IMMOBILIZATION OF OIL PALM MESOCARP LIPASE FOR THE PRODUCTION OF MONO AND DI-ACYLGLYCEROL

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ABSTRACT

Oil palm mesocarp lipase was preserved and stabilized as the natural immobilized lipase (NIL) onto the oil palm mesocarp by four different immobilization treatments. The treatments consist of phosphate buffer 50mM (pH8.0), isopropyl alcohol, 2.25% glutaraldehyde and the combination of them. The stability of immobilized lipase was found to be highest in the treatment with 2.25% glutaraldehyde, followed by treatment with phosphate buffer pH8.0. This is because glutaraldehyde acts as a crosslinking agent in the immobilization of lipase. The synthesis of methyl oleate using immobilized lipase was investigated through esterification of oleic acid and methanol as substrate and hexane as reaction medium. The highest esterification production was 2.97 mmole in 16 hours. The hydrolysis activity of fatty acid and glycerol was found to be 0.257 mmole in 8 hours. The effects of immobilization on the stability of oil palm mesocarp lipase at higher temperatures and in organic solvent were also examined. Immobilized lipase showed higher esterification activity than free lipase or untreated and higher temperature stability up to 45^{o}C. At 75^{o}C, the untreated oil palm mesocarp completely lost its esterification activity whereas the immobilized lipase still posses 20% esterification activity. As a result, the immobilized oil palm mesocarp lipase was selected for the subsequent experiment of esterification of free fatty acid from palm oil fatty acid distillate (PFAD) with glycerol in hexane to produce mono and di- acylglycerols (MAG and DAG).

Keywords: *mono/di-acylglycerols, esterification, oil palm mesocarp lipase, fatty acid*

INTRODUCTION

Lipase constitutes the most important group of biocatalysts for biotechnological application. Lipase is an enzyme that hydrolyzed fat or oil to glycerol and fatty acids, and catalyzes various other reactions, such as esterification, interesterification and transesterification. Lipase is the first enzyme involved in the degradation of stored lipids [1].The increase in free fatty acid (FFA) levels in palm oil is attributed to the action of lipase. As lipase exerts enantioselectivity, it can be used to synthesize chiral compounds [2,3].

Enzymes are fragile and operate within very specific temperatures and environments that reflect their cellular origins. This fragility has, until now, limited researcher's ability to precisely control enzyme reactions or to reuse the enzymes. Furthermore, lipase is expensive and cannot be reused because of its high solubility in water. Many studies have examined the use of immobilization techniques to overcome these constraints. Therefore in the last few years, the potential value of immobilized enzymes has captured the imagination of an increasing number of scientist and engineers. The concept of being able to create an immobilized enzyme which have long term- stability and is able to be recovered and reuse is fascinating.

Immobilized enzymes are enzymes which are either covalently bound or absorbed onto the surface of an insoluble support. Immobilized enzymes have several advantages over the soluble enzyme. It provides convenience where miniscule amounts of protein dissolve in the reaction, so workup can be much easier. Upon completion, reaction mixtures typically contain only solvent and reaction products. The extensive development of enzyme immobilization techniques now appears to be largely attracted to the design of cheap, inert, have good mechanical and flow properties carriers. An additional feature which is receiving attention is the potential for chemical modification of the carrier surface. There are also other techniques of wet spinning enzymes and inert polymers together to obtain fibre entrapped enzymes [4].

One of the newest industrial scale applications of immobilized enzymes is the production of glucose/fructose syrups from glucose utilizing immobilized glucose isomerase. There are also many other processes important to the food industry may soon be converted to the use of immobilized enzymes. For example, there are chill proofing of beer using immobilized papain, the clarification of fruit juices and wines using immobilized

pectinase, the production of invert sugar from sucrose using immobilized invertase and the production of cheese using immobilized rennin. In many of these cases there are other advantages to used immobilized enzymes, is especially important since it assures that the enzyme does not remain in the final product [4].

Monoacylglycerol, MAG and diacylglycerol, DAG can be produced from various enzymatic processes including the esterification of free fatty acid, FFA with glycerol [5,6,7], glycerolysis of a fat and glycerol [8,9,10,11] and hydrolysis of triolein [12].

In this study, lipase are used to catalyse the esterification of free fatty acid (FFA) with glycerol to produce acylglycerols such as monoacylglycerol (MAG) and diacylglycerol (DAG). Monoacylglycerols (MAG) and diacylglycerols (DAG) are important emulsifying agents. Oil which contains a high level of DAG oil can be used to suppress the accumulation of human body fat, thus, reduces the risk of obesity [13,14].

In this study, the of enzyme used is from natural sources, which is oil palm mesocarps. In the oil palm industry, the formation of free fatty acids can decrease the oil quality. Oil palm press fibre or mesocarp fibre is the fibre obtained after expressing oil from the fruit mesocarp. On average, for every tonne of fresh fruit bunches (FFB) processed, there are 200 kg empty fruit bunches (EFB), 670 kg palm oil mill effluent (POME), 120kg mesocarp fibre, 70kg shell and 30kg palm kernel cake (PKC) produced. Oil palm mesocarp fibre contains, on a dry weight basis, approximately 40% cellulose, 21% lignin, 24% pentosan and 5% ash [15]. Cellulose, a polymer of α-D-1,4-linked anhydrous glucose units, $(C_6H_{12}O_5)n$, constitutes 40%-60% of the cell walls of woody plants.

In this paper the naturally found lipase in oil pal mesocarp is subjected to different treatment methods for immobilization to obtain Natural Immobilized Lipase (NIL). The effects of temperature and pH on the stability and the performance of the enzyme through esterification and hydrolysis process were investigated.

MATERIALS AND METHODS

(a) Materials

Oil palm mesocarp fibre was obtained from the MPOB Experimental Palm Oil Mill in Labu, Negeri Sembilan. The fruit lets were from *Tenera* species where they have bigger fruit lets and thick mesocarps. Glutaraldehyde, isopropyl alcohol, natrium hydrogen phosphate, natrium hydroxide, oleic acid, methanol, ethanol, acetone and phenolptalien were purchased from Sigma-Aldrich Inc., USA. Technical grade of n-hexane was obtained from Kofa Chemical Co. Hexane, a type of organic solvent can dissolve long-chain fatty acids and has been used previously as the reaction medium for short-chain esterifications. Palm cooking oil for hydrolysis experiment was 'Pisau' brand bought from the supermarket.

(b) Chemical Analysis

Chemical analysis of the oil palm mesocarp fibre was carried out by the methods below:

- Moisture content – TAPPI Method T208 om-84 (Anon.,1984a);
- Water solubility – TAPPI Method T207 om-81 (Anon.,1981);
- Sodium hydroxide (1%) solubility – TAPPI Method T223 hm-84 (Anon.,1984b);
- Klason lignin – TAPPI Method T222 om-83 (anon.,1983b);
- Holocellulose content – Wise et al. (1946); and
- α-Cellulose content – TAPPI Method T203 om-83 (Anon., 1983c).

(c) Preparation of Natural immobilized lipase (NIL)

The nuts were removed from the obtained oil palm pressed mesocarp. Blendering was done to produce smaller pieces of mesocarps. This can ease the defatting process later.

Defatting was carried out using a Soxhlet extractor on a heating mantle and n-hexane as the solvent, for five days at 6 hour each day to ensure that all the fat and oil were totally removed from mesocarps. The colour of the hexane in the soxhlet extractor went colourless if there was no more oil remaining in the mesocarps. The mesocarps were then dried under the sunlight and defatted mesocarps underwent different treatments to obtain the Natural Immobilized Lipase (NIL).

There are four treatments methods used to prepare NIL:

i. Treatment A – 10g of mesocarp was immersed in 50mM phosphate buffer pH 8.0 for one hour and washed twice with the same solution.
ii. Treatment B – Mesocarp was washed 30 minutes with isopropyl alcohol. It was further washed three times with distilled water. Then the mesocarp underwent treatment A.
iii. Treatment C – Mesocarp was immersed in 2.25% glutaraldehyde for an hour. It was then followed with treatment A.
iv. Treatment D - Combination of the above treatments.

The NIL was dried overnight in the oven at a temperature of 45^{o}C. Precipitation of lipase on to oil palm mesocarp was as suggested by Satiawihardja et al. (2004). The immobilized lipase was then stored at -20^{o}C until further use.

(d) Esterification Assay

Lipase was used as biocatalyst in the esterification of 10.9ml oleic acid (0.25M) and 1.3ml methanol (0.4M) in 88ml hexane. 2g NIL was added in the beaker with the reaction mixture. Then the sample was incubated in water bath for 1, 4, 8 and 16 hours at 55^{o}C. Blank was prepared with the same amount but contained no NIL and was incubated for 1 hour. Reaction was exhibited by adding 20 ml of acetone and ethanol for 10ml reaction mixture. Methyl oleate and water were formed where the unreacted acid was titrated with 0.1M NaOH. A few drops of phenolphthalein were used as indicator. If the enzyme's activity was higher, less oleic acid was formed. The volume of NaOH titration for samples was compared with blank where the NaOH volume for the sample must not exceeding blank. One unit of lipase activity was defined as the amount of enzyme that liberated 1mmol of fatty acid released per hour.

(e) Hydrolysis Assay

Hydrolysis is a reverse process of esterification. Two ml of palm cooking oil and 1ml 0.05M phosphate buffer (pH 8.0) was dissolved in 2 ml ethanol to be hydrolyzed by 0.2g NIL. The reaction was carried out at 55^{o}C for 1, 4, 8, 16 hours. A Blank was also prepared (same amount of mixture but contains no NIL) at 1 hour incubation to determine the volume of NaOH needed to detect the free fatty acids. Stopping agents (5ml acetone and 5ml methanol) were added to inhibit the reaction. The mixture was titrated with 0.1 M NaOH with phenolphthalein as indicator. Titration was stopped when the mixture turned to red orange in colour. The NaOH titration volume in the sample should be more than the titrated volume in the blank, due to fatty acids released, resulting from the lipase activity.

(f) The esterification of FFA from palm oil fatty acid distillate (PFAD) by immobilized oil palm mesocarp lipase (OPML).

A typical production mixture for the esterification contained 20g of PFAD, 3.35g of glycerol (molar ratio of 2:1) in the presence of 40g of hexane in a 250ml conical flask. Twenty gram (20) g of immobilized OPML was subsequently added into the reaction mixture, followed by addition of 2g of silica gel as water absorbent. A control reaction was also performed without any addition of lipase. The reaction mixture was agitated at 100 rpm in a water bath shaker. The esterification was carried out in the temperature range 45-75^{o}C. The reaction temperature during the esterification has to be higher than 45^{o}C to prevent improper mixing due to crystallization of PFAD [16]. Each reaction was carried out in triplicate.

(g) Identification of reaction products and estimation of the degree of esterification

Product analysis

1. Qualitative analysis: Thin-layer chromatography

Thin layer chromatography (TLC) technique has been widely used for the monitoring of lipase-catalyzed esterification reactions [17,18]. Esterification products (MAG, DAG and TAG) were identified by the aid of this simple method. This method facilitates a rapid separation and identification of fatty acids, which are retained at the origin of the chromatogram as their sodium salts from the various acylglycerols, which migrate together close to the solvent front (Figure 6). The TLC enables a rapid yet clear-cut separation of unesterified fatty acids and each of the different classes of acylglycerols that are likely to occur in a lipase-catalyzed esterification reaction. Since one single 4cm x 14cm plate may be subdivided into 3 lanes and the time of development is very fast (5-6 min), this technique is very convenient for the rapid assay of a large number of

samples.

The reaction products (MAG, DAG and TAG) were identified by thin-layer chromatography (TLC) method. TLC plates (4cm x 14cm) were precoated with silica gel (Sigma-Aldrich Inc., USA) and activated by heating at 105$_o$C for 30 min. The samples were then applied onto the starting line and water was evaporated by heating the plate briefly (10-15s) on a hot plate at 90$_o$C. The samples were spotted directly on the plate with authentic standards of tri-, di- and monoolein (Nu-Check-Prep, U.S.A). The plate was developed in chloroform: acetone: methanol (90:8:2 v/v/v), to a height of about 10cm, in about 5-6 min. Spots of each lipid were visualised by staining the plate with iodine vapour.

2. Quantitative analysis: Alkaline Titration

The concentration of free fatty acid in the sample of reaction product was quantified by titration with 0.5 M NaOH. All the samples analysis was performed in triplicate. The degree of esterification were calculated based on the following equation:

Amount of esterified free fatty acid , μmol = $(V_c - V_s)$ M x 1000 (1)

$$ED = \frac{Amount\ of\ esterified\ free\ fatty\ acid\ mol}{Amount\ of\ free\ fatty\ acid\ mol} \text{ x } 100\% \quad (2)$$

where V_c = volume of NaOH used for the control, ml
V_s = volume of NaOH used for the sample, ml
M = molarity of NaOH solution
ED = degree of esterification, %

RESULTS AND DISCUSSIONS

(a) Chemical analysis

The compositions of oil palm mesocarp fibre were determined. Table 1 shows the approximate chemical analysis of the oil palm mesocarp fibre. It contains a high content of holocellulose (cellulose and hemicellulose) and lignin, like all lignocellulosic/woody materials [15]. With the high content of holocellulose, it is to be expected that considerable sugars (glucose and xylose) can be extracted from it.

Table 1: Chemical Analysis of Oil Palm Mesocarp Fibre

Parameter	(%)
Moisture	5.2
Hexane-alcohol solubility	3.6
Holocellulose	70.6
Pentosan	15.6
α – Cellulose	32.4
Lignin	20.5
1% NaOH solubility	20.0

(b) Esterification assay

In esterification assay, oleic acid is reacted with methanol to form ester and water. The excess oleic acid is titrated with NaOH 0.1M. This shows the amount of unreacted acid that did not react with methanol to form ester as catalysed by lipase. As the time increased, the difference of NaOH titration between the sample and blank tended to get higher. This means that the amount of esters formed increased with time. With increasing time the lipase is in contact longer with the oleic acid, which allowed the reaction to proceed further. Sixteen (16) hour of incubation was observed to be sufficient time for esterification (Figure 1). At this time, the amount of oleic acid left in the mixture was lowest. Lipase was at the most productive state at this time. In this esterification assay, it is observed that the NIL treated with glutaraldehyde 2.25% followed by phosphate buffer pH8.0 has the highest esterification activity.

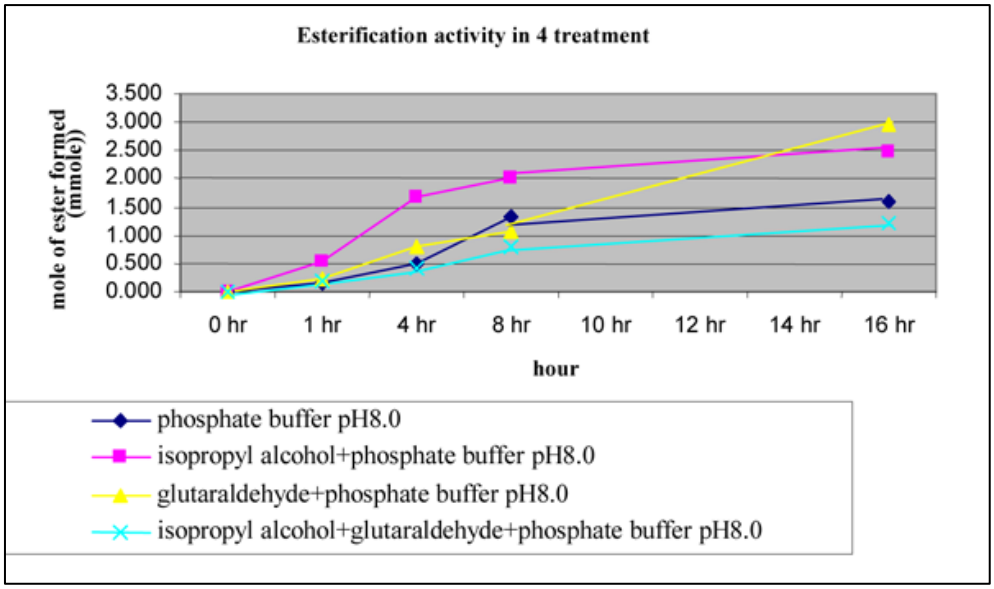


Figure 1: Ester formation by natural immobilized lipase (NIL) prepared by four treatments.

(c) Hydrolysis Assay

In hydrolysis assay, it is observed that the NIL treated with glutaraldehyde 2.25% followed by phosphate buffer pH 8.0 has better performance (Figure 2). As time increased, more fatty acid was released by lipase. The most optimum incubation time for hydrolysis was 8 hour. At this time, the amount of fatty acids produced reached the maximum. After 8 hour, the production of fatty acids decreases. This could be due to reversible reaction occurred to some extend.

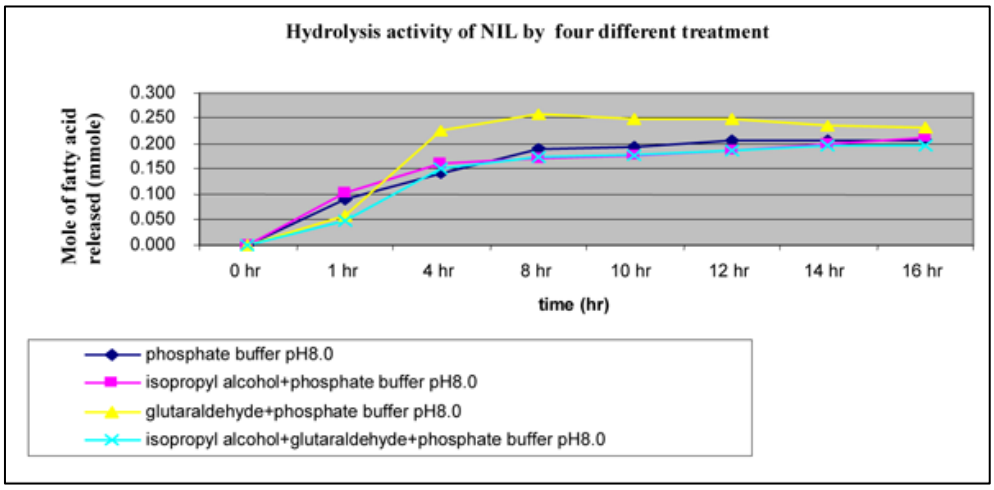
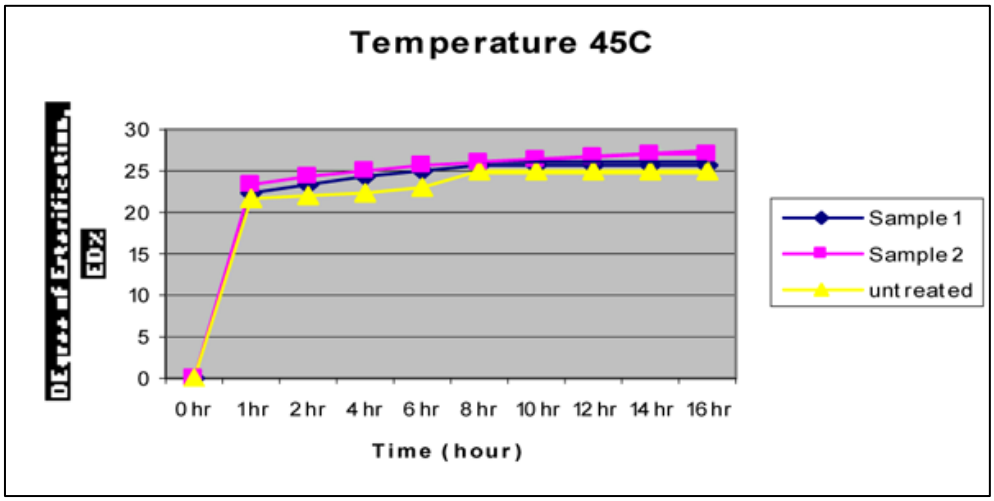


Figure 2: Fatty acid released due to hydrolysis by natural immobilized lipase (NIL) prepared by four treatments.

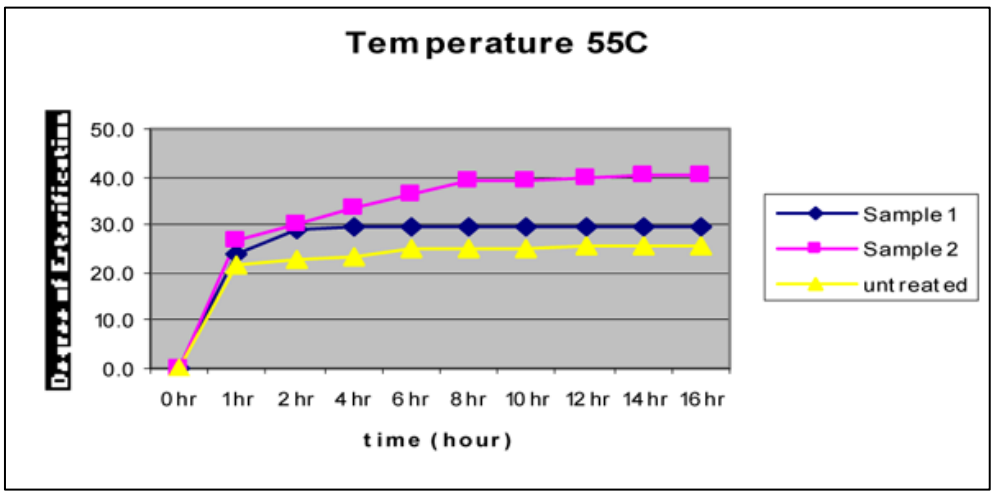
(c) Effect of temperature on the esterification activity of oil palm mesocarp lipase.

The activities of free lipase and immobilized lipase were investigated at various reaction temperatures. The change of the reaction temperature will affect enzymatic rate and functional group of substrate involved in the reaction. Therefore, reactions must be carried out to determine the optimum temperature in order to obtain the best yield. Temperature plays an important role in liquid viscosity and enzyme activity [19]. Generally, higher reaction temperature causes a decrease in viscosity of the reaction mixture and therefore, increases the rate of interaction between the substrates and enzyme molecule. The PFAD is in crystal form at temperatures below 45^{o}C, which caused improper mixing of reaction mixture. For a comparative study, the untreated and immobilized OPML were used as biocatalysts for this esterification.

The optimum reaction temperature for most immobilized lipases ranging from 45^{o}C to 65^{o}C [20,21]. The highest degree of esterification was achieved at 65^{o}C in the present study by Sample 2, where the lipase was treated with 2.25% glutaraldehyde, followed by treatment with phosphate buffer pH8.0. Further increase of temperature to 75^{o}C has resulted in a drastic decrease in esterification. The decrease of esterification at this high temperature may be due to the denaturation of lipase at elevated heat energy. Moreover, immobilized lipase was more temperature-stable, see Figure 3 than the free treatment form or untreated at temperatures higher than optimal. At 75^{o}C, the untreated oil palm mesocarp completely lost its esterification activity whereas the immobilized lipase still posses 20% esterification activity.



A



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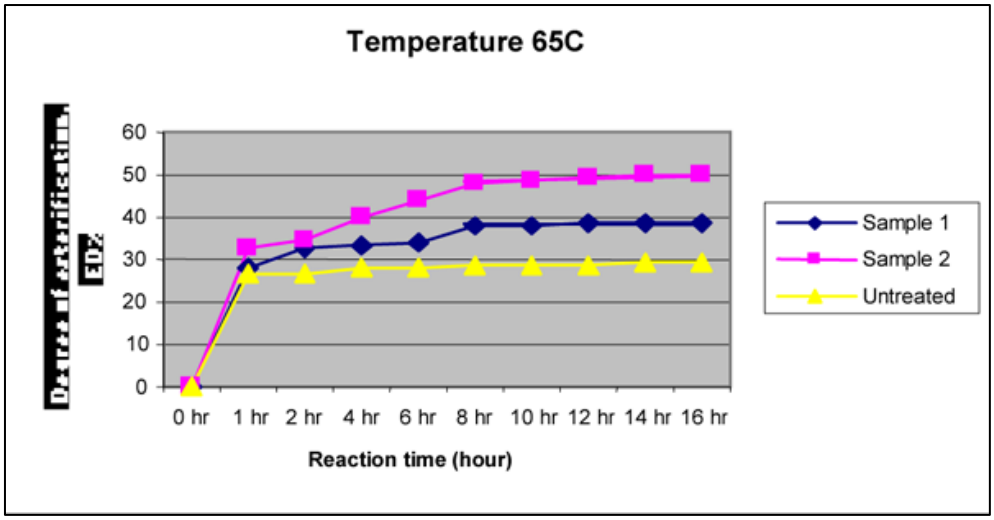

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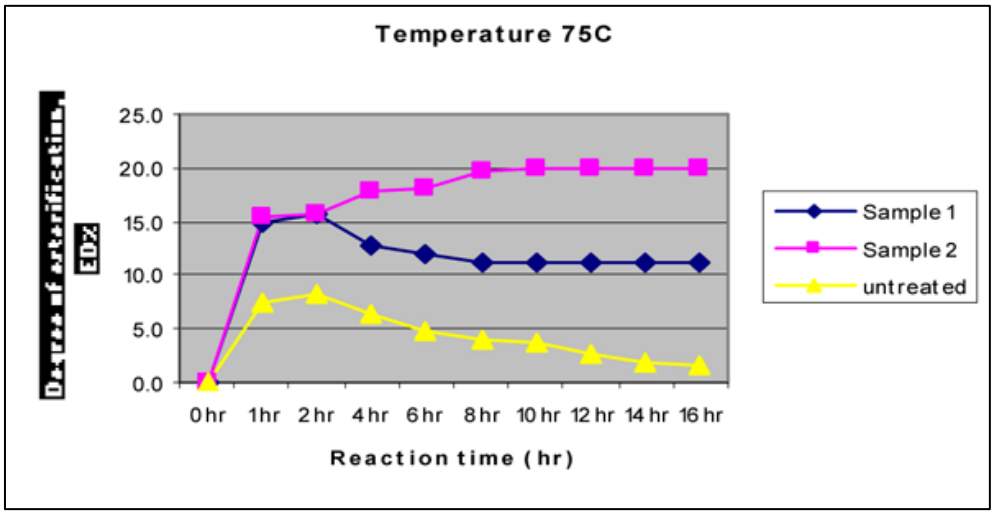


D

Figure 3: The effect of temperature, (A) 45oC; (B) 55oC; (c) 65oC; and (D)75oC of FFA from PFAD with glycerol in hexane on the degree of esterification in two best treatment (sample 1 and 2) and untreatment/blank (sample 3); sample 1: isopropyl alcohol + phosphate buffer pH8.0, sample 2: 2.25% glutaraldehyde + phosphate buffer pH8.0, sample 3: untreatment / blank sample.

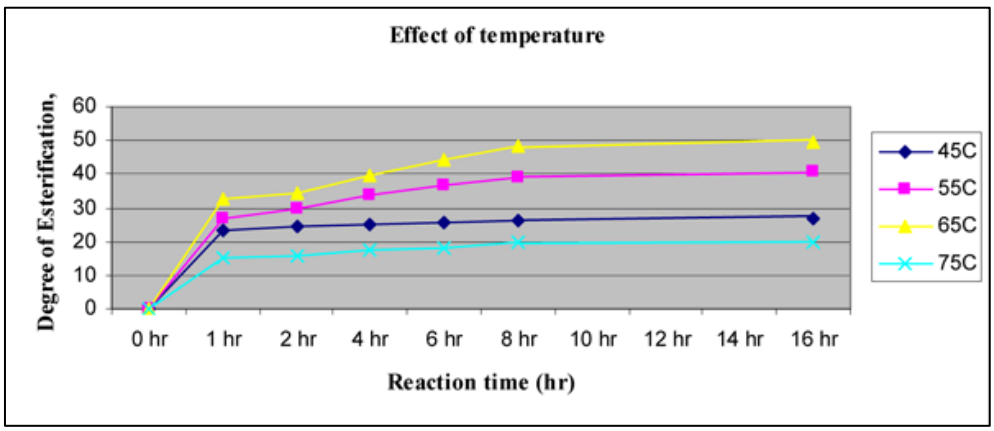


Figure 4: The effect of temperature of FFA from PFAD with glycerol in hexane and immobilized OPML (the best treatment) on the degree of esterification.

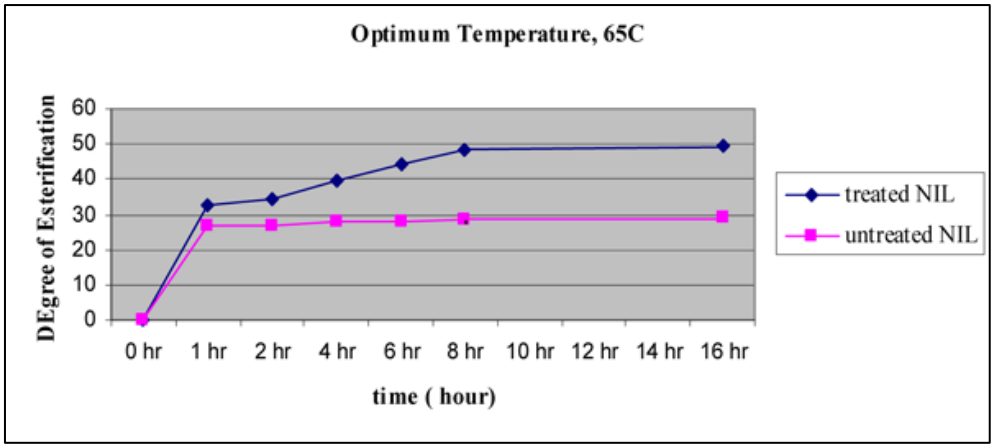
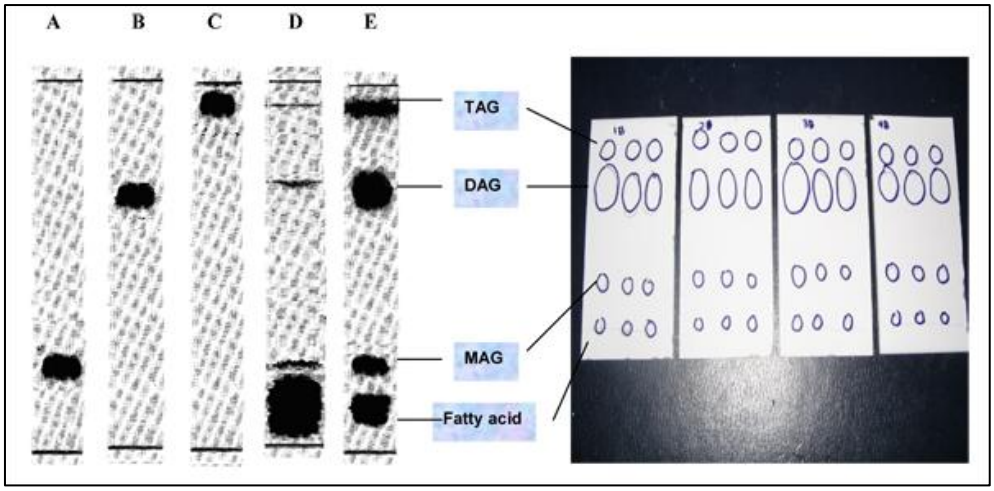


Figure 5: Comparison at the optimum temperature for treatment NIL and Untreatment NIL.

(d) Rapid thin-layer chromatographic analysis

The chromatograms in Figure 6 were developed using chloroform/ acetone/ methanol (90:8:2, v/v/v)up to a height of 10cm. Lane A, Lane B, Lane C is the standards of mono-, di- and triolein respectively. There were purchased from Nu-Check-Prep, U.S.A. Lane D shows the reaction mixture before esterification and Lane E shows the fractionation of product obtained from the esterification mixture of 20g PFAD, 3.35g glycerol (as glycerol/ fatty acid ratio 1:2 molar), 40g hexane, 2g silica gel and 20g immobilized OPML. The esterification reaction was performed at 65$_o$C and shaking at 100rpm.



Thin layer chromatograms. Lane A, Lane B, Lane C: Standards of mono-, di- and triolein were purchased from Nu-Check-Prep, U.S.A. Lane D: Reaction mixture before esterification. Lane E: Esterification product

Identification reaction with TLC using treatment

Figure 6: Chromatogram showing the fractionation of lipid classes bt TLC on sodium carbonate/ silica gel.

CONCLUSIONS

From the four treatments of immobilization, it can be concluded that the treatment with 2.25% glutaraldehyde followed by immersion in 50mM phosphate buffer (pH8.0) was the best treatment for preserving and stabilizing the activity of natural immobilized lipase in oil palm mesocarp. This is because glutaraldehyde acts as crosslinking agent which enable the NIL to form a stable structure. The application of immobilized OPML as biocatalyst for the esterification of FFA from PFAD with glycerol in hexane has been demonstrated in this work. Immobilized OPML has showed to have higher esterification activity and was more heat stable compared to the untreated OPML.

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