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Optimization of Lipase Immobilized on Chitosan Beads for Biodiesel Production

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The objective of this study was to optimize the immobilization of lipase on a chitosan support for the purpose of biodiesel production. The immobilized enzyme was selected for characterization studies. The optimum lipase loading, immobilization time and concentration of immobilized enzyme were determined. The effect of important parameters such as pH, reaction temperature, reaction time, operational and storage stabilities were studied. From the study it was found that the optimal immobilization conditions were as follows: enzyme loading 0.2g/g Chitosan, temperature of 40ºC, pH of 7.0, molar ratio of 1:4, amount of beads of 2g and immobilization time of 3 h. Thermal, operational and storage stabilities of the enzyme were found to improve after immobilization. In conclusion, optimization of immobilization conditions was found to offer additional advantage on enzyme activity.

Keywords: Chitosan bead, Immobilized enzyme, optimization and characterization.

INTRODUCTION

Lipases are enzymes that catalyze the hydrolysis of ester bonds at a lipid-water interface. They may exhibit specificity for the position of acid in a triglyceride in their natural substrate. Lipases also exhibit stereochemical specificity when reacting with a wide variety of substrates in organic solvents (Baillargeon, 1990).

About 20% of all biotransformations reported today are performed with lipases (Gitlesen *et al*., 1997). The use of specific microbial lipases to catalyze esterification reactions has generated considerable interest because of certain advantages over chemical catalysis which includes the limitation associated with synthesis of chemical intermediaries/products of commercial interest. Biotransformation can compete with optimized chemical production if improved techniques, which involve the choice of convenient methods of downstream processing, are employed.

Concerted efforts have been made over the years, to develop the catalytic activity and operational stability of industrial enzymes through the use of genetic engineering, immobilization and/or process alterations. The most commonly used strategy to impart the desirable features of conventional heterogeneous catalysts onto biological catalysts is enzyme immobilization. For many applications enzymes are preferably used in an immobilized state in order to easily separate the catalyst from the product steam. With immobilized lipases, improved stability, reuse, continuous operation, the possibility of better control of reactions and hence more favorable economical factors can be expected (Frense *et al*, 1996). Many different methods of enzyme immobilization are available, each involving a different degree of complexity and efficiency (Malcata *et al*, 1990).

Chitosan, a derivative of chitin is the most attractive choice of immobilization since it offers several advantages such as versatility of available physical forms (flakes, porous beads, gel, fiber and membrane); low biodegradability; ease of handling; high affinity for proteins and, above all, non-toxicity (Felse and Panda, 1999). It is also worthy of note that it is cheaper to use Chitosan since chitin is the second most abundant biopolymer in nature next to cellulose (Krajewska, 1991) hence underscores the challenge posed by unavailability. Also, previous studies in which chitosan was used to immobilize lipase shows good results (Itoyama *et al*, 1994; Carneiro da Cunha *et al*, 1999) and other hydrolases such as amyloglucosidase, papain, β-glycosidase and β-L arabinofuranosidase (Krajewska, 1991; Felse and Panda, 1999).

The aim of this project is to produce immobilized lipase for the purpose of biodiesel production. The immobilization criteria were based on the use of a low-cost method of loading enzyme onto the support. The chosen method of immobilization was simple adsorption, whereby the enzyme adheres to the surface of the support particles by van der Waal's forces of attraction. The efficiency of immobilization was assessed in terms of its ability to convert the substrate to the required product; ease of separation from product, as well as the environment friendly factor which enhances sustainability. The immobilized lipase was selected for further studies, including characterization of the immobilized derivative.

MATERIALS AND METHOD

MATERIALS

Bovine serum albumin (BSA), Coomasie Brilliant Blue, 0.15 M NaCl Spectrophotometer and Micropipettes. Commercial lipase and chitosan in powdered form were purchased from CLEA technologies, China with the following characteristics, according to the manufacturer's information: 95% purity, 6% moisture and particle size 30 mesh. Solvents were standard laboratory grade and other reagents were purchased either from Aldrich Chemical Co or Sigma Chemical Co., USA through Zayo-Sigma, Jos Nigeria.

METHODS

Immobilization of Lipase on Chitosan

Lipase was immobilized by physical adsorption on Chitosan following a previously developed methodology (Carneiro da Cunha *et al*, 1999) briefly, 2g of Chitosan powder was soaked in hexane under agitation conditions (100 rpm) for 1 hour. Excess hexane was removed followed by the addition of 0.5 grams of powder lipase dissolved in 10 ml of distilled water. The lipase was on the support under agitation for 3 hours at room temperature followed by an additional period of 18 hours under static conditions at 4º C. The derivative was filtered (Whatman filter paper 41) and thoroughly rinsed with hexane. Analyses of hydrolytic activities carried out with the enzyme offered for immobilization, and immobilized preparations were used to determine the activity yield by dividing catalytic activity by lipase loading (units.g^{-1}support). The effect of other factors, such as solution pH, immobilization time and enzyme amount, on immobilized enzyme activity were investigated.

Protein Assay

Lipase Concentration was determined by comparing the absorbance of lipase at 595nm wavelength with Bovine Albumin Serum standard curve. The amount of bound enzyme was determined indirectly from the difference between the amount of enzyme introduced (AEI) and the amount of enzyme remaining in the solution (AER) (Sawangpanya *et al.,* 2010)

$$AP = AEI - AER$$

Determination of Lipase Activity

Hydrolysis Assay

Hydrolytic activities of free and immobilized lipase were assayed by the olive oil emulsion method according to the modification proposed by Soares *et al.,* (1999). One unit (U) of enzyme activity was defined as the amount of enzyme necessary to produce 1 µmol of free fatty acid per min under the assay conditions (37º C, pH 7.0, 150 rpm).

Esterification Assay

Transesterification reaction was conducted following a suggested method by Devanesan *et al*. (2007). Experiments were carried out at the optimum temperature 40^{0}C, immobilized cell concentration of 2 g beads and substrate concentration of 50 ml of oil (1:3 and 1:4 molar ratio of oil to methanol) with n-hexane (3 ml) as solvent. After a period of reaction time, the reaction was stopped and the immobilized lipase was removed from the reaction mixture. The produced ester and by-product glycerol were separated using separate funnels. Lipase activity assay was determined based on the amount of fatty acid methyl ester produced by using Thin Layer chromatography (TLC) method. The solvent system was a mixture of hexane and chloroform (1:1 molar ratio). The sample was plotted on TLC plate by using a capillary tube. Then, the TLC plate was placed into the solvent system in vertical form. This was left for a while to allow the solvent adsorbed to the TLC plate. The TLC plate was dried and put onto iodine pallets in the beaker for colorized spot on the TLC plate. The spot present on the TLC plate was identified to calculate percent yield of ester. The percent yield was calculated as stated below:

$$\%\text{Yield} = \text{Highest peak of sample TLC plate} \times 100\% / \text{high of TLC plate}$$

One unit of enzyme activity is defined as the amount of the enzyme that catalyzed the liberation of 1% conversion of jatropha oil (Nasratun *et al*.,2009).

Subsequently the yields of methyl esters were calculated using the following formula;

Yield of methyl esters (%) = grams of methyl esters produced/ grams of oil used in reaction x 100 (Rashid and Anwar, 2008)

Characterizations of Immobilized Enzyme

Determination of Enzyme Loading

Further experiments were carried out to determine appropriate lipase loading by using a fixed amount of support (1gram, dry weight) for different amounts of enzyme (0.1 to 1.2 grams lipase).

Determination of Optimum Lipase Loading Time

The time taken to achieve the appropriate loading of the amount of enzyme established above was studied at the optimum reaction condition of 40ºC, 50U enzyme concentration and substrate molar ratio 1:4. Samples were taken from the bead enzyme mixture at the time interval of (1, 2, 3, 4 and 5 h) and enzyme activity was assayed as well as biodiesel yield.

Determination of Optimum pH

Effect of pH on free enzyme and immobilized enzyme was studied by assaying the enzymes at different pH values (4.0–7.5).

Determination of Optimum Temperature

To determine the optimum temperature for free enzyme and immobilized enzyme, the enzyme activities of lipase were measured at various temperatures using Jatropha oil as the substrate.

Determination of Operational Stability

The operational stability or reusability of immobilized lipase was conducted by carrying out transesterification reaction repeatedly for seven (7) different cycles using Jatropha oil. Experiments were carried out for the reaction period of 48 h at an optimum temperature of 40ºC, immobilized cell concentration of 2 g beads and substrate molar ratio of 1:4 (oil to methanol) with n-hexane (3 ml). The immobilized lipase was filtered at the end of the reaction, washed with t-butanol or hexane to remove any substrate or product retained in the matrix and again introduced with fresh reactants in order to study its operational stability in each cycle.

Determination of Storage Stability

The storage stability of both the immobilized and free lipase was studied by determining the biodiesel yield within 90 days. The enzyme was stored at 4 ºC for 90 days within which the transesterification reaction was conducted.

Determination of Effect of reaction Time on Biodiesel Yield

Effect of time on biodiesel production from Jatropha oil using immobilized Lipase was studied by conducting experiments with different periods of 12, 24, 36 and 48. Experiments were carried out at the optimum temperature 40ºC, pH 7.0, immobilized lipase concentration of 2 g beads equivalent to activity of 50U and substrate molar ratio of 1:4 (molar ratio of oil to methanol) with n-hexane (3 ml) as constant. Samples were taken from the reaction mixture at specified time intervals. The samples were centrifuged at 1500 rpm for 30 min at 4℃ to remove the carrier containing the immobilized enzyme (thus negating the possibilities of additional reaction) followed by dilution of the initial sample with n-hexane. Transesterification by free lipase was repeated with the same reaction condition with the optimum temperature of 35 ºC.

Determination of Effect of Enzyme concentration

Effect of enzyme concentration was conducted by varying the concentration from 20 to 60 U at 1:4 Jatropha oil:methanol molar ratio and at 40^0C and 35^0C respectively for both immobilized and free enzyme.

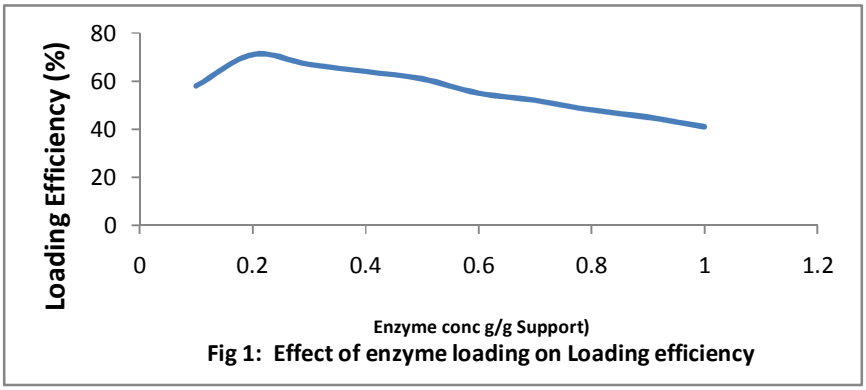

Fig 1: Effect of enzyme loading on Loading efficiency

RESULTS AND DISCUSSION

Immobilization of lipase:

Effect of Lipase Loading

The result in Fig 1 shows the loading efficiency of enzyme on Chitosan support. The highest efficiency was obtained at a loading of 0.2g of enzyme/g of support. Increased loadings beyond 0.2g of enzyme/g of support led to progressive decrease in efficiency. This result agrees with those obtained in the study conducted by Pereira *et al*., (2003). They found out that the hydrolytic activity of the immobilized enzyme increased as more lipase was loaded onto the support until it reaches a particular threshold where reduction in activity was observed. The results suggested that instead of obtaining the desired crowded upright adsorption of enzyme onto the support surface, multilayer adsorption occurred, possibly blocking or inhibiting access to enzyme active sites. Therefore, most of the other experiments were carried out with immobilized preparation at lipase loadings of 0.2g enzyme g^{-1} of dry chitosan.

Effect of Lipase Loading Time on Biodiesel Yield

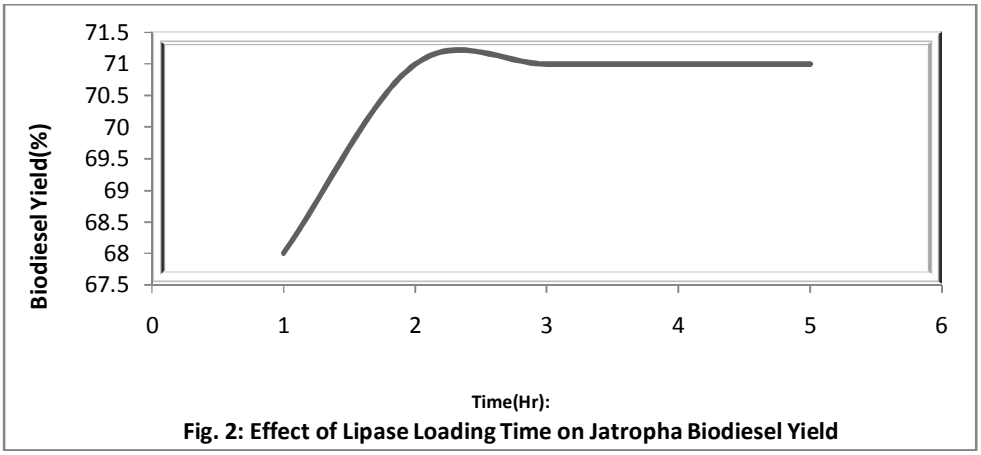

Fig. 2: Effect of Lipase Loading Time on Jatropha Biodiesel Yield

The result obtained in Fig 2 shows that increase in lipase loading time resulted in increase in the percentage yield of biodiesel. Maximum biodiesel yield was attained at 3 h of enzyme loading. Increase in enzyme loading time beyond 3 h has no further effect on percent biodiesel yield. Previous work conducted by Lu *et al.,* (2004) reported that immobilized enzyme activity attained maximum at 2.0 h and that immobilized enzyme activity remained unchanged with further increase in loading time beyond 2.0 h. The above result indicated that the available space on the immobilization carrier became saturated with prolonged loading time; hence the immobilized enzymes attained highest activity at 3 h of loading.

Protein Assay:

The amount of protein (lipase) immobilized was determined by comparing the absorbance of lipase at 595 nm wavelength with BSA standard curve. The amount of protein (lipase) enzyme was calculated as a difference of total free protein (lipase) introduced and the remaining protein in solution after immobilization as shown in table 1 and 2.

Table 1 Unknown 1: Free Lipase

Unknown(µl)	H2O (µl)	1 M NaOH (µl)	Brad. reagent (µl)	A595	Protein (µg)
0	700	100	200	0.592	-1.12
0	700	100	200	0.590	-1.16
1	699	100	200	1.234	14.02
1	699	100	200	1.215	13.57
5	695	100	200	1.409	18.14
5	695	100	200	1.412	18.21
10	690	100	200	1.728	25.66
10	690	100	200	1.694	24.88
15	685	100	200	1.945	30.77
15	685	100	200	1.926	30.33
20	680	100	200	2.001	32.09
20	680	100	200	1.987	31.76

Table 2: Unknown 2: Lipase remaining in solution

Unknown(µl)	H2O (µl)	1 M NaOH (µl)	Brad. reagent (µl)	A595	Protein (µg)
0	700	100	200	0.592	-1.12
0	700	100	200	0.590	-1.16
1	699	100	200	0.650	0.25
1	699	100	200	0.744	2.47
5	695	100	200	0.809	3.99
5	695	100	200	1.106	10.99
10	690	100	200	1.313	15.88
10	690	100	200	1.313	15.88
15	685	100	200	1.467	19.51
15	685	100	200	1.483	19.88
20	680	100	200	1.586	22.31
20	680	100	200	1.605	22.76

Equation 1: [µg protein] = $(2.113 \times A5952) + (19.18 \times A_{595}) - 12.99$

The result showed that the concentration of protein (lipase) introduced was 32.09µg/ml and the protein (lipase) remaining in solution after immobilization was 22.76µg/ml. Therefore the protein (lipase) concentration adsorbed to the surface of chitosan was 9.33µg/ml.

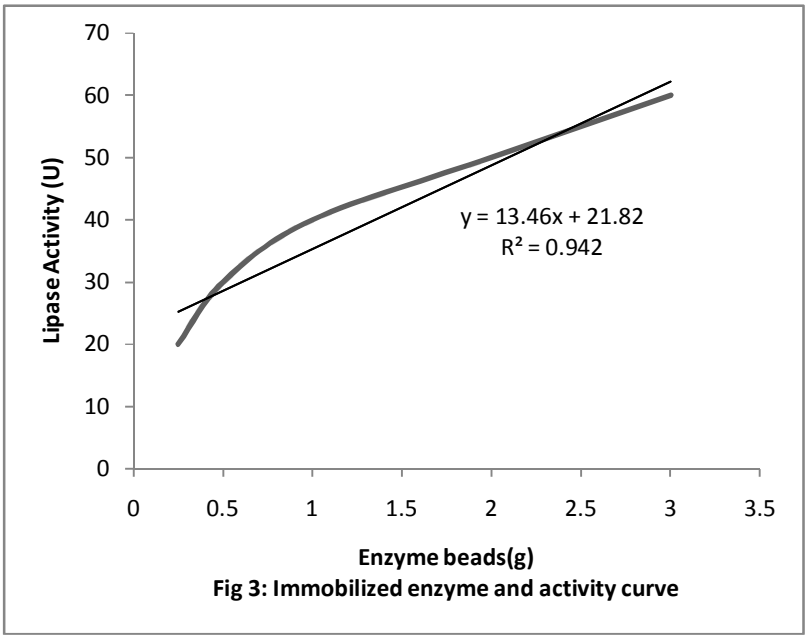

Fig 3: Immobilized enzyme and activity curve

The result in Fig 3 shows that bead load has a linear relationship with enzyme activity. The R^2 linearity is 94.2% fitting, indicating that the enzyme activity of immobilized lipase can be predicted at any bead load.

Transeterification Reaction

The immobilized lipase optimized in the sections above was thereafter applied for the transesterification reaction for the optimization of other biodiesel process variables.

Effect of reaction pH on biodiesel yield

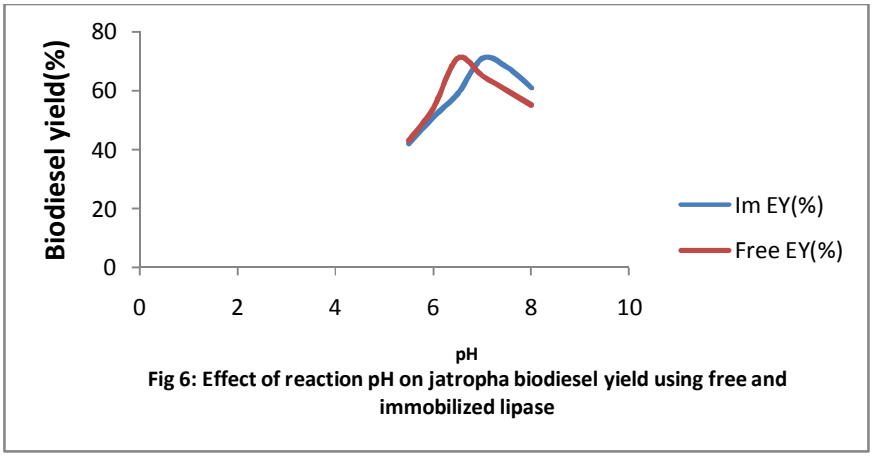

Fig 6: Effect of reaction pH on jatropha biodiesel yield using free and immobilized lipase

The result in Fig 6 shows an increase in biodiesel yield with increased pH. Maximum biodiesel yield was obtained at a pH 6.5 with free lipase and 7.0 with immobilized lipase processes, representing a 0.5 units increase in the optimum pH of immobilized enzyme when compared with that of free enzyme. This result agrees with those of Kulshrestha *et al.* (2006). They reported that the pH-optimum of free peroxidase was 5.0, while immobilized enzyme showed a pH-optimum at pH 5.6 (a 0.6 unit increase). These differences in the behaviors of free and immobilized enzyme processes could be explained by the poly-cationic nature of the enzyme supports like chitosan. They attract more OH^- ions around the immobilized enzyme, thus making the pH of the enzyme's micro-environment more than the bulk solution which eventually leads to a shift in pH towards alkalinity. Immobilized enzyme therefore requires a higher pH for optimal activity than free enzyme. In the present study, the optimum temperatures for free and immobilized lipase were 6.5 and 7.0 respectively. These pH optima were maintained to optimize other transesterification variables.

Effect of reaction temperature on biodiesel yield

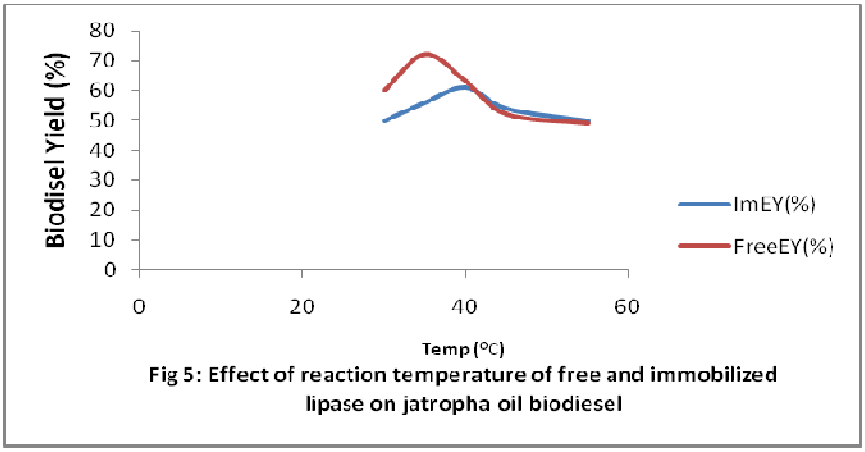

Fig 5: Effect of reaction temperature of free and immobilized lipase on jatropha oil biodiesel

The results in Fig 5 shows that as the reaction temperature increased, biodiesel yield increased up to 35^{O}C with free enzyme and 40^{O}C with immobilized enzyme processes. A further increase beyond these temperatures does not have effect on biodiesel yield. This result supports the findings of Fashmy et al. (1998) who have shown that the optimum temperature of immobilized urease increased from 55^{o}C for free enzyme to 65^{o}C for immobilized enzyme. Similarly, Reddy and Shankar (1987) reported that optimum temperature of free single-strand specific nuclease on immobilized enzyme. The temperature increases from free to immobilized enzyme processes obtained in the different studies described above suggested that immobilized enzyme has a higher resistance to change of temperature than free enzyme. On the other hand decreases observed in biodiesel yield at temperature beyond the optimum could be as a result of enzyme denaturation. It was also observed that at low temperatures, biodiesel yield with free enzyme process were higher than that of immobilized enzyme (72% and 61% respectively). This may reflect molecular diffusion

enzyme by immobilization (Reddyand Shankar, 1987). In this study, the optimum temperatures for free and immobilized lipases were taken to be 35°C and 40°C respectively.

Operational Stability of Immobilized Lipase

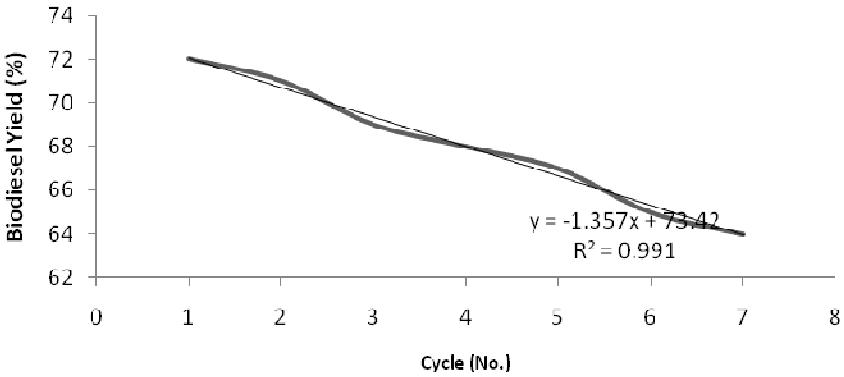

Fig 8: Operational stability/reusability of immobilized lipase enzyme.

The result in Fig 8 shows that reaction behaviour changed when an immobilized lipase was used repeatedly. Slight reduction in the percent yield (about 11%) of biodiesel was observed even after seven cycles of reuse of immobilized enzyme, the relative activity was reduced to 89%. From previous study residual activity after seven cycle of reuse have shown that there was no apparent loss in the biodiesel synthesis in t-butanol system (Novizar *et al*., 2009) The reduction in percent yield may be largely attributed to loss of enzyme during filtration and drying before reuse, prolonged interaction of the enzyme with the organic solvent which leads to enzyme denaturation and production of substantial quantities of co-product, water after each cycle as reported by Dave and Madamwar (2006). From this study, immobilization of enzyme has proven to be a useful technique for improving enzyme activity through direct interaction with the lipase besides protecting it from direct inactivation by reactants and products. The present study agrees with the findings of Persson *et al.* (2002). The study concludes that separation of enzyme from the reaction mixture is easy with immobilization hence the possibility of reuse, whereas the free enzyme is inseparable from the product at the end of the reaction. The repeated use of immobilized enzyme helped to bring down the product cost and made the enzymatic process economically viable as earlier reported by Ye *et al*. (2006). The design and construction of biodiesel reactor is therefore feasible since the operational stability is high. The prediction of operational stability at any cycle of reuse is also very possible because the R^2 is 99%.

Storage Stability of Enzyme

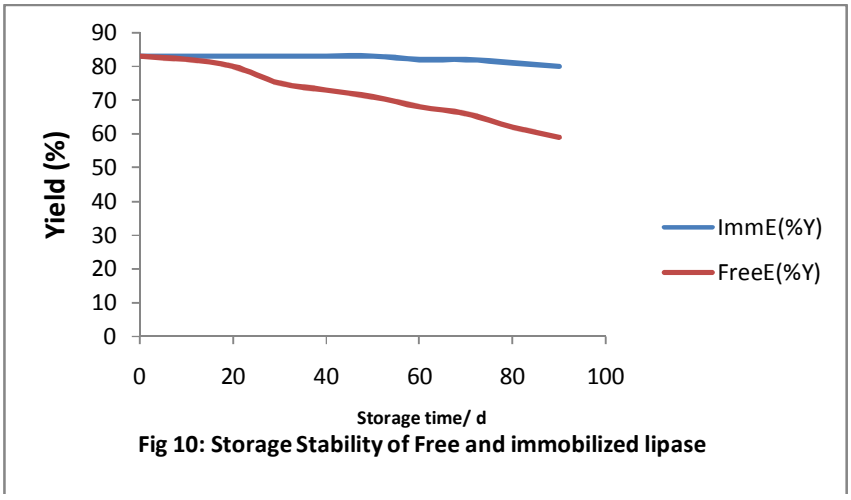

Fig 10: Storage Stability of Free and immobilized lipase

The result in Fig 10 showed that storage stability of immobilized enzyme was quite good. The rate of biodiesel yield was almost steady when stored at 4ºC after 90 days with about 96% residual activity achieved. On the other hand, almost 30% activity of free enzyme was lost after 90 days. Previously, Fashmy *et al*., (1998) studied storage stability of free and immobilized enzyme on DEAE cellulose at 4ºC and 25ºC, respectively. According to their results, storage stability of immobilized enzyme was better than that of free enzyme. Similar result was reported by Lu *et al.,* 2004 on Nuclease p1 immobilized on DEAE cellulose. Generally, the combination of immobilized carrier with enzyme increases enzyme stability, hence the reason for better storage stability of immobilized enzyme. The storage stability of immobilized enzyme in this study was very favourable for its industrial application.

Effect of Reaction Time on Biodiesel Yield

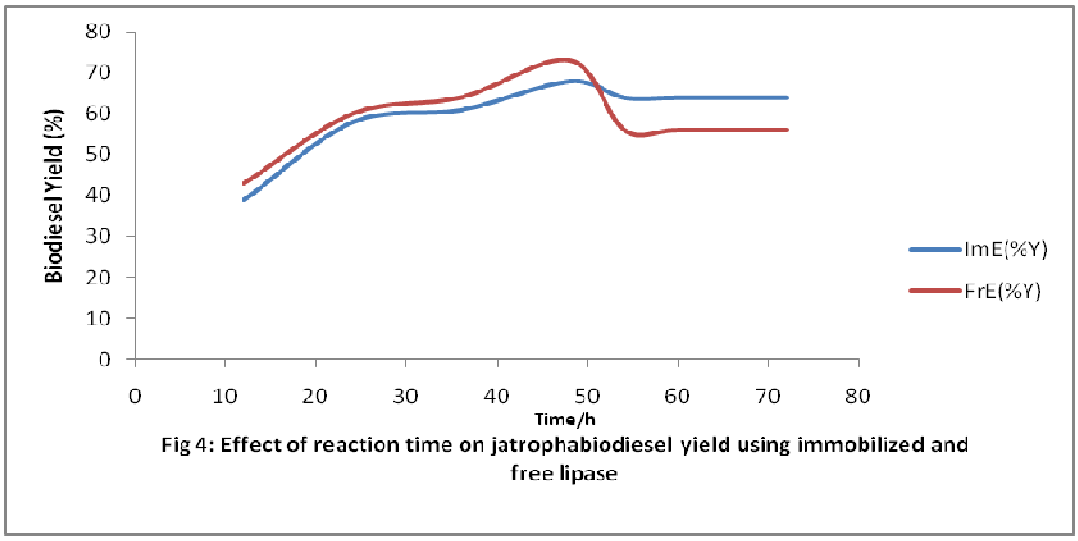

Fig 4: Effect of reaction time on jatrophabiodiesel yield using immobilized and free lipase

The result in Fig 4 shows that an increase in the transesterification reaction time caused an increase in the percentage yield of biodiesel up to 48 h and thereafter decreased until it reached 54 h for both processes using immobilized and free lipase. Further increase in reaction time beyond 54 h had no effect on the production of biodiesel. This result agrees with those obtained by Devanesan *et al*., (2007) and Nastratun *et al*., (2010) who have reported on transesterification of Jatropha oil using immobilized and free lipase. They found out that biodiesel yield was optimum at 48 h with decrease observed in biodiesel yield beyond 48 h of reaction time. The decrease in biodiesel yield observed in this study could be as a result of reduction in lipase activity due to denaturation of enzyme caused by prolonged reaction time. Therefore, the optimum reaction time for transesterification reaction in this study using both immobilized and free lipase was established at 48 h.

Effect of enzyme concentration on biodiesel yield

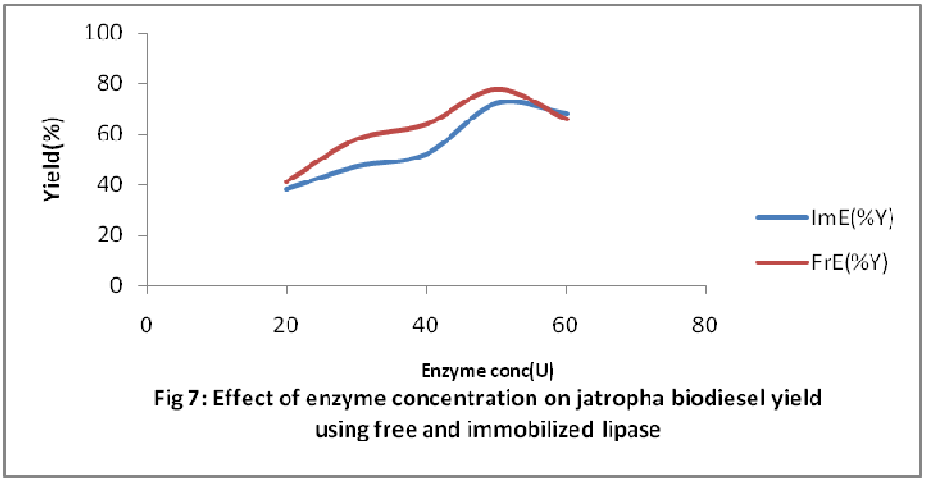

Fig 7: Effect of enzyme concentration on jatropha biodiesel yield using free and immobilized lipase

The effect of enzyme concentration on the transesterification reaction was also investigated. Fig 7 shows that the percent biodiesel yield increased with increase in the enzyme load up to 50U for both free and immobilized lipase. The maximum biodiesel yields at this enzyme load were 72% and 78% for free and immobilized enzyme respectively. Whereas upon further increase in the enzyme load up to 60U, a decrease in biodiesel yield was observed. A previous study by Ghamguia *et al.* (2004), demonstrated that the yield of 1-butyl oleate increased when the amount of lipase was increased from 30 to 60 U and remained almost constant with increase in lipase amount beyond 60 U. Similar results were reported for the methanolysis of rice bran oil catalysed by *Cryptococcus* spp. S-2 lipase (Kamini *et al*., 2001). The decrease observed with increase in enzyme load up to and beyond 60U may be due to the presence of a high amount of lipase, leading to overcrowding of enzyme molecules. The active sites of the enzyme are thus prevented from contact with the substrates. Agglomeration using immobilized lipases in a solvent-free system has been previously reported (Liou *et al*., 1998; Foresti and Ferreira, 2005). In this study the optimum enzyme amount of both free and immobilized enzyme was taken to be 50U.

CONCLUSION

The study concludes that:

- Chitosan was suitable for the immobilization of lipase with optimum enzyme loading at 0.2g/gchitosan at 3 h of loading and protein concentration adsorbed to the surface of Chitosan was 9.33mg/ml.
- Subsequent application of the immobilized enzyme on different transesterification variables/parameters showed a high percentage yield of biodiesel.
- Comparing the biodiesel yield obtained with immobilized and those of free lipase on the different transesterification parameters revealed that percent yield performance were higher with the free than immobilized.
- On the other hand, immobilized lipase provided important advantages such as easy separation from the product conferring a high potential for reuse. Immobilization also conferred on the enzyme high storage stability. The impacts of the above advantages are that immobilization of enzyme drastically reduced direct biodiesel production cost. Consequently upon the above, immobilized lipase may be used for the development of a bioreactor for use in commercial biodiesel production.

Finally, it is very convenient to conclude at the end of this study that immobilized enzyme is a better catalyst for commercial production of biodiesel for its cost effectiveness.

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