

Article

# Biodiesel Production by Lipase-Catalyzed In Situ Transesterification of Rapeseed Oil Containing a High Free Fatty Acid Content with Ethanol in Diesel Fuel Media

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Received: 14 April 2020; Accepted: 18 May 2020; Published: 20 May 2020



**Abstract:** In this study, low-quality rapeseed was used as a raw material for biodiesel fuel production. The application of such seeds with an enzyme catalyst is a green approach to producing renewable biodiesel fuel. During the in situ transesterification process, mineral diesel was selected as an extraction solvent for the simultaneous extraction and transesterification of rapeseed oil (RO). This allowed, at the end of the process, for the production of a mixture of mineral diesel and biodiesel fuel. Energy is saved using this process, as the need to extract the oil separately is eliminated and extraction and transesterification take place together in the in situ process. In this study, 11 different lipases were analyzed from which to select the most effective biocatalyst according to the chosen experimental conditions. The most suitable lipase for in situ transesterification was Lipozyme TL IM (*Thermomyces lanuginosus*). The impact of the temperature and duration of the reaction was investigated along with the concentration of the lipase. A ethanol-to-oil molar ratio of 5:1 was chosen. The optimal reaction conditions were as follows: a reaction duration of 7 h, a reaction temperature of 30 °C and a lipase concentration of 5% (based on oil weight). Under these conditions, 99.92% of oil was extracted from the rapeseed. The degree of oil transesterification acquired was 99.89%. A mineral diesel and rapeseed oil ethyl ester blend of 9:1 (w/w) was produced.

**Keywords:** biodiesel; ethanol; in situ transesterification; lipase; mineral diesel; rapeseed oil

## 1. Introduction

Biodiesel fuel is an alternative fuel to mineral diesel fuel that contains fatty acid methyl or ethyl esters and is produced from various lipid-rich raw materials. These renewable sources include food-grade vegetable oils, non-edible vegetable oils, aquatic biomass (seaweeds, microalgae) and waste feedstock (e.g., residual cooking oil, animal fats and used coffee grounds). For a long time, biodiesel has been produced in many countries using edible plant oils. In Europe, the European Union is still popular for producing biodiesel from edible rapeseed oil (RO) [1]. However, due to increasing competition with the food industry, scientists have had to find other non-edible oil raw materials suitable for biodiesel production. Some of these raw materials, including *Jatropha curcas* oil [2], jojoba oil [3], Karanja oil [4], *Madhuca longifolia* oil [5], castor oil [6], *Cynara cardunculus* L. oil [7], *Pongamia pinnata* oil [8], cottonseed oil [9] and linseed oil [10] are already in use for biodiesel production. Additionally, it is possible to produce biodiesel from raw waste materials such as animal fats [11], residual cooking oil [12], spent coffee grounds [13,14] and grapeseed oil [15].

Oil produced from low-quality rapeseed commonly contains high amounts of free fatty acids. This indicates hydrolysis of triglycerides. The oil becomes acidic due to the action of lipase enzymes and is an indicator of inadequate treatment and storage conditions such as high temperature and relative humidity or tissue damage. Waste edible oils are largely destroyed in incinerators or are abandoned in the environment, which causes problems with waste management or water pollution [16]. The advantage of using low-quality seeds to produce biodiesel is not only that there is no competition with the food industry, but there is no competition with the use of land for food crops.

Rapeseed oil containing a high amount of free fatty acids can be used for the production of biodiesel. One of the alternatives to such production is the in situ transesterification process, which involves fewer steps in biodiesel production technology compared to those in the conventional biodiesel production process. The in situ transesterification process can be described as the direct use of oil-containing raw materials without oil extraction before the transesterification process.

Some scientists have noted that the temperature of the reaction and oil acidity are two of the most important factors that can affect the transesterification process using lipase as a biocatalyst [17]. The in situ transesterification reaction is a reversible process, so an excess of alcohol is usually required to shift the reaction equilibrium towards the formation of the product [18]. For alcoholysis reactions, many different alcohols can be used, including short-chain, long-chain and cyclic monohydric alcohols, but short-chain alcohols such as methanol and ethanol are most commonly used because of their availability, low cost, high reactivity and polarity [19–21]. Currently, methanol is industrially produced from fossil fuels, but bioethanol is produced through the fermentation of a locally available and renewable raw material or biomass, giving it a strong advantage as a more sustainable reactant [15,22,23]. To obtain a high yield of biodiesel, it is important to choose the right type and amount of alcohol, as well as to choose the right type and amount of catalyst, depending on the selected raw material. Research results have shown that enzymes can be potential biocatalysts for biodiesel production because of their properties and advantages. One advantage is that using an enzyme catalyst during the biodiesel production process is a green approach to producing renewable fuel. Enzymatic catalysts are of biological origin, and their use requires less energy, as operating conditions are gentler than with chemical catalysts [24]. Enzymatic catalysts can be effective for ethyl ester production, but it is important to optimize reaction conditions (molar ratio, temperature, pH, amount of catalyst). By optimizing the reaction parameters, the ester yields can exceed 80% with the use of a co-solvent or with long reaction durations [17,25–29]. The duration of the enzymatic in situ process is longer than that of the conventional oil transesterification process, as oil extraction and transesterification are carried out simultaneously. Research has shown that the optimum duration of the biotechnological process is 19–24 h [30,31]. Scientists have investigated the production of biodiesel from spent coffee grounds via enzymatic catalysis with ethanol and a co-solvent. They determined that the use of ethanol instead of methanol is more appropriate for this process, and concluded that it is possible to obtain a yield of ethyl esters of 96.7% by using ethanol at 99.8%, an oil-to-ethanol molar ratio of 1:5, a temperature of 45 °C, a lipase Lipozyme RM IM concentration of 4.5% and 20% (v/w) hexane based on oil [32]. Other scientists have investigated the in situ transesterification process using a 10% lipase Novozyme 435 catalyst concentration (based on oil weight) and ethanol. Pistacia (*Pistacia chinensis* B.) and jatropha (*Jatropha curcas* L.) seeds were used as raw materials. The highest yields of *P. chinensis* B. ethyl ester and *J. curcas* L. ethyl ester (90.7% and 94.5%, respectively) were achieved when the reaction temperature was 50 °C and the reaction duration was from 24 to 36 h [33]. Additionally, several studies have reported results regarding the enzymatic synthesis of fatty acid ethyl esters with the in situ transesterification of acidic and low-cost oil from macauba in a solvent-free system. Lipase from *Rhizomucor miehei* was used as a catalyst. To obtain a fatty acid ethyl ester content above 90.8%, the oil-to-ethanol molar ratio was 1:6, the reaction duration was 96 h, the reaction temperature was 40 °C and the lipase content was 13 U per g of oil [34]. In other scientific publications, results have been reported for in situ transesterification using lipase Lipozyme TL IM and jatropha seeds (*Jatropha curcas* L.), and the highest biodiesel yield attained was 90.6%. The optimum conditions were as follows:

an n-hexane/seed ratio of 3.5:1 mL/g, an oil to methanol molar ratio of 1:6, a catalyst concentration of 15% (based on oil weight), a reaction temperature of 45 °C and a reaction duration of 12 h [35].

In the current study, a techno-economic analysis of the in situ process of biodiesel produced from rapeseed with a capacity of 50,000 t/yr was performed and compared with conventional biodiesel fuel production. A mass balance was developed through which to produce 1.0 t of biodiesel, and from this a preliminary economic study was conducted to produce 50,000 t/yr of biodiesel that showed promising results. The techno-economic indicators show that a total capital investment of \$16,065,000 and gross profits/year of \$14,630,300, the percentage simple rate of return (%SRR) was 79.5% for a constant estimated price of \$945/t, while the specific biodiesel prices for SRR% of 10 and 50 were \$722 and \$850, respectively [36].

In this study, unsuitable rapeseed food production was used to investigate the process of enzymatic biodiesel fuel production by applying in situ transesterification with ethanol. Mineral diesel fuel was used additionally as an extraction solvent. We aimed not only to extract the highest amount of oil from rapeseed, but also to obtain the highest degree of transesterification. At the end of the in situ transesterification process, a biodiesel-mineral diesel fuel mixture was produced. The influence of operating parameters such as reaction duration, reaction temperature and lipase concentration on oil extraction and transesterification effectiveness was evaluated.

## 2. Materials and Methods

### 2.1. Materials

Rapeseed containing oil of high acidity was received from the Educational Experimental Station of Vytautas Magnus University Agriculture Academy (Lithuania); arctic mineral diesel (Class 1) was acquired from a local (Lithuania) market; and ethanol (analytically pure) was purchased from Sigma-Aldrich. All of the 11 enzyme preparations were kindly donated from the global biotechnology company Novozymes A/S (Copenhagen, Denmark) and are listed as follows: Lipozyme RM IM (*Rhizomucor miehei*, >30 U/g); Lipozyme TL IM (*Thermomyces lanuginosus*, ≥3 kU/g); Novozyme 435 (*Candida antarctica*, 600 U/g); Lipozyme 435 (*Candida antarctica*, 600 U/g); Lipolase 100L (*Thermomyces lanuginosus*, 122 kU/g); Lecitase Ultra (*Thermomyces lanuginosus*, 150 U/g); Resinase A 2X (*Aspergillus oryzae*, 119.6 kU/g); Palatase 20000L (*Rhizomucor miehei*, ≥20 kU/g); Lipozyme CALB (*Candida antarctica* B., 5 kU/g); Lipozyme TL 100L (*Thermomyces lanuginosus*, 100 kU/g); and Lipex 100L (*Thermomyces lanuginosus*, 10 kU/g).

### 2.2. Determination of Rapeseed Quality and Its Oil Properties

The properties of rapeseed and its oil (e.g., the content of oil in the seeds, oil acidity and moisture of the seeds) were determined by following standard procedures (Table 1). To determine the rapeseed oil content, rapeseeds were crushed with a laboratory mill (IKa M 20 Universal), placed in a Soxhlet thimble and extracted with n-hexane for 24 h. The solvent was removed from the extracted oil using a vacuum rotary evaporator at 60 °C, and the oil content was determined according to the standard ISO 659.

In this study, a rapeseed oil content of  $45 \pm 2\%$  was determined. The acidity of the extracted oil was determined by acid–base titration according to the standard ISO 660 using a standard solution of 0.5 N KOH. The acidity of the extracted rapeseed oil was determined to be 2.63 mg KOH/g. The moisture content of the rapeseed was determined according to the standard ISO 665. It was found that the rapeseed contained 6.3% moisture. The fatty acid composition of rapeseed oil was determined by a gas chromatography method using a Restek MXT-Biodiesel TG column according to the standards EN ISO 5509 and EN ISO 5508.

**Table 1.** The properties of rapeseed and its oil.

Property	Unit	Value	Standard
Oil content in seeds	%	45 ± 2.0	ISO 659
Acid value	mg KOH/g	2.63 ± 0.25	ISO 660
Moisture content in seeds	%	6.3 ± 1.5	ISO 665
Fatty acid composition	%		ISO 5508
C14:1		0.04 ± 0.01	
C15:0		0.33 ± 0.06	
C16:0		3.73 ± 0.12	
C16:1		0.20 ± 0.01	
C18:0		1.80 ± 0.21	
C18:1		64.84 ± 1.03	
C18:2		18.47 ± 0.94	
C18:3		7.42 ± 0.66	
C20:0		0.61 ± 0.05	
C20:1		1.33 ± 0.02	
C22:0		0.43 ± 0.06	
C22:1		0.45 ± 0.02	
C24:0		0.19 ± 0.02	
C24:1		0.16 ± 0.01	

### 2.3. Selection of Lipase for *in Situ* Transesterification Process

For this research, 11 types of lipases were used as biocatalysts for the *in situ* transesterification process (see Section 2.1). It was important to select the most effective biocatalyst for the transesterification of rapeseed oil. The selection was performed using the following steps: the rapeseed was ground (to a particle size of 0.315 mm) and mixed with mineral diesel, which acted as the extractor solvent. To obtain 30 g of the final product, 6.52 g of ground rapeseed containing 3 g of oil (according to the amount of oil in the seeds) was poured into a reaction flask, and 27 g of mineral diesel was added to obtain a mixture of 90% mineral diesel and 10% oil for a ratio of diesel fuel-to-biodiesel of 9:1 (w/w) at the end of the reaction. The reaction flask was connected to a condenser and placed on a magnetic stirrer–heater with constant stirring at a rotation speed of 250 min<sup>−1</sup>. The screening of lipase stage reactions was carried out at 40 °C for 5 h. When the rapeseed–mineral diesel mixture reached 40 °C, ethanol (0.78 g (an ethanol-to-oil molar ratio of 5:1)) and lipase (0.21 g (7% based on oil weight)) were added to the reaction flask, and the reaction time began. The ethanol-to-rapeseed oil molar ratio was selected to be 5:1. At the end of the reaction time, the syntheses were completed, and the suspensions were filtered and washed with distilled water. Residues of water were removed by applying rotary evaporation.

#### 2.3.1. Thin-Layer Chromatography

Effectiveness of transesterification was evaluated by thin-layer chromatography (TLC). The reference samples were rapeseed oil (RO) and rapeseed oil ethyl esters (REEs) in diethyl ether. The mobile phase consisted of petroleum ether, diethyl ether and acetic acid at a ratio of 80:20:1. Sample preparations for thin-layer chromatography were performed according to a previously reported and modified method [24]. Biodiesel content was evaluated by comparing the spot position, brightness and area of sample and reference spots.

#### 2.3.2. Gas Chromatography

Samples selected for quantitative analysis were further thoroughly evaluated via gas chromatography to verify whether the produced rapeseed ethyl esters (REEs) had a high degree of transesterification (more than 96.5%) and met the requirements of standard EN 14214 for the glyceride (monoglyceride (0.8%), diglyceride (0.2%) and triglyceride (0.2%)) content in the biodiesel produced. Analyses were performed with a Perkin Elmer Clarus 500 gas chromatograph (detector—FID; column—Restek MXT-Biodiesel TG (15 m × 0.32 mm × 0.10 μm)), according to the standard EN 14105.

The flow gas was hydrogen. The flow rate of hydrogen was 1 mL/min. The pressure was held constant at 80 kPa. The initial injector temperature of 50 °C changed when the oven temperature changed. The detector temperature was maintained at 380 °C. The oven temperature programmed was as follows: 50 °C held for 1 min, followed by an increase of 15 °C/min to 180 °C, then 7 °C/min to 230 °C and finally 10 °C/min to 370 °C. The temperature was held at 370 °C for 7 min. The peaks corresponding to the different glycerides were identified by comparing the reaction times of each detected component in the sample with pure glyceride standards.

After the gas chromatography results were evaluated, the most effective lipase was selected for further optimization of the enzymatic *in situ* transesterification process. The same chromatographic method was used to optimize the process. The glyceride content and degree of transesterification were calculated only for the biological/oil phase (since it was important to estimate the amount of oil converted to esters) and not for the total product obtained (i.e., for the mixture with mineral diesel).

#### 2.4. Optimization of the Enzymatic *In Situ* Transesterification Process

Additional experiments (according to Section 2.3) using the lipase Lipozyme TL IM were carried out to optimize the reaction conditions and achieve the highest yield of rapeseed oil (RO) extracted from rapeseed and formed rapeseed ethyl esters (REEs) and the highest degree of transesterification with the required amounts of glyceride content in the biodiesel produced.

To optimize the temperature of the reaction, temperatures of 25, 30 and 40 °C were chosen. To optimize the duration of the reaction, durations of 1, 3, 5, 7 and 9 h were chosen. For the experiments of optimization of reaction duration and temperature conditions, a Lipozyme TL IM concentration (based on the amount of oil) of 7% was chosen. When optimum reaction duration and temperature conditions were selected, the next step was to optimize the amount of lipase added to the samples. To optimize the lipase Lipozyme TL IM concentration, 3%, 4%, 5% and 6% lipase (based on the amount of oil) in the samples was chosen.

All samples prepared for reaction optimization were analyzed with a gas chromatograph (according to Section 2.3.2) to determine the degree of transesterification and the glyceride contents in the biodiesel produced.

#### 2.5. Fourier-Transform Infrared Spectroscopy

The yield of rapeseed oil (RO) and rapeseed ethyl esters (REEs) in the reaction products were determined for all samples prepared (according to Section 2.3). Fourier transform infrared spectroscopy (FTIR) was used to perform quantitative analysis of the samples produced. An FTIR Spectrum RX-I spectrophotometer from PerkinElmer was used for the analyses. Samples were prepared according to the standard EN 14078. The selected measurement was in the range of 1600 to 1900  $\text{cm}^{-1}$ . The ester functional group containing a carbonyl had a key C = O absorption signal. In biodiesel, fatty acid ethyl/methyl ester molecules have a characteristic absorption band detected at approximately 1745  $\text{cm}^{-1}$  (5.4 micrometers) due to their ester carbonyl bonds. The same absorption band is characteristic of rapeseed oil due to the carbonyl group of triglycerides. According to this peak and calibration curve prepared by using a known concentration of rapeseed oil in fossil diesel fuel, the total amount of rapeseed methyl ester and rapeseed oil in the reaction product was determined. Using this amount and knowing the amount of total oil contained within the rapeseed used for experiments, the total yields of oil and rapeseed oil ethyl esters were calculated as a percentage.

### 3. Results and Discussion

#### 3.1. Selection of Lipase for *In Situ* Transesterification

Samples were prepared with 11 different types of biocatalysts and analyzed by thin-layer chromatography. The results showed that five lipases, two immobilized (Lipozyme RM IM and Lipozyme TL IM) and three not immobilized (Lipolase 100L, Lipozyme TL 100L and Lipex 100L),

were effective for transesterification of RO with ethanol via in situ transesterification. The other six lipases were ineffective or not sufficiently effective, according to the experimental design. To compare selected lipases with one another and select the most effective lipase for further studies, validation of the degree of transesterification and the glyceride content in biodiesel fuel was performed. For this further evaluation, gas chromatography was chosen.

To evaluate the five effective (selected by thin-layer chromatography results) lipases and to select the most effective one, it was important to determine the degree of transesterification and glyceride content (Table 2) of the biodiesel produced in each sample. The evaluation was performed by applying gas chromatography.

**Table 2.** Dependence of the degree of transesterification and glyceride content in biodiesel fuel on the different types of lipases when the oil-to-ethanol molar ratio was 1:5, the duration of reaction was 5 h, the temperature of reaction was 40 °C and the lipase concentration was 7%.

Lipase	Degree of Transesterification, %	Glyceride Content in Biodiesel Fuel, %		
		Monoglycerides	Diglycerides	Triglycerides
Lipozyme RM IM	84.40 ± 1.32	2.26 ± 0.15	0.50 ± 0.05	9.50 ± 1.17
Lipozyme TL IM	97.74 ± 0.62	0.91 ± 0.04	0.02 ± 0.01	0.00 ± 0.01
Lipolase 100L	50.82 ± 3.04	3.27 ± 0.08	8.40 ± 0.45	29.86 ± 3.28
Lipozyme TL 100L	30.10 ± 2.17	3.51 ± 0.11	10.97 ± 1.04	93.56 ± 1.43
Lipex 100L	73.35 ± 1.03	2.51 ± 0.08	5.56 ± 0.27	12.72 ± 1.04

As shown in Table 2, when the duration of the reaction was 5 h, the temperature of the reaction was 40 °C and the lipase concentration was 7%. The highest degree of transesterification for biodiesel fuel was obtained when only Lipozyme LT IM was used. The content of monoglycerides, diglycerides and triglycerides in biodiesel must not exceed 0.8, 0.2 and 0.2 w%, respectively. Under these reaction conditions, only the glyceride content in biodiesel produced with Lipozyme TL IM met the requirements for diglyceride (0.02%) and triglyceride (0.00%) concentrations. The monoglyceride (0.91%) concentration with this lipase did not meet the requirements for biodiesel fuel, but it was sufficient for continuing the optimization studies by choosing this lipase. In other samples with different lipases, glyceride contents were too high compared to the requirements for biodiesel fuel.

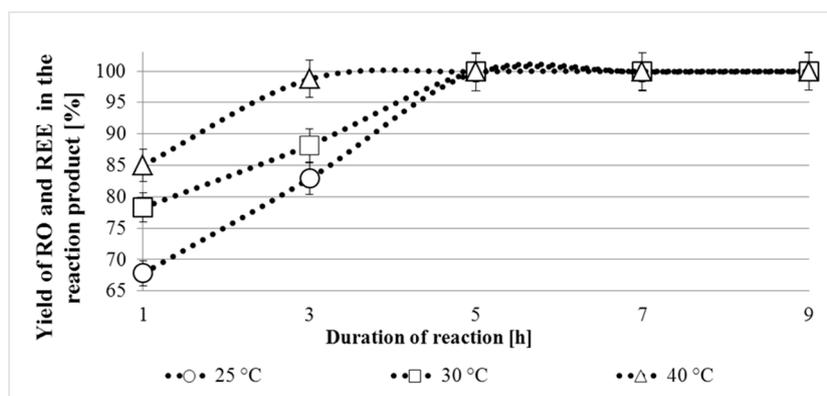
Other researchers have also determined that lipase Lipozyme TL IM can be used for enzymatic syntheses to produce biodiesel fuel from sea mango oil by applying the transesterification process [37]. The catalytic effectiveness of the lipases Lipozyme TL IM and Novozyme 435 using the alcohols methanol and ethanol and different raw materials such as sunflower, borage, olive and soybean oils was also studied. It was found that alcoholysis is more effective with methanol when using lipase Lipozyme TL IM [38]. In our study, after comparing the results of the degree of transesterification and the glyceride content in biodiesel fuel in each of the samples, the most effective lipase for the in situ transesterification process, lipase Lipozyme TL IM, was selected for further studies.

### 3.2. Optimization of the Enzymatic In Situ Transesterification Process

The in situ transesterification process using lipase Lipozyme TL IM and ethanol in a blend with mineral diesel was applied to produce biodiesel from rapeseed unsuitable for food production. The duration and temperature of the reaction and the concentration of the biocatalyst are important variables that affect the yield of biodiesel. Additionally, when the reaction inputs are changed, such as the use of another raw material, alcohol or catalyst, the optimal reaction conditions needed to produce the biodiesel also change accordingly. Consequently, we studied the effects of these variables on biodiesel yield when oil from rapeseed unsuitable for food production is transesterified by ethanol using lipase Lipozyme TL IM as a catalyst.

### 3.2.1. Optimal Temperature and Duration of Reaction

For this study, reaction temperatures of 25, 30 and 40 °C and reaction durations of 1, 3, 5, 7 and 9 h were chosen. Samples prepared for the optimization of the duration and temperature of the reaction time were investigated to determine the yield of RO and REE in the reaction product obtained at the end of the in situ transesterification process. Quantitative analysis was performed to obtain experimental results, which are presented in Figure 1.

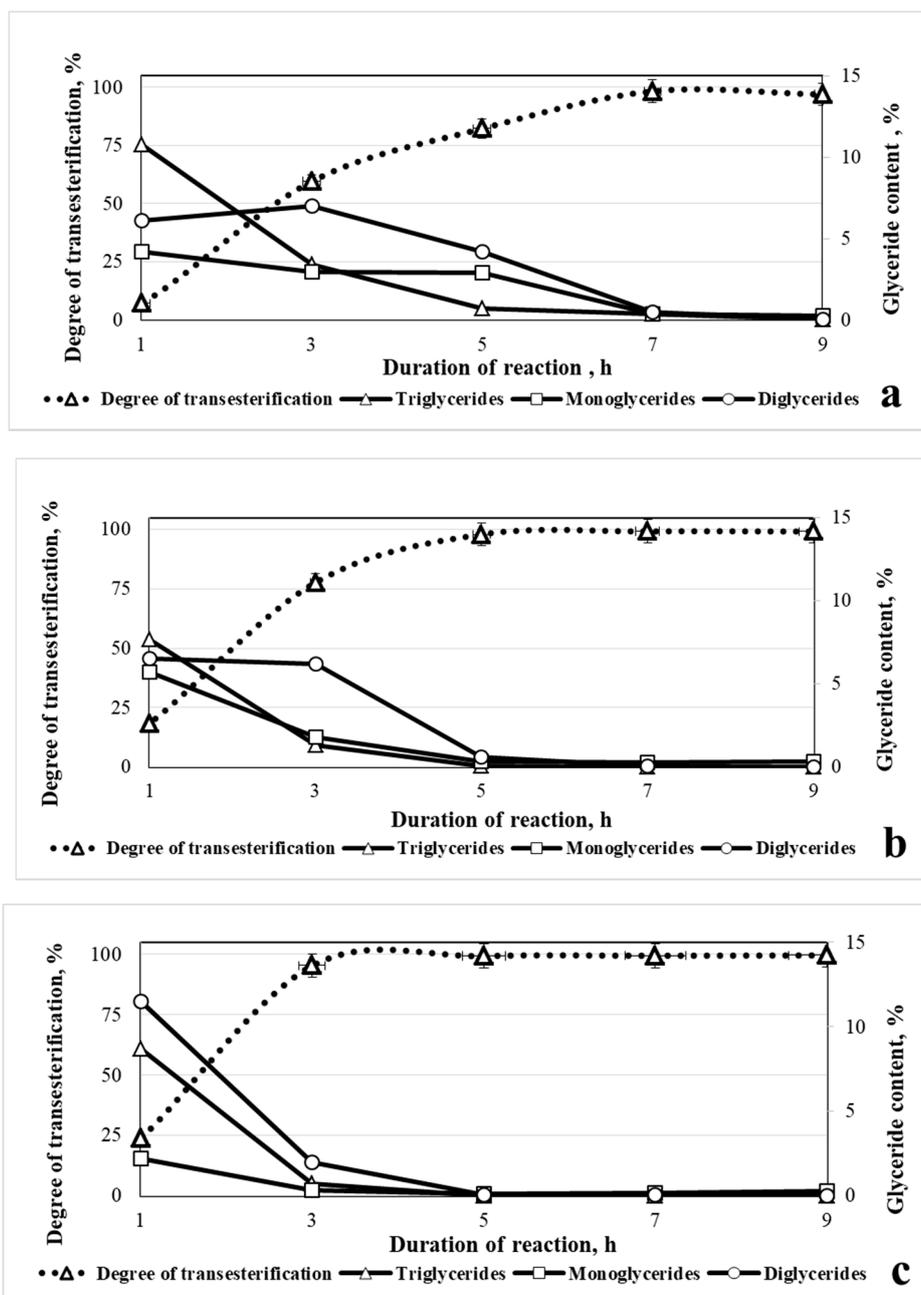


**Figure 1.** The dependence of the yield of rapeseed oil (RO) and rapeseed ethyl esters (REEs) on the reaction time and temperature when the mineral diesel and rapeseed oil blend ratio was 9:1 (w/w), the oil-to-ethanol molar ratio was 1:5 and the lipase (Lipozyme TL IM) concentration was 7%.

As shown in Figure 1, when the reaction duration was 1 h and the reaction temperature was 25 °C, the yield of RO and REEs was the lowest and reached only 67.82%. When temperatures were 30 °C or 40 °C, the yields of RO and REEs were 78.29% and 85.03%, respectively. When the reaction duration was increased to 3 h and the reaction temperature was 25 or 30 °C, the yields of RO and REEs were 82.91% and 88.21%, respectively; at 40 °C, the yield increased to 98.72%. At reaction times of 5, 7 or 9 h, the yields of RO and REEs were very similar at all the chosen reaction temperatures and reached over 99.85%. The maximum yields of RO and REEs were obtained at a reaction duration of 9 h and a reaction temperature of 40 °C, and reached 99.97%. This indicates that extraction efficiency was high when the duration of the reaction was 5 h or longer at all temperatures chosen for this research. The results show that mineral diesel is a suitable rapeseed oil extraction solvent for use in the in situ transesterification process with a lipase catalyst and ethanol.

The dependence of the degree of transesterification and glyceride content in biodiesel on the duration and temperature of the reaction when the oil-to-ethanol molar ratio was 1:5 and the concentration of lipase was 7% was studied. The optimized duration and temperature of the reaction accordingly require a lower energy input, which makes this biodiesel production process more economically attractive and environmentally friendly. The results of the dependence of degree of transesterification and glyceride content in biodiesel on the duration and temperature of the reaction are demonstrated in Figure 2a–c.

When the temperature of the reaction was 25 °C (Figure 2a) and reaction durations were 1, 3 and 5 h, the degree of transesterification reached 7.09%, 59.55% and 82.22%, respectively. The requirements for glyceride contents in biodiesel fuel were not achieved. At reaction durations of 7 and 9 h, the degree of transesterification reached 98.30% and 96.90%, respectively. However, when the reaction duration was 7 h, only the monoglyceride content (0.35%) in the biodiesel fuel met the requirements of standard EN 14214 as the diglyceride (0.45%) and triglyceride (2.51%) contents were too high. When the reaction duration was 9 h, the monoglyceride (0.23%) and diglyceride (0.04%) contents met the requirements for biodiesel fuel, but the diglyceride (0.21%) content was still too high.



**Figure 2.** The temperature of the reaction: (a) 25 °C, (b) 30 °C and (c) 40 °C. The degree of transesterification (vertical (value) axis) and the glyceride content (secondary vertical (value) axis) dependence on the reaction duration and temperature when the oil-to-ethanol molar ratio is 1:5 and the lipase (Lipozyme LT IM) concentration is 7%.

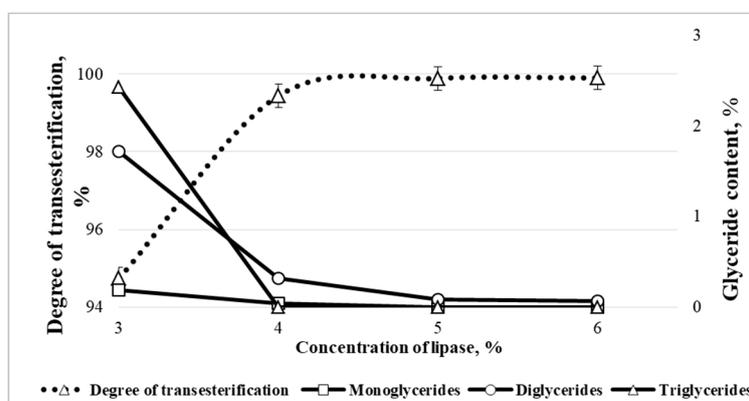
When the temperature of the reaction was 30 °C (Figure 2b) and reaction durations were 1 and 3 h, the degree of transesterification reached 18.44% and 77.75%, respectively, and the glyceride content requirements in biodiesel fuel were not achieved. With a reaction duration of 5 h, 97.99% transesterification was obtained, but by evaluating the glyceride content, only the monoglyceride (0.35%) content in biodiesel fuel met the requirements. The diglyceride (0.62%) and triglyceride (0.30%) contents were too high. At reaction durations of 7 and 9 h, either the requirements for the degree of transesterification (99.27% and 99.20%, respectively) or the requirements for glyceride content in biodiesel fuel were achieved.

When the temperature of the reaction was 40 °C (Figure 2c), the lowest degree of transesterification of 23.91% was obtained at a 1-h reaction duration. The requirements for the glyceride content in biodiesel fuel were also not achieved. With a reaction duration of 3 h, the degree of transesterification reached 95.37%, and the glyceride content in biodiesel fuel met the requirements only for monoglyceride (0.35%), as the diglyceride (1.98%) and triglyceride (5.06%) contents in the biodiesel fuel were too high. At reaction durations of 5, 7 and 9 h, either the degree of transesterification (99.35%, 99.49% and 99.64%, respectively) or the glyceride content in biodiesel fuel met the requirements for biodiesel fuel.

Therefore, it can be concluded that, to optimize the in situ transesterification process using ethanol and a lipase biocatalyst (Lipozyme TL IM), the reaction should be carried out by selecting a reaction duration of 7 h and a reaction temperature of 30 °C, because the lower reaction temperature allows for lower energy requirements, which is economically beneficial. Other scientists have studied how temperature affects the metanalysis of crude palm oil catalyzed by Lipozyme TL IM and concluded that a reaction temperature of 30 °C is ideal for achieving a high biodiesel yield of 96.15% [39].

### 3.2.2. Optimal Lipase Concentration

Selecting an optimal lipase concentration for the in situ transesterification process is no less important than optimizing the reaction duration and reaction temperature. To investigate the effect of the dosage of lipase on the yield of RO and REEs in the reaction product, on the degree of transesterification and on the glyceride content in biodiesel fuel, concentrations of the lipase Lipozyme TL IM in reaction media of 3%, 4%, 5% and 6% based on the amount of oil were chosen. First, the samples were investigated to determine the total yield of RO and REEs in the reaction product obtained at the end of the in situ transesterification process. Experimental results are presented in Figure 3. During the reaction, the oil-to-ethanol molar ratio of 1:5, a reaction duration of 7 h and a reaction temperature of 30 °C were maintained.



**Figure 3.** The dependence of degree of transesterification (vertical (value) axis) and glyceride content in biodiesel fuel (secondary vertical (value) axis) on the lipase (Lipozyme TL IM) concentration when the oil-to-ethanol molar ratio is 1:5, the duration of reaction is 7 h and the temperature of reaction is 30 °C.

As shown in Table 3, the results of the lipase amount optimization experiment at different lipase concentrations were very similar. When the lipase concentration was 3%, the yield of RO and REEs in the reaction product was 99.81%. When the lipase concentration in the reaction media was 4% or 5%, the yields of RO and REEs in the reaction product were 99.95% and 99.92%, respectively. When the lipase concentration in the reaction media was 6%, the yield of RO and REEs in the reaction product was 99.97%. It is known that Lipozyme TL IM is a laboratory-synthesized biocatalyst from *Thermomyces lanuginosus* that is immobilized on a non-compressible silica gel carrier, and that it is highly effective catalyst for transesterification and can successfully rearrange fatty acids. It has been reported that catalytic activity is higher when biocatalysts are immobilized compared to that of free biocatalysts [40]. These results indicate that the lipase concentration in the sample had no significant

effect on the RO and REE yields. However, studies have shown that lipase concentration affects the degree of transesterification and the glyceride content in biodiesel fuel (Figure 3).

**Table 3.** The dependence of the yield of rapeseed oil (RO) and rapeseed ethyl esters (REEs) in the reaction product on the lipase (Lipozyme TL IM) concentration when the mineral diesel and rapeseed oil blend ratio is 9:1 (w/w), the oil-to-ethanol molar ratio is 1:5, the duration of reaction is 7 h and the temperature of reaction is 30 °C.

Concentration of Lipase, %	Yield of RO and REE in the Reaction Product, %
3	99.81 ± 0.04
4	99.95 ± 0.02
5	99.92 ± 0.02
6	99.97 ± 0.01

For the sample with a lipase concentration of 3%, the degree of transesterification was 94.74%. In this case, the glyceride content in the biodiesel fuel only met the requirements for monoglyceride (0.19%), whereas the diglyceride (1.72%) and triglyceride (2.43%) contents were too high. However, for the samples with lipase concentrations of 4%, 5% and 6%, there was a high degree of transesterification (99.44%, 99.89% and 99.90%, respectively). When the lipase concentration in the sample was 4%, only the diglyceride content (0.32%) in the biodiesel fuel was too high; the monoglyceride (0.04%) and triglyceride (0.00) contents met the requirements for glyceride content in biodiesel fuel. When the lipase concentrations in the samples were 5% or 6%, the glyceride content ideally met the requirements for biodiesel fuel. Taking this into account, it can be concluded that a lipase concentration of 5% (based on the amount of oil) is the most appropriate for the in situ transesterification process, as it led to a high yield of RO and REEs in the reaction product (99.92%), a high degree of transesterification (99.89%) and optimal glyceride contents in the biodiesel fuel.

#### 4. Conclusions

A mixture of biodiesel-mineral diesel (9:1 w/w) was produced from rapeseed unsuitable for food production, as it contained a high amount of free fatty acids. The in situ transesterification process was performed using a mixture of ethanol and mineral diesel (as an extraction solvent) and the lipase biocatalyst Lipozyme TL IM. The evaluation of the study results leads to the conclusion that to produce a biodiesel-mineral diesel mixture by an in situ transesterification process with a lipase catalyst and ethanol, the optimal conditions for ethanolysis when the oil-to-ethanol molar ratio is 1:5 are as follows: duration of reaction, 7 h; temperature of the reaction, 30 °C; and lipase concentration, 5%. Under these optimal conditions, 99.90% of the oil was extracted from rapeseed and transesterified, and a degree of transesterification of 99.89% with optimal glyceride contents in biodiesel fuel could be obtained. These results indicate that rapeseed unsuitable for food production is a suitable raw material to produce biodiesel via in situ transesterification using the lipase Lipozyme TL IM as the catalyst and with ethanol.

**Author Contributions:** Conceptualization, E.S.; methodology, E.S. and V.M.; investigation M.S., E.S., V.M., and K.K.; data curation and methodology M.S. and E.S.; resources E.S. and K.K.; writing—original draft preparation M.S., E.S. and V.M.; writing—review and editing, E.S. and V.M.; supervision, E.S. and V.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Acknowledgments:** Authors are thankful for biocatalyst to JSC “Biopolis”.

**Conflicts of Interest:** The authors declare no conflict of interest.

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