CHEMICAL ENGINEERING TRANSACTIONS
VOL. 80, 2020
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Eliseo Maria Ranzi, Rubens Maciel Filho

ISBN 978-88-95608-78-5; **ISSN** 2283-9216
DOI: 10.3303/CET2080051

Enzymatic Production of Biodiesel from Grapeseed Oil

Maria Sarno[ab], Mariagrazia Iuliano[c,*]

[a]Department of Physics, University of Salerno, Via Giovanni Paolo II, 132 - 84084 Fisciano (SA), Italy
[b]Centre NANO_MATES, University of Salerno Via Giovanni Paolo II, 132 - 84084 Fisciano (SA), Italy
[c]Department of Industrial Engineering, University of Salerno, via Giovanni Paolo II, 132 - 84084 Fisciano (SA), Italy
msarno@unisa.it

Magnetic nanoparticles (Fe_3O_4/Ag NPs) covered with tartaric acid (TA) have been synthesized through an "eco-friendly" approach and used for direct physical immobilization of lipase from *Thermomyces lanuginous* (TLL). The immobilized lipase was tested for the production of biodiesel from grape seeds oil, and in a solvent-free system. The maximum yield to biodiesel was about 94%, at the methanol/oil molar ratio of 1:6. In the same operating conditions free lipase exhibited, after 24 h, a maximum yield of 77%. Moreover, the FAME content in biodiesel was ~96.54% in accordance with European Standards.

1. Introduction

In recent years, biofuel's interest has continued to grow. It is because of new technological developments to guarantee good agricultural production and its successive transformation through suitable energetic methods with little economic investment. Thus, agricultural waste used as fuel is competitive with fossil sources, opening a new opportunity for the use of renewable resources available globally and more particularly at the regional level. World grapes production was over 67 million metric tons in 2005, with Spain, France, and Italy being the dominant world grape producers providing almost half of the total output. Grape seeds represent between 2-5% of the grape weight and constitute approximately 38-52% of solid wastes generated by wine industries. In general, they contain about 40% fiber, 10-20% lipid, 10% protein, complex phenolics, as well as sugars and minerals. Indigestible fractions, mainly cellulose and pectins, are other constituents of grape seeds. In particular, the main characteristic of the grapeseed oil is its high content of unsaturated fatty acids, such as linoleic acid (72–76%, w/w) (Martinello et al., 2007). Biodiesel production from grape seed constitutes an economical alternative for the valuation of by-product obtained from the wine manufacture. Moreover, the wineries will have new economic difficulties with winery waste management.
The most established method for biodiesel production is the chemically catalyzed transesterification. However, the costs and complicated procedures lead to the need for alternative methods for biodiesel production. Enzyme-catalyzed transesterification is a relatively new method (Hama et al., 2013). Lipase due to their ability to catalyze transesterification of oils and fats can be used for this purpose (Fernandez-Lafuente et al., 2010). Enzymes' poor stability towards pH, temperature and time and their costs encourage the use of immobilizations to facilitate separation, recovery and enhance activity (Basso et al., 2019). For enzyme immobilization two central factors must be considered: immobilization methods and the support. Immobilization methods can be divided into chemical and physical. Ideal support properties include inertness towards enzymes, biocompatibility, hydrophilicity, resistance to microbial attack and compression, and accessibility at a low cost (Sarno et al., 2017; Sarno et al., 2016; Sarno and Ponticorvo 2017). It is challenging to predict the performance of immobilized enzymes. The immobilization of enzymes onto nanomaterials is today a topic of great interest. Indeed the reduction of the enzyme support size can provide a larger surface area for the attachment, leading to higher loading and activity. In the present paper, for the first time, Fe_3O_4/Ag nanoparticles have been synthesized through an "eco-friendly approach" and subsequently used for direct physical immobilization of the lipase from *Thermomyces lanuginous* (TL). The activity of lipase was investigated in different conditions. Finally, the new immobilized lipase nanocatalyst was used in a solvent-free condition to produce biodiesel, demonstrating the suitability of our simple and easy approach, i.e. scalable and

Paper Received: 17 November 2019; Revised: 1 February 2020; Accepted: 27 April 2020
Please cite this article as: Sarno M., Iuliano M., 2020, Enzymatic Production of Biodiesel from Grapeseed Oil, Chemical Engineering Transactions, 80, 301-306 DOI:10.3303/CET2080051

simple nanomaterials synthesis, easy enzyme immobilization and high activity to produce biodiesel from abundant grapeseed oil waste.

2. Materials & Methods

Grape seeds of the white wine variety Moscato were obtained from the local (Roccadaspide, Campania) wine cellar and were stored wet until extraction.
Ferric chloride hexahydrate ($FeCl_3•_6H_2O$, Sigma Aldric >99%) salts and silver nitrate ($AgNO_3$, Sigma Aldric >99%) were used for the synthesis of Fe_3O_4/Ag nanoparticles. L-(+)-Tartaric Acid (Sigma Aldric ≥99.7) was used for the coating agent. All other chemicals were of reagent were acquired from Aldrich Chemical Co. All chemicals were of analytical grade.

2.1 Grape seeds oil extraction

10 g of grape seeds were dried at 50 °C for two days just before extraction. After the sample was grounded in a mortar and added in a soxhlet extractor with hexane with a ratio of 1/2 wt./v for 6 h. Later the solvent was removed within a rotavapor to obtain the clear yellowish oil (see Figure 1).

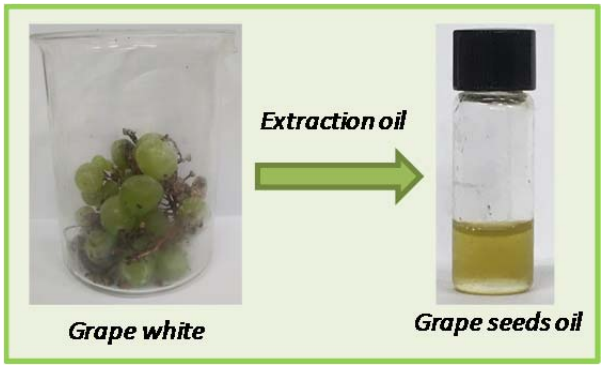

Figure 1: oil extracted from grape seeds

The physical-chemical properties of the oil, such as acid value, free fatty acid content, iodine number, moisture, and saponification index, were determined and reported in Table 1.

Table 1: Physical-chemical properties of the grape seeds oil

Property	Value	Unit
Acid value	2.21±0.20	mgKOH/g
Free fatty acid content	1.11±0.16	%
Moisture	0.08±0.05	%
Saponification Index	175.80±0.2	mgKOH/g
Iodine Value	159.11±0.10	gI_2/100g oil

2.2 Synthesis Nanoparticles

The nanoparticles were synthesized using solvothermal method (Sarno et al., 2019), in particular, 3 mmol of ferric chloride hexahydrate, 0.1 mmol of silver nitrate, 1 mmol of L-(+)-tartaric acid and 30 mmol of urea were added entirely in 30 ml of ethylene glycol and mixed for 30 min a room temperature. The orange solution was added in an autoclave (capacity, 50 mL). The temperature was increased up to 200 °C and kept for 6 h. The pressure generated in the autoclave was measured at approximately 9 bar. After the synthesis time, the solution was cooled at room temperature, and the black mixture was washed with 80 ml of ethanol, and subsequently, cleaning with ethanol and deionized water several times. Finally, dried at 60 °C for 24 h.

2.3 Enzyme immobilization and Hydrolytic activity

0.05 g of Fe_3O_4/Ag nanoparticles were introduced into glass flask containing 10 mL of *Thermomices lanuginous* lipase (0.1 mg/mL) dissolved in 1 M citrate buffer pH= 3. The glass flask was transferred into a temperature-controlled water bath shaker and shaken at 4 °C for 3 h. The solid product was collected using a magnet and washed several times with citrate buffer to remove free enzyme. The concentration of lipase in the solution was determined by a UV–visible spectrophotometer (Evolution 60S, Thermo Scientific) at

wavelength 595 nm (Bradford, 1997). The immobilization efficiency (%) (IE) was calculated according to the following expression:

$$\text{Immobilization efficiency} = \frac{(c_0 - c_f)v_1}{c_0 v_2} \ (\%) \quad (1)$$

where c_0(mg/mL) is the initial concentration of lipase solution, c_f (mg/mL) is the final concentration of lipase in solution after adsorption. v_1 and v_2 are the volumes (mL) of initial lipase solution and final lipase solution.
The hydrolytic activity of the free and immobilized lipase was measured by using titrimetric assays based on an olive oil emulsion method (Abrami et al., 1999; Sarno et al., 2019; Sarno et al., 2018). The amount of the hydrolyzed fatty acids was determined by the titration of the fatty acids derived from the hydrolysis of olive oil with a standard KOH solution (0.1 M). The activity recovery (%) (AR) was evaluated as the ratio between the enzymatic activity of the immobilized lipase and the activity of the free lipase (Sarno et al., 2019).

2.4 Production of fatty acid methyl esters (biodiesel) from grape seed oil

The transesterification reactions were carried out in a 150 ml screw-capped vessel and heated to 45 °C in a temperature-controlled water bath shaker at 200 rpm/min. Briefly, 10 g of grape seed oil, three-step addition of methanol (1:6 molar ratio of oil/methanol), and 10 % immobilized lipase or free lipase (wt. enzyme/ wt. oil) were added in a screw-capped vessel. After a 24 h, the enzyme immobilized was removed with a magnet and the sample was centrifugated to remove glycerol and purified with hot water for further analysis. The yield of biodiesel was evaluated as reported by Sarno et al. (Sarno et al., 2018). Methyl heptadecanoate which served as the internal standard and an aliquot of the sample was measured for GC analysis to determine fatty acid methyl esters (FAMEs). The methyl ester contents of the reaction mixture were measured on a gas chromatograph Thermo-Fischer gas chromatography equipment, capillary column (Trace-GOLD TG-POLAR GC Columns 0.25 µm×0.25 mm×60 m). Helium was used as carrier gas with a flow rate of 1.2 ml/min. The start temperature of the column was 150 °C and it was gradually raised at the rate of 15 °C/min to temperature of 190 °C, after it was progressively raised at the rate of 4 °C/min to the final temperature of 230 °C, while the injector and detector were maintained at 250 °C.

3. Results & Discussion

3.1 Characterization of Support and Biocatalyst

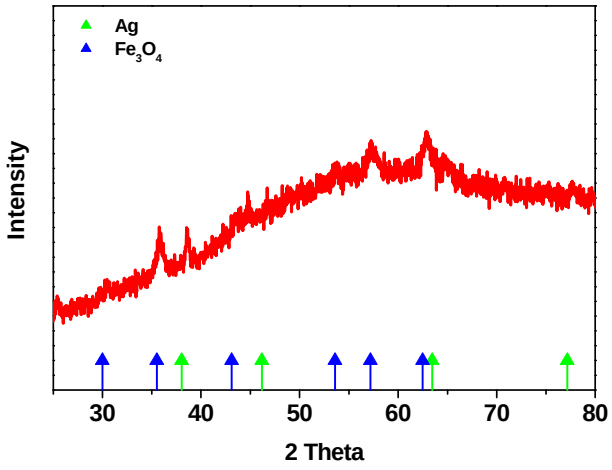

Figure 2: XRD profile of Fe_3O_4/Ag nanoparticles.

The XRD analysis was performed using Bruker D8 X-ray diffractometer using CuKα radiation. The XRD profile of Fe_3O_4/Ag (Figure 2) NPs evidences the typical peaks of the magnetite at 30.1° (220), 35.6° (311), 43.1° (400), 53.4° (422), 57.2° (511) and 62.5° (440). While the four major peaks of Ag NPs can be clearly observed at 2θ values of 38.5, 44.7and 64.8° and 77.8° which correspond to the reflections of (1 1 1), (2 0 0), (2 2 0) and (311) crystal planes of Ag (JCPDS card no. 87-0720), indicating the face-centered cubic structure of the Ag NPs (Xiong et al., 2013).
The Scherrer equation was used to estimate the average crystallite size (D) of the NPs (Yong et al., 2008):

$$D = \frac{k\lambda}{\beta \cos\theta} \quad (2)$$

where the k=Sherrer constant, λ=incident X-ray wavelength, β= full width at half maximum intensity observed in the pattern, and θ=Bragg diffraction angle. The calculated values of D for the synthesized Fe_3O_4 and Ag NPs were of 9.5±1.1 nm and 5.4±1.0 nm, respectively.

3.2 Factors affecting TLL immobilization on Fe_3O_4/Ag

In Figure 3a immobilization efficiency as a function of initial lipase, concentration was reported. The immobilization efficiency on the Fe_3O_4/Ag support decreases slowly when the initial enzyme concentration increase from 0.1 mg/ml to 0.2 mg/ml while decreases more quickly with initial lipase concentration > 0.2 mg/ml. This is probably due to saturated binding sites on the support, which eventually prevent further enzyme acceptance (Lei et al., 2009).

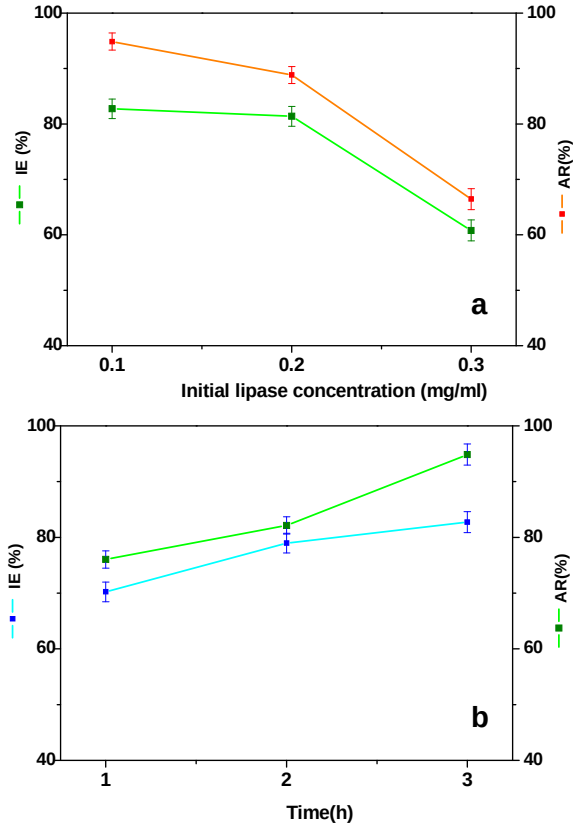

Figure 3: Effect of the initial lipase concentration on lipase loading, immobilization efficiency and activity recovery (the measurements were performed by setting the coupling time to 3 h, coupling pH 3) (a). These parameters were also measured by varying the coupling time of the immobilization procedure (initial lipase concentration: 0.1 mg/ml) (b).

The activity recovery of lipase decreased with increasing the initial concentration of lipase from 0.1 to 0.3 mg/mL. Diffusion restrictions observed in these situations exercise further strain on enzyme catalysis, hampering substrate entrance to the active site, and product release (Liu et al., 2011). Decreased enzyme activity is the expected outcome (Figure 3a). Figure 3b shows the relationships between activity recovery and immobilized efficiency at increasing immobilization times. The coupling time is indicative of the occurrence of a correct binding between the enzyme and support.

3.3 Effect of immobilized and free TLL for biodiesel synthesis

The result in Figure 4 shows the yield to biodiesel for free and immobilized TLL with an oil/methanol molar ratio of 1:6 and a temperature of 45°C. Maximum biodiesel yield was obtained for immobilized lipase with a yield of 94 %; yield to biodiesel of 77 % was achieved by free lipase. The different behavior of free and

immobilized enzyme can be due to a major resistance of immobilized lipase to methanol deactivation. Furthermore, the presence of the small Ag NPs induced enhanced stability, which has been supposed able to induce favorable conformational changes and electrostatic interaction (Sarno et al., 2019b). Indeed, Ag NPs can act as conduction centers to facilitate the transfer of an electron, helping enzymes to assume an advantageous orientation.

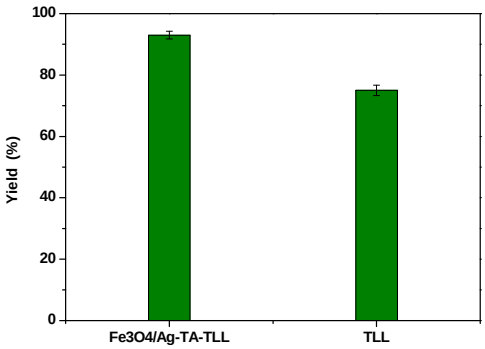

Figure 4: Biodiesel yield (%) for immobilized and free TLL. Immobilization condition: coupling temperature 4°C; coupling time 3 h; coupling pH 3; lipase amount 0.1 mg/ml. Reaction conditions: reaction time 24 h; reaction temperature 45°C; lipase concentration 10%; oil/methanol molar ratio 1:6 M.

Finally, a typical chromatogram of biodiesel from grape seed oil (Figure 5) showed that there were four fatty acid methyl esters (FAMEs), which were further analyzed with a mass spectrometer (MS).

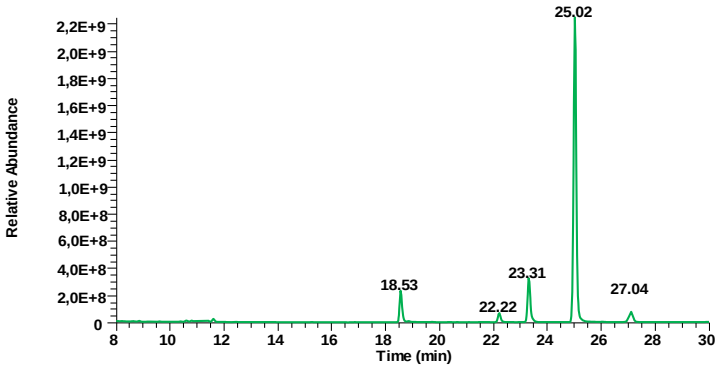

Figure 5. GC spectrum of Biodiesel from grape seed oil with immobilized TLL. Immobilization condition: coupling temperature 4°C; coupling time 3 h; coupling pH 3; lipase amount 0.1 mg/ml. Reaction conditions: reaction time 24 h; reaction temperature 45°C; lipase concentration 10%; oil/methanol molar ratio 1:6 M.

Table 2: FAME composition of Biodiesel from grape seed oil

FAME		Value (wt.%)
Palmitic acid methyl ester	C16:0	6.97±0.07
Stearic acid methyl ester	C18:0	2.90±0.11
Oleic acid methyl ester	C18:1	14.50±0.08
Linoleic acid methyl ester	C18:2	75.10±0.10
Linolenic acid methyl ester	C18:3	0.53±0.12
ΣSFA		9.87
ΣUFA		90.13

Data are mean ± SD (n=3). ΣSFA = sum of saturated fatty acids; ΣUFA = sum of unsaturated fatty acids.
The composition of the biodiesel analyzed by GC–MS suggested that grape seed oil biodiesel (see Table 2) was composed of: palmitic acid methyl ester, stearic acid methyl ester, oleic acid methyl ester, and linoleic acid methyl ester. The two main components account for ~89% of the total biodiesel. The determined

components were in accordance with the composition of the extracted grape seed oil, as previously reported in other cases (Fernandez et al., 2010). Ester content was in agreement with the European Standard, result equal to 96.54% ± 0.28.

4. Conclusion

Fe_3O_4/Ag nanoparticles constituted of a Fe_3O_4 NPs with a diameter of about 9 nm supporting faceted Ag NPs exposing a diameter of ~6 nm were coated by tartaric acid and used for direct immobilization of lipase by ionic interaction. The immobilization efficiency and activity recovery on the Fe_3O_4/Ag support decrease with the initial enzyme concentration increase due to the binding sites on the saturated support and diffusion restrictions. The immobilized efficiency of ~90 % was achieved after 3 h, with a lipase concentration of 0.1 mg/ml, the corresponding activity recovery was about 92 %. Indeed, the different work functions of magnetite and silver NPs enhance the affinity of the Fe_3O_4 surface with polar molecules, favoring strong ionic interaction. Moreover, in the reaction media, Ag NPs can act as conduction centers to facilitate the transfer of an electron, inducing enzymes to adopt an advantageous alignment. A very high conversion yield, of 94%, was achieved for immobilized lipase, thanks to the interaction between the enzyme and the support. The MS analysis of biodiesel from grape seeds oil showed that two main components (oleic acid methyl ester, linoleic acid methyl ester) account for ~89% of the total biodiesel, in accordance with the oil composition. For the biodiesel prepared at lipase concentration of 10%, reaction temperature 45 °C, oil/methanol ratio 1:6 M, reaction time 24 h, total FAME of 96.54 % ± 0.26 in accordance with the specification reported in EN14124.

References

Abrami M, Lešˇci I., Korica T., Vitale L., Saenger W., Pigac J., 1999, Purification and properties of extracellular lipase from Streptomyces rimosus, Enzyme and Microbial Technology, 25, 522–529.

Basso A., Serban S., 2019, Industrial applications of immobilized enzymes—a review, Molecular Catalysis, 479, Article 110607

Bradford M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry Journal, 72, 248–254.

Fernandez C.M., Ramos M.J., Perez A., Rodriguez J.F., 2010, Production of biodiesel from winery waste: extraction, refining and transesterification of grape seed oil, Bioresource Technology, 101, 7030-7035

Fernandez-Lafuente R., 2010, Lipase from *Thermomyces lanuginosus*: uses and prospects as an industrial biocatalyst, Journal of Molecular Catalysis B: Enzymatic, 62, 197-212,

Hama S., Kondo A., 2013, Enzymatic biodiesel production: an overview of potential feedstocks and process development, Bioresource Technology, 135, 386-395

Lei L., Bai Y., Li Y., Yi L., Yang Y., Xia C., 2009, Study on immobilization of lipase onto magnetic microspheres with epoxy groups, Journal of Magnetism and Magnetic Materials, 321, 252-258

Liu X., Lei L., Li Y., Zhu H., Cui Y., Hu H., 2011, Preparation of carriers based on magnetic nanoparticles grafted polymer and immobilization for lipase, Biochemistry Engineering Journal, 56, 142-149

Martinello, M., Hecker, G., Pramparo, M.C., 2007, Grapeseed oil deacidification by molecular distillation: analysis of operative variables influence using the response surface methodology, Journal of Food Engineering, 81, 60–64.

Sarno M., Iuliano M., 2018, Active biocatalyst for biodiesel production from spent coffee ground, Bioresource Technology, 266, 431-438.

Sarno M., Iuliano M., 2019, Optimized procedure for the preparation of an enzymatic nanocatalyst to produce a bio-lubricant from waste cooking oil, Chemical engineering journal, 377, 120273.

Sarno M., Iuliano M., Polichetti M., Ciambelli P., 2017, High activity and selectivity immobilized lipase on Fe_3O_4 nanoparticles for banana flavor synthesis, Process Biochemistry, 56, 98-108.

Sarno M., Ponticorvo E., 2017, Much enhanced electrocatalysis of Pt/PtO_2 and low platinum loading Pt/PtO_2-Fe_3O_4 dumbbell nanoparticles, International Journal of Hydrogen Energy, 42, 23631-23638.

Sarno M., Cirillo, C., Scudieri, C., Polichetti M., Ciambelli P., 2016, Electrochemical Applications of Magnetic Core-Shell Graphene-Coated FeCo Nanoparticles, Industrial and Engineering Chemistry Research, 55, 3157-3166

Xiong R., Lu C., Wang Y., Zhou Z., Zhang X., 2013, Nanofibrillated cellulose as the support and reductant for the facile synthesis of Fe_3O_4/Ag nanocomposites with catalytic and antibacterial activity, Journal of Materials Chemistry A, 1, 14910-14918

Yong Y., Bai Y., Li Y., Lin L., Cui Y., Xia C.,2008, Preparation and application of polymer-grafted magnetic nanoparticles for lipase immobilization, Journal of Magnetism and Magnetic Materials, 320, 2350-2355.