

# The Influence of Qualitative Parameters of Waste Cooking Oils and Fat on Fatty Acid Methyl Esters Yield

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## Abstract

Due to the growing demand for alternative green fuels, biodiesel remains a focal point of scientific research. While chemical biodiesel production, mostly from edible oils, has been established worldwide for years, enzymatic synthesis and the utilisation of raw materials like waste or inedible oils and fats are increasingly preferred, primarily due to economic and environmental considerations. The aim of this study was to perform enzymatic biodiesel synthesis with *Burkholderia cepacia* lipase using selected waste cooking vegetable oils and animal fat, and to correlate the resulting fatty acid methyl esters (FAME) with lipase substrate specificity and main specific characteristics of the selected waste feedstock (such as fatty acid profile, free fatty acid content, peroxide and iodine values). The listed characteristics of the oils/fat were determined using standard methods, while biodiesel was synthesised through transesterification of the selected waste oils/fat with methanol in the presence of a lipase catalyst. Analysis of FAME during 24 h of biodiesel synthesis from waste cooking vegetable oils revealed that a one-hour reaction time was sufficient to achieve a yield of over 97 % of FAME. Conversely, when utilising waste cooking animal fat as a substrate, the minimum yield of 96.5 %, as per European standard (EN 14214), was not achieved even after 24 h of synthesis. According to the correlation matrix, the highest positive correlation was found between iodine value and the resulting FAME. The waste cooking vegetable oil with the highest iodine number ( $123.88 \pm 2.41 \text{ g I}_2/100 \text{ g}$ ) yielded the highest proportion of methyl esters after 1 h of synthesis. A statistically significant positive correlation was found between FAME and the iodine and peroxide values, suggesting these parameters as indicators of waste oil suitability for biodiesel synthesis. This study is a contribution to further research on potential substrates for biodiesel synthesis from waste streams.

## Keywords

Biodiesel synthesis, iodine value, waste cooking vegetable oil, waste cooking animal fat, *Burkholderia cepacia* lipase

## 1 Introduction

Chemical synthesis of biodiesel has been developed on an industrial scale for many years. However, the high selling price of biodiesel stems from the use of edible oils as raw materials,<sup>1,2</sup> accounting for up to 95 % of the total cost.<sup>3</sup> Nowadays, there are many other raw materials available that can be used for biodiesel production. Depending on the raw material used, biodiesel is categorised into different generations of biofuels. While first-generation biodiesel is derived from edible oils primarily intended for human consumption, the third and fourth generations involve algae or modified algae, offering distinctive yields<sup>4</sup> but often proving economically unfeasible due to the requirements of algae cultivation.<sup>1,3</sup> On the other hand, second-generation biodiesel, sourced from waste vegetable oils or fats, stands out as the most cost-effective variant globally. Apart from the reduced cost of raw materials for biodiesel production, addressing disposal and exploring beneficial utilisation instead of disposal are also essential. However, the composition of oils as raw material is the most important criterion for assessing their suitability in biodiesel production.<sup>2</sup> Through frying, oil undergoes a series of reactions such as

thermal oxidation, polymerisation, and hydrolysis, leading to increased free fatty acid content (FFA) and peroxide value (PV), among other changes.<sup>5,6</sup> According to Budžaki *et al.*,<sup>7</sup> waste cooking oils and animal fats typically contain higher FFA levels, which can significantly affect biodiesel production when certain chemical catalysts are employed. Indeed, a base catalyst becomes unsuitable for biodiesel production if the oil contains more than 3 % FFA,<sup>8</sup> leading to foaming and necessitating oil pretreatment, thereby further increasing production costs. While acid catalysts exhibit greater tolerance to FFA content than base catalysts<sup>9</sup>, enzymatic biodiesel production emerges as the best solution. Lipases, in this context, can convert all FFA content into fatty acid methyl esters (FAME),<sup>10</sup> eliminating the need for oil pretreatment.<sup>7</sup> Alongside FFA, peroxide (PV) and iodine values (IV) serve as important indicators of oil quality. The IV indicates the oil's degree of unsaturation and aids in monitoring the thermo-oxidative changes during frying, allowing for the prediction of the biodiesel's IV and correlation with other parameters such as viscosity and cetane number.<sup>11,12</sup> On the other hand, PV indicates the oxidation degree and is an indicator of freshness or rancidity of oils or fats, and can adversely affect the enzymatic synthesis of biodiesel due to the inhibitory effect of peroxides on lipase activity.<sup>12</sup>

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Continuing our efforts toward the commercialisation of enzymatic biodiesel synthesis, this research explores the possibility of producing biodiesel enzymatically using *Burkholderia cepacia* lipase as a biocatalyst and selected waste cooking vegetable oils and animal fat as feedstock. Transesterification reactions were conducted under optimal conditions as reported in our previous research.<sup>13</sup> Moreover, we investigated the effect of lipase substrate specificity toward the selected oils and animal fat, as well as the levels of FFA, PV, and IV of the examined oils and fat on FAME yield.

## 2 Experimental

### 2.1 Materials

Amano lipase from *Burkholderia cepacia*, intended for industrial application, Supelco F.A.M.E. Mix (C4-C24), and sodium chloride were purchased from Sigma Aldrich (St. Louis, USA). Ethanol 96 %, phenolphthalein, potassium iodide, potassium hydroxide, *o*-phosphoric acid 85 %, and sodium hydroxide were purchased from Kemika (Zagreb, Croatia). Acetic acid, iodine monobromide, and sodium thiosulphate were procured from Kefo (Ljubljana, Slovenia). Chloroform and *n*-heptan were purchased from Carlo Erba (Val de Reuil, France). Potassium dihydrogen phosphate and starch were purchased from Fisher Chemical (Geel, Belgium). Boric acid was obtained from T.T.T. (Sveta Nedelja, Croatia). Diethyl ether was purchased from Macron (Gliwice, Poland). Glycerol was purchased from Gram-mol (Zagreb, Croatia). Gum Arabic was purchased from Acros Organics (Geel, Belgium). Methanol was purchased from J. T. Baker (Gliwice, Poland). Potassium chloride was purchased from Merck-Alkaloid (Skopje, Macedonia). Waste cooking vegetable oils (WCO) of different origin and composition and waste cooking animal fat (lard) (WCF) were obtained from households.

### 2.2 Methods

#### 2.2.1 Characterisation of waste cooking oils and fat: Free fatty acids (FFA), peroxide (PV), and iodine (IV) values

The selected waste cooking oils (WCOs) and waste cooking animal fat (WCF) underwent analysis for their FFA, PV, and IV contents. The FFA content in WCOs and WCF was determined according to ISO 660<sup>14</sup> and expressed as a percentage of oleic acid. The PV was determined according to ISO 3960<sup>15</sup> and expressed as mmol O<sub>2</sub>/kg of oil, while IV was determined according to ISO 3961<sup>16</sup> and expressed as g I<sub>2</sub>/100 g of oil.

#### 2.2.2 Lipase substrate specificity

The substrate specificity of *B. cepacia* lipase toward selected WCOs and WCF was determined using a titrimetric assay for lipase activity as described in a previous work<sup>13</sup> at a pH of 10 and a temperature of 50 °C.

### 2.2.3 Biodiesel synthesis

Biodiesel was synthesized under optimal conditions previously described<sup>13</sup>, where it was determined that the lipase activity load of 250 U per 1 g of the reaction mixture and 200 mmol l<sup>-1</sup> Britton-Robinson buffer at pH 10 for maintaining the water phase of the reaction mixture, was sufficient for the production of over 99 % FAME within one hour. Three separate syntheses were carried out with each selected feedstock in a batch reactor (*V* = 250 ml) under continuous stirring at 850 rpm in a water bath (Thermomix 1420, Germany) at 50 °C for 24 h. Biodiesel samples were collected after 1, 3, 6, and 24 h of reaction, and prepared for FAME determination according to the procedure described by *Ostojčić et al.*<sup>13</sup>, using gas chromatography. The samples from the WCF syntheses underwent additional filtration (PTFE Syringe filter, pore size: 0.45 μm, *i.d.* = 25 mm) to prevent potential blockage of the GC column.

### 2.2.4 FAME analysis

FAME analysis of WCOs, WCF, and the biodiesel was performed using a Shimadzu GC-2010 Plus gas chromatograph (Kyoto, Japan) equipped with a flame ionisation detector (FID) and fitted with an SH-FAMEWAX<sup>TM</sup> capillary column (30 m, 0.32 mm ID, and 0.25 μm thick stationary phase). The injector temperature was 240 °C, and the sample volume was 2 μl (split ratio 1 : 100). The initial column temperature of 120 °C was maintained for 5 min, followed by an increase to 220 °C at a rate of 5 °C min<sup>-1</sup>, held for 20 min. Nitrogen was used as the carrier gas with a flow rate of 1.26 ml min<sup>-1</sup>. The detector temperature was set at 250 °C. The results were expressed as a percentage of identified fatty acid on total fatty acids (%). Prior to GC analysis, fatty acids in WCOs and WCF were transesterified to fatty acid methyl esters using a cold potassium hydroxide solution and heptane as solvent.<sup>17</sup>

### 2.2.5 Statistical analysis

Mean values and standard deviations were calculated using Microsoft® Excel® 2016 MSO, while data correlation and statistical significance were assessed using Statistica 14.0.0.15 (TIBCO Software Inc.).<sup>18</sup> Under the natural assumption of normal data distribution for measurements under the same controlled conditions, ANOVA was used for statistical inference.

## 3 Results and discussion

### 3.1 Characteristics of WCOs and WCF

To determine the suitability of WCOs and WCFs as feedstocks for enzymatic biodiesel synthesis, the fatty acid profiles of selected WCOs and WCF were determined, followed by the contents of FFA, PV, and IV. It was expected that the properties of WCOs and WCF would resemble those of edible oils, with variations dependent on the age and multiple uses of the oil, as indicated by *Gui et al.*<sup>2</sup>

### 3.1.1 Fatty acid profile

The fatty acid profiles of selected oils and fat included four different samples. WCOs 1 and 3 were sunflower oils used for frying meat, fish and potatoes in two different households, likely sourced from different producers. On the other hand, WCO 2 was a mixture of sunflower, rapeseed, and poppy vegetable oil, used in a deep fryer. WCF consisted of waste cooking animal fat/lard from households used for frying meat, fish and potatoes. The analysis of the fatty acid profiles of the examined oils and fat is presented in Table 1. The WCOs contained a significant amount of C18 unsaturated fatty acids (C18:1 and C18:2), with other fatty acids present in much lower amounts. *Demirbas*<sup>19</sup> also found the highest proportion of these two fatty acids in waste sunflower oil, while *Klofutar et al.*<sup>20</sup> reported the highest proportion of C18:2 fatty acid and absence of C18:1 fatty acid. Other quantified fatty acids were present in similar proportions as reported by *Demirbas*<sup>19</sup> and *Klofutar et al.*<sup>20</sup> According to *Awogbemi et al.*<sup>21</sup>, sunflower oil contains 49–57 % linoleic (C18:2) and 14–40 % oleic acid (C18:1), consistent with the results for the tested oils, where WCO 1 contained  $30.70 \pm 0.08$  % oleic and 58.60 % linoleic acid, and WCO 3 contained 32.90 % oleic and  $55.86 \pm 0.01$  % linoleic acid. For WCO 2, the proportions of these two fatty acids were approximately the same, amounting to  $43.77 \pm 0.06$  % for oleic acid and  $41.43 \pm 0.19$  % for linoleic acid. Also, in WCO 2, there was a slightly higher proportion of linolenic (C18:3) acid, unlike the other two investigated waste oils. WCF, in addition to a significant amount of C18 unsaturated fatty acids, contained higher levels of saturated fatty acids C14:0, C16:0 and C18:0, consistent with the findings of *Shin et al.*<sup>22</sup> *Sarantopolous et al.*<sup>23</sup> *Stojković et al.*<sup>24</sup> *Srinivasan*

and *Jambulingam*,<sup>25</sup> and *Miladinović et al.*<sup>26</sup>, stating almost identical percentages for individual fatty acids for waste lard or yellow and brown grease. Considering the data<sup>27</sup> on the amount of individual fatty acids (C14:0–C24:0) available for sunflower and rapeseed oil as well as animal fats, it can be safely concluded that the obtained fatty acid profiles (Table 1) confirmed that WCOs 1 and 3 were sunflower oils, and WCF was of animal fat origin. However, which type of oil dominated in WCO 2, considering the fatty acid profile, sunflower or rapeseed, could not be determined with certainty. None of the tested oils and fat exhibited a total fatty acid content of 100 %, indicating the presence of impurities ranging from 0.53 to 1.53 % for WCOs, and over 3 % for WCF. The maximum permitted values for water and volatile substances, and insoluble impurities in Regulation<sup>27</sup> are 0.2 and 0.05 % for refined oils, 0.4 and 0.05 % for mixtures of vegetable oils, and 0.2 and 0.5 % for animal fats. Therefore, none of the tested oils and fat met the regulatory requirements for impurities and/or water content in edible oils and fats. Statistical analysis of the results (Table 1) revealed a statistically significant difference in the fatty acids C16:0, C16:1, C18:0 and the total fatty acids (%) in the composition of the WCOs and WCF used. However, for the other fatty acids, determining the Welch's *p* coefficient was not possible due to equal variances, a condition not supported by this test. Therefore, a non-parametric analysis (Kruskal-Wallis ANOVA) was performed, which generally did not show a statistically significant difference for any fatty acid (data not shown). The post-hoc analysis (Scheffe test) demonstrated differences in the composition of individual fatty acids between the raw materials used (Table 1), again demonstrating the high similarity between WCO 1 and WCO 3.

Table 1 – Fatty acid profiles of selected waste cooking oils (WCO 1-3) and waste cooking fat (WCF). Results present average values (%)  $\pm$  SD of three independent determinations, each performed in triplicate

Tablica 1 – Profil masnih kiselina odabranih otpadnih jestivih ulja (WCO 1-3) i otpadne jestive masti (WCF). Rezultati predstavljaju prosječne vrijednosti (%)  $\pm$  SD triju neovisnih određivanja izvedenih u trima ponavljanjima

Sample Uzorak	C14:0	C16:0	C16:1	C18:0	C18:1n9c+t	C18:2n6c	C18:2n6t	C18:3n3	C20:0	C20:1	C22:0	C24:0	Total fatty acids Ukupne masne kiseline (%)
WCO 1	n.d.	$5.87 \pm 0.01^a$	$0.14 \pm 0.01^a$	$2.94 \pm 0.01^a$	$30.70 \pm 0.08^a$	$58.60 \pm 0.00^a$	n.d.	$0.10 \pm 0.01^a$	$0.21 \pm 0.01^a$	$0.12 \pm 0.00^a$	$0.61 \pm 0.01^a$	$0.17 \pm 0.05^a$	$99.43 \pm 0.03^a$
WCO 2	n.d.	$6.87 \pm 0.07^a$	$0.14 \pm 0.01^a$	$1.90 \pm 0.04^b$	$43.77 \pm 0.06^b$	$41.43 \pm 0.19^b$	n.d.	$3.28 \pm 0.02^b$	$0.29 \pm 0.00^b$	$0.50 \pm 0.00^b$	$0.30 \pm 0.00^b$	n.d.	$98.47 \pm 0.01^b$
WCO 3	n.d.	$6.27 \pm 0.04^a$	$0.14 \pm 0.01^a$	$3.00 \pm 0.02^a$	$32.90 \pm 0.00^a$	$55.86 \pm 0.01^c$	$0.12 \pm 0.01$	$0.10 \pm 0.00^a$	$0.21 \pm 0.01^a$	$0.14 \pm 0.01^a$	$0.60 \pm 0.00^a$	$0.15 \pm 0.08^a$	$99.47 \pm 0.01^a$
WCF	$1.73 \pm 0.31$	$23.07 \pm 0.56^b$	$3.57 \pm 0.23^b$	$7.48 \pm 0.01^c$	$46.18 \pm 1.25^b$	$12.77 \pm 0.28^d$	n.d.	$0.66 \pm 0.01^c$	n.d.	$1.23 \pm 0.05^c$	n.d.	n.d.	$96.67 \pm 0.08^c$
Welch's <i>p</i>	–	0.004730*	0.008627*	0.000036*	–	–	–	–	–	–	–	–	0.000202*

n.d. not detected; Means with the same letter within the column were not significantly different at  $p \geq 0.05$ , \* significant at  $p < 0.05$

Fatty acid methyl esters: C14:0 methyl myristate, C16:0 methyl palmitate, C16:1 methyl palmitoleate, C18:0 methyl stearate, C18:1n9c+t methyl oleate + methyl elaidate, C18:2n6c methyl linoleate, C18:2n6t methyl linolelaidate, C18:3n3 methyl linolenate, C20:0 methyl arachidate, C20:1 methyl cis-11-eicosenoate, C22:0 methyl behenate, C24:0 methyl tetracosanoate



Table 2 – Free fatty acids (FFA) content, peroxide value (PV), and iodine value (IV) of selected waste cooking oils and fat. Results present average values  $\pm$  SD of three independent determinations, each performed in triplicate

Tablica 2 – Slobodne masne kiseline (FFA), peroksidni (PV) i jodni broj (IV) odabranih otpadnih jestivih ulja i otpadne jestive masti. Rezultati predstavljaju prosječne vrijednosti  $\pm$  SD triju neovisnih određivanja izvedenih u trima ponavljanjima

Sample Uzorci	FFA Slobodne masne kiseline /% of oleic acid	PV Peroksidni broj /mmol O <sub>2</sub> /kg	IV Jodni broj /g I <sub>2</sub> /100 g
WCO 1	0.37 $\pm$ 0.02 <sup>a</sup>	3.98 $\pm$ 0.33 <sup>a</sup>	123.88 $\pm$ 2.41 <sup>a</sup>
WCO 2	2.92 $\pm$ 0.04 <sup>b</sup>	1.29 $\pm$ 0.26 <sup>b</sup>	116.68 $\pm$ 2.11 <sup>b</sup>
WCO 3	0.23 $\pm$ 0.02 <sup>c</sup>	1.40 $\pm$ 0.42 <sup>b</sup>	120.34 $\pm$ 3.23 <sup>a,b</sup>
WCF	0.51 $\pm$ 0.01 <sup>d</sup>	1.60 $\pm$ 0.42 <sup>b</sup>	74.72 $\pm$ 2.89 <sup>c</sup>
Welch's p	0.000000*	0.000003*	0.000000*

Means with the same letter within the column were not significantly different at  $p \geq 0.05$ , \*significant at  $p < 0.05$

### 3.1.2 Free fatty acid content, peroxide value, and iodine value

To find possible correlations between the FAME yield after *B. cepacia* lipase-catalysed biodiesel production and quality parameters of the examined oils and fat used as lipase substrates, we determined the fatty acid content, peroxide value, and iodine value of WCOs and WCF (Table 2). The FFA content in WCOs ranged from  $0.23 \pm 0.02$  to  $2.92 \pm 0.04$  %, while WCF contained  $0.51 \pm 0.01$  % FFA. These values were only slightly higher than those defined by the regulation for edible oils and fats,<sup>27</sup> where FFA content for refined, mixed vegetable oils, and animal fats should not exceed 0.3, 2, 0.75 % oleic acid, respectively. This suggests that the examined oils and fat had not been used repeatedly for frying, with the exception of slightly elevated FFA content in WCO 2, attributable to multiple uses in the deep fryer. The obtained data on FFA content in waste cooking oils and fat (Table 2) are well in accordance with literature reports, which often find relatively higher amounts of FFA in WCO and WCF compared to fresh oils and fats, attributable to the frying process.<sup>2,9,28</sup> Moreover, given that the examined oils and fat contained < 15 % FFA, they can be classified as “yellow grease” with a price ranging from \$0.04 to \$0.09 per kg,<sup>29</sup> potentially greatly reducing the overall cost of biodiesel production, and ultimately, its selling price. Analysis of the peroxide values of WCOs and WCF (Table 2), revealed that they were within defined limits for refined, mixed vegetable oils, and animal fats, which should not exceed 5, 7, 2 mmol O<sub>2</sub>/kg, respectively.<sup>27</sup> This additionally proved the earlier assumption that the examined oils and fat had not been used repeatedly for frying, especially considering that frying at high temperatures typically increases peroxide value.<sup>30</sup> The iodine values in the examined WCOs ranged from  $116.68 \pm 2.11$  to  $123.88 \pm 2.41$  g I<sub>2</sub>/100 g, while WCF contained  $74.72 \pm 2.89$  g I<sub>2</sub>/100 g (Table 2), partially in agreement with defined limits for refined, mixed vegetable oils, and animal fats.<sup>27</sup> Namely, the IV should be in the range of 118–141 g I<sub>2</sub>/100 g for sunflower, and 105–126 g I<sub>2</sub>/100 g for rapeseed oil, while for animal fats within 45–70 g I<sub>2</sub>/100 g. Both WCO belonging to sunflower oil (WCO 1 and 3) had IV values within the defined limits, while WCO 2, a mixture of sunflower, rapeseed, and poppy vegetable oil, showed an IV of  $116.68 \pm 2.11$  g I<sub>2</sub>/100 g,

suggesting a possibly higher content of rapeseed oil. Only WCF exhibited an IV exceeding the defined limits, indicating thermo-oxidative changes of the fat during frying. Although *Alireza et al.*<sup>31</sup> stated that IV could decrease after heating due to more intensive thermo-oxidative transformations, it is less likely that this occurred, especially considering the aforementioned that the examined oils and fat had not been used repeatedly for frying. The statistical analysis (Welch's test) revealed a statistically significant difference ( $p < 0.05$ ) for all three parameters tested (FFA, PV, IV) between the WCOs and WCF used (Table 2). The post-hoc analysis (Scheffe test) (Table 2) showed a statistically significant difference for FFA between all raw materials used, while for PV, between WCO1 and other raw materials. For IV, a statistically significant difference was found between WCF and WCOs, as well as between WCO 1 and WCO 2.

### 3.2 Lipase substrate specificity

The substrate specificity of *B. cepacia* lipase toward selected WCOs and WCF is presented in Fig. 1. *B. cepacia* lipase exhibited the highest substrate specificity toward WCO 3, followed by WCF and WCO 1, with activity levels almost the same as those observed with edible refined fresh sunflower oil, published as in our previous paper.<sup>13</sup> It is noteworthy that the correlation matrix between substrate specificity and FFA revealed a significant negative correlation between these two parameters (Table 3). This is particularly evident from the fact that lipase from *B. cepacia* exhibited the lowest substrate specificity toward WCO 2, which also displayed the highest proportion of FFA (Table 2).

Although there are no data in the available literature on the substrate specificity of *B. cepacia* lipase toward waste oil, except for our previously published work,<sup>13</sup> several studies have demonstrated high substrate specificity of other lipases toward waste oil. For instance, *Sen et al.*<sup>32</sup> reported high substrate specificity of lipase from *Streptomyces lienomycini* toward waste edible oil, *Uttatree et al.*<sup>33</sup> lipase from *Acinetobacter baylyi* toward waste palm oil and lard, *Sahoo et al.*<sup>34</sup> recombinant lipase (RK-lip479) toward various waste oils (olive, sunflower, coconut, palm, etc.), *Bhatti and Amin*<sup>35</sup> lipase from *G. lucidum* toward waste oil. Con-

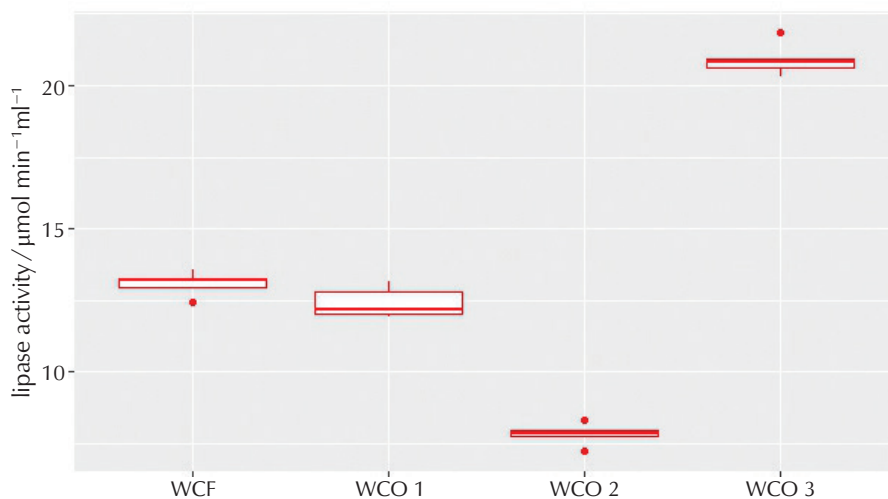


Fig. 1 – Substrate specificity of *Burkholderia cepacia* lipase toward selected waste cooking oils and waste cooking fat. Results present average values  $\pm$  SD of three independent determinations, each performed in triplicate

Slika 1 – Supstratna specifičnost lipaze *Burkholderia cepacia* prema odabranim otpadnim jestivim uljima i otpadnoj jestivoj masti. Rezultati predstavljaju prosječne vrijednosti  $\pm$  SD triju neovisnih određivanja izvedenih u trima ponavljanjima

Table 3 – Spearman's rank order correlation coefficients between analysed parameters (free fatty acids (FFA), peroxide value (PV), iodine value (IV), fatty acid methyl esters (FAME), substrate specificity)

Tablica 3 – Spearmanov koeficijent korelacije između analiziranih parametara (slobodne masne kiseline (FFA), peroksidni broj (PV), jodni broj (IV), metilni esteri masnih kiselina (FAME), supstratna specifičnost)

Variable Varijabla	FFA Slobodne masne kiseline	PV Peroksidni broj	IV Jodni broj	FAME (1 h) Metilni esteri masnih kiselina	Substrate specificity Supstratna specifičnost
FFA Slobodne masne kiseline	1.0000				
PV Peroksidni broj	-0.0769	1.0000			
IV Jodni broj	-0.4406	0.6154*	1.0000		
FAME (1 h) Metilni esteri masnih kiselina	-0.4336	0.6294*	0.9930*	1.0000	
Substrate specificity Supstratna specifičnost	-0.7063*	0.1119	0.1399	0.0839	1.0000

\* Significant at  $p < 0.05$

sidering that the tested waste oils exhibited no quality deviations compared to edible oils (Table 2), it is evident that the substrate specificity of *B. cepacia* lipase toward waste oils is also very high.

### 3.3 Biodiesel synthesis and FAME content

Based on the optimal reaction conditions established in our previous paper,<sup>13</sup> biocatalytic biodiesel synthesis was conducted with a *B. cepacia* lipase loading of  $250 \text{ U g}^{-1}$  in Britton-Robinson buffer at pH 10 and a temperature of  $50 \text{ }^\circ\text{C}$  for 24 h. FAME was monitored over time at intervals of 1, 3, 6, and 24 h of the reaction. Regardless of the WCO used, it was observed that more than 97 % of FAME was produced after the first hour of the trans-

esterification reaction (Table 4), fully complying with the standard;<sup>36</sup>  $\text{FAME} \geq 96.5 \%$ . The share of FAME after the first hour of synthesis was  $99.17 \pm 0.15 \%$  for biodiesel synthesised from WCO 1,  $97.91 \pm 0.13 \%$  for biodiesel from WCO 2 and  $98.49 \pm 0.46 \%$  for biodiesel from WCO 3. However, even after 24 h of synthesis, biodiesel from WCF did not achieve this percentage, yielding  $92.63 \pm 2.26 \%$ . Considering the fatty acid profiles of WCOs and WCF (Table 1), it is not surprising that the largest fraction of FAME, after the first hour of the reaction (Table 4), originated from unsaturated long-chain C18:2 fatty acids, ranging from  $41.84 \pm 0.23$  to  $59.18 \pm 0.66 \%$  for WCOs, and  $15.25 \pm 0.68 \%$  for WCF, followed by C18:1 ranging from  $28.98 \pm 1.08$  to  $42.29 \pm 0.26 \%$  for WCOs, and  $54.41 \pm 1.27 \%$  for WCF, and even C16:0 saturated fatty acids ranging from  $7.06 \pm 0.20$  to  $7.36 \pm 0.04 \%$

Table 4 – Time-dependent profile of fatty acid methyl esters (FAME) and total yield during biodiesel synthesis at 50 °C using *B. cepacia* lipase from waste cooking oils and fat. Results present average values (%) ± SD of three independent determinations, each performed in triplicate

Tablica 4 – Vremenska ovisnost profila metilnih estera masnih kiselina (FAME) i ukupni prinos biodizela tijekom sinteze pri 50 °C s lipazom *B. cepacia* iz otpadnih jestivih ulja i masti. Rezultati predstavljaju prosječne vrijednosti (%) ± SD triju neovisnih određivanja izvedenih u trima ponavljanjima

Sample Uzorak	Time Vrijeme /h	C14:0	C16:0	C16:1	C18:0	C18:1n9c+t	C18:2n6c	C18:2n6t	C18:3n3	C20:0	C20:1	C22:0	C24:0	TOTAL FAME Ukupni metilni esteri masnih kiselina /%
WCO 1	1	0.09 ± 0.01	7.19 ± 0.49	0.13 ± 0.05	2.90 ± 0.05	28.98 ± 1.08	59.18 ± 0.66	n.d.	0.11 ± 0.00	0.15 ± 0.02	0.11 ± 0.01	0.41 ± 0.06	0.08 ± 0.00	99.17 ± 0.15
	3	0.08 ± 0.00	8.22 ± 2.16	0.16 ± 0.01	3.07 ± 0.12	28.51 ± 1.35	57.34 ± 2.70	n.d.	0.11 ± 0.01	0.15 ± 0.04	0.12 ± 0.01	0.42 ± 0.06	0.09 ± 0.00	98.09 ± 1.98
	6	0.08 ± 0.01	7.97 ± 2.14	0.17 ± 0.04	3.18 ± 0.31	29.09 ± 1.67	56.76 ± 2.74	n.d.	0.11 ± 0.01	0.14 ± 0.06	0.13 ± 0.02	0.45 ± 0.11	0.13 ± 0.00	98.00 ± 2.11
	24	0.08 ± 0.01	7.38 ± 1.30	0.16 ± 0.02	3.14 ± 0.24	29.36 ± 1.26	57.90 ± 0.67	n.d.	0.12 ± 0.03	0.18 ± 0.02	0.12 ± 0.00	0.51 ± 0.10	0.14 ± 0.00	98.89 ± 0.60
WCO 2	1	0.08 ± 0.01	7.36 ± 0.04	0.13 ± 0.00	1.88 ± 0.02	42.29 ± 0.26	41.84 ± 0.23	n.d.	3.29 ± 0.01	0.27 ± 0.01	0.48 ± 0.01	0.30 ± 0.01	n.d.	97.91 ± 0.13
	3	0.08 ± 0.00	8.39 ± 0.69	0.14 ± 0.01	2.13 ± 0.15	40.60 ± 0.58	40.93 ± 1.29	n.d.	3.12 ± 0.20	0.30 ± 0.01	0.51 ± 0.03	0.36 ± 0.03	n.d.	96.54 ± 1.02
	6	0.09 ± 0.01	8.72 ± 1.24	0.14 ± 0.01	2.12 ± 0.09	40.39 ± 0.83	40.20 ± 2.42	n.d.	3.01 ± 0.39	0.28 ± 0.06	0.51 ± 0.02	0.37 ± 0.03	n.d.	95.82 ± 2.16
	24	0.08 ± 0.01	8.12 ± 0.83	0.13 ± 0.01	2.04 ± 0.12	41.54 ± 1.14	40.70 ± 1.21	n.d.	3.11 ± 0.20	0.28 ± 0.02	0.49 ± 0.01	0.35 ± 0.02	n.d.	96.85 ± 1.52
WCO 3	1	n.d.	7.06 ± 0.20	0.13 ± 0.00	3.09 ± 0.04	31.09 ± 0.77	55.90 ± 0.31	0.10 ± 0.00	0.11 ± 0.01	0.19 ± 0.01	0.15 ± 0.00	0.57 ± 0.02	0.10 ± 0.08	98.49 ± 0.46
	3	n.d.	7.11 ± 0.26	0.13 ± 0.00	3.21 ± 0.12	30.94 ± 0.91	56.01 ± 0.23	0.10 ± 0.01	0.11 ± 0.01	0.20 ± 0.01	0.15 ± 0.01	0.60 ± 0.02	0.15 ± 0.01	98.70 ± 0.35
	6	n.d.	7.04 ± 0.16	0.13 ± 0.00	3.17 ± 0.09	31.37 ± 0.61	55.86 ± 0.18	0.10 ± 0.00	0.11 ± 0.01	0.20 ± 0.01	0.14 ± 0.01	0.59 ± 0.01	0.14 ± 0.01	98.85 ± 0.24
	24	n.d.	6.90 ± 0.04	0.13 ± 0.01	3.10 ± 0.06	32.10 ± 0.14	55.44 ± 0.11	0.11 ± 0.01	0.11 ± 0.01	0.20 ± 0.00	0.15 ± 0.01	0.61 ± 0.11	0.16 ± 0.11	99.01 ± 0.10
WCF	1	0.72 ± 0.04	8.33 ± 0.43	3.26 ± 0.09	5.68 ± 0.06	54.41 ± 1.27	15.25 ± 0.68	n.d.	0.69 ± 0.03	0.22 ± 0.01	1.59 ± 0.06	n.d.	n.d.	90.15 ± 1.06
	3	0.55 ± 0.03	5.75 ± 0.19	3.49 ± 0.13	3.93 ± 0.08	56.66 ± 1.10	16.06 ± 0.23	n.d.	0.75 ± 0.01	0.20 ± 0.01	1.67 ± 0.01	n.d.	n.d.	89.06 ± 1.15
	6	0.45 ± 0.02	4.93 ± 0.34	2.91 ± 0.09	3.41 ± 0.53	51.12 ± 2.16	18.14 ± 1.26	n.d.	0.81 ± 0.05	0.27 ± 0.01	1.65 ± 0.23	n.d.	n.d.	83.69 ± 2.03
	24	n.d.	9.23 ± 1.81	5.12 ± 0.27	3.47 ± 0.69	44.82 ± 1.32	32.44 ± 1.96	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	92.63 ± 2.26
<i>p</i> (sample) <i>p</i> (sirovina)		0.000000*	0.141669	0.000000*	0.000000*	0.000000*	0.000000*	-	0.000000*	0.000006*	0.000000*	0.000000*	0.000000*	0.000000*
<i>p</i> (time) <i>p</i> (vrijeme)		0.007550*	0.586436	0.010970*	0.152073	0.093820	0.025240*	0.109144	0.039484*	0.085066	0.007900*	0.077434	0.000541*	0.002885*

Fatty acid methyl esters: C14:0 methyl myristate, C16:0 methyl palmitate, C16:1 methyl palmitoleate, C18:0 methyl stearate, C18:1n9c+t methyl oleate + methyl elaidate, C18:2n6c methyl linoleate, C18:2n6t methyl linoleaidate, C18:3n3 methyl linolenate, C20:0 methyl arachidate, C20:1 methyl cis-11-eicosenoate, C22:0 methyl behenate, C24:0 methyl tetracosanoate

for WCOs and  $8.33 \pm 0.43$  % for WCF, given their abundance in the examined waste oils and fat. Methyl esters of other fatty acids accounted for up to 6 % in all samples, regardless of whether WCOs or WCF were used. *Samuel and Gulum*<sup>37</sup> also reported the highest amounts of C18:1, C18:2 and C16:0 fatty acids in waste sunflower oil biodiesel. More than 99 % of FAME was produced after one hour when using WCO 1 and after 24 h when using WCO 3.

These results were expected since these two WCOs were sunflower oils, which, according to the determined characteristics (Table 2), actually corresponded to fresh refined oil, thus proving to be highly effective in biodiesel production, as previously reported.<sup>13</sup> Additionally, the substrate specificity results indicated that lipase from *B. cepacia* exhibited the greatest activity precisely in these two waste oils (Fig. 1). Moreover, these oils had lower FFA content (Table 2), although the correlation matrix (Table 3) did not reveal a significant correlation between FFA and the resulting FAME. According to the correlation matrix (Table 3), the highest positive correlation was found between IV and the resulting FAME. Since IV serves as an indicator of unsaturation, a measure of the unsaturated fatty acids, it is not surprising that IV was the highest in WCO 1 (Table 2), since the analysis of the fatty acid profile also showed that this WCO contained the highest proportion of unsaturated fatty acids (Table 1). It is evident that WCO 3 showed a slightly lower IV compared to WCO 1, which was consistent with the slightly lower percentage of FAME produced in the synthesis with WCO 3 compared to WCO 1.

On the other hand, the synthesis with WCO 2, which recorded the highest proportion of FFA (Table 2), exhibited the lowest FAME yield of  $97.91 \pm 0.13$  % after the first hour of reaction. These findings suggest that higher FFA content has a greater effect on the transesterification reaction than higher PV content in terms of obtaining a higher percentage of FAME, also proven by the correlation matrix (Table 3).

The statistical analysis of the results (Table 4) revealed that for most of the FAMEs obtained, C14:0, C16:1, C18:2n6c, C18:3n3, C20:1, C24:0, and the total FAME content, both predictors (raw material and time), were statistically significant. For C18:0, C18:1n9c+t, C20:0 and C22:0, only the raw material (WCO/WCF) was statistically significant, while for C16:0, neither predictor showed statistical significance.

While *Yaakob et al.*<sup>10</sup> reported that the enzymatic production of biodiesel from WCO contained FAME ranging from 55 to 95 %, this paper demonstrates a slightly higher conversion (Table 4). Notably, the results (Table 4) indicate that using waste cooking sunflower oil can yield FAME of over 99 %, equivalent to the percentage yield of fresh edible refined sunflower oil.<sup>13</sup> *Rauf et al.*<sup>38</sup> also observed no difference in biodiesel production between fresh and waste sunflower oil. Other studies have reported lower FAME yields for biodiesel from waste oils but using different lipases, such as *Pseudomonas fluorescens* 63.84 %, <sup>39</sup> *Candida sp.* 88 %, <sup>40</sup> *Candida antarctica* ( $94.6 \pm 1.4$  %), <sup>41</sup> and *Thermomyces lanuginosus*  $93.8 \pm 0.5$  %.<sup>42</sup> Regarding WCF, approximately 90 % conversion was achieved after the first hour, increasing to approximately 92 % after 24 h. There-

fore, when using WCF, the minimum FAME yield required by the standard cannot be achieved. This is not surprising, given that the share of total fatty acids in WCF (Table 1) was the lowest among all analysed waste feedstock, at only  $96.67 \pm 0.08$  %, requiring a 100 % conversion of fatty acids into FAME to meet the standard. Similar FAME yield results with WCF but with different reaction conditions have been reported by *Shin et al.*<sup>22</sup> and *Lawan et al.*<sup>43</sup> However, *Adewale et al.*<sup>44</sup> reported a 96.8 % FAME conversion when using immobilised *Candida antarctica* and waste lard.

## 4 Conclusion

This study investigated the possibility of utilising WCOs and WCF for enzymatic biodiesel synthesis employing *B. cepacia* lipase. The results indicate that, under optimal conditions for biodiesel synthesis with *B. cepacia* lipase, more than 99 % FAME can be produced after one hour of reaction when using WCO. On the other hand, when using WCF, even after 24 h of synthesis, the minimum of 96.5 % FAME set by the standard could not be achieved. The percentage of FAME obtained by enzymatic catalysis was found to be influenced by the chemical characteristics of the oil, such as the FFA, PV, and IV content. The IV of the feedstock (oil or fat) had the highest statistical significance on FAME content - the higher the iodine value, the higher the final FAME content. Therefore, IV can be one of the indicators of the suitability of waste oil for biodiesel synthesis. These results provide valuable data that could be used for further research into the utilisation of other waste oil types for enzymatic biodiesel synthesis, thereby contributing to reducing dependence on edible oils or other expensive raw materials.

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## SAŽETAK

### Utjecaj kvalitativnih parametara otpadnih jestivih ulja i masti na prinos metil estera masnih kiselina

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Zbog sve veće potrebe za alternativnim zelenim gorivima biodizel ne napušta fokus znanstvenih istraživanja. Dok se u svijetu već godinama provodi kemijska proizvodnja biodizela, uglavnom iz jestivih ulja, sve se više daje prednost primjeni enzimске sinteze, kao i sirovina u obliku otpadnih ili nejestivih ulja i masti, ponajprije zbog ekonomskih i ekoloških razloga. Cilj ovog istraživanja bio je provesti enzimsku sintezu biodizela s *Burkholderia cepacia* lipazom uporabom odabranih otpadnih biljnih ulja i otpadne životinjske masti te korelirati dobivene metilne estere masnih kiselina sa supstratnom specifičnošću lipaze i glavnim karakteristikama odabranih otpadnih sirovina (profil masnih kiselina, sadržaj slobodnih masnih kiselina, peroksidni i jodni broj). Navedena svojstva ulja/masti određena su standardnim metodama, dok je biodizel dobiven reakcijom transesterifikacije odabranih otpadnih ulja/masti s metanolom u prisutnosti lipaze kao katalizatora. Analiza metilnih estera masnih kiselina tijekom 24 h sinteze biodizela iz otpadnih biljnih ulja pokazala je da je jedan sat sinteze dovoljan za proizvodnju više od 97 % metilnih estera masnih kiselina. S druge strane, upotrebom otpadne životinjske masti ni nakon 24 h nije postignut minimum od 96,5 %, zadan europskim standardom. Prema korelacijskoj matrici, najveća pozitivna korelacija utvrđena je između jodnog broja i nastalih metilnih estera masnih kiselina. Otpadno jestivo biljno ulje u kojem je izmjeren najveći jodni broj od  $123,88 \pm 2,41$  g I<sub>2</sub>/100 g nakon jednog sata sinteze dalo je najveći udio metilnih estera od  $99,17 \pm 0,15$  %. Utvrđena je statistički značajna pozitivna korelacija između metilnih estera masnih kiselina i jodnog i peroksidnog broja, stoga ti parametri mogu biti jedan od pokazatelja prikladnosti otpadnog ulja za sintezu biodizela. To se nameće kao doprinos daljnjem istraživanju potencijalnih supstrata za sintezu biodizela iz područja otpada.

#### Ključne riječi

Sinteza biodizela, jodni broj, otpadno jestivo biljno ulje, otpadna životinjska mast, *Burkholderia cepacia*

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