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Enzymatic Transesterification of Waste Frying Oil from Local Restaurants in East Colombia Using a Combined Lipase System

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Abstract: The search for innovation and biotechnological strategies in the biodiesel production chain have become a topic of interest for scientific community owing the importance of renewable energy sources. This work aimed to implement an enzymatic transesterification process to obtain biodiesel from waste frying oil (WFO). The transesterification was performed by varying reaction times (8 h, 12 h and 16 h), enzyme concentrations of lipase XX 25 split (14%, 16% and 18%), pH of reaction media (6, 7 and 8) and reaction temperature (35, 38 and 40 °C) with a fixed alcohol–oil molar ratio of 3:1. The optimum operating conditions were selected to quantify the amount of fatty acid methyl esters (FAMEs) generated. The highest biodiesel production was reached with an enzyme concentration of 14%, reaction time of 8 h, pH of 7 and temperature of 38 °C. It was estimated a FAMEs production of 42.86% for the selected experiment; however, best physicochemical characteristics of biodiesel were achieved with an enzyme concentration of 16% and reaction time of 8 h. Results suggested that enzymatic transesterification process was favorable because the amount of methyl esters obtained was similar to the content of fatty acids in the WFO.

Keywords: biodiesel; transesterification; waste frying oil; biotechnology

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1. Introduction

The environmental concerns associated with the use of fossil fuels have encouraged the research for biofuel production alternatives [1]. Biodiesel is the most popular alternative energy source to replace fuel owing its sustainability, renewability and reduced emissions [2]. This biofuel is a *n*-fuel composed of mono-alkyl esters of long-chain fatty acids that come from vegetable oils or animal fats [3]. Its main characteristics are biodegradability, low sulfur content, no aromatic compounds, a high flash point, characteristic lubrication, miscibility with petroleum diesel and higher cetane number [4]. The main drawback of biodiesel production is the relatively higher production costs in comparison to conventional fossil fuel; however, such costs can be reduced by the use of cheap and available feedstocks such as waste cooking oil, animal fats, palm fatty acid distillate (PFAD) and Jatropha curcas oil [5]. Among these, waste frying oils (WFO) have been widely employed as raw material for biodiesel production in order to provide waste valorization solution [6–9].

Waste frying oil or cooking oil is inedible oil generated by catering industry and food processing industry. To reduce costs, these industries fry the oils several times under high temperature and in the presence of oxygen and light [10]. This process causes both physical and chemical property changes such as change in color, odor, viscosity and calorie count [11]. It is estimated that the global production of WFO amounts to 5 million tonnes every year [12]. In East Colombia (Cúcuta), a large local restaurant consumes in average 80 L of frying oil per month, and 71% of these restaurants reuse the oils three times or more [13]. The cost of WFO is two to three times lower than refined vegetable oils [14]. It is considered a hazardous material with harmful impacts on the environment and has created serious problems for disposal due to its slow degradation [15]. The human consumption of WFO may cause serious health hazards, e.g., potential gastrointestinal disorders and even mutagenesis in the human body [16].

Transesterification is a common technique to provide biodiesel as of glycerides current in oleaginous biomass sources, which react with alcohol to produce alkyl esters and glycerol [17]. Methanol and ethanol are the most used alcohols in the process forming methyl esters or ethyl esters, respectively [18]. During conventional transesterification process, the high amount of free fatty acids (FFA) in WFO are converted to soap and reduce the purity of biodiesel [10]. To date, different types of catalysts have been used to enhance transesterification process and increase biodiesel production yield. Berrios et al. [19] studied the acidity removal and subsequent transesterification at different temperatures and mole ratios to enhance the biodiesel production from WFO. They reported nonpolar FAME content of 88.5 wt.% and acid value below 1 mg KOH·g^{-1} oil. Al-Hamamre et al. [20] converted WFO into biodiesel by alkali-catalyzed transesterification and evaluated the effect of operating variables such as reaction temperature, methanol (MeOH)/oil ratio and type of catalyst. The optimum conditions reported by such contribution were MeOH/oil ratio 0.4 *v/v*, 1.0% *w/v* KOH, a temperature of 50 °C and reaction time between 20 and 40 min. Cordero-Ravelo et al. [21] used different type of WCO (sunflower, maize, olive and a blend of soybean, palm and sunflower) to study the influence of oil source on biodiesel quality and suggested transesterification method for WFO with densities (at 15 °C) of up to 930 kg/m^3.

The use of enzymes to catalyze transesterification reaction is being widely studied because they can promote esterification of FFA and transesterification of triacylglycerols simultaneously [22]. Lopresto et al. [23] used waste vegetable oils to produce biodiesel via enzymatic transesterification using lipase from *Pseudomonas cepacia*. They investigated the effect of biocatalyst/substrate feed mass ratios and the waste oil quality and reported final conversion of 46%–47% when using biocatalyst/oil mass ratio of 3% and 5%. Poppe et al. [24] conducted enzymatic transesterification of oils with combi-lipases as biocatalyst and supported by an ultrasound system. Authors reported a biodiesel yield of 70% when using waste oil and 90% using soybean oil, which suggested that ultrasound technology and enzyme mixtures could be a promising route to reduce costs of biodiesel production. Souissi et al. [25] evaluated the use of two low-cost feedstocks such as WFO and wastes of beef fats to produce biodiesel via both chemical and enzymatic transesterification and reported a richer FAME biodiesel with the biological method. Kumar et al. [26] analyzed the feasibility of biodiesel production from hybrid oil using bio-support beads immobilized with lipase from *Pseudomonas cepacia*. They obtained a biodiesel yield of 78% under optimized conditions of 24 h, enzyme loading of 10% *w/w* and temperature of 50 ± 1 °C.

In this work, the feedstock for biodiesel production is a mixture of WFOs collected from local restaurants in East Colombia (Cúcuta), which are reused several times and have low fatty acids composition (49.19%). Due to the frying conditions and reusability, there are different substances in WFO formed as result of frying process mainly condensation, oxidation and hydrolysis products that may affect lipase action in enzymatic transesterification. In this sense, it is challenging to obtain high biodiesel yield from low quality fat source. In order to reach relatively high biodiesel production, it was used a combined lipase system from *Candida rugosa* and *Thermomyces lanuginosus*. The effect of enzyme concentration, reaction time, pH of reaction media and temperature were evaluated to estimate optimum operating conditions. Physicochemical characterization of biodiesel was performed to determine if such product is suitable for application in diesel engines meeting national and international standards.

2. Materials and Methods

2.1. Materials

The waste frying oil was collected from local restaurants in San Jose Cúcuta (East Colombia) and blended to obtain the oil sample. The enzyme ENZECO® LIPASE XX 25 split was provided by proenzimas®. Methanol was used as an acyl acceptor and was provided by Sigma-Aldrich.

2.2. Pretreatment and Characterization of WFO

The WFO was first filtered with a polyester mesh filter cloth of a 14-mm pore size (from Changzhou Xiahua Envirotech Co., Ltd., China) and without compression in order to remove suspended matter that may interfere the process. Then, sample was dried for 2 h in an oven at 110 °C to reduce water content and filtered a second time. The physicochemical characterization is required to evaluate the conditions of feedstock. The following properties were measured: density (ICONTEC N 432), moisture content (ICONTEC N 254), acidity index (ICONTEC N 218), pH (indicator paper), fatty acid profile (Gas chromatography–mass spectrometry, GC-MS). The molecular weight of the frying oil was also calculated using the Equation (1) reported by Phan and Phan [27]:

$$Mw_{WFO} = 3\sum(Mw_i * \%m) + 38 \tag{1}$$

where Mw_{WFO} is the molecular weight of the WFO; Mw_i is the molecular weight of the fatty acids in the oil and $\%m$ is the composition of fatty acids in the oil.

2.3. Biodiesel Production by Enzymatic Transesterification

Figure 1 depicts the experimental methodology followed to determine optimum conditions for biodiesel production from WFO. The experiments were repeated in duplicate (n = 2) because of the limitations in the amount of feedstock as it comes from different used oils collected from local restaurants in Cúcuta, which depended on frying conditions and their fatty acid profiles change significantly affecting the properties of the mixture. In the first stage, the effect of enzyme concentration and reaction time was evaluated while fixing temperature and pH. To this end, substrate must be present in excess amount to ensure that reaction rate is independent of WFO concentration [28]. The amount of WFO was fixed in 500 mL based on the results of previous researches with the same value for such operating parameter [8,29]. The methanol to oil ratio was 3:1 because of the high conversion performance reached by Acevedo et al. [8].

The typical reaction time for enzymatic transesterifications of various oils ranged from 4 h to 72 h [30], hence, reaction times of 8, 12 and 16 h were selected for WFO as feedstock. It was considered different concentrations (14%, 16% and 18%) of lipase XX 25 split, which is a combination of lipase from *Candida rugosa* and *Thermomyces lanuginosus*. Such enzyme was selected in this work owing its ability to efficiently hydrolyze edible oils [31]. The lipase combination system can provide higher hydrolysis yields because of the strong affinity to certain groups towards the ester synthesis such as unsaturated acids and long-chain fatty acids [32,33]. Studies reported that mixture of two or more lipases contributes to increase ester yield in biodiesel, which is explained by the different specificities of each lipases [34]. *Candida rugosa* is able to catalyze all the ester bonds of triglycerides to obtain fatty acids and glycerol, while, *Thermomyces lanuginosus* is stable and able to maintain activity at 50–60 °C [35].

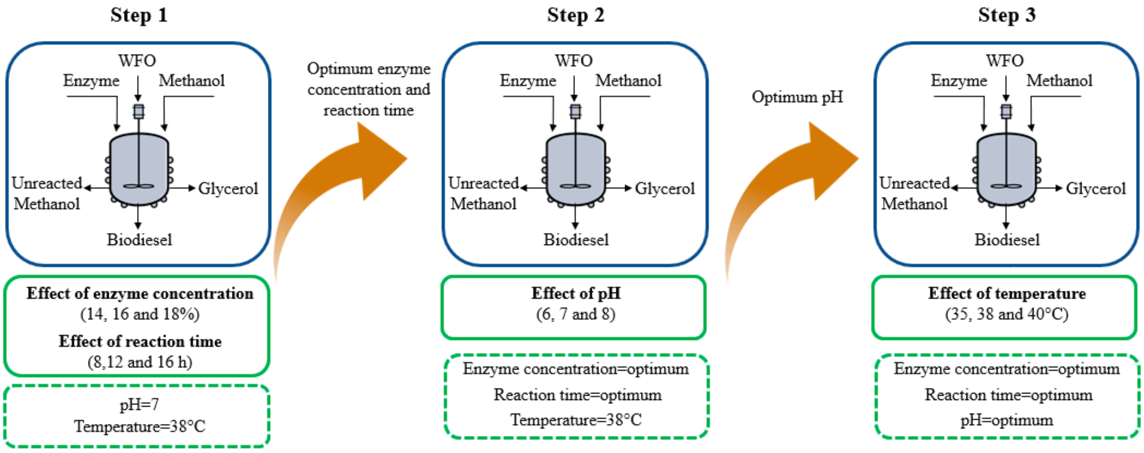

Figure 1. Schematic representation of the experimental methodology.

The reaction took place in four-input reactors, in which the feedstock, methanol and enzyme were mixed. This was achieved by adding the enzyme to the oil after establishing the reaction temperature (38 °C) as well as pH = 7.0 and mixing for 15 min. Then, methanol was divided into three equal parts and one by one was added to the reaction at intervals of 15 min as suggested by Rangel et al. [29]. After reaction time, process temperature was increased to 60 °C for 15 min in order to inhibit the enzyme and thus end the reaction mechanism. The mixture was centrifuged at 5000 rpm to separated glycerol from biodiesel. The separated mixture containing biodiesel was washed thoroughly with distilled water at 60 °C and subjected to decantation. Finally, biodiesel was heated at 130 °C to remove unreacted methanol and water droplets [29].

After selecting the optimum conditions for reaction time and enzyme concentration, the effect of pH (6, 7 and 8) was assessed in the second stage with the temperature set in 38 °C. These pH values were selected according to previous works reporting the effect of pH on transesterification performance [36]. The final stage is related to determining optimum temperature condition for the biodiesel production. Temperature influences reaction rate and yield of esters [37], hence, it was important to evaluate such parameter on transesterification at 35, 38 and 40 °C.

2.4. Physicochemical Characterization of Biodiesel

The resulting biodiesel from WFO was characterized according to the techniques summarized in Table 1. In addition, quantification of fatty acid methyl esters was performed by GC-MS chromatography in order to determine the highest biodiesel yield.

Table 1. Physicochemical characterization of biodiesel.

Property	Method	Reference
Density at 25 °C	Gravimetric method	[29]
Kinematic viscosity at 40 °C	Laboratory guide for fluids and hydraulic machines UFPS	[29]
Acidity index	ICONTEC N 218 *	[38]
Moisture content	NTC 287 *	[39]
Flash point	Open cup method	[39]
Copper corrosion test	ASTM D130 *	[29]

* ICONTEC: Instituto Colombiano de Normas Técnicas y Certificación; NTC: Norma Técnica Colombiana; ASTM: American Society for Testing and Materials.

3. Results and Discussion

3.1. Physicochemical Characterization of Waste Frying Oil

As reported in Table 2, the density achieved a slightly high value compared to density value obtained by Ramírez [40] in an oil of similar origin (0.926 g/mL). The moisture content was low that is attributed to the reusability of oil several times eliminating presence of water in the sample [8]. The acidity index indicates a similar value to the reported by Phan and Phan [27], 2.36 mg KOH/g, and much lower than the value reported by Bulla [7], 7.65 mg KOH/g. The variation in acidity index may be due to the degradation of the oil after frying and the storage time [41]. The pH of oil indicated that it was in favorable conditions for the enzyme, since the effective pH range for this enzyme is 5.0 to 8.0.

Table 2. Physicochemical characterization of WFO. Values are mean +/− standard deviation (n = 2).

Properties	Result
Density (g/mL)	0.9572 ± 0.00014
Moisture (mass fraction)	0.0600 ± 0.016
Acidity index (mg KOH/g)	2.5624 ± 0.3945
pH	5.7 ± 0

Fatty Acids Profile

As reported in Table 3, the fatty acids content in the WFO collected from local restaurants is 30.35% of palmitic acid and 18.84% of oleic acid (49.19% in total), so the substrate contains 50.8% of compounds that would not produce esters at the end of the reaction (e.g., dimers, polycyclic components, hydroperoxides, glycerol, mono glycerides, and diglycerides, among others). Other contributions stated a higher content of C16:0 and C18:1 in WFO samples, hence, better biodiesel yields are expected due to the higher availability of fats to be converted into FAMEs. Recent studies have quantified fatty acids content in WFOs of 100%, which differs from the characterization of the feedstock selected in this work [7,8]. As Sánchez and Sarmiento [42] stated, adverse conditions of WFO such as high temperatures and a large reusability leads to changes in composition as well as physicochemical properties. It was found that fatty acids in the sample are the main ones in the composition of palm olein [8]. According to fatty acids profile, this WFO is suitable for biodiesel production based on the quantity and quality of its fats.

Table 3. Fatty acids composition of WFO reported in literature.

References	Fatty Acid Composition (%)	
	Palmitic Acid (C16:0)	Oleic Acid (C18:1)
This work	30.35	18.84
Eze et al. [43]	6.1	64.2
Yahya et al. [44]	34.80	53.30
Tacias Pascacio et al. [45]	17.82	40.98
Mansir et al. [46]	60.1	27.20

3.2. Biodiesel Production by Enzymatic Transesterification (First Stage)

For a better understanding of the results, Table 4 summarizes the nomenclature employed to distinguish experiments in the first stage. Figure 2 shows the biodiesel production and yield for all experiments considering the effect of reaction time and enzyme concentration. An interesting trend was observed for E1, E4 and E7 experiments as well as E2, E5 and E8 experiments. For reaction time of 8 h and 12 h, higher biodiesel yield was reached in the following order of enzyme concentration 14%, 18% and 16%. For reaction time of 16 h, higher production of esters was observed in the following order of enzyme concentration 16%, 14% and 18%. According to these results, longer reaction times affected transesterification process when using catalyst concentration of 14 and 18% that may be attributed to the formation of undesired products such as dimers or the reaction of impurities from WFO with reagents. Also, a slight recombination of esters and glycerol to monoglycerides may occur [47]. Lipase activity may decrease over time as a result of the accumulation of substances inhibiting the enzyme or the substrate is being used up [48]. The low biodiesel production in the E4 experiment leads to the lower consumption of WFO in comparison to experiments E1 and E7. Hence, substrate availability is high enough not to inhibit enzyme activity over time. The mechanism governing enzymatic transesterification of WFO is shown in Figure 3.

Table 4. Nomenclature of experiments in the first stage.

Enzyme Concentration	8 h	12 h	16 h
14%	E1	E2	E3
16%	E4	E5	E6
18%	E7	E8	E9

The E1 experiment reached the highest production with 437.863 g (91.49% biodiesel yield), followed by the E7 experiment with a production of 419.827 g (87.72% biodiesel yield), which can be explained by the effect of catalyst concentration on biodiesel production. As is well known, enzyme concentration is an essential parameter for improving transesterification process [49]. The lower biodiesel production for experiments with higher enzyme concentration (16% and 18%) is supported by other works with similar trends [50]. These results suggested an excess of enzyme over required owing the limitations in liquid volume for reaction, hence, liquid phase was insufficient to suspend the solid catalyst causing external mass transfer resistance [51]. The excess solid enzyme available in the reactive mixture can limit glycerol separation due to the noninteraction between methanol and oil [52]. The existence of a minimum at concentration of 16% in the correlation of biodiesel yield and enzyme concentration may be explained by the formation of dimers with lower activity through the union of two molecules of lipase in the regions of their active centers, which is a common phenomenon when using *Thermomyces lanuginosus* [53]. The complexity of the substrate and the state of reuse of the oil allows to infer the difficulty of the lipase for the transesterification reaction, generating a possible inhibition in the active site of the enzyme at concentration of 16% or in its allosteric center [54]. For reaction time of 16 h, this minimum (396.08 g biodiesel) turned into a maximum (408.82 g biodiesel) because of the breaking of such dimers and higher accessibility of reactants to the active sites.

The reaction time used in the transesterification process is a relevant variable affecting the level of target conversion. This operating factor significantly affects process performance and limits reaction mechanism. Ognjanovic et al. [55] reported that it is feasible to achieve high FAMEs yields using low enzyme concentrations; however, these reactions would take longer than chemical method. As shown in Figure 2, biodiesel yield decreased when increasing reaction time for enzyme concentration of 14% and 18%, which is explained by the presence of more unreacted methanol over time interfering separation of glycerol from methyl ester and increasing its solubility. When glycerol remained in solution it contributes to drive the equilibrium back to the left, lowering the yield of biodiesel [51,56]. For enzyme concentration of 16%, biodiesel production increased during the course of the reaction, which suggested that the equilibrium was driven to the right for all the reaction times (products side). Based on the above, the low presence of unreacted methanol in E4, E5 and E6 experiments suggested that the separation of glycerol and esters was not significantly interfered.

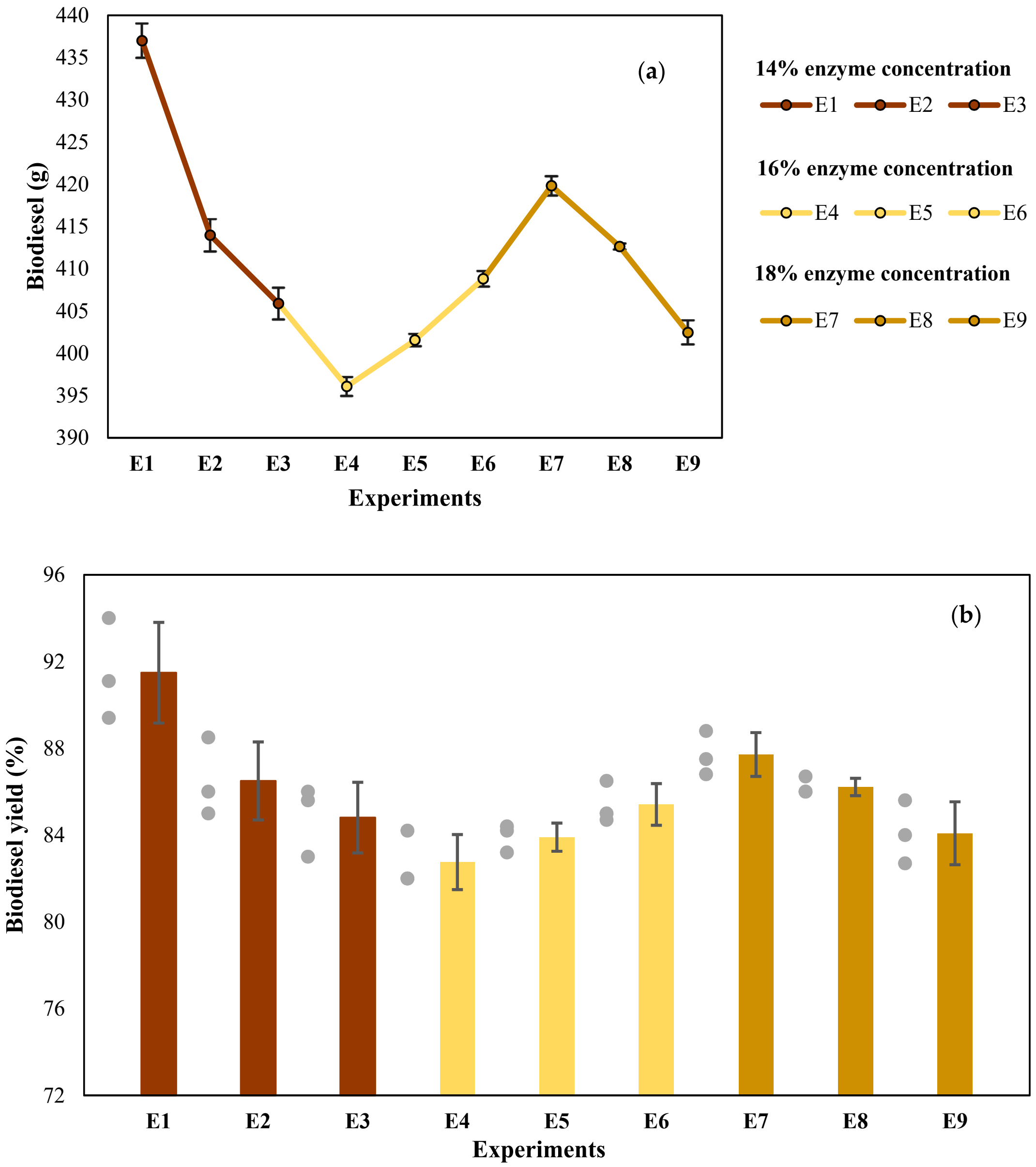

Figure 2. Biodiesel production per experiment: (**a**) yield (%) and (**b**) weight (g). Values are mean +/− standard deviation (n = 2).

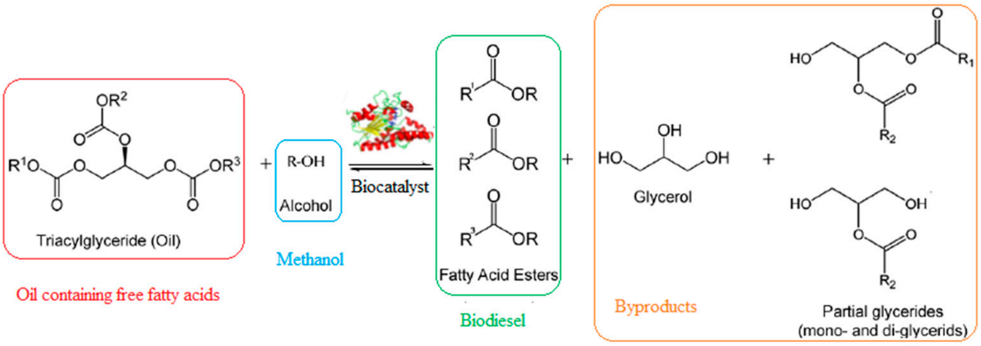

Figure 3. Schematic representation of reaction mechanism during enzymatic transesterification

The analysis of variance (ANOVA) was performed in nested one-way to study the interactions of reaction time and enzyme concentration on biodiesel yield. The *p*-value reached 0.49 that is higher than

0.05 showing no statistically significant effect of the treatments for enzyme concentration and reaction time. After transesterification process, the enzyme is difficult to retrieve because it was free dissolved in the bioreactor and the complexity of waste frying oil also affects its reusability. Studies have shown that using supporting materials such as nanoparticles to immobilize the enzyme allow to maintain and even increase the activity and stability of the enzyme for further recoveries [57,58]. The implementation of enzymatic immobilization during the enzymatic transesterification of waste frying oils still requires extensive research associated with the synthesis of novel materials, which has motivated the search for low-cost and efficient immobilization techniques.

3.2.1. Physicochemical Characterization of Biodiesel

Table 5 reports the results for characterization analysis of the biodiesel obtained in each experiment. The densities of the biodiesel was quantified with an average value of 0.92 g/mL, which is close to the density of the biodiesel produced by alkaline transesterification using WFO as reported by Acevedo et al. [8]; these numbers were higher than the range required by ASTM 6751, EN (European standards) 14214 and NTC 5444 (0.86–0.90 g/mL). The high density value of diesel could be due to the high density of the oil used [29]. It was found a difference between biodiesel density in experiment E4 and experiment E6 suggesting that biodiesel with lower density is produced during experiments with less reaction time because there are still unreacted amounts of methanol inferring in the density.

Table 5. Physicochemical characterization of biodiesel. Values are mean +/− standard deviation (n = 2).

Exp.	Density (g/mL)	Moisture Content (%)	Acidity Index (mg KOH/g Sample)	Flash Point (°C)	Kinematic Viscosity (mm^2/s)
E1	0.922 ± 0.0041	0.279 ± 0.01	2.84 ± 0.28	252.5 ± 5	10.53 ± 0.04
E2	0.925 ± 0.0042	0.381 ± 0.06	3.38 ± 0.69	240.0 ± 0	9.29 ± 0.07
E3	0.928 ± 0.0013	0.256 ± 0.04	4.61 ± 1.17	250.0 ± 0	9.66 ± 0.98
E4	0.921 ± 0.0021	0.111 ± 0.10	3.65 ± 0.55	257.5 ± 5	10.06 ± 0.09
E5	0.929 ± 0.0008	0.239 ± 0.098	3.49 ± 0.82	242.5 ± 5	9.58 ± 0.90
E6	0.929 ± 0.0048	0.315 ± 0.05	3.79 ± 0.49	227.5 ± 5	8.66 ± 0.27
E7	0.923 ± 0.0006	0.137 ± 0.13	3.41 ± 0.35	242.5 ± 5	8.59 ± 0.098
E8	0.922 ± 0.0008	0.199 ± 0.12	2.07 ± 0.20	252.5 ± 5	9.44 ± 0.20
E9	0.925 ± 0.0033	0.499 ± 0.29	4.59 ± 0.33	227.5 ± 5	7.08 ± 0.86

Moisture content obtained for each experiment were different with values between 0.11 and 0.5%, a range greater than the maximum value required by standards 0.05% (500 mg/Kg). Although the moisture content was expected to be lower owing the final drying stage at 130 °C, the repetitive washing of separated mixture and the low efficiency of drying contributed to reach higher moisture than allowed for biodiesel. Also, the remaining ternary mixture methanol-water-glycerol after separation stages may interfere water evaporation [59]. The acidity index reported values between 2.0667 and 4.6129 mg KOH/g, where lowest results were reached during experiments E8 and E1. All these values were significantly higher than those allowed by NTC 5444, EN 14214 and ASTM 6751, which may be attributed to the unknown handling of raw material in the frying and storage process and impurities may affect this property [9].

The flash point is very important in the handling and storage of fuels; therefore, to reduce the risk of fire, it is required high flash point of substances [60]. The flash points obtained in each experiment (from 227.5 to 257.5 °C) are considered favorable because they are significantly higher than the minimum value required by the standards, specifically 120 °C for standards EN 14214 and NTC 5444, and 130 °C for ASTM 6751.

The kinematic viscosity showed higher values than the established by standards of 3.5–5 mm^2/s for EN 14214, and 1.9–6 mm^2/s for ASTM 6751 and NTC 5444. This is an undesired result for the biodiesel, since viscosity exceeding the limits lead to poor fuel atomization and incomplete combustion [60]. Viscosity is related to the presence of unreacted triglycerides, showing the performance of transesterification [27]. In this sense, it was expected that experiments with higher viscosity results were E1 (10.53 mm^2/s) and E4 (10.06 mm^2/s) due to the short reaction times to conduct enzymatic transesterification. For longer reaction times, it was observed that experiments E6 (8.66 mm^2/s) and E9 (7.08 mm^2/s) reached lower viscosity at same enzyme concentration. For reaction times of 8 h and 16 h, kinetic viscosity decreased when increasing enzyme concentration similar to the trend reported by Jilse et al. [52] for enzyme concentration range of 6–9 wt%. To overcome the high viscosity of biodiesel, additives such as Diethyl Ether/DDE and the commercial additive Viscoplex 10-330 CFI can be used according to the results of Fajar et al. [61] for reduced viscosity of blends. Also, Arumugan et al. [30] suggested that preheating before injection can be useful to improve operation of biodiesel.

Biodiesel is more corrosive to automotive materials than diesel because of the presence of oxygen moieties, autooxidation, increased polarity of biodiesel and its hygroscopicnature [62]. Hence, it was expected to observe this issue in corrosion tests from biodiesel from WFO. To analyze the results of the copper foil corrosion test (Figure 4), the standard strip of the ASTM D130 was used (Figure 5). Research suggested that ester components, moisture absorption and oxidation can enhance the corrosion of copper in biodiesel [63]. It was found that each experiment reported corrosion test of 1a, complying with the standards of ASTM 6751 with a minimum corrosion value of copper foil of 3b, and EN 14214 and NTC 5444 with a maximum corrosion value of copper foil of 1a. Results proved that the biodiesel from WFO is free of components that can corrode engine parts or storage tanks that are made of copper material or copper alloys [64]. This favorable result may be attributed to few reactions resulting from metal contact that convert esters into acids owing the lower availability of FAMEs in the product in comparison to biodiesel from other sources such as palm [62].

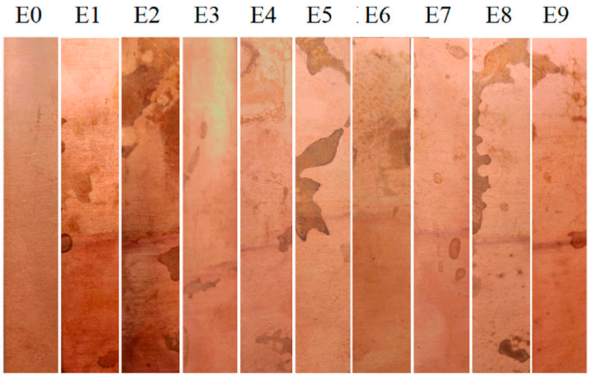

Figure 4. Copper corrosion test for biodiesel.

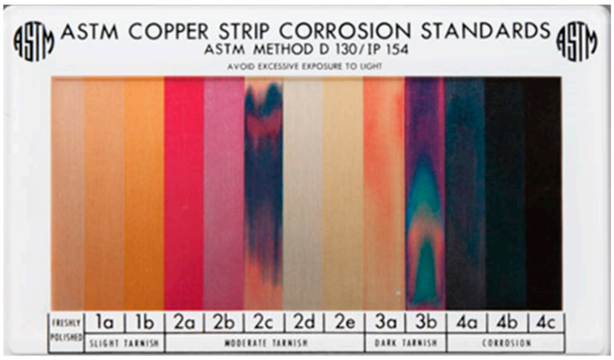

Figure 5. ASTM copper strip corrosion standards.

3.2.2. Analysis of Methyl Esters in the Biodiesel

This analysis was applied to the biodiesel obtained in the experiment E1 because of the high production yield. Figure 6 shows the GC-MS spectra of the biodiesel from WFO, in which FAMEs were identified by the retention time data over 60 min of samples running. The sample was directly injected in the equipment under the operating conditions defined in Appendix A. The peak at retention time of 31.21 corresponds to methyl palmitate (C16:0) with a composition of 20.25% (see Table 6). It was identified a substance of methyl oleate (C18:1) in methyl ester with the retention time of 33.30 and the composition of 17.91%. The results of GC-MS analysis indicate that there is a substance of methyl linolelaidate with the retention time of 21.43 and composition of 2.80%. The peak at 21.58 corresponds to methyl tridecanoate with composition of 1.90%. These results indicate that 42.86% of the sample represents methyl esters in the biodiesel. However, the fatty acids content in WFO was only 49.2% representing the total amount suitable for transesterification. In this sense, the conversion of fatty acids into methyl esters were 87.11% $\frac{total\ biodiesel\ (g)}{total\ WFO\ (g)}$.

Different FAMEs profiles are available in literature for biodiesel from WFO and other sources (e.g., palm oil, sunflower oil). For example, Thi et al. [65] determined the FAMEs profile of the waste cooking oil biodiesel and reported highest contributions for methyl oleate (11.24 of reaction time and 37.59% of composition), followed by methyl palmitate (6.78 of retention time and 25.14% of composition). Nurdin et al. [66] performed GC-MS analysis to determine FAMEs composition of biodiesel from industrial wastes of palm oil processing and identified a substance of methyl hexadecanoate in methyl ester with the retention time of 19.74 and the composition of 12.87%. Also, a sub-stance of methyl octadecanoate was identified with the retention time of 21.63 and the composition of 5.71%.

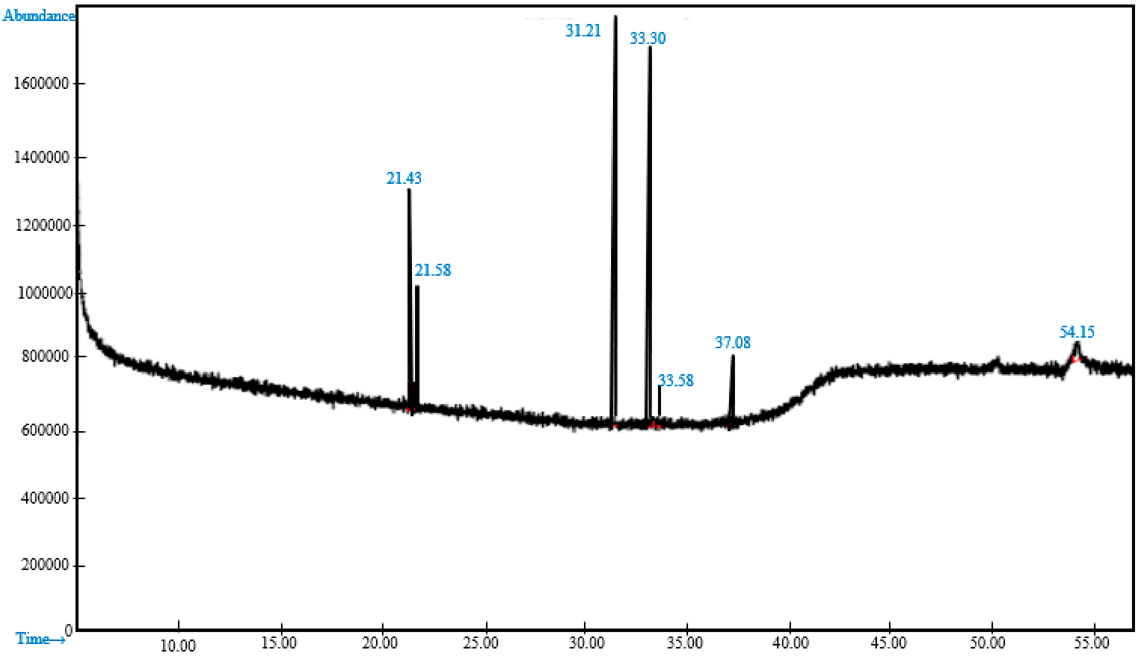

Figure 6. Gas chromatography–mass spectrometry spectra of biodiesel.

Table 6. Fatty acids composition.

FAME	Retention Time	Composition (%)
Methyl palmitate	31.21	20.25
Methyl linolelaidate	21.43	2.80
Methyl oleate	33.30	17.91
Methyl tridecanoate	21.58	1.90

Rangel et al. [29] reported a reaction yield of 85.49%, while Acevedo et al. [8] reached a value of 50% using the same enzyme as a catalyst. These results were significantly lower than those obtained in this work. Acevedo et al. [8] also performed the analysis with alkaline catalyst and obtained yield of 89.4%, which is higher than 87.11% (see Table 7).

Table 7. Transesterification yield of different researches.

Reference	Rangel et al. [29]	Acevedo et al. [8]	Acevedo et al. [8]	This Work
Oil type	Seaweed oil	WFO	WFO	WFO
Catalyst	Enzymatic	Enzymatic	Alkaline	Enzymatic
Conditions	10% of lipase enzyme XX split Oil-alcohol 1:3 33 °C 6 h	5% of lipase enzyme XX 25 split Oil-Alcohol 1:3 38 °C 3 h	1% of KOH Oil-Alcohol 1:6 60 °C 70 min	14% of lipase enzyme XX 25 split Oil-Alcohol 1:3 38 °C 8 h
Yield	85.49%	50%	89.40%	87.11%

3.3. Effect of pH on Enzymatic Transesterification (Second Stage)

Figure 7 shows the effect of pH of the reaction media on transesterification process as key parameter for biodiesel production. The pH value of the enzyme solution at concentration of 14% (E1 experiment) was measured in 6.5. The pH = 7.0 reached the highest yield (91.49 ± 2%), followed by pH = 6.0, which were in the effective range for the lipase (5.0–8.0). Similar results were reported by other contributions. For example, Touqueer et al. [67] evaluated the production of biodiesel from waste cooking oil using free lipase and immobilized lipase. For the free lipase system, they reported that maximum lipase activity was reached at pH = 7.0 and above this value activity was reduced. Devanesan et al. [36] also reported a maximum production yield of biodiesel from Jatropha oil (72%) at pH = 7.0 and the yield decreased when increasing pH beyond 7.0.

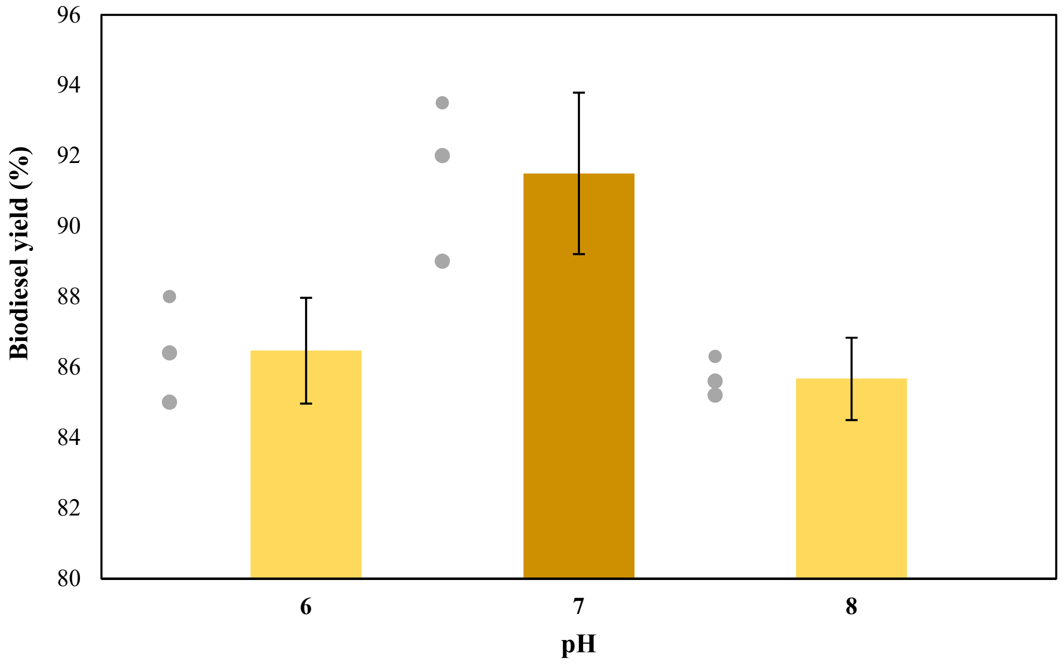

Figure 7. Effect of pH of the reaction media on biodiesel yield. Values are mean +/− standard deviation (n = 2).

3.4. Effect of Temperature on Enzymatic Transesterification (Third Stage)

The temperature was varied to 35 °C, 38 °C and 40 °C in order to analyze its effects on biodiesel production. As shown in Figure 8, highest biodiesel yield was reached at reaction temperature of 38 °C followed by 35 °C. The reaction rate increases with temperature due to the reduction of viscosity of the oil that increases its solubility in methanol. However, decrease in yields is observed after a defined temperature suggesting the deactivation of enzymes [68]. The statistical analysis reported *p*-value of 0.24 (>0.05) indicating that treatments with temperature variations are not statistically significant. Similar trend was reported by Charpe et al. [69] for the production of biodiesel from waste sunflower frying oil. They found an increase in conversion for reaction temperature between 25–45 °C and a decrease in conversion after 55 °C.

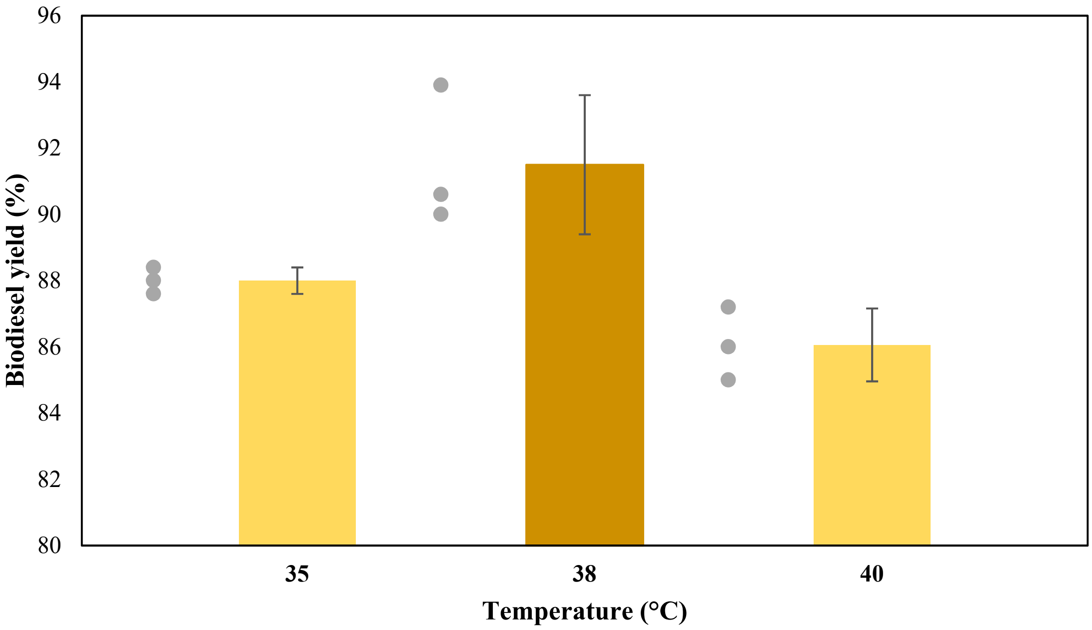

Figure 8. Effect of temperature on biodiesel yield. Values are mean +/− standard deviation (n = 2).

Table 8 summarizes the biodiesel fatty acid compositions for additional reaction temperatures considered in this assessment (35 °C and 40 °C) while FAMEs composition of biodiesel at reaction temperature of 38 °C was previously reported in Section 3.2.2. The composition of methyl palmitate decreased from 25.4 to 26.4 when increasing temperature from 35 °C to 40 °C. At 38 °C, the methyl palmitate composition was 20.25% suggesting a minimum on the transformation of triglycerides on this ester. The methyl oleate showed a composition varying from 17.40 to 20.30 for 35 °C and 40 °C, respectively. At 38 °C, this ester reached a composition of 17.91 suggesting that temperature contributes to increase methyl oleate production.

Table 8. Fatty acids composition at different temperature conditions.

FAMEs Composition (%)	Composition (%)	
	35 °C	40 °C
Methyl palmitate	25.4	26.4
Methyl linolelaidate	0	0
Methyl oleate	17.40	20.30
Methyl tridecanoate	0	0

4. Conclusions

In this work, biodiesel is produced from waste frying oil via enzymatic transesterification and the effects of reaction time, enzyme concentration, pH and temperature were evaluated. The WFO was collected from local restaurants in East Colombia (Cúcuta) and is mainly composed of palmitic acid with 30.35% and oleic acid with 18.84%. Due to the frying process of such oil and its reusability more than three times, the fatty acid content was significantly low, and the presence of impurities features a challenge to reach high conversion yields. Despite these limitations in feedstock quality, the conversion of fatty acids into methyl esters were 87.11% total biodiesel (g)/total WFO (g). The highest biodiesel production of 437.863 g (91.49% yield) was reached with an enzyme concentration of 14%, reaction time of 8 h, pH of 7 and temperature of 38 °C. The resulting biodiesel from WFO reported composition of 20.25% for methyl palmitate and 17.91% for methyl oleate. The flash points of biodiesel ranged from 227.5 to 257.5 °C, meeting the standards. Also, corrosion tests reported favorable results with minimum value of 1a. The moisture content as well as viscosity of biodiesel from WFO reached values that exceed national and international standards. These issues represent the main limitations in the use of biodiesel from such source. To overcome high viscosity, it was suggested the use of additive to reduce viscosity or preheating before injection. Finally, it can be concluded that the implementation of combined lipase system as a catalyst for the transesterification process was promising, since the proportion of methyl esters in the biodiesel was close to the content of fatty acids in the wasting oil.

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Appendix A. GC-MS Chromatography (Column/Parameters/Conditions)

Equipment Characteristics	Operating Conditions
Model: 6890N Agilent Detector: 5973N Agilent Column: DB-1MS Injector: Split/Splitless	Carrier gas: Helium Operating mode: Splitless Injection volume: 3.0 µL Injector temperature: 250 °C Detector temperature: 320 °C Initial temperature: 70 °C Heating rate: 8.00 °C/min Recording time: 60 min

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