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Immobilization Optimization and Characterization of Immobilized Lipase from *Lysinibacillus macroides* FS1 for Biodiesel Production

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A B S T R A C T

Enzyme Immobilization favours operational stability, reuse, easy separation and enhanced stability than free enzyme. Lipase from *Lysinibacillus macroides* FS1 was entrapped into Ca-alginate gel beads and effect of independent variables such as alginate concentration of 1-4% (w/v) and $CaCl_2$ concentration of 50-200mM on immobilization efficiency and activity were investigated. After optimization of immobilization conditions, maximum immobilization efficiencies of 69% and activity of immobilized lipase was 6.0 U/ml were recorded at optimum concentrations of 4% (w/v) sodium alginate and 200mM $CaCl_2$. The optimum temperature of both free and immobilized lipase was 45°C and optimum pH of free and immobilized lipase was pH 5 and 8 respectively. The lipase activity of 46% was recovered by immobilized lipase after 6 cycles of reusability. Stability studies revealed that immobilized lipase was more stable than free lipase at optimum pH (8) and temperature (45°C) when incubated for 3 hr. Furthermore, the immobilized lipase showed enhanced stability to methanol than ethanol compared to free lipase. The biodiesel was produced by using immobilized lipase and it was confirmed by glycerol assay. These findings advise the efficient and sustainable use of the developed immobilized lipase as a biocatalyst for production of biodiesel.

Introduction

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are hydrolyses group of enzyme which catalyses the hydrolysis triglycerides to glycerol and free fatty acids. In addition to that lipases are also able to catalyze a wide range of reactions like esterification, trans-esterification, inter-esterification, alcoholysis, acidolysis, and aminolysis with high substrate specificity. (Nawal *et al.,* (2019), Yucel *et al.,* (2013). Bacterial lipases have wide industrial applications like in food industry, textile, cosmetics, paper and pulp, pharmacy industry and also in synthesis of biodiesel. (Sirisha *et al.,* 2010). The lipases being grouped as third largest class of commercialized enzymes owing to catalyze different reactions coupled

with the high activity and stability in organic solvents (Adetunji and Olaniran., 2018). Microbial lipases are the most common significant source for biodiesel production due to their ease in production and purification steps(Kareem *et al.,* 2020). The cost barrier of lipases remains a technological and economic constricts and this makes use of lipases for industrial scale less viable. As per studies carried out by Zavarise and Pinotti, 2019 suggests some alternatives like improvement in fermentation technologies, application of immobilization techniques and protein engineering for more effective lipase production. The way of making more effective lipase production process is modification in fermentation process and immobilization process for repeated reuse of enzymes.

Use of free lipases as industrial biocatalysts is not much advisable because of too costly for biotechnological applications along with drawbacks like soluble in homogenous catalysis system, product contamination, difficulty in recovery for reuse in the active form, unstable and inactivated by different environmental conditions. In order to make efficient enzyme utilization in bioprocesses and to abate the problems associated with free lipase immobilization has been recognized as an important strategy in enzyme technology as it enhances the enzyme activity, selectivity, stability and specificity and allow multiple reuses, and continuous operation of enzymatic processes. Furthermore, it improves process economy by lipase recycling and product purity (Helal *et al.,* 2021, Adetunji and Olaniran, 2018). The term Immobilization of enzymes refers to physically confined or localized in a certain defined region of space with retention of their catalytic activities and which can be used repeatedly and continuously (Brena and Batista-Viera, 2006).

Immobilization can be done in numerous ways, adsorption, cross-linking or entrapment. Entrapment is a physical restriction of enzymes within a confined space or network of support materials. Among the different techniques entrapment is one of the most efficient technique owing to its potential to improve enzyme stability, reduce biocatalyst leakage, and prevent chemical coupling of the enzyme with support material and create ideal microenvironment for the enzyme (Adetunji and Olaniran.,2018).This immobilization technique allows movement of both substrate and product through the matrix and is applicable to a wide range of carriers and lipases for biodiesel production (Kareem *et al.,* 2020). Different organic (e.g. polymers) or inorganic support materials are used for entrapment among that sodium alginate is most widely used polymer due to its low cost, easy to use, mild gelling properties, biocompatibility, thermostability, effective particle size, availability and non toxicity(Ding *et al.,*2020). Hence lipase entrapment in calcium alginate beads has shown to be relatively safe and straightforward techniques so in this study lipase was immobilized using calcium alginate beads (Zusfahair *et al.,* 2020). Energy is key factor of economic development of nation. The increasing demand of energy that results more dependence on fossil fuels (Tripathi *et al.,* 2014). Currently world is facing scarcity of fossil fuels and large scale burning of fossil fuels leads to major environmental changes (Thangaraj *et al.,* 2018). The need to find alternate for fossil fuels take turn to renewable energy sources which are more ecofriendly which are produced from biomass and can replace the existing energy sources. One of such renewable energy source is Biodiesel. Biodiesel is a mixture of Fatty Acid Methyl Esters (FAMEs) which is produced from renewable resources by transesterification process by the action of chemical catalyst or biocatalyst. To abate the issues related to chemical catalyst the use of biocatalyst was encouraged. Due to the high stability of

enzymes as well as their convenient production, they are the best biocatalysts for producing biodiesel (Helal *et al.,* 2021). Owing to the robust versatility and specificity of lipase to catalyze wide range of bioconversion reactions, the focus is required to explore indigenous bacterial strains with high catalytic efficiency and cost effective biocatalyst. This study therefore focused on optimization of lipase immobilization of Lysinibacillus *macroides* FS1 and characterise the immobilized lipases to investigate the potential application in biodiesel production.

Materials and Methods

Lipase Production by submerged fermentation

Lysinibacillus macroides FS1 was previously isolated from oil rich temple soil and identified using 16Sr DNA. The isolates were sub cultured routinely in the minimal media composed of 0.3% of yeast extract 0.5% NaCl,0.5% peptone,2% agar at pH 7 and preserved in agar slants at 4°C in the refrigerator (Sirisha *et al.,* 2010).The lipase producing *Lysinibacillus macroides* FS1 was grown in optimized medium containing 3gm beef extract,3gm galactose,3gm ammonium chloride,0.01gm $CaSO_4$,0.05gm KH_2PO_4, 0.01gm $MgSO_4.7H_2O$,1gm honge oil in 100ml distilled water and pH set to 7. The flask were incubated at 37°C for 48 hr at 120rpm. The sample were collected after 48hr of incubation and centrifuged at 10000rpm for 30min at 4°C to collect supernatant as crude lipase source for lipase immobilization. (Bharathi *et al.,* 2018, Babatunde and Sulaimon., 2017).

Immobilization of lipase

Varying concentrations (1%–4%, w/v) of sodium alginate were prepared by adding different quantities to 0.05 M Tris HCL buffer (pH 7) and boiled for 5min to form uniform slurry then cooled. The crude lipase was mixed with sodium alginate in equal proportion (1:1) by continuous stirring to obtain homogenous suspension. The enzyme-alginate mixture was added drop wise to cold $CaCl_2$ solution (50-200mM) using a hypodermic syringe. The obtained beads were preserved for curing at 4°C for 1 hr(Adetunji and Olaniran, 2018). After curing beads (3 mm diameter) were collected from the solution by filtration and then washed with cold Tris–HCl buffer (0.05 M, pH 7) and distilled water to remove unbound enzyme. These beads were stored in distilled water at 4°C till further use.

Lipase assay

Lipase activity of free and immobilized lipase was measured by Titrimetric method using olive oil as substrate. 1ml/1g of free and immobilized beads were added to reaction mixture containing 2ml of 0.05M phosphate buffer of pH 7.0 and 1ml of olive oil, incubated at 37°C for 60min. The reaction was stopped by adding 1ml of acetone: ethanol solution in 1:1 ratio. The amounts of fatty acids were estimated by titrating with 0.05M NaOH in presence of phenolphthalein as a indicator until pH 10.5. Amount of NaOH consumed indicates the amount of fatty acids liberated. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1µmol of equivalent fatty acid (Patel *et al.,* 2018).Lipase activity was calculated by using following formula

$$\text{Lipase Activity (U/ml)} = \frac{\text{Vol. of alkali consumed x Strength of alkali x 1000}}{\text{Vol. of sample x Time in min}}$$

Determination of Immobilization efficiency

The immobilization efficiency is the percentage of bound enzyme activity observed in the immobilized beads. It was calculated

using the equation that is described by Talekar and Chavare, 2012.

$$\text{Immobilization efficiency \%} = \frac{\text{Activity of Immobilized Enzyme (IU)}}{\text{Activity of Free enzyme (FU)} - \text{wash water activity (RUS)}} \times 100$$

Characterization of free and immobilized lipase

Effect of temperature and pH on free and immobilized lipase activity

The effect of temperature on free and immobilized lipase activity was assessed by carrying out lipase assay at different temperatures ranging from 25 to 75°C at pH 7.

The optimum pH of free and immobilized lipase activity was determined by carrying out lipase assay at optimum temperature in buffer solutions of pH values ranging from 5.0 to 10.0.

The buffers used include 0.05 M acetate buffer (pH 5.0), 0.05 M phosphate buffer (pH 6.0), 0.05 M Tris–HCl buffer (pH 7.0–9.0) and 0.05 M Glycine–NaOH buffer (pH 10.0). The lipase activities were calculated and the values were plotted against the respective temperature or pH(Adetunji and Olaniran, 2018).

Stability of free and immobilized lipase to optimum temperature and pH

The temperature and pH stability of free and immobilized lipase was determined by keeping lipase in substrate solution at 45°C for a period of 3 hrs. Lipase activity was checked with a time interval of 30min (Hombalimath *et al.,* 2020).

Stability of immobilized lipase to organic solvents

Stability to organic solvent is desirable feature in the process of esterification and transesterification, as these organic solvents may inhibit the lipase activity during tranesterification reaction. Biodiesel production can be greatly enhanced by evaluating stability of lipase to organic solvents. Hence to investigate stability of produced lipase to organic solvents, immobilized lipase was incubated with 1ml methanol and ethanol for 1 hour and residual lipase activity was assayed, as methanol and ethanol are the most commonly used organic solvents in biodiesel industry (Tripathi *et al.,* 2014).

Reusability potential

The reusability of the immobilized lipase was evaluated by carrying out the lipase assay for six cycles at 45°C for 30 min. After each cycle, the beads were separated by filtration using filter paper then washed with cold distilled water and Tris–HCl buffer (pH 8) and re-introduced into the reaction medium consisting of fresh substrate solution. Lipase activity was measured after every cycle under standard assay conditions. The residual lipase activity was calculated by taking the lipase activity of the freshly prepared beads in the first run as 100%. (Adetunji and Olaniran, 2018 and Hombalimath *et al.,* 2020).

Biodiesel production

Biodiesel was produced by using *Pongamia* seed oil. The tranesterification reaction was performed in 100ml conical flask containing 10ml *Pongamia pinnata* seed oil, 1.3ml methanol (1:3 ratio) and 5% (0.5g) immobilized lipase and kept in shaking incubator at 37°C for 24 hr with agitation speed of 120rpm. After 24hr reaction mixture

were collected and filtered to remove immobilized beads, collected filtrate were added to separating funnel and left overnight for separation of biodiesel and glycerol (Vallari *et al.,* 2015). The biodiesel layer was separated from the sediment layer of glycerol. The production of biodiesel was confirmed by glycerol assay. The glycerol assay was carried by following method:

Reagents required

Working Solution= 95% ethanol and distilled water in 1:1 ratio

Sodium periodate solution=10mM sodium periodate in 1.6 M acetic acid solution)

Acetyl acetone solution =0.2 M acetyl acetone was mixed with 1:1 solution of 1.6 M acetic acid and 4M ammonium acetate.

Glycerol Assay: 0.5 ml of sample was mixed with 1.5 ml working solution and 1.2 ml of sodium periodate was added followed by shaking for 30 seconds. After that 1.2 ml of acetyl acetone was added and kept in hot water bath at 70°C for 1 min and immediately cooled in water. Formation of yellow colour indicates presence of glycerol. Absorbance was taken at 410 nm. (Hombalimath *et al.,* 2020).

Results and Discussion

Lipase production and activity assay

Production of lipase from *Lysinibacillus macroides* FS1 were carried in optimized medium as submerged fermentation process mentioned in methodology. The Media optimization was performed in previous studies and formulated optimised media for enhanced lipase activity. (Bharathi *et al.,* 2018). The production of enzymes at industrial scale mainly uses the submerged fermentation (SmF) technology, as this method of fermentation process easy to monitor and to control (Melani *et al.,* 2019). The use of submerged fermentation for lipase production using bacterial sp. gives satisfactory results studied by Vishwanatha *et al.,* 2019.Culture supernatant were collected and lipase activity of 14.1U/ml was assayed by titrimetric method as this is simplest method mentioned by Patel *et al.,* 2018 and Sirisha *et al.,* 2010.

Optimization of Lipase Immobilization

Immobilised lipase offers many advantages over free lipase in terms of reusability, operational stability and cost effective process. Entrapment is one of immobilization techniques in which enzymes are physically restricted within a confined space or network. Immobilization by using Ca-alginate provides many benefits like its cost-effective process and environmentally friendly nature as per Qamar *et al.,* 2020 and the concentrations of alginate and calcium chloride formed a key factor for enzyme immobilization Since cross linking between alginate and calcium chloride results in gelation (Adetunji and Olaniran, 2018). Therefore in this study the influence of sodium alginate and $CaCl_2$ concentration on immobilization efficiency of lipase from *Lysinibacillus macroides* FS1 was investigated. Produced lipase was successfully immobilized and produced beads using various concentrations of sodium alginate and $CaCl_2$ (Fig.1). As shown in the Table.1, the highest immobilization efficiency and yield of 76% and 38.2% was observed for T7 at 2% sodium alginate and 150mM $CaCl_2$ but the beads were too soft may be due to dilution of sodium alginate with equal amount of lipase in 1:1 ratio, hence it is difficult to be use in reaction mixture of biodiesel production and may be difficult for reuse of these beads therefore next highest immobilization efficiency and yield of 69% and 43% which

was shown by T12 at 4% sodium alginate and 200mM calcium chloride were selected for further studies since these beads were physically stable. There was an increase in immobilization efficiency as the concentration of alginate increases from 1% to 4% (w/v). The lower alginate concentration of 1% resulted in the formation of fragile beads which got disrupted easily and had larger pore sizes resulting in leakage of the enzyme from the beads thereby lowering enzyme activity (Malhotra and Basir, 2020 and Zusfahair *et al.,* 2020).The concentration calcium chloride influences the stability and porosity of the beads. Maximum immobilization efficiency and yield (69% & 43%) was recorded from beads prepared from 200mM calcium chloride. The similar finding were reported by Adetunji and Olaniran, 2018 in his studies.

Characterization of Immobilized Lipase

Effect of Temperature and pH on activity of free and immobilized lipase

The effect of temperature on both free and immobilized lipase was studied and it was found that maximum activity of both free and immobilized lipase was observed at 45°C as indicated in Fig. 2. Optimum temperature of 45°C for free and immobilised lipase has been reported. This may be because of the enzyme retain stable structure at this temperature. Beyond 45°C activity decreases up to 75°C. The optimum temperature of free lipase was found to be quite similar with results obtained by Hombalimath *et al.,*2020 and Helal *et al.,* 2021.Both enzyme preparations showed considerable activity in the broad pH range of 5.0–10.0, the results suggested that optimal pH of the immobilized lipase shifted to pH 8 and free lipase at pH5(Fig.3). As per Fig. 3 it was observed that, there was much difference in optimum pH of free lipase and immobilized lipase. The optimum pH of free lipase found to be at pH 5 and for immobilized lipase at pH8, these findings revealed that at alkaline pH values, the immobilized lipase exhibited better activity than the free lipase, suggesting that the immobilization enhanced the tolerance of the lipase to alkaline conditions similarly reported by Ding *et al.,* 2020.

Stability of free and immobilised lipase to optimized temperature and pH

As indicated in Fig.4 both free and immobilized lipase showed the residual activity of 60% and 71% after 3 hrs of incubation at 45°C respectively. It was observed that the thermal stability of immobilized lipase was more than that of free lipase. The greater thermal stability of immobilized lipase may be due to the protection provided by the support material, which stabilized the enzyme and maintained its bio catalytic activity. When comparing the activities of free and immobilized lipase, immobilization process considerably improves the thermal stability of enzymes, which states its importance for wide biotechnological applications. Such increased thermal stability of lipase entrapped in alginate beads has been reported by many studies (Kumar *et al.,* 2014, Shafei and Allam, 2010, Adetunji and Olaniran, 2018, Hombalimath *et al.,* 2020)

Stability to organic solvents

Enzymes are inactivated or denatured in presence of organic solvents which are the co substrates in transesterification reactions thereby limiting their use in some cases. Therefore Solvent stable lipases are one of the leading biocatalysts in non-aqueous environment due to their unique property of catalysing a wide variety of useful transformations (Ryan *et al.,*2019). The lipase from *Lysinibacillus macroides* FS1 demonstrated significant stability and activity in the presence of organic solvents.

Table.1 Immobilization efficiency of lipase from *Lysinibacillus macroides* FS1 at different concentration of sodium alginate and calcium chloride.

Test	Sodium Alginate (%)	Calcium chloride(mM)	Immobilization efficiency (%)	Immobilization yield(%)
T1	1	50	14.2	9.2
T2	1	100	31	13.4
T3	1	150	26	14.1
T4	1	200	33	18
T5	2	50	38	23.4
T6	2	100	50	29
T7	**2**	**150**	**76**	**38.2**
T8	2	200	61	32
T9	3	50	37	26.2
T10	3	100	48	33.3
T11	3	150	41.2	28.3
T12	3	200	52	35.4
T13	4	50	36.4	25
T14	4	100	39	27
T15	4	150	55	36.1
T16	**4**	**200**	**69**	**43**

Table.2 Stability of free and immobilized lipase to the organic solvents.

SI.No.	Organic solvent	Residual Activity of immobilized lipase (%)
1	Control	100
2	Methanol	129
3	Ethanol	57

Fig.1 Immobilized beads of lipase from *Lysinibacillus macroides* FS1

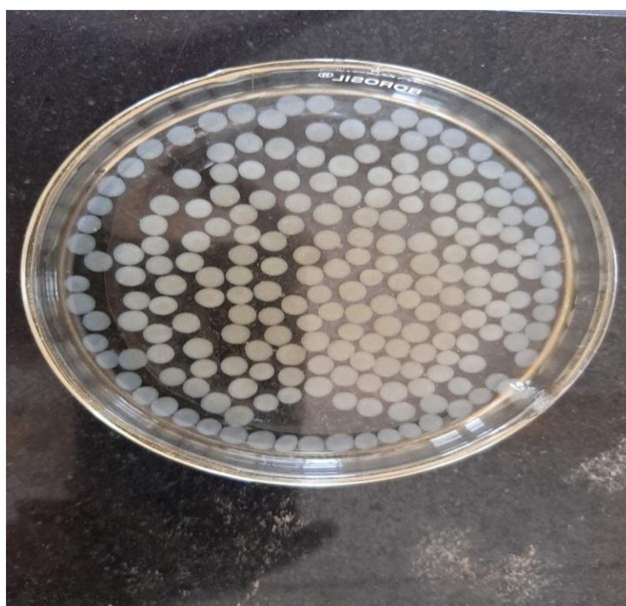


Fig.2 Effect of Temperature on activity of free lipase and immobilized lipase.

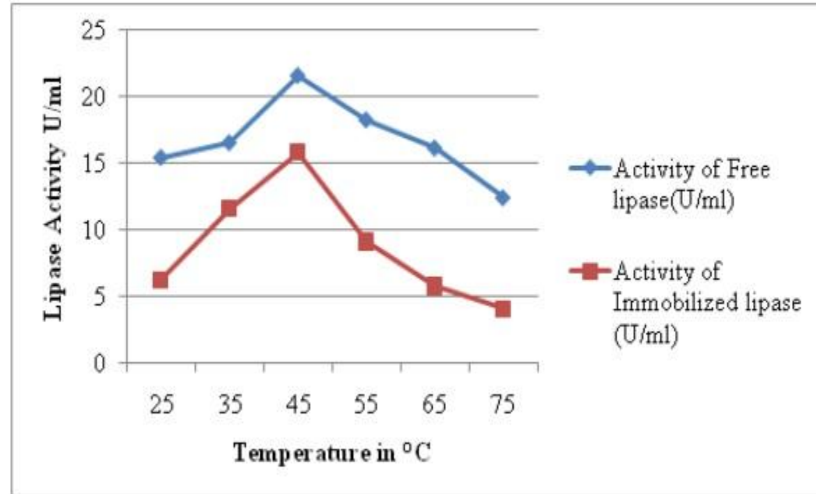


Fig.3 Effect of pH on activity of free lipase and immobilized lipase.

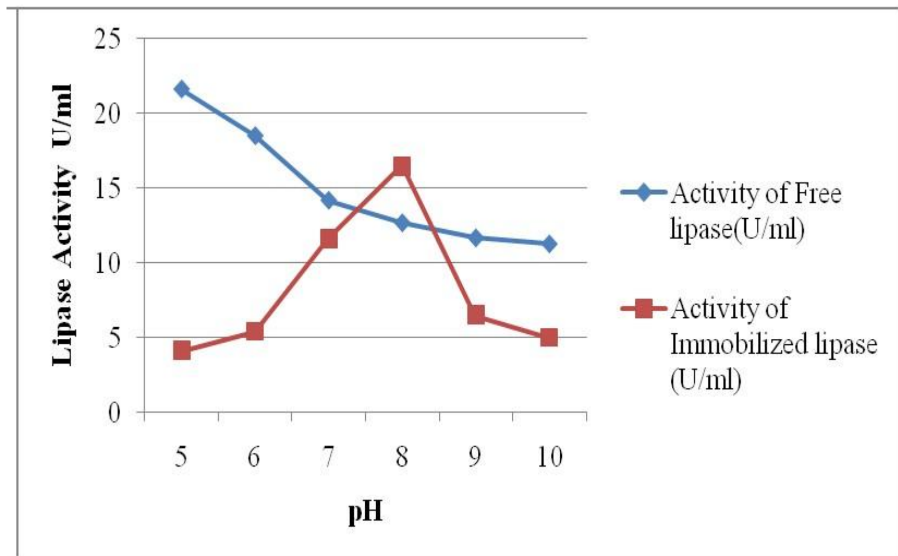


Fig.4 Stability of Immobilized lipase to optimum temperature and pH.

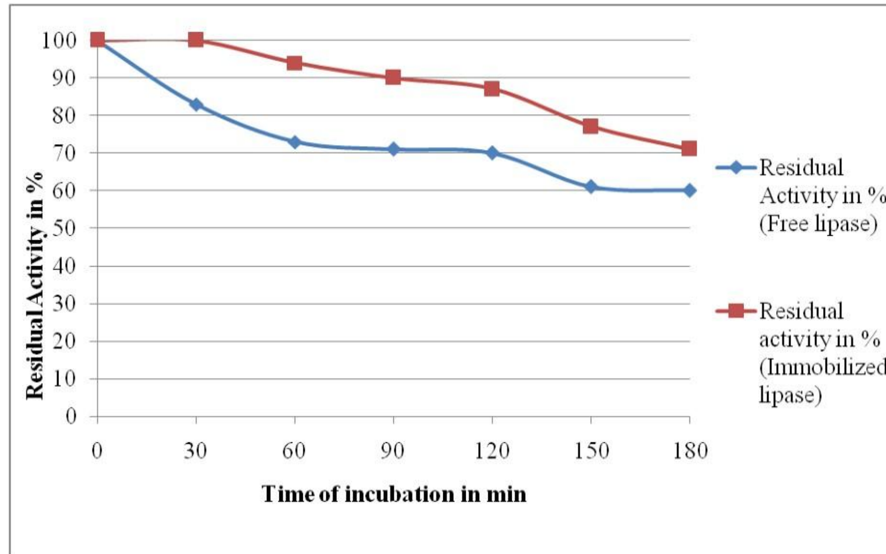


Fig.5 Reusability of Immobilized lipase

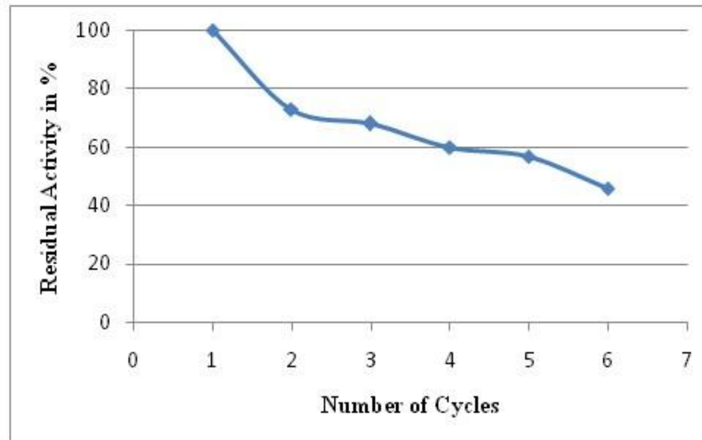
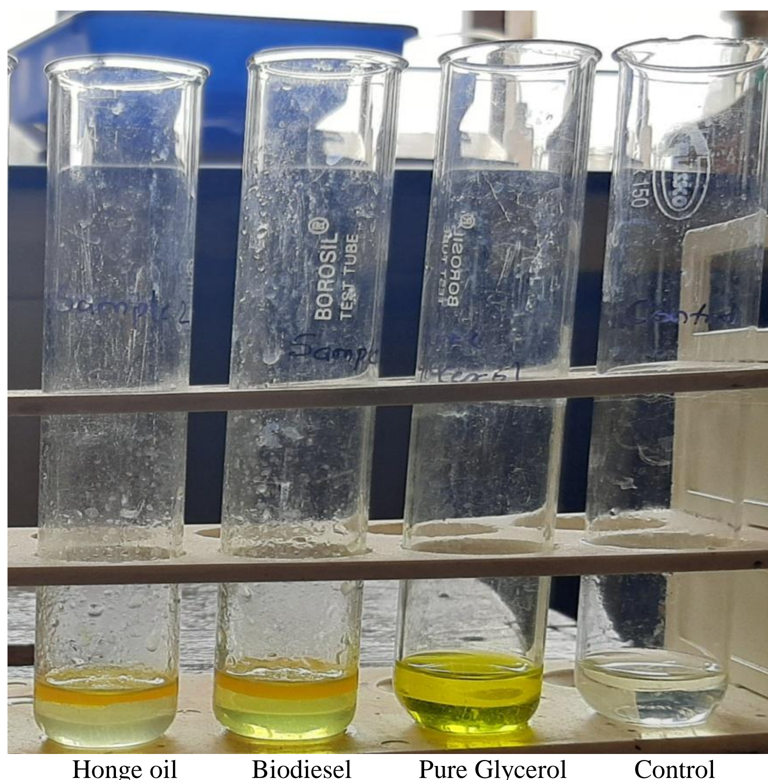


Fig.6 Glycerol Assay



The stability of produced lipase to organic solvents was tested and results reveal that lipase was stable in presence of methanol than ethanol.

As per Table 2 it was noted that residual activity of immobilized lipase in presence of methanol and ethanol was 129% and 57% respectively, it indicates methanol has induces activity and ethanol inhibits the activity.

Ethanol affects negatively on lipase activity. Hence methanol is most suitable acyl acceptor for transesterification reaction in which lipase from *Lysinibacillus macroides* FS1 were used as biocatalyst (Table 2).

Reusability of Immobilized Lipase

The most important characteristic of immobilized enzyme is its stability and reusability for extended periods of time which can be reduce the operational cost for large scale applications. In the present study, lipase immobilized in alginate gel beads was assayed continuously for six cycles using olive oil as a substrate. The immobilized lipase retained the residual activity of 46% after six cycles as indicated in Fig.5. The residual activity of the immobilized lipase reduced with increase in the number of cycles this may be due to leakage of the lipase from the beads. Similar results have been reported by Zhang *et al.,* 2013, Adetunji and Olaniran, 2018, Hombalimath *et al.,* 2020 in which lipase immobilized in alginate beads was used for six cycles.

Biodiesel Production

Biodiesel production was assayed by using immobilized lipase as mentioned in methodology and analysed by glycerol assay. Biodiesel produced by the process of

transesterification where Glycerol is by-product of reaction. Biodiesel formation was confirmed by glycerol assay in which the sample is treated with sodium periodate, it reacts with free glycerol in the sample to generate formaldehyde. Reaction between this formaldehyde and acetyl acetone produces the yellow complex, 3, 5- diacetyl- 1,4-dihydrolutidine. This yellow compound exhibits a maximum absorbance peak at 410 nm. As shown in Fig.6 the formation of yellow colour in pure glycerol and biodiesel sample indicates the presence of glycerol which shows that immobilized lipase can catalyse the tranesterification process to produce the biodiesel. (Hombalimath *et al.,* 2020)

In the present study, calcium alginate gel beads were prepared and used as support material for immobilization of lipase from *Lysinibacillus macroides* FS1 via entrapment method. Maximum immobilization efficiency and lipase activity was recorded at optimal conditions of 4% (w/v) and 200mM for sodium alginate and calcium chloride, respectively.

Both free and immobilized lipase had maximum activity at optimum temperature (45°C) and pH (8). Conversely, the entrapped lipase exhibited improved pH and temperature stability over optimized conditions for 3 hr. Furthermore, the immobilized lipase showed better reusability for up to six consecutive cycles and able to convert the *Pongamia* oil to biodiesel, suggesting the efficient and cost-effectiveness of the developed immobilized biocatalyst for biodiesel production.

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