World Applied Sciences Journal 8 (6): 719-724, 2010
ISSN 1818-4952


# Esterification of Free Fatty Acids by *Rhizopus oryzae* as Cell-Catalyzed from Used Cooking Oil for Biodiesel Production

[1]*Mohammad Pazouki,* [1]*Farzane Zamani,* [1]*Amir Hossein Zamzamian,*
[1]*Maryam Fahar and* [2]*Ghasem Najafpour*

[1]Materials and Energy Research Center, Meshkindasht, Karaj, Iran
[2]Faculty of Chemical Engineering, Babol Noshirvani University of Technology, Babol, Iran

**Abstract:** Biodiesel fuel, as fatty acid methyl ester is produced by esterification of plant oil or animal fat with methanol. This renewable fuel resource is an attractive alternative for the replacement of petroleum based fuels. Utilization of the whole cell as biocatalyst instead of free or immobilized enzyme is a new approach to reduce the catalysts costs in lipase-catalyzed biodiesel production. In this research work, the immobilized cell of *Rhizopus oryzae* (PTCC5174) in biomass support particles (BSPs) was used for the methanolysis of used cooking oil (UCO). The inhibitory effect of the undissolved methanol on lipase activity was eliminated by stepwise addition of methanol to the reaction mixture. Initially, the UCO was filtered by a Whatman 42 filter paper and then the filtered UCO was heated to 100°C. The pretreated UCO was converted to biodiesel using methanol to fatty acid molar ratio of 3:1, at 35°C. In the final step, BSPs of the immobilized cells of *R. oryzae were* added for 72 h. The free acid to methyl esters conversion for the filtered UCO was only 46%. The free acids were to methyl esters conversion for the heated and filtered UCO enhanced to 88%. The untreated UCO and the methyl ester produced were analyzed using GC-MS chromatography. The heated UCO is cost effective and simple method for the large scale biodiesel production of whole cell-catalyzed methanolysis.

**Key words:** Biodiesel · Whole-cell biocatalyst · Esterification · UCO · *Rhizopus oryzae*

## INTRODUCTION

Energy use is the most fundamental requirement for human existence. A high percentage of the world's total energy output is generated from fossil fuels and it has been universally conceded that fossil fuels are finite. The world is no longer endowed with new sources of cheap fossil fuels and experts have warned about the depletion of the present sources in the near future. Furthermore, the threat of supply instabilities and increased public awareness on impacts of fossil fuel emissions on environment and their potential health hazards triggered governments around the world to impose restrictions on fossil fuel combustion emissions [1]. The disadvantages of fossil fuels for the environment, created an interest for alternative fuel sources. One of the most promising renewable sources is biomass [2]. Among the biomass sources, vegetable oils and animal fats have attracted much attention as a potential resource for production of an alternative fuel such as biodiesel for the replacement of the petroleum-based diesel fuel. The biodiesel is renewable, biodegradable, non-inflammable and non-toxic and it also has a favorable combustion emission profile, producing much less carbon monoxide, sulfur dioxide and unburned hydrocarbons than petroleum based diesel [3]. The biodiesel fuel (fatty acid methyl esters), which is produced by methanolysis (transesterification) of triglycerides [4] obtained from vegetable oils like soybean oil, jatropha oil, rapeseed oil, palm oil, sunflower oil, corn oil, peanut oil, canola oil and cottonseed oil [5]. Apart from vegetable oils, biodiesel can also be produced from other sources like animal fat (beef tallow, lard), waste cooking oil, greases (trap grease, float grease) and algae [6]. Because of the high price of high-quality virgin oils, the cost of biodiesel from these resources is higher than that of petroleum-based diesel [7]. The increasing production of used cooking oil (UCO) from household, restaurants and industrial sources is a growing problem in the world. This residue is regularly poured down the drain, resulting in problems for wastewater treatment plants and energy loss, or is integrated into the food chain through animal feeds,

**Corresponding Author:** Mohammad Pazouki, Materials and Energy Research Center, Meshkindasht, Karaj, Iran

thus becoming a potential cause of human health problems. The production of biodiesel from waste cooking oil to partially substitute petroleum diesel is one of the measures for solving the twin problems of environment pollution and energy shortage. Also, used cooking or frying oils are the increasing interest as inexpensive feedstock for biodiesel production [8].

Transesterification of fatty acid is carried out in a number of ways such as using an alkali catalyst, acid catalyst, biocatalyst, heterogeneous catalyst or using alcohols in their supercritical state [9]. Presently, industrial production of biodiesel from waste cooking oil is performed by chemical alkaline or acidic processes. Chemical catalysts including alkaline have been employed most widely since they give a high conversion of triglycerides to methyl esters in a short reaction time. However, chemical transesterification has some unavoidable drawbacks such as high energy and methanol consumption, difficulty in glycerol recovery, the need to eliminate the catalyst and salt and a large amount of alkaline wastewater from the catalyst [10-13]. In the case where supercritical alcohol was used, higher rates of reaction were observed when compared to conventional transesterification. Another advantage of this process is that the FFAs are converted completely into esters. However, the requirements of high temperature, high pressure and high molar ratio of alcohol to oil make the process costly for industrial scale [3].

In recent times, there has been a growing interest in the use of enzymes such as lipases as biocatalyst for biodiesel production. Some of the advantages lipase biocatalyst over the chemical-catalyzed reactions include the generation of no by-products, easy product removal, mild reaction conditions (reaction temperature of 35-45°C) and catalyst recycling [14]. It has been reported that enzymatic reactions are insensitive to FFA and water content in waste cooking oil [14-16]. Hence, enzymatic reactions can be used in transesterification of used cooking oil [17]. But the cost of enzyme remains as a challenge for its industrial implementation. In order to enhance the cost effectiveness of the process, the enzyme (both intracellular and extracellular) is reused by immobilizing in a suitable biomass support particles of polyurethane and that has resulted in considerable improvements in process efficiency [13].

In this work, used cooking oils obtained from MERC restaurant was used to produce biodiesel employing immobilized *R. oryzae* cells within biomass support particles of polyurethane foam. Furthermore, the effect of pretreated used cooking oil on whole cell biocatalyst esterification was investigated.

## MATERIALS AND METHODS

**Preparation of Whole Cell Biocatalyst:** All lipase catalyzed experiments were carried out using the filamentous fungus *R. oryzae PTCC5174*. Basal medium for growth of *R. Oryzae* which contained of polypepton 70 g; $NaNO_3$ 1.0 g; $KH_2PO4$ 1.0 g; $MgSO_4 \cdot 7H_2O$ 0.5 g and olive oil 30 g in 1 l of distilled water. Flasks (500 ml) containing 100 ml of the basal medium with biomass support particles (BSPs) were inoculated by aseptically transferring spores from a fresh agar slant enriched with 4% potato dextrose agar and 2% agar and incubated at 30°C for 90 h on a reciprocal shaker (150 oscillations/min, amplitude 70 mm). The *R. oryzae* cells became well immobilized within the BSPs as a natural consequence of their growth during shake-flask cultivation. Immobilization was performed by placing 150 particles inside a flask together with the medium, subjected to prior sterilization. The pH of the medium was initially adjusted to 5.6 and then allowed to follow its natural course. Reticulated polyurethane foam with a particle voidage of more than 97% and a pore size of 50 pores per linear inch (ppi) was cut into 6mm×6mm×3mm cuboids. After cultivation, the BSP-immobilized cells were separated from the culture broth by filtration, washed with tap water and dried at room temperature for around 24 h. To stabilize the lipase activity, the dried cells were treated with a 0.1% (v/v) glutaraldehyde solution at 25°C for 1 h, washed with tap water, dried at room temperature for more than 24 h and then used as whole-cell biocatalyst for methanolysis reaction [18-19].

**Measurement of Cell Mass Immobilized Within BSPs:** The cell concentration immobilized within one BSP was measured as follows: In the first step, the weight of 20 polyurethane foam particles was measured before immobilization. In the next step, Specified BSPs after immobilization process were washed with acetone for several times to remove unwanted materials and were dried for 2h at 105°C. The particles plus dried cells were then weighed and the cell mass was calculated from the difference between the weights.

**Pretreatment of UCO:** In the early stage of experiment, the raw used cooking oil was filtered by applying a reduced pressure system using a filter paper (Whatman 42) to eliminate the indiscerptible impurities. In the second stage, the filtered UCO was heated up to 100°C for 15 min using a magnetic stirrer, to eliminate extra water that has an influence on the estrification reactions yield with methanol as an alcohol.

**Whole Cells Esterification:** Methanolysis of used cooking oil was carried out at 35°C for 72 h in 50 ml Erlenmeyer flasks while incubated on a reciprocal shaker (150 oscillations/ min, amplitude 70 mm). The reaction mixture (UCO 9.65 g, 0.1M phosphate buffer (pH 6.8) 1.5 ml and methanol 0.35 g) was dispensed with 50 BSPs into an Erlenmeyer. To convert the entire oil to its corresponding methyl esters, at least three molar equivalents of methanol were necessary. Consequently, 0.35 g of methanol was successively added to the reaction mixture at 24 and 48 h reaction time, respectively.

Methanolysis reactions have performed with UCO before and after pretreatment under conditions such as methanol to UCO molar ratio 3:1, temperature 35°C in last-step addition of methanol. After the reaction, the whole-cell biocatalyst was separated from the reaction mixture by filtration. Samples (200 µl) were withdrawn from the reaction mixture at specified time, centrifuged at 12,000 rpm for 5 min, to obtain the upper layer and analyzed by capillary gas chromatography.

**Gas Chromatography (GC) Analysis:** The methyl esters content in the reaction mixture were quantified using a gas chromatography/mass spectrometer (GC-MS) which equipped with a HP-5 column with 30 meter long, internal diameter 0.25mm. The column temperature was held at 160°C for 2 min, heated to 300°C with 8°C/min rate and then maintained for 5min. The temperatures of the injector and detector were set at 280 and 230°C; respectively. The total time required to process one sample was 24.5 minutes. For GC-MS analysis, 5µl of the aforementioned mixture and 300 µl of 1.4 mmol/l heptadecanoic acid methyl ester (hexane as the solvent) which is served as the internal standard were precisely measured and mixed thoroughly. A 1.0 µl of the treated sample was injected into a gas chromatograph column.

## RESULTS AND DISCUSSION

With regards to the possibility of enzyme immobilization using intracellular or whole cell biocatalyst, the later one has been adapted more due to the reduction of recovery and separation of product and which will produce more yield compared to the free Enzyme. The advantages of using whole cells immobilized are reuse of enzyme in continuous or multiple step processes, supplying the possibility of recovery, favorite reaction conditions, purity and control of end products and

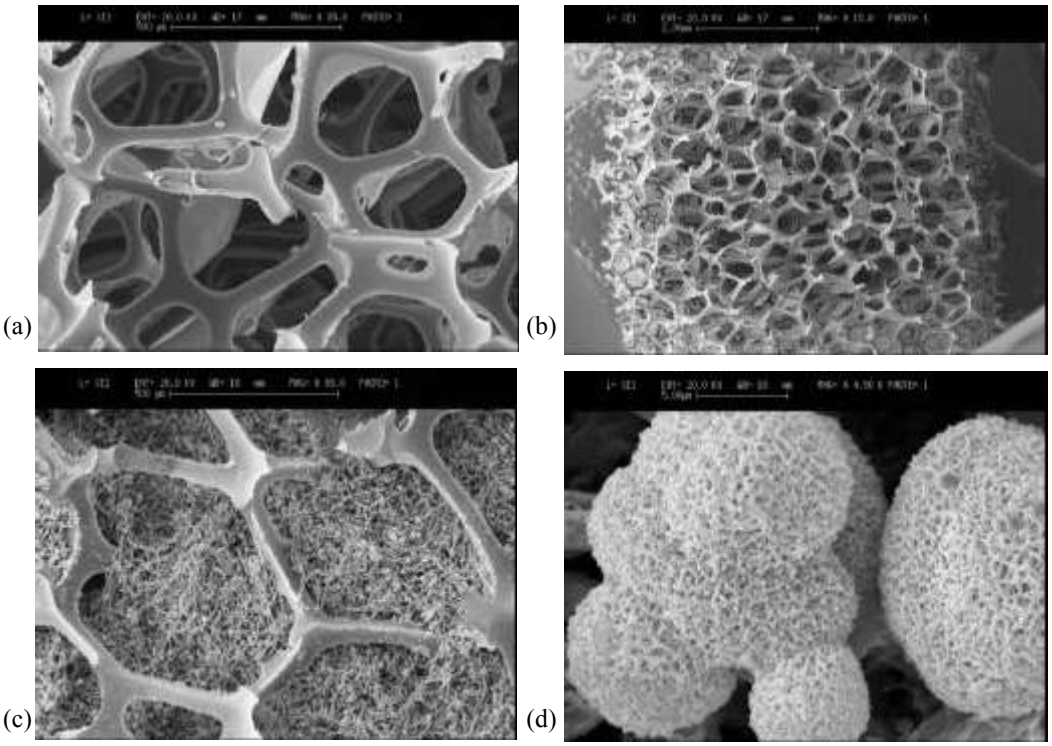


Fig. 1: SEM micrographs of foam particles
a. High magnification and b. Low magnification, before immobilization
c. High magnification and d. Low magnification, after immobilization

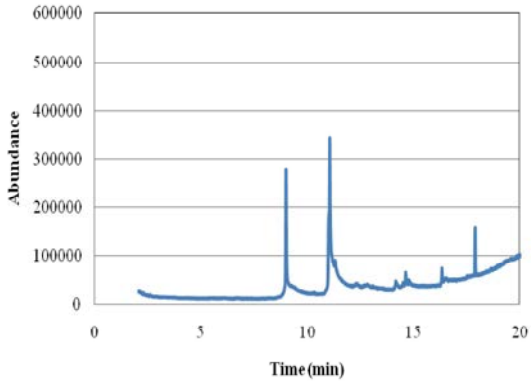


Fig. 2: GC-MS analysis of the preliminary UCO (filtered)

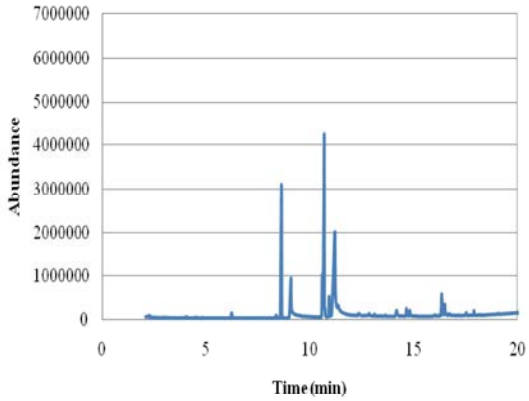


Fig. 3: GC-MS analysis of biodiesel without heating the filtered UCO

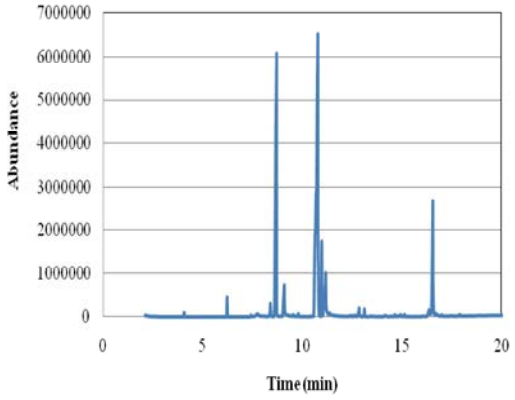


Fig. 4: GC-MS analysis of biodiesel filtered and heated UCO

the increasing of enzyme containing whole cell stability [20]. In this research work, the weight of 20 polyurethane foam particles before immobilization was 0.0849 g and the weight of 20 aforementioned BSPs after immobilization was increased to 0.1653 g. Therefore, it was concluded that the weight of polyurethane foam particles after immobilization have increased almost twice.

Figure 1(a, b) and (c, d) shows the SEM micrographs of polyurethane foam particles surface with 2 magnifications before and after cell immobilization. The images are shown that the immobilization process was successfully preformed. The biomass deposited on polyurethane foam particles is clearly observed through SEM micrographs.

The efficiency of the process was checked by employing GC-Mass analysis of the UCO and biodiesel product. The primary sample was withdrawn before any transesterification reaction occurred. Figure 2 shows the components of the UCO filtered sample before any sort of lipase enzymatic pretreatment took place. Also the initial sample had no heat treatment. The major peaks presented the chemical constituents of preliminary oil (Figure 2), these peaks are relevant to n-Hexadecanoic acid, 9, 12-Otadecadienoic acid and 9-Octadecenoic acid.

In Figures 3 and 4 depict analysis of transesterification reactions performed on the filtered and filtered/heated oil feed, respectively. Similarly the reaction of methanol and fatty acid without and with heating were carried out in presence of immobilized lipase. At initial stage with filtration of oil, according to our base pattern, by addition of methanol to oil ratio of 3:1, a 46% conversion of free fatty acids to methyl esters was obtained (see Figure 3). While the filtered oil along with heating the reaction solution, the conversion of free fatty acids to methyl esters rose to 88% (Figure 4).

GC-Mass analysis of the sample without heating showed that, the main peaks are with relation to the retention time (RT):8.62 min is for the production of Hexadecanoic acid methyl ester with 15.20% abundance. The peak with relation to the RT: 10.6 min is for the production of 9, 12-Octadecadienoic acid methyl ester with 4.69% abundance. The peak with relation to the RT: 10.69 min is for the production of 8-Octadecenoic acid methyl ester, 11-Octadecenoic acid methyl ester and 9-Octadecenoic acid methyl ester with 23.18% abundance (Figure 3). These components with low percentages account for 46% of FAME.

GC-Mass analysis for the sample with heating, the main peaks with relation to the RT: 8.69 min is for the production of Hexadecanoic acid methyl ester with 27.73% abundance. The peak with relation to the RT: 10.68 min is for the production of 9, 12-Octadecadienoic acid methyl ester, 10, 13-Octadecadienoic acid methyl ester with 14.03% abundance. The peak of relation to retention time (RT):10.77 min is for the production of 8-Octadecenoic acid methyl ester, 11-Octadecenoic acid methyl ester and 7-Octadecenoic acid methyl ester with 31.06% abundance. The result indicated that the all components in the

UCO-derived biodiesel were methyl octadecenoate, methyl hexadecenoate, methyl octadecadienoate, methyl octadecanoate, methyl tetradecanoate, methyl heptadecanoate, methyl dodecanoate, methyl pentadecanoate. These components account for 88.09% of FAME (Figure 4).

## CONCLUSIONS

Biodiesel as a supersede fuel for diesel engines has become increasingly important due to the environmental consequences of fossil fuel based diesel engines and the decreasing petroleum resource. The main challenges in the production of biodiesel are its cost and availability of fats and oils resources. By collecting used frying oils and converting them to biodiesel fuel, the cost of biodiesel is significantly lowered and the negative impact of disposing used oil to the environment reduced. However, in the process of frying, oil undergoes many reactions leading to the formation of many undesirable compounds such as polymers, free fatty acids and many other chemicals. These impurities create a lot of challenges in the transesterification of UCO. The pretreatment of the UCO to remove these chemicals is not practical; hence the oil is heated and filtered to remove solid particles prior to transesterification reaction. Therefore heating the oil may enhance the yield and productivity of the transesterification reaction, that may increase the biodiesel production. In the present study, possibility of production of biodiesel fuel by methanolysis of used cooking oil by the use of enzymatic cells of *Rhizopus oryzae* immobilized within biomass support particles (BSPs) of polyurethane as a biocatalyst were investigated. Furthermore the effect of pretreated oil was also investigated. The result showed that heating of the preliminary UCO have a possibility to increase the reaction conversion of free fatty esters to methyl esters (biodiesel). The fatty acid conversion was enhanced by 40%.

## REFERENCES


1. Tashtoush, G.M., M.I. Al-Widyan and A.O. Al-Shyoukh, 2004. Experimental study on evaluation and optimization of conversion of waste animal fat into biodiesel. Energy Conversion and management, 45: 2679-711.
2. Geyae, S., M. Jacobus and S. Letz, 1984. Comparison of diesel engine performance and emissions from heat and transesterified vegetable oil. Transactions of the ASAE, 27(2): 375-81.
3. Meher, L.C., D.V. Sagar and S.N. Naik, 2006. Technical aspects of biodiesel production by transesterification-a review. Renewable and Sustainable. Energy Reviews, 10: 48-68.
4. Ma, F.R. and M.A. Hanna, 1999. Biodiesel production: a review. Bioresource Technol., 70: 1-15.
5. Peterson, C.L., 1986. Vegetable oil as a diesel fuel: Status and research priorities. Transactions of the ASABE, 29(5): 1413-1422.
6. Pearl, G.G., 2002. Animal Fat Potential for Bioenergy use. Bioenergy 2002, The Biennial Bioenergy Conference, Boise, ID, September, pp: 22-26.
7. Zheng, S., M. Kates, M.A. Dubé and D.D. Mclean, 2006. Acid-catalyzed production of biodiesel from waste frying oil. Biomass and Bioenergy, 30: 267-72.
8. Chen, Y., B. Xiao, J. Chang, Y. Fu, P. Lv and X. Wang, 2009. Synthesis of biodiesel from waste cooking oil using immobilized lipase in fixed bed reactor. Energy Conversion and Management, 50: 668-673.
9. Zhang, Y., M.A. Dub´e, D.D. McLean and M. Kates, 2003. Biodiesel production from waste cooking oil. 1. Process design and technological assessment. Bioresource Technol., 89: 1-16.
10. Haas, M.J., P.J. Michalski, S. Runyon, A. Nunez and K.M. Scott, 2003. Production of FAME from acid oil, a by-product of vegetable oil refining. J. American Oil Chemists Society, 80: 97-102.
11. He, Q., Y. Xu, Y. Teng and D. Wang, 2008. Biodiesel production catalyzed by whole-cell lipase from Rhizopus chinensis. Chinese J. Catalysis, 29: 41-46.
12. Lu. J., K. Nie, F. Xie, F. Wang and T. Tan, 2007. Enzymatic synthesis of fatty acid methyl esters from lard with immobilized Candida sp 99-125. Process Biochemistry, 42: 1367-1370.
13. Ranganathan, S.V., S.L. Narasimhan and K. Muthukumar, 2008. An overview of enzymatic production of biodiesel. Bioresource Technol., 99: 3975-3981.
14. Wu, W.H., T.A. Foglia, W.N. Marmer and J.G. Phillips, 1999. Optimizing production of ethyl esters of grease using 95% ethanol by response surface methodology. Journal of the American Oil Chemists' Society, 76(4): 571-621.
15. Kulkami, M.G. and A.K. Dalai, 2006. Waste cooking oil-an economical source for biodiesel: a review. Industrial and Engineering Chemistry Research, 45: 2901-13.

16. Hsu, A., K.C. Jones and W.N. Marmer, 2001. Production of alkyl esters from tallow and grease using lipase immobilized in pyllosilicate sol-gel. J. American Oil Chemists' Society, 78(6): 585-8.
17. Hsu, A., K.C. Jones, T.A. Foglia and W.N. Marmer, 2002. Immobilized lipase-catalyzed production of alkyl esters of restaurant grease as biodiesel. Appl. Biochem., 36(3): 181-6.
18. Ban, K., S. Hama, K. Nishizuka, M. Kaieda, T. Matsumoto, A. Kondo, H. Noda and H. Fukuda, 2002. Repeated use of whole-cell biocatalysts immobilized within biomass support particles for biodiesel fuel production. Journal of Molecular Catalysis B: Enzymatic, 17: 157-165.
19. Hama, S., H. Yamaji, T. Fukumizu, T. Numata, S. Tamalampudi, A. Kondo, H. Noda and H. Fukuda, 2007. Biodiesel-fuel production in a packed-bed reactor using lipase-producing Rhizopus oryzae cells immobilized within biomass support particles. J. Biochemical Engineering J., 34: 273-278.
20. Nelson, L.A., T.A. Foglia and W.N. Marmer, 1996. Lipase-catalyzed production of biodiesel. Journal of the American Oil Chemists' Society, 73: 1191-1195.