Enzyme-catalysed Biodiesel Production from Edible and Waste Cooking Oils

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Biodiesel synthesis was performed as transesterification of edible and waste cooking sunflower oil catalysed by free lipase from Thermomyces lanuginosus (Lipase 100L). Experiments were performed at three different temperatures (T = 40, 50 and 60 °C) as one-step and four-step reactions with methanol.

The highest fatty acids methyl esters (FAME) content (C = 95 %) was achieved in the one-step transesterification reaction of edible sunflower oil performed at 40 °C.

Key words:
lipase, Thermomyces lanuginosus, transesterification, biodiesel, waste cooking oil, sunflower oil

Introduction

Biodiesel is a mixture of alkyl esters of fatty acids produced from vegetable oils, micro algal oils, animal fats, waste products of vegetable oil refineries, or waste cooking oils (WCO). It can be used in diesel engines. Industrial production of biodiesel is mostly conducted from high-quality oils by the transesterification process with methanol in the presence of acid or basic catalysts where biodiesel and glycerol are produced. A disadvantage of this process is the soap formation that needs to be separated from the product mixture leading to an increased amount of wastewater generation and additional energy consumption. Therefore, downstream processing costs, by-product recovery and environmental problems have imposed the need to explore alternative methods. Application of enzymes such as immobilized or free lipases originating from different microorganisms for biodiesel production brings several advantages. Beside the well-known fact that enzymatic processes are performed under mild conditions and without additional energy consumption, enzymatic biodiesel production leads to the production of food-grade glycerol without soap generation. However, enzymes have certain disadvantages which move them away from wider application in the synthesis of biodiesel, such as high costs. Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyse the hydrolysis of triacylglycerols to di- and monoacylglycerols, free fatty acids and glycerol, and are commonly applied in the production of food, detergents, paper, textile, leather and cosmetics. However, they can be used in biodiesel production due to their ability to simultaneously catalyse hydrolysis, esterification and transesterification.

As already mentioned, high quality edible vegetable oils are usually used for commercial biodiesel production. In order to make the process more economical and environmentally friendly, a considerable number of articles suggest the use of WCO in enzymatic biodiesel productions. WCO exists in a significant amount (1.85–2.65 million litres/day in EU) and there is no permanent solution for its proper disposal. Research has been conducted in order to find the optimal process conditions giving maximal conversion and good quality biodiesel, such as optimal reaction temperature, stirring rate, molar ratio of oil to alcohol with or without solvent and excess water in single or multi-step reactions, using lipases originating from different sources (fungi, bacteria, and yeasts), as well as the purification and quality analysis procedures. According to the literature, there are different approaches in enzymatic biodiesel production using free enzymes. Some authors have separately performed hydrolysis and esterification using enzyme in the first phase to obtain free fatty acids from oils without the addition of emulsifiers, and adding alcohols and heterogeneous catalysts in the second phase to obtain corresponding esters. However, other approaches are different: simultaneous hydrolysis and esterification reactions are performed with different organic solvents (petroleum ether, tert-butanol, n-hexane) using immobilized lipases.

In this paper, biodiesel was produced from edible and waste cooking oil in one-step and four-step
reactions catalysed by free lipase from *Thermomyces lanuginosus* (Lipolase 100L), where in the latter reaction, methanol was added in small portions to the reaction mixture, without the use of emulsifiers. Initial conditions (enzyme concentration, oil to methanol ratio) were chosen based on preliminary experiments (data not shown). Since the reaction of lipase-catalysed biodiesel production from oil occurs at the interface between oil and water, the enzyme was prepared in buffer ensuring the enzyme’s demand for water, while stirring in the reactor was set up in order to create an emulsion. The four-step reaction was performed to prevent possible inactivation of the enzyme by methanol, ensuring the final methanol concentration equal to that in the one-step reaction.

**Materials and methods**

**Substrate, enzyme and chemicals**

Edible sunflower oil (Oil Refinery Čepin, Croatia) was purchased at a nearby market. Waste cooking oil (WCO) used in this research was produced from edible sunflower oil in a laboratory fryer after the deep-frying of potatoes at 190 °C according to the already published method\(^\text{23}\). Lipase from *Thermomyces lanuginosus* (Lipolase 100L), phosphate buffer saline – PBS (pH = 7.4), heptane, F.A.M.E. mix GLC-10 and heptadecanoic acid methyl ester as internal standard were purchased from Sigma-Aldrich Handels GmbH (Vienna, Austria). Methanol was purchased from Kemika (Zagreb, Croatia).  

**Experimental set-up**

The experimental set-up consisted of a bioreactor constructed as a vessel with double walls, Liebig’s condenser to avoid methanol loss, magnetic stirrer, septum, and separation funnel.

**Biodiesel production in batch and fed-batch reactors**

Biodiesel production from edible and waste cooking sunflower oil using Lipolase 100L was performed in laboratory batch and fed-batch reactors. The experiments were conducted as one-step and four-step reactions. To investigate the influence of the temperature on the biodiesel production process, experiments with edible and waste cooking oil were performed at three different temperatures (\(T = 40, 50\) and 60 °C). The four-step reaction was carried out in order to avoid possible inactivation of the enzyme with methanol\(^\text{16}\). In order to ensure a sufficient amount of methanol in the one-step reaction, the molar ratio of oil to methanol was 1:3.4. In the four-step reaction experiment, the molar ratio of oil to methanol at the beginning of the process was 1:1 after which the methanol was fed into the reaction mixture every 12 hours (molar ratio of oil to methanol was 1:0.8)\(^\text{16}\). All experiments were performed for four days (96 h) with constant stirring (600 rpm). The initial lipase concentration (dissolved in phosphate buffer saline pH 7.4), was the same in all experiments (\(γ_{E,0} = 0.1 \text{ mg cm}^{-2} \cdot \text{s} \cdot \text{A} = 100000 \text{ U mg}^{-1}\)).  

The reaction started by the addition of the enzyme into the reaction mixture. The total mass of the reaction mixture was 550.95 g, and comprised 450 g of oil, 55.95 g of methanol, and 45 g of Lipolase 100L stock solution diluted with 0.01 mol dm\(^{-3}\) phosphate buffer at pH 7.4 in molar ratio 1:10. Samples of biodiesel were taken at certain time intervals (after 1, 2, 3, 6, 12, 24, 36, 48, 60, 72, 84 and 96 hours), carefully withdrawn from the upper layer of the reaction mixture and analysed by gas chromatography.

Upon completion of the experiments, glycerol was separated from the biodiesel by a separation funnel.

**Analytics**

The edible oil, waste cooking sunflower oil, and the obtained methyl esters were analysed by gas chromatograph Shimadzu GC-2014 (Kyoto, Japan) equipped with FID and Zebron ZB-wax GC capillary column (length 30 m, I.D. 0.53 mm and film thickness 1.00 µm) using heptadecanoic acid methyl esters as an internal standard. The method consisted of holding the temperature at 180 °C for one minute and then heating up to 230 °C at a rate of 5 °C min\(^{-1}\). Total time of determination was 20 minutes with helium as carrier gas at 1.97 cm\(^3\) min\(^{-1}\). Peaks identification was carried out using standard F.A.M.E. mix GLC-10\(^\text{23}\). The retention times for corresponding esters of fatty acids were 7.494 min for palmitic, 10.192 min for stearic, 10.545 min for oleic, 11.257 min for linoleic, 12.336 min for linolenic acids, and for internal standard 9.07 min. Total fatty acid methyl esters (FAME) content (\(C, \%\)) was calculated according to the equation

\[
C = \frac{\sum A - A_\text{st} (C_{17:0}) \cdot V_\text{s} \cdot C_\text{m}}{m_s} \cdot 100 \% \quad (1)
\]

where \(A\) denotes total peak area \(C_{14:0} - C_{24:1}, A_\text{st}\) internal standard peak area, \(V_s\) volume of the internal standard solution used (cm\(^3\)), \(C_m\) concentration of the internal standard solution (mg cm\(^{-3}\)), and \(m_s\) mass of the sample (mg).

Biodiesel density was determined according to the European regulations of European Committee for Standardization (EN 14214)\(^\text{26}\) for quality assurance of pure biodiesel.
Results and discussion

Dynamic changes in FAME content during the transesterification of the edible sunflower oil catalysed by free Lipolase 100L performed at three different temperatures are presented in Fig. 1 for the one-step reaction, and in Fig. 2 for the four-step reaction.

Dynamic changes in FAME content during the transesterification of WCO catalysed by free Lipolase 100L performed at three different temperatures are presented in Fig. 3 for the one-step reaction, and in Fig. 4 for the four-step reaction.

Literature data on *T. lanuginosus* lipases temperature optimum are diverse and indicate that the lipase temperature optimum can vary from 37 °C to 55 °C, moreover, to obtain higher activity at higher temperatures, lower lipase concentration is needed\[^{10,16,24,27,30}\]. According to the results in this study, FAME content in the one-step reaction of both feed-stocks (edible and waste cooking sunflower oil) at 50 and 60 °C was negligible compared to the FAME content accomplished at 40 °C. The maximum FAME content (94.8 % with edible oil and 82.57 % with WCO) was reached in experiments performed at 40 °C after 96 h. On the other hand, there was almost no difference in FAME content after the four-step transesterification of edible oil between the results of experiments performed at 40 and 50 °C, while a significantly lower FAME content was obtained in experiments performed at 60 °C. A similar situation occurred in WCO experiments for the four-step reaction, even though the difference between results obtained at 40 and 50 °C was significantly higher.

In the four-step reactions performed with edible oil, an increase in FAME content occurs after every addition of methanol. The deviation in this trend was observed in the experiment performed with waste cooking oil; the decrease in FAME con-

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**Fig. 1** – FAME content (C, %) of edible sunflower oil to biodiesel in one-step reaction of transesterification catalysed by *Thermomyces lanuginosus* at 40, 50 and 60 °C

**Fig. 2** – FAME (C, %) content of edible sunflower oil to biodiesel in four-step reaction of transesterification catalysed by *Thermomyces lanuginosus* at 40, 50 and 60 °C

**Fig. 3** – FAME content (C, %) of waste cooking oil (WCO) to biodiesel in one-step reaction of transesterification catalysed by *Thermomyces lanuginosus* at 40, 50 and 60 °C

**Fig. 4** – FAME content (C, %) of waste cooking oil (WCO) to biodiesel in four-step reaction of transesterification catalysed by *Thermomyces lanuginosus* at 40, 50 and 60 °C
tent between the 6th and 12th hour, and 36th and 48th hour can be explained by an insufficient amount of methanol that would ensure a shift reaction in the direction of product formation, and with the hydrolysis that drives the equilibrium back to the formation of free fatty acids and alcohol.  

The biodiesel produced from WCO had lower FAME content (82.57% and 72.02% for one-step and four-step reaction, respectively) in comparison to the biodiesel produced from edible oil. The maximum FAME content (94.8%) of all performed experiments was gained with the one-step reaction using edible oil. Rodrigues et al. showed that lipase from Thermomyces lanuginosus had lower affinity for WCO than for fresh sunflower oil. Since the molar ratio of oil and methanol was the same in all reactions (1:3:4), it can only be assumed that, for a higher conversion of waste cooking oil, it is necessary to increase the molar ratio of oil and methanol in favour of alcohol, as indicated in literature.  

According to the results in this study, maximal ester content (95.02%) was obtained from edible oil at 40 °C after 96 h in the one-step reaction. Under the same process conditions, lower ester content (82.57%) was gained when WCO was used (Fig. 4). According to the literature, high FAME content can be produced (total conversion, 100%) by immobilized Thermomyces lanuginosus after 4 h hours from crude palm oil.  

To be declared as biodiesel and used in internal combustion engines, a fatty acid methyl esters mixture must meet the quality criteria prescribed by the legislation according to EN 14214, and density is a key physical property of biodiesel directly influencing engine performance. According to Croatian Standard HRN EN 142014:2012, which is based on the European Standard EN 142014, the density of biodiesel needs to be in the range from 860 to 900 g dm⁻³.  

The density of biodiesel produced from edible oil was 886.0 and 886.2 g dm⁻³, for the four- and one-step reactions, respectively. The density of biodiesel obtained from WCO was 907.2 g dm⁻³ in the one-step reaction, and 908.2 g dm⁻³ in four-step reaction, and exceeded the limits. The reason for such high density values is low ester content and high amount of waste cooking oil presented in the final mixture. Therefore, this process should be further explored.

Conclusions

Reactions conducted in a batch reactor are preferable in comparison to reactions conducted in a fed-batch reactor due to the accomplished higher final content of FAME (95%) in the same reaction time. The use of waste cooking oil as a cheaper feedstock can reduce the costs and is environmentally friendly, since the waste oils from the food industry make up a significant amount of pollutants.

Further research will be directed towards process intensification by reactor configurations to maximize fatty acid methyl esters content, and improve the quality of the biodiesel obtained from waste cooking oil.

ACKNOWLEDGMENTS

The authors would like to thank the colleagues of the biodiesel plant “Biodizel Vukovar Inc. Vukovar, Croatia” for their kind advice and practical help in the experimental part of this work.

List of symbols and abbreviations

- A – total peak area C_{4:0} - C_{22:1}
- A_{st} – internal standard peak area
- C – content of fatty acids methyl esters, %
- C_{st} – concentration of the internal standard solution, mg cm⁻³
- EC – Enzyme Classification
- EU – European Union
- F.A.M.E. mix GLC-10 – analytical standard
- FAME – fatty acids methyl esters
- FID – flame ionization detector
- GC – gas chromatography
- m – mass of sample, mg
- PBS – phosphate buffer saline
- S.A. – enzyme activity, U mg⁻¹
- T – temperature, °C
- V_{st} – volume of the internal standard solution used, cm³
- WCO – waste cooking oil
- γ – enzyme concentration, mg cm⁻³

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