Biodesel Production from *Pseudomonas Fluorescens* Lp1 Lipase Immobilized on Amino-silane Modified Super Paramagnetic Fe₃O₄ Nanoparticles

S Kanimozhi¹,a and K Perinbam²

Department of Biotechnology, Sathyabama University, Jeppiaar Nagar, Old Mamallapuram Road, Chennai - 600 119, Tamil Nadu, (INDIA)
Faculty of Plant Biology and Biotechnology, Nandanam Arts College (Men), Chennai 600 035 - 600 Tamil Nadu, (INDIA)

Email: skanimo@gmail.com, drperinbam73@gmail.com

Abstract. An extracellular lipase from *Pseudomonas fluorescens* Lp1 isolated from oil contaminated soil was immobilized onto amino silane modified superparamagnetic Fe₃O₄ nanoparticles. The magnetic nanoparticles, magnetite was synthesized chemically by co-precipitation and characterized by Scanning Electron Microscopy (SEM), Fourier Transformed Infrared Spectroscopy (FT-IR) and Powder X-ray diffraction studies (XRD). The structure of the synthesized magnetic nanoparticles was uniform, spherical and the size was determined around 31 nm by powder XRD. The biodiesel production mixture was prepared by addition of waste cooking oil, lipase immobilized magnetite and methanol. The transesterified products were analyzed by Gas Liquid chromatography-Mass spectroscopy (GC-MS). The methyl esters such as Oxiraneundecanoic acid, 3-pentyl-methyl ester, Hexadecanoic acid, methyl ester and 10-Octadecenoic acid, methyl ester were obtained. The study experimentally proved the use of amino silane modified superparamagnetic Fe₃O₄ nanoparticles in biodiesel production from waste cooking oil.

1. Introduction

Biodesel production has received considerable attention in the recent past as a biodegradable and nonpolluting fuel [1]. Vegetable oil and animal fat (m) ethyl esters, commonly referred to as “biodiesel,” are prominent candidates as alternative Diesel fuels [2]. Biodiesel can be processed from any type of vegetable oils and animal fats: (1) food grade vegetable oils, such as soybean, canola, palm, sunflower and peanut; (2) animal fats, such as lard, tallow, chicken fat and fish oils and (3) used cooking oils from restaurants. Alternative fuels of petroleum solve many of the current social problems and concerns, from air pollution and global warming to other environmental improvements and sustainability issues [3, 4]. The used cooking oils are used as raw material, adaption of continuous transesterification process and recovery of high quality glycerol from biodiesel by-product (glycerol) are primary options to be considered to lower the cost of biodiesel [5].

Lipases (triacylglycerol acyl ester hydrolases; EC 3.1.1.3) are biocatalysts that hydrolyse long chain triglycerides at the water/oil interphase to yield free fatty acids, monoglycerides, diglycerides and glycerols [6]. Lipases find promising applications in organic chemical processing, detergent formulations, synthesis of biosurfactants, the oleochemical industry, the dairy industry, the
agrochemical industry, paper manufacture, nutrition, cosmetics, and pharmaceutical processing. Development of lipase-based technologies for the synthesis of novel compounds is rapidly expanding the uses of these enzymes [6, 7]. Lipases are widely distributed in nature and found in many species of plants, animals, bacteria, yeast and fungi. The *Pseudomonas* lipases constitute a major group; they have been reported from *P. aeruginosa; P. fluorescens, P. glumae* and other *Pseudomonas* sp. [8, 9].

Magnetic nanoparticles offer abundant attractive possibilities in biotechnology. The use of magnetic nanoparticles offers many advantages due to their unique size and physical properties [10]. Besides, Magnetic nanoparticles exhibits the superparamagnetism, not keeping magnetized after the action of magnetic field, provides the highest advantage of reducing risk of particle aggregation [11]. Among various magnetic nanoparticles, Fe₃O₄ nanoparticles have been considered as suitable for biological applications due to superparamagnetic behavior and low toxicity [12]. It is a promising alternative which provide outstanding support materials for the enzymes immobilization because of its striking characteristics, such as large surface area, mobility and high mass transference [13]. Immobilization provides convenient handling of the enzyme, provides for its facile separation from the product, thereby minimizing or eliminating protein contamination of the product. Immobilization also facilitates the efficient recovery and reuse of costly enzymes with enhanced stability under storage and operational conditions [14]. In the present study the lipase from *Pseudomonas fluorescens* Lp1 was immobilized on to the aminosilane modified superparamagnetic Fe₃O₄ nanoparticles and characterized. The biodiesel production was carried out and analyzed by GL-MS.

2. Materials and Method

2.1. Lipase production and purification

Extracellular lipase was obtained from *Pseudomonas fluorescens* Lp1 by submerged production in the medium containing olive oil 1% v/v (emulsified with gum acacia- 0.5%) as the carbon source and inducer of lipase, peptone-5 g/l; yeast extract-5 g/l; NaCl-0.5 g/l; CaCl₂-0.05 g/l; pH-8.0. The fermentation was carried out in 100 ml of sterile production broth seeded with 3% inoculum and incubated at 40°C and 150 rpm agitation. Crude lipase was obtained by centrifuging the culture broth 10,000 g, 10 min at 4 °C. The lipase purification was carried out by ammonium sulphate precipitation followed by dialysis against 0.05 M Sodium phosphate buffer solution (pH 7.2). Dialyzed lipase was further purified by Sephadex G -100 previously equilibrated with 0.1M Tris- HCl (pH 7.0). Extracellular lipase activity was estimated by photometric assay [15].

2.2. Synthesis of magnetite nanoparticles, amino functionalization and lipase immobilization

Fe₃O₄ nanoparticles (Magnetite) was prepared through chemical co precipitation method by mixing FeCl₂ (0.2 M) and FeCl₃ (0.3 M), stearic acid and NaOH (4M) and stirred vigorously. A black precipitate obtained was filtrated, washed and dried. Surface modification of magnetite was done by mixing magnetite, 3-(2-aminoethylamino propyl trimethoxysilane (APTS), methanol and sodium fluoride (NaF) and stirred vigorously for 10 min. To this tetraethyl orthosilicate (TEOS) was added drop wise slowly into the flask and stirred vigorously at room temperature for 24 hrs. The black precipitate of amino functionalized magnetic nanoparticles (AFMNPs) obtained was collected by magnetic decantation, washed thrice with ethanol and water and dried.

The immobilization of lipase was performed using glutaraldehyde as a cross linking agent. To the magnetite, glutaldehyde was added and stirred at room temperature. Then, *Pseudomonas fluorescens* Lp1 lipase in phosphate buffer solution was added and stirred for several minutes at room temperature. The immobilized lipase was separated by centrifugation and washed with phosphate buffer and dried. The immobilization efficiency (η %) was calculated according to the following equation

\[ \eta (\%) = \frac{U_s \times 100}{U_o} \]

where Us is the total enzyme activity recovered in the support and Uo is the enzyme units offered for immobilization.
2.3. Biodiesel Production
The biodiesel production mixture was prepared by the addition of waste cooking oil and immobilized
magnetite and incubated at 40°C for 48 hours with 150 rpm agitation. For the transesterification
process, methanol was added thrice at different interval of time. After 48 hrs, content was mixed
vigorously with hexane and centrifuged. The upper layer was subjected to Gas chromatography (GC-
MS) analysis for fatty acid methyl esters.

2.4. Characterization of nanoparticles
XRD patterns of magnetic nanoparticles obtained with X-ray Powder Diffractometer model Bruker
AXS D8 Advance with Cu (Kαλ=1.5406 Å) radiation, scanning range from 10°–90°. The morphology
of the magnetic nanoparticles was revealed by scanning electron microscopy SEM JEOL Model JSM -
6390LV. FT-IR spectra of magnetic nanoparticles, was obtained using Nicolet FT-IR Avatar 370 with
the wavenumber ranged from 4,000 cm⁻¹ to 400 cm⁻¹ using KBr beam splitter.

3. Materials and Method
Extracellular lipase from Pseudomonas fluorescens Lp1 was purified to 18 fold with the specific
activity of 1016.78 U/mg by sequential purification methods as described. Pseudomonas fluorescens
MTCC 2421 lipase was purified 184.37-fold with a specific activity of 424.04 U/mg after anion
exchange and gel exclusion chromatography with an apparent molecular mass of 65.3 kDa [16].
Lipase from Candida rugosa was purified by ammonium sulphate precipitation, dialysis, ultra-
filtration and gel filtration using Sephadex-200 to a 43-fold purification and 64.35 mg/ml specific
activity [17]. The magnetite as support was selected because it has some advantages: (i) higher
specific surface (ii) lower mass transfer resistance and less fouling, and (iii) the selective separation of
immobilized enzymes from a reaction mixture by the application of a magnetic field [18]. Magnetite
is one of the famous magnetic materials in common use. As a result of strong magnetic property and
low toxicity, its applications in biotechnology and medicine have gained significant attention [19].

In the present study the synthesized magnetite was analyzed by XRD (Fig.1) showed the
characteristic spinel structure of Fe₃O₄ confirmed the synthesis of magnetic nanoparticles. The average
size of the magnetic nanoparticles was calculated to be about 31nm by Debye–Scherrer equation using
the (311) peak, and there are no changes after the consequent lipase adsorption. The results were
coinciding the results of [20].
The SEM analysis revealed that magnetite has uniform spherical structures (Fig. 2) and was well dispersed. The grain size of the agglomerated spherical magnetite was found to be around 100 nm.

FT-IR vibrational analysis (Fig. 3) revealed the structure of Fe₃O₄. The vibrational peak at 582 cm⁻¹ in the naked nanoparticles corresponds to the Fe–O bond, O-H stretching vibration near 3419 cm⁻¹ and O–H deformed vibration near 1563 cm⁻¹ [21, 22]. The efficiency of immobilization of Pseudomonas fluorescens Lp1 lipase on to magnetic nanoparticles was found to be 58%. The binding efficiency of lipase was 90% and the corresponding activity recovery was 70% [23].
The transesterification of waste cooking oil into methyl esters was analyzed by GC-MS. The results revealed that the majority of the oil was converted to biodiesel and GC-MS analysis demonstrated the synthesis of Fatty acid methyl esters (FAMEs) such as Oxiraneundecanoic acid, 3-pentyl-methyl ester, Hexadecanoic acid, methyl ester and 10-Octadecenoic acid, methyl ester were obtained (Fig. 4). The result was correlated with biodiesel produced by *Photobacterium lipolyticum* lipase [17]. FAMEs were produced by the enzymatic transesterification of soyabean oil using immobilized *Pseudomonas cepacia* [24]. Waste cooking oil used as a substrate for biodiesel synthesis using immobilized lipase from *Penicillium expansum* [25]. By lipase transesterification process palm oil yielded biodiesel [26].
References

[1] S. V. Ranganathan, S.L. Narasimhan, K. Muthukumar, An overview of enzymatic production of biodiesel, Biores. Technol. 99 (2008) 3975–3981.

[2] G. Vicente, M. Martinez, J. Aracil, Integrated biodiesel production: a comparison of different homogeneous catalysts systems, Biores. Technol. 92 (2004) 297–305.

[3] H.L. MacLean, L.B. Laveb, Evaluating automobile fuel/propulsion system technologies, Prog. Energy. Combust. Sci. 29 (2003) 1–69.

[4] A.S. Ramadhass, S. Jayaraj, C. Muraleedharan, Use of vegetable oils as IC engine fuels-A review, Renew. Energy. 29 (2004) 727–42.

[5] F. Ma and M.A. Hanna, Biodiesel production: a review, Biores. Technol. 70 (1999) 1–15.

[6] R.K. Saxena, P.K. Ghosh, R. Gupta, W.S. Davidson, S. Bradoo, R. Gulati, Microbial lipases: Potential biocatalysts for the future industry, Curr. Sci. 77 (1999) 101–115.

[7] A Liese, K. Seelbach, C. Wandrey, editors. Industrial biotransformations - Weinheim: Wiley-VCH, 2000.

[8] E. Kageyama, M. Hirata, T. Nibira, and Y. Yumada, Purificaton, characterizaton and molecular cloning of lecoting lipase from Pseudomonas species, J. Biol. Chem. 266(1991) 18135-18140.

[9] H. Kumura, S. Hirose, H. Sakurai, K. Mikawa, F. Tomita, and K. Shimazaki, Molecular cloning and analysis of a lipase gene from Pseudomonas fluorescenc, BioSci. Biotechnol. Biochem. 62 (1998) 2233–2235.

[10] M.A. El-Sayed, Some Interesting Properties of Metals Confined in Time and Nanometer Space of Different Shapes, Acc. Chem. Res. 34 (2001) 257–264.

[11] T.K. Indira and P.K. Lakshmi, Magnetic Nanoparticles – A Review, Int. J. Pharma. Sci. Nanotechnol. 3 (2010) 1035–1042.

[12] A.K. Gupta and M. Gupta, Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications, Biomater. 26 (2005) 3995–4021.

[13] A.H. Lu, W. C. Li, A. Kiefer, W. Schmidt, E. Bill, G. Fink, and F. Schuth, Fabrication of Magnetically Separable Mesostructured Silica with an Open Pore System, J. Am. Chem. Soc. 126 (2004) 8616–8617.

[14] R.A. Sheldona, Enzyme Immobilization: The Quest for Optimum Performance, Adv. Synth. Catal. 349 (2007) 1289.

[15] U.K. Winkler, M. Stuckmann, Glycogen, hyaluronate and some other polysaccharides greatly enhance the formation of exolipase by Serratia marcescen, J. Bacteriol. 38 (1979) 663–670.

[16] K.R. Chakraborty, Purification and Biochemical Characterization of an Extracellular Lipase from Pseudomonas fluorescens MTCC, J. Agric. Food. Chem. 57 (2009) 3859–3866.

[17] S. Benjamin and A. Pandey, Isolation and Characterization of Three Distinct Forms of Lipases from Candida rugosa produced in solid state fermentation, Braz. Arch. Biol. Technol. 43 (2001) 453–460.

[18] S.H Huang, M.H. Liao, Direct Binding and Characterization of Lipase onto Magnetic Nanoparticles, Biotechnol. Prog. 19 (2003) 1095–1100.

[19] A. Curtis and C. Wilkinson, Nanotechniques and approaches in biotechnology, Trends Biotechnol. 19 (2001) 97–101.

[20] J. Dussan, O. H. Giraldo and C. A. Cardona, Application of magnetic nanostructures in biotechnological processes: Biodiesel production using lipaseimmobilized on magnetic carriers, paper presented at the European Congress of Chemical Engineering (ECCE-6), Copenhagen, 2007.

[21] B. Hu, J. Pan, H.L. Yu, J.W. Liu, J.H. Xu, Immobilization of Serratia marcescen lipase onto amino-functionalized magnetic nanoparticles for repeated use in enzymatic synthesis of Diltiazem intermediate, Process. Biochem. 44 (2009) 1019–1024.

[22] L.H. Andrade, L.P. Rebeloa, C.G.C.M Nettob, H.E. Toma, Kinetic resolution of a drug precursor by Burkholderia cepacia lipase immobilized by different methodologies on
superparamagnetic nanoparticles, J. Mol. Catal B: Enzym. 66(2010) 55–62.

[23] K.H. Mak, C.Y. Yu, I.C. Kuan and S.L. Lee, Immobilization of *Pseudomonas cepacia* lipase onto magnetic nanoparticles for biodiesel production, Sci. Technol. Vision. 5 (2009) 19-23.

[24] H. Noureddini, X. Gao and R.S. Philkana, 2005. Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil, Biores. Technol. 96 (2005) 769-777.

[25] N.W. Li, M.H. Zong and H. Wu, Highly efficient transformation of waste oil to biodiesel by immobilized lipase from *Penicillium expansum*, Process. Biochem. 44(2009) 685-688.

[26] L. Zhang, S. Sun, Z. Xin, B. Sheng, and Q. Liu, Synthesis and component confirmation of biodiesel from palm oil and dimethyl carbonate catalyzed by immobilized-lipase in solvent-free system, Fuel. 89(2010)3960-3965.