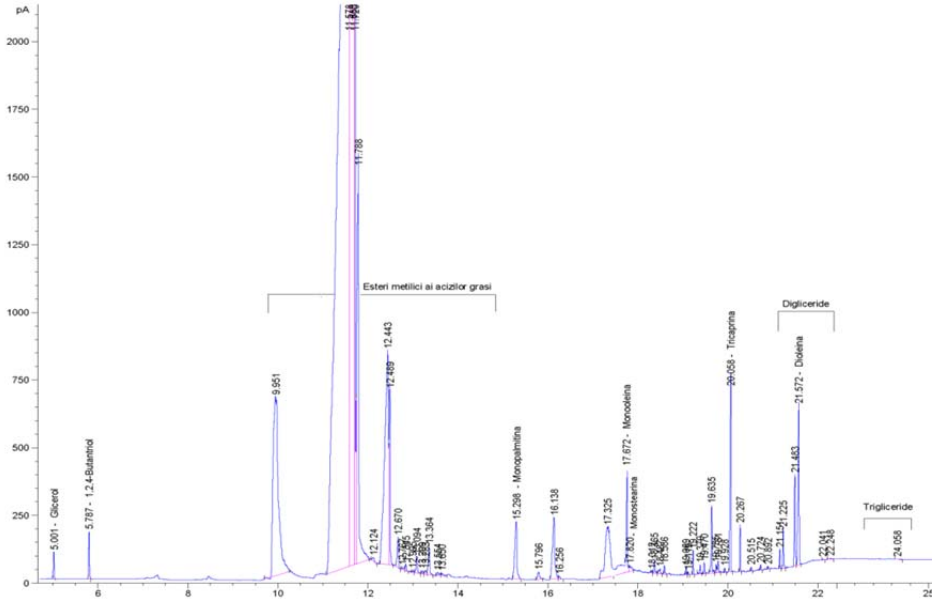

Figure 6.3 *The chromatogram obtained for the analysis of glycerol, mono-, di- and triglycerides content from the biodiesel obtained by the enzymatic methanolysis of sunflower oil*

The free glycerol content for biodiesel obtained by enzymatic methanolysis of sunflower oil was 0.015% under the maximum limit imposed by the European biodiesel standard, EN 14214:2010 and the American standards for biodiesel, ASTM D 6751, 0.02%.
The total glycerol content of enzymatic biodiesel was 0.21%, close to the maximum limit imposed by the quality standards for biodiesel. Thus, the European biodiesel standard, EN 14214:2010, sets a limit on total glycerol content up to 0.25% while the limit imposed by the ASTM D 6751 standard, is 0.24%.

3.11 Na, K content

In the case of biodiesel produced by enzymatic methanolysis of sunflower oil, this analysis just completed the rest of the features. Because in this method are not used alkaline catalysts, these metals can only suggest the presence of some contaminants during production or storage process. The values obtained for Na and K are very small, for Na is lower than the limit of method quantification the while the K value is 0.11 mg/kg. The European biodiesel standard, EN 14214:2010, sets a maximum limit for the content of metals in Group I (Na, K) up to 5 mg/kg and for Group II (Ca, Mg) up to 5 mg / kg. The American standard for biodiesel, ASTM D 6751, does not set a limit for the content of alkali metals.

3.12 Energy content (Calorific power)

For the biodiesel obtained by enzymatic methanolysis of sunflower oil an energy value of 32.7 MJ/kg was obtained, a value that corresponds to data reported in the literature. [18]
Although the presence of oxygen lowers the energy value of biodiesel comparatively to that of diesel (an average of 37.2 MJ/kg compared with 43.8 MJ/kg for diesel), the energy value of biodiesel is much less variable, and depends especially on the feedstock used and not on the production process.

Chapter VII
Conclusions

After browsing the theoretical and experimental research activity of the present thesis entitled *Study of biodiesel fuel production through enzymatic methods*, the following conclusions can be presented regarding the production of biodiesel fuel by the enzymatic methanolysis of sunflower oil:

- The sunflower oil has a relatively high content of polyunsaturated fatty acids which gives some disadvantages with respect to oxidation stability and storage. This higher degree of unsaturation of sunflower oil is retrieved also in the iodine value. The value determined for acid value indicates a very low content of free fatty acids and the values obtained for the water content and sulphur content are far below the limits imposed by the quality standard for biodiesel EN 14214:2010. Meanwhile, the high content of unsaturated fatty acids gives advantages such as increasing the possibility of using the biodiesel in winter, which is of great interest for the countries with cold climate together with a better performance engine due to higher energy content.
- Of the enzymes tested in the screening process for the enzymatic methanolysis of sunflower oil, *Candida antarctica* lipase B, immobilized on acrylic resin (Novozym 435) was found to be most effective for triglycerides conversion into biodiesel.
- The optimum reaction conditions for the methanolysis of sunflower oil catalyzed by Novozym 435 were found to be: methanol/oil molar ratio 6:1, *tert*-butanol/oil volumetric ratio 6:1, 10% Novozym 435 by oil weight.
- Using these optimum conditions the reaction was monitored two different reaction systems: a continuous stirring reaction system using a batch type reactor, and a plug-flow reaction system using a packed-bed column reactor. For both systems high yields of more than 95% were obtained, after 24 h reaction time. However, the batch reactor gave the highest yield of 98.6%, while for the packed-bed column the yield was 95.6%. The advantage of the batch reactor was substantially higher in the beginning of the reaction (first 4 h) when, due to shaking, the enzyme is in contact with the entire amount of the oil present, leading to a higher reaction rate.
- The biodiesel obtained from the enzymatic methanolysis of sunflower oil was tested to determine the following physico-chemical characteristics: the ester content, the density at 15°C, viscosity at 40°C, flash point, sulphur content, water content, acid value, iodine value, methanol content, mono-, di-, triglycerides content, free and total glycerol content, Na, K content, the energy value. The obtained values for these physico-chemical characteristics showed that the biodiesel meets the quality requirements of European standard SR EN 14214: 2010, except iodine index. This however is due to raw material - sunflower oil - not production process, the European standard for biodiesel in one particular for the rapeseed oil, limiting the raw material that can be used for biodiesel production.

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