# PREPARATION AND CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* LIPASE IMMOBILIZED ON CALCIUM ALGINATE MICROSPHERES

**GARBA, A.[1]*, SALLAU, A.B.[1], IBRAHIM, S.[1], UMAR, I.A.[1], DANTSOHO, F.A.[2] AND AHMED A.S.[1]**

[1]Department of Biochemistry Ahmadu Bello University, Zaria, Nigeria.
[2]Department of Public Health, Maryam Abacha American University, Maradi, Niger

## ABSTRACT

Lipases are promising industrial biocatalysts because they are stable and active enzymes obtained from numerous sources. Lipase from *Pseudomonas aeruginosa* was immobilized on calcium alginate and characterized. Sodium alginate was mixed with purified lipase and the enzyme/alginate mixture was pumped drop wise in 0.4M $CaCl_2$. The molecular mass of the lipase was found to be 32 kDa as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The immobilized lipase had pH and temperature optima in the range of 7.5 to 8.0 and 45 to 55°C respectively and was stable at pH (7.5 to 8.5) and the temperature range of 45 to 60°C as compared to the free lipase. The $K_M$ and $V_{max}$ values were 0.13mmole higher than that of free lipase and 50.0µmole/minutes respectively as compared to free lipase which had $K_M$ and $V_{max}$ values of 0,07mmole and 55.5µmole/minutes. The enzyme activity was enhanced in the presence of $Mg^{2+}$and $Ca^2$ with the residual activities of 97.1% and 111.4% as compared to free lipase that had residual activity of 97.2^ and 110%. Immobilization improved the residual activity from 61.9% and 26.1% in the presence of $Fe^{2+}$ and $Ni^{2+}$ in free lipase to 64.5% and 50.0%in immobilized lipase. Slight inactivation of immobilized lipase occurred in the presence of $Cu^2$ with the residual activity of 62.8% as compared to free lipase which had 64.1%. In the presence of methanol and *n*-hexane, the enzyme had residual activities of 113.1% and 113.1%, respectively. The immobilized lipase was recycled up to 5 cycles and the result showed that calcium alginate\immobilized lipase could be used as an operative immobilized enzyme system.

**Keywords:** Calcium-alginate, characterization, immobilization, lipase, residual activity
***Correspondence:** garbaauwalu2011@gmail.com, auwalgarba@abu.edu.ng, 2348130329089

## INTRODUCTION

Lipases are ubiquitous in nature and are distributed widely in plants, animals and micro-organisms) [1]. However, lipases, of microbial origin are mostly explored in different biotechnological applications [2]. Over the past few decades lipases have gained special attention as a result of their ability to act in lipid-aqueous interface and catalyse esterification, trans-esterification, aminolysis and acidolysis reactions [2]. Many industrial applications of lipase focus on its regio-and enantio-selective properties [3]. Lipases do not require cofactors to catalyse hydrolytic reactions and remain active in the presence of organic solvents. Enzymes are largely soluble and therefore unstable against some conditions like temperature, pH, organic solvents and surfactants [4]. In order to make good use of this enzyme, many of its properties need to be improved so as to increase its industrial utilization. Immobilization is therefore considered as an exceptional solution for such barriers as it enhances the enzyme activity, selectivity, stability, and specificity.

Immobilization means retaining enzymes in a proper geometry to improve their recycling and product purity that subsequently improves the process economy for economic reusability under stabilized condition by associating it with an insoluble matrix [5]. The operational and temperature stability of lipases could be enhanced by immobilization which also induces change in the conformation of enzyme thus allows substrate access to the active site .Methods of enzyme immobilization can be classified as physical adsorption [6], entrapment or microencapsulation [7] and covalence binding to a solid matrix [8]. A very important factor for obtaining an efficient lipase activity is achieved by proper selection of method and support material. The material used for immobilization should be readily available, biodegradable, toxic free and of low cost. In this study entrapment in calcium alginate was used for immobilization of lipase from *P. aeruginosa* which entails mixing a soluble enzyme with a polymer solution and then cross linking the polymer to form a lattice structure that captures the enzyme [9].
Entrapment method is fast, cheap and can be carried out under mild condition and has wider industrial applications [10]. Alginate, celite, carrageenan, resins and acrylic polymers are the most commonly used carriers for entrapment. The use of *Bacillus cepacia* lipase encapsulated on *β*-carrageenan for biodiesel production from palm oil was reported by Jegannathan *et al*. [11]. Moreover, Sawangpanya *et al.* [12] reported the production of biodiesel from palm oil by calcium alginate immobilized *Candida rugosa* lipase.

## MATERIALS AND METHOD

### Materials

*P. aeruginosa* was obtained from Department of Microbiology, Ahmadu Bello University, Zaria. All the reagents used were of analytical grade.

### Preparation of lipase immobilized on calcium alginate gel spheres

The calcium alginate beads immobilization of the purified lipase was carried out according to the method of Bhushan *et al.* [13]. Exactly 5 g of sodium alginate was dissolved in 30ml distilled water and autoclaved at 121$^{o}$C for 15 minutes. After cooling at room temperature 20ml of sodium alginate was mixed with 5ml of purified lipase (19.2 U/ml) and then allowed to stand for 10minutes.The enzyme/alginate mixture was pumped drop wise in 0.4M $CaCl_2$ and kept for 1 hour to ensure its complete hardening. The alginate beads were washed with distilled water and finally with 50mM tris-HCl buffer pH 8.0. The lipase / calcium alginate beads were kept at 4$^{o}$C in tris-HCl buffer pH 8.0 until used.

### Determination of protein concentration of calcium alginate immobilized lipase

Protein concentration of calcium alginate immobilized lipase was determined according to Bradford 1976 [14]. One alginate bead containing 15µl lipase solution entrapped was put in 285µl distilled water and 3 ml of the Coomassie brilliant blue was added and allowed to stand for 5 min at room temperature. Absorbance was read at 595nm and the concentration was extrapolated from the standard curve

### Assay of activity of calcium alginate immobilized lipase

This was carried out according to the method of Gopinath *et al.,* 2005 [ [15]. One calcium alginate bead containing 15 µl lipase solution (0.13 mg/ml) was taken in 8.905ml of substrate *p*NPL solution (0.03 mM) and put into each test tube and incubated for 15 minutes and absorbance was read at 410 nm. One unit of lipase activity was defined as the amount of enzyme releasing 1µmol of *p*-nitrophenol per minute, under the assay condition

### Determination of $K_M$ and Vmax of immobilized lipase

This was carried out by determining initial velocity studies on immobilized lipase. Varying concentration of substrate *p*NPL (0.01 mM to 0.06 mM) were incubated with one bead of calcium alginate immobilized lipase each for this and the activity was determined. From the Lineweaver- Burk plot, $K_M$ and Vmax were determined.

### Effect of pH on the activity and stability of immobilized lipase

The optimal pH of calcium alginate immobilized lipase activity was determined in accordance with Zhang *et al*. [16] by incubating one bead in each case with *p*NPL (0.03 mM) at different pH ranging from 5 to 9 for 15 minutes. For stability studies 1ml of buffer (phosphate buffer and tris-HCl) at various pH (5.0 -9.0) was incubated in each case with three beads of calcium alginate immobilized lipase for 2 hrs. and residual activity was determined by incubating one of the beads with *p*NPL for 15 mins and expressed in terms of percentage

### Determination of the effects of temperature on the activity and stability of immobilized lipase

The optimal temperature of calcium alginate immobilized lipase activity was determined by incubating one bead with *p*NPL at different temperature ranging from 25$^{o}$C to 70°C for 15 mins. and the activity was determined. For thermostability studies 1 ml of buffer each containing three beads of calcium alginate immobilized lipase was incubated at different temperature ranging from 25 to 70$^{o}$C for 2 hrs. The residual activity of the pre incubated immobilized lipase was then determined by incubating one bead with substrate and expressed in terms of percentage

### Determination of the effects of some cations on immobilized lipase activity

The effect of $Mg^{2+}$, $Co^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Fe^{2+}$, $Cu^{2+}$ and $Ni^{2+}$ at final concentration of 1 mM on the activity of immobilized lipase was determined by incubating 2 ml of various cations with three beads each of calcium alginate immobilized lipase for two hours. The residual activity was determined against the control.

### Determination of stability of immobilized lipase in some organic solvents

The lipase stability in the presence of organic solvents 15% (v/v), 30% (v/v) and 45% (v/v) was determined in accordance with the method of Zhang *et al*. [16]. Exactly 2ml each of the varied concentration of the solvents were incubated with three beads each of calcium alginate immobilized lipase for two hours. The residual activity was determined against the control

### Determination of reusability of calcium alginate immobilized lipase

This was determined by carrying out several cycles of trans-esterification of *Acacia nilotica* oil with 3 beads of calcium alginate immobilized lipase. Trans-esterification reaction was carried out according to the method of Kim *et al*. [17]. The initial reaction mixture contained (98.55 ml) oil / methanol (1:10), three beads

of calcium alginate immobilized lipase, 1.20 ml *n*-hexane. After each period of transesterification, the beads were removed, washed with water at room temperature and used to carry out another round of trans-esterification process

## RESULTS AND DISCUSSION

As shown in Plate 1, the molecular weight of purified lipase from *P. aeruginosa* was found to be 32 kDa. Hiol *et al*. [18] reported that lipase from *Mucor fhiemalis* had a molecular weight of 49 kDa. Fullbrook [19] reported a lipase from *B. stearothermophilus* having 43 kDa. A molecular weight of 58 kDa was obtained for lipase from *P. aeruginosa* [20]. The difference in the molecular weight of lipases could be due to the differences in the specie of the organism with which the enzymes were isolated.

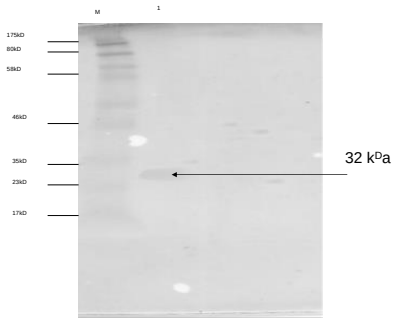


**Plate 1**: SDS-PAGE of purified lipase: Lane 1 is molecular weight marker and lane 2 is the purified lipase

Figure 1 shows the initial velocity studies of immobilized lipase from. The $K_M$ and $V_{max}$ values of 0.13 mmole and 50.0 µmole/minutes were obtained as compared to free lipase which had the $K_M$ and $V_{max}$ values of 0.07 mmole and 55.5 µmole/mins respectively. Most industrially relevant enzymes such as lipases and proteases have the $K_M$ values in the range of $10^{-5}$ and $10^{-1}$M [21]. An increase in $K_M$ after immobilization indicates that the enzyme has an apparent lower affinity for the substrate which may be caused by the loss of enzyme flexibility necessary for substrate binding or by diffusional resistance to substrate transport [22].

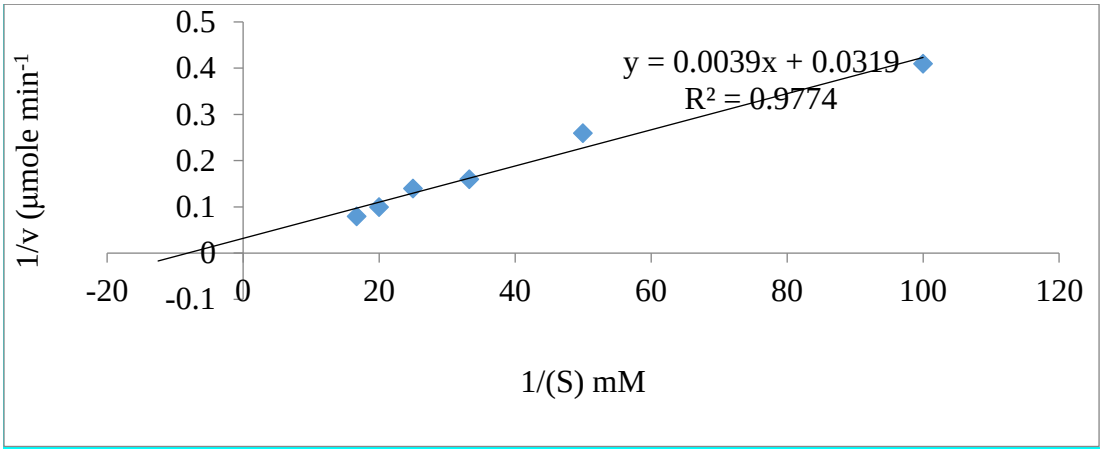


**Figure 1:** Lineweaver-Burk Plot for immobilized lipase

The effect of pH on the activity of immobilized lipase is shown in Figure 2. The enzyme had a broad pH range of activity with 19.0 U/ml at pH 7.5 and 8.0. No activity was detected at pH below 5.5 and above 9.0. The pH

stability of immobilized lipase is also shown in Figure 3. At pH 6, (76.3%) of the activity was retained, but the enzyme retained 100% activity in the pH range of 7.5 to 8.5. Immobilization has shifted the optimum pH and pH stability from 7.0 to 8.5 as compared to free lipase. Most lipases have optimal activity at neutral or slightly basic pH values [23]. The changes in the optimum pH for immobilized lipase could be due to the polarity of surface of the beads in which the enzyme is localized

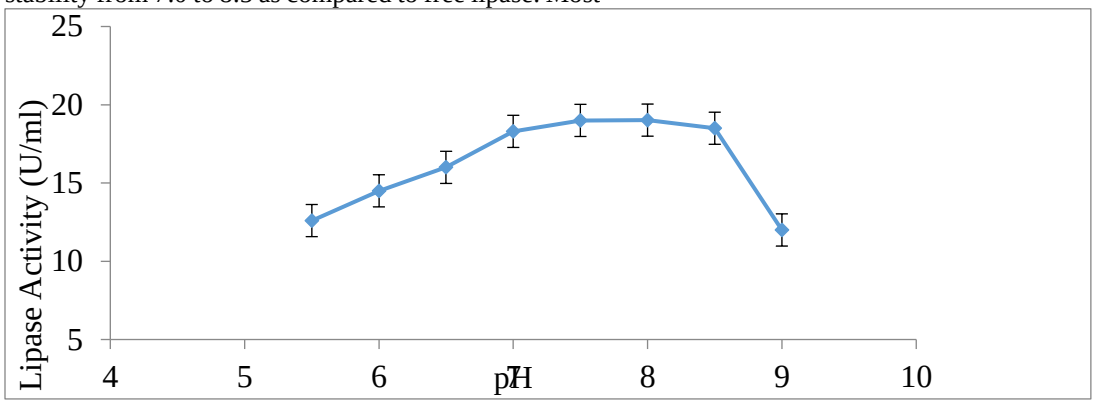


**Figure 2**: Effect of pH on the activity of immobilized lipase

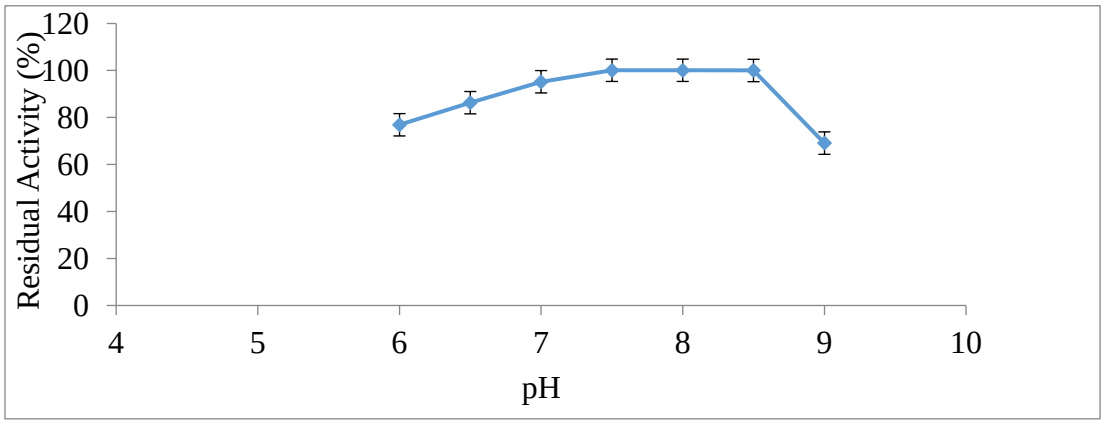


**Figure 3**: Effect of pH on the stability of immobilized lipase

The enzyme had maximum activity of 19.2 U/ml at 45°C, 50°C and 55°C but decreased to 18.4 U/ml and 17.2 U/ml at 60°C and 65°C respectively as shown in (Figure 4). The thermostability profile is shown in (Figure 5), there was a steady increase in activity of lipase at 30°C, 35°C and 40°C which was at its peak (100 % activity) at 45°C, 50°C and 55°C. Subsequently a decrease was observed at 60°C and 65°C. The solubility of globular proteins increases with the temperature up to 40ºC - 65ºC. Above this temperature the thermal agitation tends to disrupt the tertiary structure leading to denaturation and sharp decrease in solubility which in turns affect the enzyme activity. Shefai and Allam [24] reported that immobilized lipase remained active up to 50°C. This shift in the enzyme's optimum temperature after immobilization could be an added advantage in biodiesel production which might be due to the possible protection of immobilized enzyme from the bulk temperature through the formation of a molecular cage around the enzyme [25]. Greater optimum activity and temperature stability are attractive and desirable characteristics for industrially relevant enzymes [25].

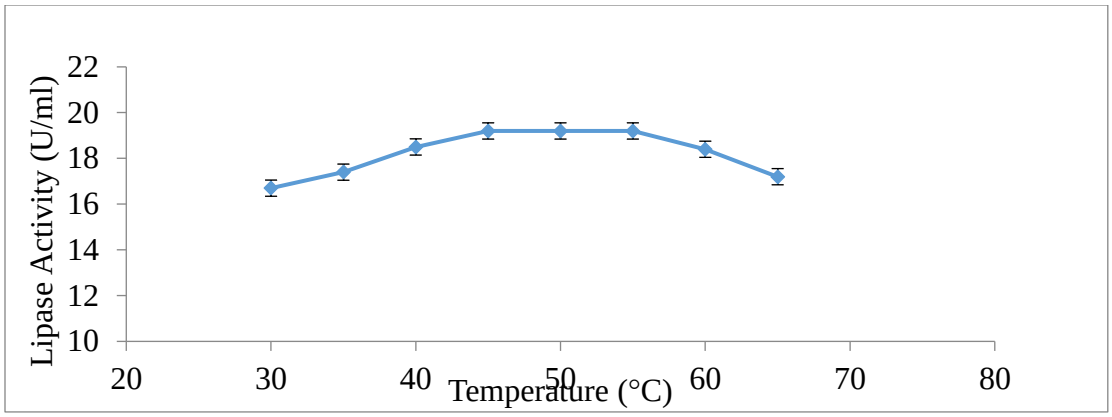


**Figure 4:** Effect of temperature on the activity of immobilized lipase

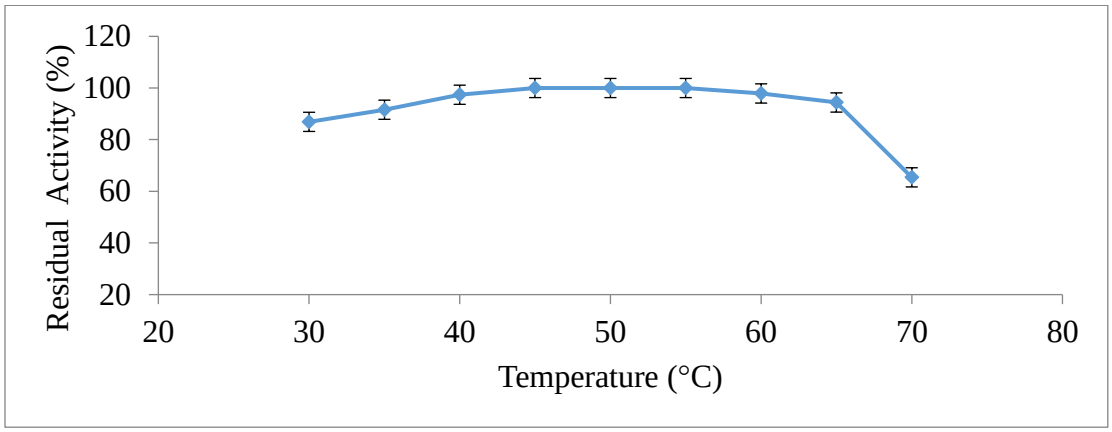


**Figure 5**: Profile of stability of immobilized lipase at varying temperature (30ºC to 70ºC)

The effect of cations on the activity of immobilized lipase is shown in Figure 6. The enzyme activity was enhanced in the presence of $Mg^{2+}$ and $Ca^{2+}$ with the residual activities of 97.1% and 111.4% as compared to free lipase which had residual activity of 97.2^ and 110% in the presence of $Mg^{2+}$and $Ca^{2}$. Immobilization improved the residual activity from 61.9% to 64.5%, and from 26.1% to 50.0% in the presence of $Fe^{2+}$ and $Ni^{2+}$ respectively. In the presence of $Cu^{2+}$ the residual activity of 62.8% was obtained as compared to free lipase which had 64.1%. The activation of lipase in the presence of calcium ions is supported by Rashid *et al.* [26] who reported maximum lipase activity in the presence of $Ca^{2+}$. Moreover, Dong *et al*. [27] reported increased in the activity by 250% in lipase from *Pseudomonas spp* in the presence of $Ca^{2+}$. Matsumae and Shibatani [28] suggested that the effect of metals could be attributed to a change in the solubility and the behaviour of the ionized fatty acids at interfaces, and from a change in the catalytic properties of the enzyme itself. The result agrees with Mehwish *et al*. [29] who reported improved residual activity of immobilized lipase in the presence of $Mg^{2+}$, $Ca^{2+}$, $Fe^{2+}$ and $Ni^{2+}$ respectively.

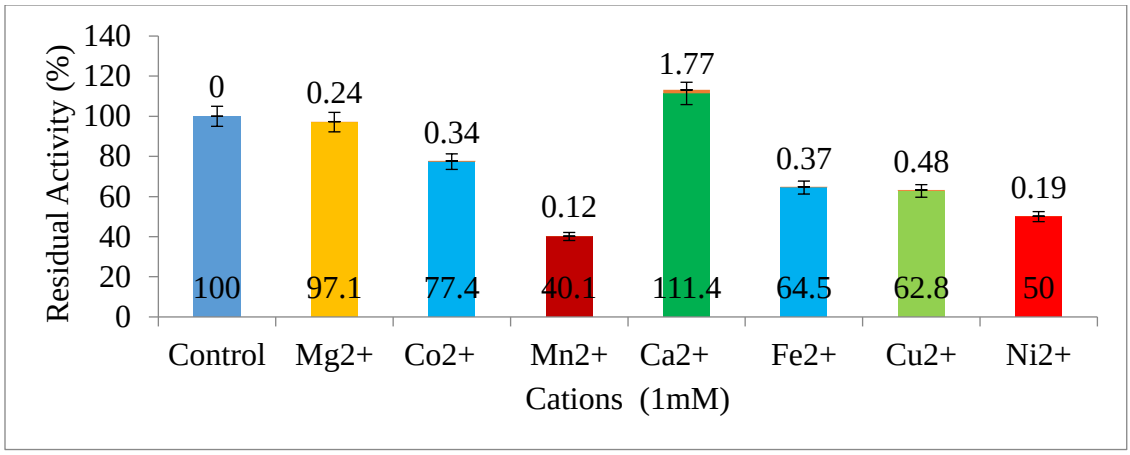


**Figure 6:** Effect of some cations on the activity of immobilized lipase

Figure 7 shows the effects of organic solvents on the activity of immobilized lipase. The enzyme had residual activities of 104.1% in 15% methanol, 60.1% in 30% methanol and 49.3% in 45% methanol. The residual activities in the presence of 15% ethanol was (83.4%), in 30% ethanol it was 61.3% as well as 39.7% in 45% ethanol. While in the presence of 15%, 30% and 45% isopropanol, the residual activities of 81.7%, 60.3% and 53.1% respectively were obtained. The enzyme also had residual activities of 113.1%, 84.0% and 62.5% in the presence of 15%, 30% and 45% *n*-hexane respectively. Lipase residual activities retained in 15%, 30% and 45% butanol were 77.4%, 61.3% and 51.5% respectively. As compared to free counterpart, the immobilized lipase had higher inactivation resistance to these solvents. The sensitivity of lipases to solvents varies depending on their polarity, with polar solvents being more destabilizing [30]. When enzymes are exposed to organic solvents, the water bound to protein on their surface can be easily stripped off and results to damage of its native structure [31]. Salihu *et al*. [32] reported the ability of methanol even at concentration of 60-70% to enhance the activity of lipase.

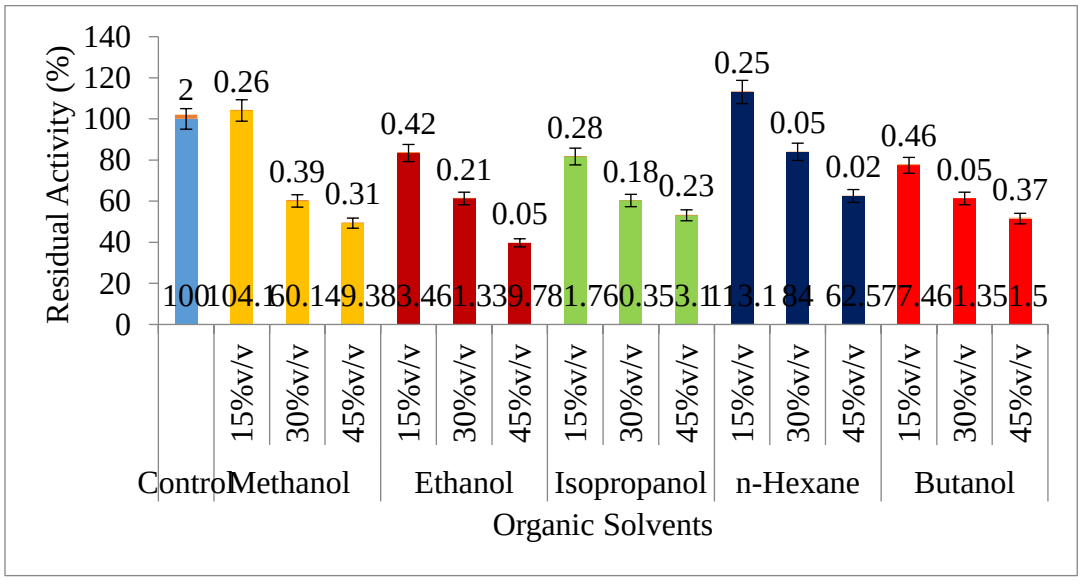


**Figure 7:** Effect of some organic solvents at concentration of 15%, 30% and 45% (v/v) on the activity of immobilized lipase

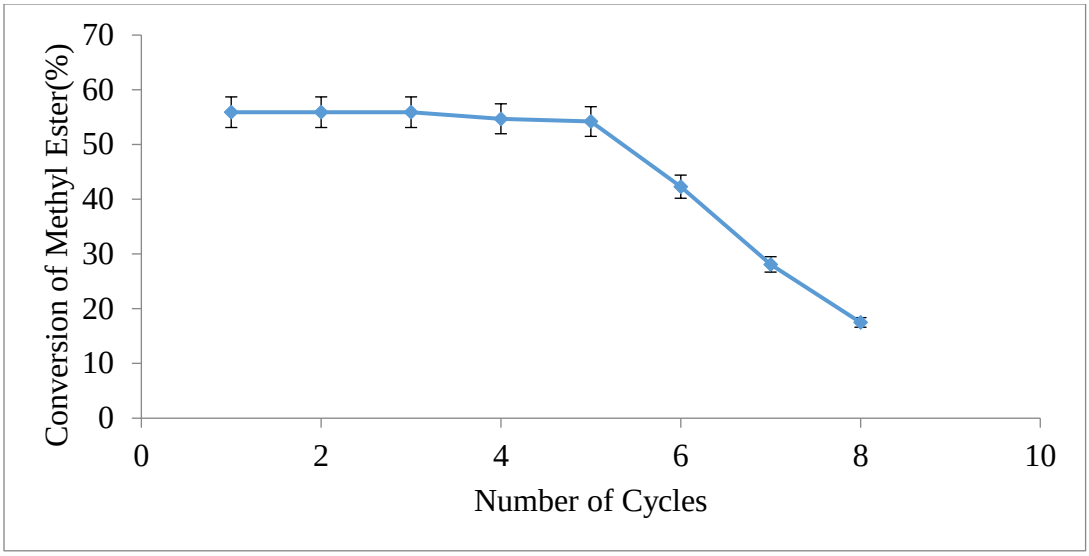


**Figure 8:** Effect of number of cycles immobilized lipase mediated trans-esterification of *Acacia nilotica* seed oil

The effect of number of cycles on trans-esterification mediated by immobilized lipase on methyl ester conversion is shown in Figure 8. Methyl ester conversion of 55.9% was found in cycles 1, 2 and 3. Cycles 4 and 5 had methyl ester conversion of 54.7% and 54.2% respectively. A percentage methyl ester conversion of 42.3% and 28.1 % was obtained in cycles 6 and 7 respectively. The lowest methyl ester conversion of 17.5% was recorded at cycle 8. One of the important characteristics of an immobilized enzyme is its stability and re-usability over an extended period of time. The repeated use of calcium alginate immobilized *P* lipase in transesterification of *A. nilotica* oil was studied. Zorica *et al*. [33] reported the use of alginate-immobilized lipase for up to three reaction cycles with little loss of activity. Elnashar [34] reported that alginate immobilized lipase from *Penicillium chrysogenum* retained 72.09% activity after six cycles. The ability of lipase to be reused for a number of transesterification cycles might be due to its entrapment in calcium alginate beads. However, the decreased methyl ester conversion observed after five cycles could be due to the leakage of enzyme from the beads as a result of the washing at the end of each cycle as no make-up quantities of enzyme was added [35].

## CONCLUSION

The findings from this work have shown that the pH, temperature, organic solvent stability as well as the ability of lipase from *P. aeruginosa* to be recycled improved with its immobilization on calcium alginate beads thus making the production of biodiesel from vegetable oil cost effective which could be used to power machines by blending it with petro-diesel.

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## REFERENCES

1. BARROS, M., FLEURI, L.F. & MACEDO, G.A. (2010). Seed lipase: Sources, applications and properties a review. *Brazilian Journal of Chemical Engineering*, **27**: 15-29.
2. CHOUDHURY, P. & BHUNIA, B. (2015). Industrial application of lipase: Review. *Biopharmaceutical Journal*, **1** (2): 41-47.
3. HE, P., GREENWAY, G. & HASWELL, S.J. (2010). Development of a monolith based immobilized lipase micro-reactor for biocatalytic reactions in a biphasic mobile system. *Process Biochemistry*, **45**: 593-597.
4. JAVED, S., AZEEM F., HUSSAIN S., RASUL I., SIDDIQUE, M.H. & RIAZ, M. (2018). Bacterial lipases: A review on purification and characterization. *Progress in Biophysics &. Molecular Biology*, **132**: 23-34.
5. JOSEPH, B., RAMTEKE, P.W. & THOMAS, V. (2008). Cold active microbial lipases: Some hot issues and recent developments. *Biotechnology Advance*, **26**: 457-470.
6. KHAN, A.K., MUBARAK, N.M., ABDULLAH, E.C., KHALID, M., NIZAMUDDIN, S. & BALOCH, H.A. (2019). Immobilization of Lipase Enzyme Carbon Nanotubes via

Adsorption. IOP Conference Ser. *Material. Science Engineering*, **495**: 012055.

7. PEREIRA D.S., FRAGA J.L., DINIZ M.M., FONTES-SANT'ANA G.C. & AMARAL, P.F.F. (2018). High catalytic activity of lipase from *Yarrowia lipolytica* immobilized by microencapsulation. *International Journal of Molecular Science*, **19**: 3393.
8. CARVALHO, N.B., VIDAL, B.T., BARBOSA, A.S., PEREIRA, M.M., MATTEDI, S. & FREITAS, L.D.S. (2018). Lipase immobilization on silica xerogel treated with protic ionic liquid and its application in biodiesel production from different oils. *International Journal of Molecular Science*, **19**: 1829.
9. COSTA, S.A., AZEVEDO, H.S. & REIS, R.L. (2005). Biodegradable systems in tissue engineering and regenerative medicine. Enzyme immobilization in biodegradable polymers for biomedical applications, CRC. Press LLC. London, ISBN **978**: 0-203-49123-2.
10. IKEDA, Y., KUROKAWA, Y., NAKANE, K. & OGATA, N. (2002). Entrap-immobilization of biocatalysts on cellulose acetate-inorganic composite gel fiber using a gel formation of cellulose acetate–metal (Ti, Zr) alkoxide. *Cellulose*, **9**: 369-379.
11. JEGANNATHAN, K.R., JUN-YEE, L., CHAN, E.S. & RAVINDRA, P. (2010). Production of biodiesel from palm oil using liquid core lipase encapsulated in K-carrageenan. *Fuel*, **89**: 2272-2277.
12. SAWANGPANYA, N., MUANGCHIM, C. & PHISALAPHONG, M. (2010). Immobilization of lipase on $CaCO_3$ and entrapment in calcium alginate bead for biodiesel production. *Science Journal*, UBU, **1**(2): 46-51.
13. BHUSHAN, I., PARSHAD, R., QAZI, G.N. & GUPTA, V.K. (2008). Immobilization of lipase by entrapment in Ca-alginate beads. *Journal of Biotechnology, Compatibility Policy*. **23**: 552-562.
14. BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, **72**(1-2): 248-254.
15. GOPINATH, S.C.B., HILDA, A., LAKSHMI, P.T. & ANNADURAI, G. (2005). Purification of lipase from *Cunninghamella verticillata* and optimization of enzyme activity using response surface methodology. *World Journal of Microbiology and Biotechnology*, **18**: 449-458.
14. ZHANG, A., GAO, R., DIAO, N., XIE, G., GAO, G. & CAO, S. (2009). Cloning, expression and characterization of an organic solvent tolerant lipase from *Pseudomonas fluorencens. Journal of Molecular Catalysis B: Enzymatic*, **56**: 78–84.
15. KUMARI, A., MAHAPATRA, P., GARLAPATI, V.K. & BANERJEE, R. (2009). Enzymatic transesterification of Jatropha oil. *Biotechnology Biofuels*, **2**(1): 1-7.
16. HIOL, A., JONZO, M.D., RUGANI, N., DRUET, D., SARDA, L. & COMEAU, L.C. (2000). Purification and characterization of an extracellular lipase from a thermophilic *Rhizopus oryzae* isolated from palm fruit. *Enzyme and Microbial Technology*, **26**: 421-430.
17. KIM, H.K., PARK, S.Y., LEE, J.K. & OH, T.K. (1998). Gene cloning and characterization of thermostable lipase from *Bacillus stearothermophilus* L1. *Biological science, Biotechnology and Biochemistry*, **62**(1): 66-71.
18. HESHAM, M.S., TAHA, I.Z., AHMED, I.K. & MOHAMMED, T.A. (2005). Purification and characterization of two extracellular lipases from *Pseudomonas aeruginosa* Ps-x. *Polish Journal of Microbiology*, **54**(3): 233-240.
19. FULLBROOK, P.D. (1996). Practical applied kinetics. In Godfrey, T. & West, *S.* (Eds). *Industrial Enzymology* (2nd edition), New York: Stockton Press 483-540.
20. ŞAHIN, F., DEMIREL, G. & TUMTÜRK, H. (2005). A novel matrix for the immobilization of acetylcholinesterase International *Journal Biological Macromolecule*, **37**: 148-153.
21. BUSSAMARA, R., FUENTEFRIA, A.M., DE OLIVEIRA, E., BROETTO, L., SIMCIKOVA, M., VALENTE, A. & VAINSTEIN, M.H. (2010). Isolation of a lipase-secreting yeast for enzyme production in a pilot plant scale batch fermentation. *Bioresource Technology*, **101**: 268-275.
22. SHEFAI, M.S. & ALLAM, R.F. (2010). Production and immobilization of partially purified lipase from *Penicillium chrysogenum*. *Malaysian Journal of Microbiology*, **6**(2): 196-202.
23. ASGHER, M. & IQBAL, H.M.N. (2011). Characterization of a novel manganese peroxidase purified from solid state culture of *Trametes versicolor* IBL-04. *Bioresources*, **6**: 4317-4330.
24. RASHID, N., SHIMADA, Y., EZAKI, S., ATOMI, H. & IMANAKA, T. (2001). Low temperature lipase from psychotropic Pseudomonas sp. strain KB700A. *Applied Environmental Microbiology*, **67**: 4064-4069.

25. DONG, H., GAO, S., HAN, S. & CAO, S. (1999). Purification and characterization of a *Pseudomonas sp.* lipase and its properties in non-aqueous media. *Applied Microbiology Biotechnology*, **30**: 251-256.
26. MATSUMAE, H. & SHIBATANI, T. (1994). Purification and characterization of lipase from *Serratia marcescens* Sr41 8000 responsible for asymmetric hydrolysis of 3-phenylglysidic acid esters. *Journal Fermentation Bioengineering*, **77**: 152-158.
27. MEHWISH, R., AAMER, A.S., ABDUL, H. & FARIHA, H. (2010). Characterization of lipase produced by Bacillus sp. FH5 in immobilized and free state. *Annals of Microbiology.* DOI - 10.1007/s13213-009-0012-9169 -175.
28. AHMAD, A.L., CHAN, C.Y., ABD-SHUKOR, S.R. & MASHITAH, M.D. (2010). Optimization of carotene recovery from extracted oil of POME by adsorption chromatography using response surface methodology. *Separation and Purification Technology*,**73**: 279-285.
29. YU, C.Y., LI, X.F., LOU, W.Y. & ZONG, M.H. (2013). Cross-linked enzyme aggregates of mung bean epoxide hydrolases: a highly active, stable and recyclable biocatalyst for asymmetric hydrolysis of epoxides. *Journal of Biotechnology*, **166**: 12-19.
30. SALIHU, A., ALAM, M.Z., ABDULKARIM, M.I. & SALLEH, H.M. (2012). Lipase production: an insight in the utilization of renewable agricultural residues. *Resource conservation and recycling*, **58**: 36-44.
31. ZORICA, K., SVETLANA, B., ALEKSANDRA, M., BOJANA, O., LJILJANA, M. & BRANKO, B. (2002). Alginate-immobilized lipase by electrostatic extrusion for the purpose of palm oil hydrolysis in lecithin/isooctane system. *Process Biochemistry*, **38**(3): 313-318.
32. ELNASHAR, M. M. M. (2010). Review article: Immobilized molecules using biomaterials and nanobiotechnology. *Journal of Biomaterials and Nanobiotechnology*, **1**(1): 61-77.
33. RODRIGUES, D.S., MENDES, A.A., ADRIANO, W.S., GONÇALVES, L.R.B. & GIORDANO, R.L.C. (2010). Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods. *Journal of Molecular Catalysis B: Enzymatic*, **51**(3-4): 100-109.