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SPECIALTY OLEOCHEMICALS FROM PALM OIL VIA ENZYMATIC SYNTHESES

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ABSTRACT

Palm-based specialty oleochemicals are special industrial chemicals from palm oil which are highly priced with high profit margins. These oleochemicals exhibit interesting properties such as excellent emolliency, surface activity, emulsifying properties as well as beneficial biological properties. As such, these compounds find many applications in the cosmetic, pharmaceutical and food industries. Enzyme catalysed syntheses of these chemicals are preferable as compared to their usual petro-chemical counterpart as these processes are nature identical and 'green'. Lipase-catalysed syntheses of specialty oleochemicals were carried out. The oleochemicals include amino acid esters, fatty alkanolamides, fatty esteramines, various wax esters, medium chain triglycerides and biologically active esters. The optimisation of the reaction conditions was discussed. The effects of the parameters influencing the reactions including temperature, reaction time, substrate molar ratio, amount of enzyme and solvents used were presented. The characteristics of the reaction system and the products were determined.

Keywords: specialty, oleochemicals, palm oil, enzymatic, syntheses.

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INTRODUCTION

Southeast Asian countries have dominated the export trade of palm oil, as Malaysia and Indonesia exported over 35 million tonnes of oil palm in 2010 (MPOB, 2011). The Malaysian palm oil industry has flourished with a favourable growth in export demand due to the limited supply of world oils and fats. The high demand due to depleting world stock levels results in higher prices of oils and fats as well as palm oil products.

The export of palm oil products includes palm oil, palm kernel oil, palm kernel cake and oleochemicals. The major markets for oleochemicals are the European Union, China, USA and Japan (MPOB, 2011). Oleochemicals achieve a much higher price as compared to palm oil. The main oleochemicals presently exported are fatty acids, fatty alcohols, methyl esters, glycerine and soap noodles. One sector of the oleochemical market that has great potential is that of specialty oleochemicals. However, not much work in oleochemicals production has been carried out. Thus, research in the processing of palm oil into higher value-added products such as specialty oleochemicals would be of great importance to an oil palm producing country such as Malaysia.

Palm-based specialty oleochemicals can be defined as those high priced industrial palm-based chemicals that can be sold with high profit margins. These high value oleochemicals are used in the cosmetics, food, drugs, pharmaceuticals and other chemical industries. Traditionally, these oleochemicals are produced chemically and thus undergo processes that are energy intensive, sometimes toxic and non-environmental-friendly. The alternative process of using enzymes as catalysts in the production of these products, for instance esters, has many advantages due to the favourable properties of enzymes. These

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include their high selectivity and consequent high product purity; their ability to undergo reaction at ambient condition, and the overall environmental friendliness of their processes. Research into enzymatic catalysis has been on-going in our laboratory since the 1980s. The general focus has been towards utilising our natural resource, that is palm oil, to acquire high value-added products as described.

The purpose of this review is to present an updated summary of the enzymatic synthesis research on specialty oleochemicals mainly carried out at Universiti Putra Malaysia. The oleochemicals studied include palm-based amino acid esters, palm-based fatty alkanolamides, palm-based fatty esteramines, palm-based wax esters and medium chain triglycerides.

PALM-BASED AMINO ACID ESTERS

Amino acid esters are amongst a group of 'bio-based' surfactants, the synthetic amphipathic structures of which are based on the natural structures of microbial lipo-amino acid/peptide biosurfactants and have multifunctional abilities (Clapes and Infante, 2002). Their simpler derivatives are constructed from a hydrophobic moiety most often represented by a long hydrocarbon chain and linked via an alkyl, acyl, ester, or amide bond to its hydrophilic partner, the amino acid. Depending on reaction conditions, such bonds linking the fatty chain to the amino acid can be located either at the α-amine or –carboxylic groups, or, at various functionalities in the side chain of the amino acid. When both the α- and side chain groups are substituted, dialkyls, diesters and diamides are also produced.

The functional properties of an amino acid surfactant are conferred by its three main structural components: the hydrophobic long chain, the amino acid, and the chemical linkage. The length of a fatty chain and its degree of unsaturation, in particular, have been shown to influence the melting, micelle forming, detersive, foaming, emulsifying and solubilising behaviours of the fatty acyl amino acid derivatives.

Collectively, the antimicrobial, surface-active and biocompatible properties of the amino acid surfactants have led to their being particularly useful in applications requiring biological contact. The properties of several families of amino acid-based surfactants with different structures resulted in high biodegradability, low aquatic toxicity and moderate cytotoxicity. The functionality of the amino acids allows obtaining a wide range of amino acid-based surfactants tailored to specific needs which have shown that surfactants with high efficiency, antimicrobial activity and moderate hemolytic activity can be prepared (Pinazo *et al.*, 2011).

Likewise, cosmetic and personal care applications include the use of acyl peptides in hair conditioning formulations and anionic N-acyl glutamate surfactant in syndet bars, anti-bacterial soaps and medicated shampoos. Palmitoyl hydroxyproline (LIPACIDE DPHP) has also been specifically used in anti-ageing creams as a result of the ability of the compound to prevent premature loss of skin elasticity (Akhtar and Yazan, 2008).

Enzymatic Synthesis

Enzymatic synthesis of amino acid-based surfactants has been widely attempted using a variety of amino acids and biocatalysts (proteases and lipases) either in the absence or presence of organic solvents. Of the proteases, papain was most frequently employed. Clapes *et al.* (1999) produced a range of arginine N-alkyl esters and amides using papain immobilised onto polyamide, while Piera *et al.* (2000) extended the study to the more complex arginine-based gemini surfactants.

Equimolar amounts of amino acids and fatty acyl donors were used with appropriate amounts of lipozyme as biocatalyst and hexane as solvent at 60°C (Soo *et al.*, 2003). Thin layer chromatography then facilitated the monitoring of the reactions and quick detection of any ninhydrin-positive products formed at R_f values of 0.51. Product spots from reactions with palm olein and palm kernel olein also demonstrated the feasibility of producing acyllysines by transamidation reactions between palm oil fractions and L-lysine. With the compounds of interest well separated and detected on thin layer chromatograms, the intensity of its colouration could then be directly measured, and correlated to the amount of the product formed via standard calibration curves. Photodensitometry was used as a quick and reliable tool for procuring such quantitative data on enzymatic reactions as exemplified by the works of Chamouleau *et al.* (2001), Soo *et al.* (2003) and Bidin *et al.* (2010).

Optimisation of reaction condition. A range of enzymes was first screened at various temperatures for their ability to catalyse the reactions between the selected substrates. The individual effects of organic solvents, incubation period, fatty substrate/L-lysine molar ratio, enzyme amount and water removal on the reactions were then alternately analysed while keeping the other parameters constant. The study, as described in our publications (Soo *et al.*, 2003; 2004), revealed that the immobilised Mucor miehei lipase, lipozyme, is to be the most efficient biocatalyst for the synthesis of palm-based acyllysines. Moderate yields were achieved with the other immobilised lipases, Novozyme 435 and Lipase 'Amano' AK, in

TABLE 1. OPTIMISED CONDITIONS FOR THE SYNTHESIS OF PALM-BASED AMINO ACID ESTERS USING LIPOZYME

Substrate pair	Parameter					
	Temperature (°C)	Organic solvent	Substrate mole ratio (fatty substrate: L-lysine)	Time (hr)	Enzyme amount (mg)	Molecular sieves amount (mg)
PA + L	80	Hexane	5:1	96	186.2	160
OA + L	80	Hexane	3:1	96	133.0	160
TP + L	70	Hexane	1:1	96	26.6	0
TO + L	80	Toluene	1:3	96	186.2	80
PO + L	70	Hexane	1:1	144	186.2	20
PKO + L	80	Hexane	1:1	144	186.2	0

some cases (as in reactions of triolein, palm olein and palm kernel olein with L-lysine) while poor results were generally obtained with the native *Candida rugosa* lipase and the protease trypsin in all reactions.

The corresponding temperature range over which lipozyme performed most efficiently in all six reactions was 70°C-80°C (*Table 1*). Lower temperatures not only resulted in low enzyme activity but also allowed crystallisation of high melting free fatty acids, which in turn caused mass transfer limitations. Higher temperatures, on the other hand, supported higher enzyme activity and promoted liquefaction of free fatty acids to improve substrate solubility, fluidity and mixing.

Other similarities shared by the six model reactions include their preference for hexane (Log P 3.5) as solvent and relatively large amounts of enzyme except for TP + L. In contrast, slight excesses of free fatty acids over L-lysine were required compared to pure and palm-based triglycerides, which were adequate at equimolar amounts. Acylation reactions with both free fatty acids were also enhanced with the use of increasing quantities of molecular sieves for water removal while transacylation reactions with pure- and palm-based triglycerides were, in general, adversely affected. This may be attributed to the latter's need for water to initiate the hydrolysis of triglycerides into the free fatty acids required for subsequent reaction with L-lysine. With no net generation of water in transacylation reactions, addition of more desiccant led to more water being removed resulting in less hydrolysis and less free fatty acids for reaction with L-lysine.

Compared to free fatty acids and pure triglycerides, reaction with the more complex palm oil fractions took longer to reach equilibrium (six days instead of four days). The average yields at optimum condition for free fatty acids and pure triglycerides were 32±2% and 48±2%, respectively This was possibly due to the content of mixed chain fatty acids in the oils with some chain lengths reacting slower than others and so resulting in an overall requirement of six days to reach equilibrium. Despite being more time-consuming, the direct use of palm oil fractions gave higher yields (>80%) in most cases (Bidin *et al.,* 2009). It also offers the benefit of producing the mixed chain compounds believed to display better surfactant performance than single chain length versions at low cost of raw materials.

PALM-BASED FATTY ALKANOLAMIDES

Fatty alkanolamides are nonionic surface-active agents (surfactants) in which the hydrophilic (amine) and hydrophobic (fatty acid) moieties are linked via an amide bond (Rahman *et al.,* 2003). They belong to the fatty amide group and are nitrogen-based compounds that contain both a peptide group ($CONH_2$) and a hydroxyl group (OH). There are three important structural elements in fatty alkanolamides ($RCONHC_2H_4OH$). The first is the acyl group derived from the long chain fatty acid. The second is the amide group, involving oxygen, nitrogen, hydrogen and carbon atoms. The third element is the presence of at least one 2-hydroxyl alkyl group with a backbone where the hydroxyl group is separated from the nitrogen by two carbon atoms. The basic characteristic of the amino group (NH_2) and acidic characteristic of the carboxylic group (COOH) are lost due to the functional presence of the amide ($CONH_2$). Therefore, alkanolamides are nonionic surfactants, which do not have a charged group. Fatty alkanolamides are derived from the reaction of triglycerides or fatty acid methyl esters (FAME) from vegetable oils or animal fat with various primary alkanolamines in the presence of a catalyst.

Chemical properties of fatty alkanolamides are primarily determined by the length and configuration of the hydrocarbon chain (Basri *et al.,* 2001a). Alkanolamides generally have high thermal stability but low reactivity. The melting points of the compounds range from 68°C to 104°C and the compounds are quite stable physically and

chemically. The melting point is increased due to the replacement of the hydroxyl in the fatty acid carboxyl with an amino group in the resulting amide. At ambient temperature, fatty alkanolamides possess limited solubility in common organic solvents. Monoalkanolamides are usually in crystal or waxy form. For example, the monoalkanolamide of lauric acid (C_{12}) which is a waxy solid at room temperature, is not soluble in water and is mainly used in detergent products.

Alkanolamides are a well established group of nonionic surfactants. They are complex mixtures, the physical properties of which can vary widely depending on the choice of raw materials. As a result of the diversity in their properties, alkanolamides have a broad spectrum of uses in industrial technology such as in detergent formulations, cosmetics, biocides, bubble baths and lubricants. Fatty alkanolamides are principally important in the detergent and cosmetic industries as ingredients in liquid and powder products. This is due to the wetting properties of the compounds, and their ability to boost detergency and foam stability. The OH group of fatty acids is insoluble in water and its hard waxy form confers easier-flowing properties, leading to its use as an ingredient in spray dried light- and heavy-duty detergent powders. Alkanolamides can also be used as hard surface cleaners. In shampoo and bubble baths, alkanolamides are used to modify viscosity as well as to accelerate generation and maintain stability of foam. Alkanolamides are furthermore important as an anti-static agent for plastics in textile processing, in cosmetic and pharmaceutical emulsions, as metal working fluids, in dry cleaning systems, and in pigment dispersion.

Enzymatic Synthesis

A wide range of enzymes can function perfectly well under near anhydrous conditions and have been used to react substrates such as triglycerides or FAME with alkanolamine to form alkanolamides (Rahman *et al.,* 2003). Lipases are a class of hydrolases widely employed for hydrolysis and synthesis of esters. In recent years, the employment of lipases as biocatalysts in enzymatic amidation has emerged as a potential route to replace conventional chemical procedures. In the enzymatic synthesis of fatty alkanolamides, reactions proceeded under milder conditions than the energy intensive chemical reaction. The enzyme-catalysed reaction could be carried out under mild temperature and atmospheric pressure, leading to lower production costs. The enzyme was also more selective, forming specific chemical bonds at specific sites within a molecule. This led to the production of purer products with higher efficiency and less unwanted by-products (Mat Radzi *et al.,* 2006).

Lipases have been used for synthesising alkanolamides from palm oil fractions (natural gylcerides) (Basri *et al.,* 2001a, Rahman *et al.,* 2003).

Lipases are 'mechanically' well understood, highly stable, co-factor independent and commercially available. They are an ideal biocatalyst for the modification of lipids and a broad range of other substrates as they show excellent stereo- and region-selectivity. Furthermore, lipases are active under mild reaction conditions (Mat Radzi *et al.,* 2010; Keng *et al.,* 2008).

The synthetic reactions were between triglycerides, palm kernel olein or fatty acid with monoethanolamine. The reaction mixture, which consisted of fatty acid/triglycerides (palm kernel olein), monoethanolamine, solvent (hexane) and lipase, was incubated in a water bath shaker (150 rpm) at temperatures ranging from 37°C to 70°C for variable periods of time. The synthesis was also performed at different substrate mole ratios. The products were separated and purified by adding warm hexane and water (40°C) to the reaction mixture (Rahman *et al.,* 2003). Alkanolamides produced were re-crystallised and analysed using gas chromatography (GC), thin layer chromatography (TLC), infra-red spectroscopy (IR) and nuclear magnetic resonance (NMR).

Optimisation of reaction condition. Various lipases (native and immobilised) were screened for their ability to catalyse the amidation reaction between free fatty acids or palm kernel olein with monoethanolamide. The immobilised enzymes such as Lipozyme IM, Novozyme 435, Amano PSC-lipase and AH-lipase produced better yields than the native enzyme from *Candida rugosa*. The Amano PSC-lipase was also more selective towards the C_{18} fatty acid when both free fatty acid and triglycerides (PKL) were used as fatty substrates in the amidation reaction.

Yields for the enzymatic reactions increased from 30°C to 60°C but decreased when the temperature was further raised to 70°C. The decrease in yield could be due to the denaturation of enzymes as a result of thermal disruption of bonds required for maintaining the active enzyme conformation. In contrast, the yield obtained for the reaction in the absence of enzyme was higher at the higher temperature of 70°C. This could be due to the fact that fatty monoethanolamide can be produced via an autocatalytic reaction (without the enzyme) that is enhanced by increasing temperatures. The study on the effect of reaction time on the yield of PKL monoethanolamide showed that enzymatic reactions proceeded rapidly and reached equilibrium within 24 hr. The rate of the non-enzymatic reaction (control), on the other hand, was slower and only reached equilibrium after 48 hr.

The composition of the monoethanolamide produced can vary considerably depending on the mole ratio of reactants. A slight excess of MEA over PKL (1:2, PKL:MEA) was adequate to give high yields. The excess of amine prevented the reaction mixture from solidifying and permitted the reaction to go to completion. The yields for the PKL monoethanolamide mixture increased with increasing amounts of MEA over PKL and only decreased after achieving the optimum mole ratio. The optimum mole ratio for both the non-enzymatic (control) and enzymatic reactions was 1:5 (PKL:MEA). The decrease of the yield at higher mole ratios of 1:6, 1:8 and 1:10 (PKL:MEA) could be due to the inhibitory effect of alcohol and product on the enzyme activity in organic solvent. An excess of amine may also led to lower reaction rates and gave rise to a variety of undesirable products and increase the production cost of the compounds.

A range of solvents with differing polarities (acetonitrile, butanol, chloroform, hexane and isooctane) were tested for use as an organic medium in the synthesis of palm-based alkanolamides. Solvent effects on a reaction can be mainly apportioned to its effect on the enzyme as well as on the solubility of the substrates and products. The best yields were achieved in all three reactions (control, novozyme, Amano PSC-lipase) when the non-polar hexane (log P = 3.5) was used as solvent. The enzyme was not denatured by the solvent as hydrophobic solvents did not interact with the protein and alter its stucture. In contrast, solvents with lower log P values (acetonitrile, butanol and chloroform) may have stripped the essential water of the enzyme molecule, thus decreasing the enzyme activity and reaction yield. The use of solvents with higher log P values such as isooctane also resulted in lower yields. This could be due to the increase in solvent viscosity, which might in turn lead to decrease in interaction between substrate and enzyme.

Water plays an essential role in enzyme structure and function especially when enzyme is used in nearly anhydrous organic solvents (Adnani *et al.*, 2011a, b). The activity of enzyme depends on the amount of water content in the whole system. The small amount of water content in the medium must be shared between all components of the reaction medium. The amount of water present will influence the biocatalysis (Adnani *et al.*, 2011c) and a minimal water content is necessary for the enzymatic reaction. Addition of some desiccants (molecular sieves) to remove some of the water formed in the enzymatic reaction systems was seen to increase the yield by shifting the equilibrium towards synthesis. The presence of <3% molecular sieves was enough to maximise the yield. The yield decreased with the addition of more desiccants in the reactions.

Table 2 shows the optimised condition for the synthesis of palm kernel olein with monoethanolamine.

PALM-BASED FATTY ESTERAMINES

Fatty esteramines can be classified into primary, secondary and tertiary amines depending on the number of alkyl groups attached to the nitrogen atom. Primary and secondary esteramines are used as starting materials for the production of tertiary esteramines, which can in turn be classified into mono-, di- or tri-fatty alkyl tertiary esteramines. The most significant application of fatty esteramines is as raw materials in the production of esterquats, a new generation of cationic surfactants.

Fatty esteramines are nonionic compounds and hence, do not have a charged group. The hydrophilic function of the compounds is provided by a water-soluble ester group that does not ionise to any great degree. When reacted with a quarternising agent, however, a positive charge is introduced onto the molecules to result in the cationic esterquats. The positive charge on the nitrogen atom confers to the compounds the ability to adsorb onto surfaces, most of which are negatively charged and can easily attract a positively charged molecule. Once adsorbed onto a surface, any hydrocarbon chain present in the molecule will extend outwards from the surface. Such a configuration results in both lubricating and anti-static properties due to the hydrocarbon chain and neutralisation of the surface charge, respectively (Idris *et al.*, 1995).

Cationic materials having two long alkyl chains used as active softening compounds are substantially water-insoluble. Hence, textile softening products are conventionally prepared with only a 3%-5% content of di-stearyl di-methyl

TABLE 2. OPTIMISED CONDITIONS FOR THE SYNTHESIS OF PALM-BASED ALKANOLAMIDES

	Control (no enzyme)	Novozyme	Amano PSC lipase
Temperature (°C)	70	60	60
Time (hr)	48	24	24
Mole ratio (PKL:MEA)	1:5	1:5	1:5
Solvent	Hexane	Hexane	Hexane
Amount of molecular sieves (g)	1.0	0.1	0.1

TABLE 3. OPTIMISED CONDITIONS FOR THE ESTERIFICATION OF OLEIC ACID AND TRIETHANOLAMINE USING LIPOZYME AND NOVOZYME (analytical scale synthesis)

	Lipozyme IM	Novozyme 435
Time	24 hr	24 hr
Substrate mole ratio (OA:TEA)	3:1	1:1
Substrate concentration	1.0 M	1.0 M
Water activity (a_w)	0.32	0.09
Temperature	50°C	50°C
Organic solvent	Heptane (Log P=4.0)	Heptane (Log P=4.0)
Enzyme concentration	5%	5%
Fatty acid chain length	C12-C16	C6-C16
Amine	TEA	TEA

Note: OA – oleic acid. TEA – triethanolamine.

ammonium chloride as the softening agent since aqueous preparations with more than 10% cationic materials would result in viscosity and stability problems. With esterquats, however, it is possible to prepare stable and low viscosity dispersions containing up to 50% active softening compounds. This in turn reduces shelf space, packaging and transportation costs, and utilises smaller and easier-to-handle containers.

Ester linkages connecting the core nitrogen atom to the hydrocarbon chain can furthermore be easily hydrolysed, thus providing potential points for breaking the compound into smaller molecules of fatty acids and parent amines. These smaller molecules can then be easily attacked by microorganisms to facilitate their biodegradation.

Enzymatic synthesis. Enzyme-based synthetic routes to fatty esteramines enable reactions under milder temperatures and pressures with use of less corrosive chemicals and more selective catalysts. Enzymes with the largest potential application in the oleochemical industry are lipases (Keng *et al.,* 2009; Basri *et al.,* 2007).

Lipases have been shown to exhibit regio-specificity (Li *et al.,* 2008) which is useful in the modification of fats and oils. This class of enzymes furthermore catalyses reactions at low temperatures, enabling reactions to proceed rapidly under mild conditions and reducing energy uptake. Together, high selectivity and mild reaction conditions minimise formation of by-products, which in turn reduces downstream processing. Equally important, lipases are able to catalyse both the forward hydrolytic reaction and the backward synthetic reaction such that synthesis of esteramines from triglycerides substrates can be carried out in a simple single-step reaction.

Feasibility of preparing fatty esteramines as intermediates for esterquats via an enzymatic route was carried out (Masoumi *et al.,* 2011). The model reaction selected for study was the esterification of triethanolamine (TEA) with oleic acid (OA). Reactions were carried out at equimolar substrate ratios (TEA, 0.7 mmol; OA, 0.7 mmol) in hexane as solvent using 40 mg of lipase. The reactions were terminated after 4 hr by filtering off the enzyme and evaporating off the solvent. Quantification of lipase activity and substrate conversion (%) was further performed by measuring the amount of unreacted fatty acid in sample (with enzyme) and control (without enzyme) reaction mixtures after dilution with an acetone:ethanol (1:1) solution at the end of the defined reaction period. Unreacted fatty acids were assayed by titrating with sodium hydroxide (0.1 N) to an endpoint of pH 12.00.

Optimisation of reaction condition. Based on the foregoing enzyme screening, lipozyme and novozyme were selected for further studies on the parameters affecting their catalysis of the esterification between TEA and OA. The optimum conditions for reaction with both enzymes are summarised in *Table 3*. The conversion of substrates into products were seen to escalate rapidly and peaked within the first 5 hr for novozyme and 24 hr for lipozyme. Beyond these peaks, the amount of water generated from the esterification reactions reversed the synthetic reactions towards hydrolysis, impeding the overall reaction and substrate conversion. The percentage yield of the products formed at the optimum condition was 45$\pm$2%.

For lipozyme, the highest % yield was achieved at an OA:TEA ratio of 3:1 while for novozyme, maximum activity was obtained at equimolar ratios of the two substrates. Excess TEA was seen to have a strong inhibitory effect on lipozyme and a lesser effect on novozyme. Substrate concentration was subsequently investigated, with maximum activities achieved at 1.0 M TEA and 1.0

M OA for both enzymes. A sharp decline in activity was seen with higher substrate concentrations in both cases. Such a decline could be due to substrate or product inhibition, as well as to high viscosity of the concentrated reaction mixtures which hindered effective mixing of the substrates.

Pre-adjusting the thermodynamic water activity (a_w) of the enzymes, substrates and solvent also appeared to affect the activity profiles of novozyme and lipozyme in catalysing the esterification of TEA with OA. The activity of novozyme was decreased when the reaction components were pre-equilibrated with salt hydrates having an a_w above 0.09, stabilising only when the a_w approached 0.69. The activity of lipozyme, on the other hand, increased when pre-equilibrated with salt hydrates of increasing a_w up to 0.32, after which a linear decline was seen. The difference in behaviour of the two enzymes indicated that their catalytic activities vary according to their dependence on water activity.

Nevertheless, reactions using both enzymes were seen to share several similarities with respect to the effects of temperature, organic solvent and enzyme concentration. Highest substrate conversions were obtained with the enzymes when the reactions were performed at a relatively mild temperature of 50°C. Both enzymes also displayed maximum activity when the reactions were carried out in a solvent with a non-polar log P of 4.0 (heptane). Activities of both enzymes decreased slightly thereafter when solvents with higher log P values were used. Increasing enzyme concentration from 2% to 5% (2.5-fold) was paralleled with increments in substrate conversion by 1.3-fold for novozyme and 1.1-fold by lipozyme. Further increasing the enzyme concentration to 10% (5.0-fold) did not significantly improve the reactions, possibly as a result of substrate limitations.

A detailed study on the structural variations of the fatty and amine substrates was also carried out. Novozyme catalysed the esterification of all fatty acids (C6-C16) almost to the same extent while lipozyme showed preference towards medium and long chain derivatives (C12-C16). The activities of both enzymes also reduced significantly when the degrees of unsaturation in the fatty acid were increased.

Mono-, di- and tri-ethanolamine were also alternately used as the alkanolamine substrate for esterification to OA, with the results suggesting the preference of lipozyme and novozyme for TEA over mono- and di-ethanolamine. This preference may be attributed to a pH effect since mono- and di-ethanolamine (pH 11.32 and 11.00, respectively) were more alkaline than TEA (pH 8.36). The pH values of mono- and di-ethanolamine therefore significantly exceeded the pH 7.0-8.0 range prescribed as optimal by the enzyme manufacturer (Novo Nordisk A/S Information Leaflet). Furthermore, pH 7.0-8.0 is the range within which salts of fatty acid and TEA form as intermediates en route to esterification. Hence, a mixture of OA and TEA provides the most suitable pH environment for conversion of substrates into fatty esteramines.

PALM-BASED WAX ESTERS

Wax esters consist of long chain fatty acids esterified to long chain alcohols. They are produced by a variety of plants, animals, fungi and bacteria, and serve a variety of biological functions. Wax esters are major constituents of bees wax, and plants such as jojoba. Naturally occurring wax esters are chemically diverse. Wax esters are usually harder, less greasy and more brittle than fats. The compounds have many potential applications due to their excellent wetting behaviour at interfaces and a non-greasy feeling when applied on skin surfaces. Physical properties of wax esters are very important from the cosmetic formulator point of view. Therefore, the physical properties such as melting point, viscosity, specific gravity and refractive index were measured for pure single wax ester and mixed wax ester.

The melting points of long chain wax esters (*e.g.* oleyl palmitate, oleyl oleate) are below 0°C while the boiling points are up to 300°C. Patel *et al.* (2001) has reported that the melting temperatures of saturated waxes range from 38°C for dodecyl myristate (C12:0-14:0) to >75°C for tetracosanyl tetrasanate (C20:0-24:0). And the melting temperatures increased by 1°C-2°C with each single carbon increase in the total number of carbon atoms in the molecule. Whereas, the refractive index and specific gravity of wax esters usually increase with increase in the molecular weight and decrease with the increase in temperature.

Wax esters derived from natural sources such as jojoba oil and sperm whale oil have been widely used in the cosmetics and lubricant industries (Keng *et al.,* 2009). For cosmetics, wax ester is a highly effective cleanser, conditioner, moisturiser and softener for the skin and hair. Wax ester is a superior lubricant in high speed machinery, work tools and metal cutters. It requires no refining and yet will enable a car to be run 20°-30° cooler when added to the crankcase oil. Wax esters significantly reduce friction, thus extending the life of all moving parts. In pharmaceuticals, wax ester is an anti-foaming agent in the production of penicillin. Due to its purity and indigestibility, it does not interfere with biological processes, and therefore wax ester has wide application as a carrier for medications used in the treatment of minor rashes, cuts, acne, psoriasis and neurodermatitis. Other examples of the commercial application of waxes

are in the production of detergent and polishes used for the cleaning and protection of surfaces. Wax polishes for floors, furniture, motor cars and shoes are mixtures of waxes and solvents which may or may not include water, together with special additives such as resins, silicone oils, pigments, abrasives, anti-septics and emulsifying agents.

Since the naturally occurring wax esters are expensive and limited in access, the need to synthesise the compound has grown. Wax ester has been synthesised via chemical and enzymatic reaction. Enzymatic synthesis uses lower temperatures than chemical synthesis and a single product is produced at a higher yield (Awang *et al.,* 2007a). Wax esters can be produced by alcoholysis of vegetable oils such as palm oil (Gunawan *et al.,* 2004). Palm oil consists of triacylglycerides, which are a combination of glycerol and different fatty acids.

Enzymatic Synthesis

Enzymatic synthesis of wax esters offers milder reaction conditions in an environmental-friendly process. Energy consumption is reduced and thermal damage to the product is avoided. Enzyme-catalysed synthesis can produce different types of product depending on the specifity or stereospecifity of the lipase used (Awang *et al.,* 2007b). Esterification and alcoholysis reactions can be catalysed by lipases to produce various esters of important commercial value. Direct esterification reaction between long chain alcohol and fatty acid were performed using immobilised lipase for producing wax esters (Mat Radzi *et al.,* 2005a,b). However, alcoholysis of triacylglycerols (TAG) like oils and fats was shown to be simpler than direct esterification with cheaper starting materials (Gunawan *et al.,* 2004; 2005).

Studies using lipase for the synthesis of alkyl oleate by esterification in organic solvent were carried out (Basri *et al.,* 2008). Enzymatic alcoholysis of triolein with oleyl alcohol to produce the wax ester oleyl oleate was also reported by Mat Hadzir *et al.* (2001). Lipozyme could be used for a high yield production of oleyl oleate at the optimised reaction condition such of incubation time, 5 hr; temperature, 50°C; weight of enzyme, 0.3 g and molar ratio of oleyl alcohol to triolein, 6:1. Steinke *et al.* (2001) studied the lipase-catalysed (Novozyme 435 and Lipozyme IM) alcoholysis of crambe oil and camelina oil with long-chain alcohols such as oleyl alcohol or alcohols derived from these oils for production of those of wax ester.

The alcoholysis reaction of palm oil and oleyl alcohol was carried out to produce wax ester. The first part of the work was screening of commercially available lipases for their ability to synthesise wax ester. The product of the synthesis reaction was identified on a GC (Hitachi model G-3000, Tokyo, Japan), which was equipped with a DB-HT 17 medium polar column (30 m x 0.32 mm). The initial temperature used was 200°C, increased at 20°C per min to a final temperature of 300°C. This was then followed by optimisation of the reaction conditions. Enzymatic synthesis of wax esters from palm oil was compared to those from other vegetable oils.

Optimisation of reaction condition. Lipases from different sources were tested for the alcoholysis reactions of palm oil and oleyl alcohol for 5 hr-7 hr. The commercial lipases tested for the synthesis of wax esters were lipozyme, novozyme, *Candida rugosa* lipase, *Rhizopus niveurs* lipase, and *Aspergillus niger* lipase. The highest yield was achieved with by lipozyme (>80%). The percentage yield of ester increased with increasing temperature to reach the maximum percentage at 50°C-60°C as the energy from the heat was used to increase the frequency of interaction between the lipase and substrates. Lower percentage yields were observed at lower temperatures (30°C) because of the lower enzyme activity. The percentage yields of wax esters were also lower at 60°C -70°C due to enzyme denaturation at relatively higher temperatures.

The molar ratio of 1:3 or 1:4 (oil: alcohol) produced highest percentage yields compared to the other molar ratios**.** This corresponds to stoichiometric mixtures of palm oil and oleyl alcohol. Steinke *et al.* (2001) reported that lipase-catalysed esterification of stoichiometric mixtures of long-chain and very long-chain fatty acids with the corresponding mixtures of alcohols gave quantitative yields of wax esters. Increasing the molar equivalent of alcohol up to 4, improved alcoholysis. The decrease in percentage yield at higher alcohol concentrations (1:5) may have been caused by the excess alcohol distorting the essential water layer that stabilises the immobilised enzyme. Alcohols are known to inhibit lipase-catalysed transesterification probably by disturbing the water bound to the enzyme or deactivating the lipase through osmotic dehydration.

Polarity of various solvents plays an important role in the alcoholysis of vegetable oils with alcohol. The percentage yields were higher in solvents with higher log-P values. The most suitable organic solvents for ester synthesis are solvents with relatively higher log-P values. Basri *et al.* (1994) also reported that lipases functioned better in the more hydrophobic solvents as such solvents did not distort the essential water layer of the enzymes. In the work reported by Keng *et al.* (2005), hexane was found to be the best solvent for synthesising palm-based wax ester. This finding is in agreement with the work of Rahman *et al.* (2001) who reported that the best solvent for lipozyme-catalysed synthesis of the liquid wax ester, oleyl oleate, was hexane.

Increasing lipase quantity up to 1.5% (w/v) increased the ester yield. An excess amount of enzyme (2-2.5% w/v) did not improve the percentage yield of the product. This may be due to substrate limitation, where excess enzyme molecules are not in contact with the substrate and remained unreactive. Mat Hadzir *et al.* (2001) also showed that a higher load of enzyme in the alcoholysis of triolein with oleyl alcohol led to higher product yields up to a point above which increasing enzyme amount did not further increase the yield.

HYDROXY FATTY ACID ESTERS

Wax esters functionalised with hydroxyl groups in the alkyl chain of the fatty acid or alcohol moiety are called uncommon wax esters. These compounds show interesting surface-active properties and are used as plasticisers, mold release agents, emulsifiers and chemical intermediates. Sulphonated wax esters of ricinoleic acid are also promising surface-active compounds. The polyethylene glycol (PEG) ester of hydroxy acid has good solubility as well as significant surface-active properties. The surface tension of this compound at 25°C is about 36-37 dynes cm^{-1}. Based on a standard closed patch test method, hydroxy stearate was found to be non-irritant to the skin, which indicates that this compound can be used as a cosmetic ingredient (Awang *et al.*, 2009).

Hydroxystearic acid ester is probably the most important of these types of esters, and has been substantially studied (Awang *et al.*, 2004; 2006; 2009). Procedures for preparing various alkyl esters of hydroxystearic acid have been documented (Awang *et al.*, 2000; 2003) for use in many industries such as lubricating oils with good oxidation stability and low temperature properties, and also for its anti-microbia. Isopropyl hydroxystearate can be used in plastics and rubber where it acts as an anti-static, and in lipsticks where it acts as a pigment wetting agent and emollient and enhances colour development. Octyl hydroxystearate is useable in eye cream bases, usually at a content of 4%. Methyl hydroxystearate can be cyanoethylated and used as a slurry aid during recovery of butyl polymer from co-polymerisation reactions.

Enzymatic Synthesis

Our work was focused on the synthesis of palm-based dihydroxystearic acid (DHSA) ester from renewable palm oil resources using enzyme-based clean technology. The synthesis of DHSA ester from DHSA can be carried out in a one step reaction without destroying or transforming any reactive functionalities on the acid. Gas chromatographic analysis of octyl dihydroxystearate after conversion to trimethylsilane (TMS) derivatives revealed the presence of other compounds along with octyl dihydroxystearate. The major peak was seen at a retention time (R_t) of 19.12 min and was assigned to octyl dihydroxystearate (Awang *et al.*, 2000). Octyl dihydroxystearate was also subjected to ^{1}H NMR and ^{13}C NMR analyses: ^{1}H NMR of octyl dihydroxystearate in $CDCl_3$ exhibited the following peaks: 4.07 ppm (OH), 3.96 ppm (CHOH), 2.73 ppm [CH_2-CH(O)], 1.28-1.80 ppm (CH_2) and 0.94 ppm (CH_3). The ^{13}C NMR confirmed the presence of the following groups: carbonyl at 174.08 ppm, two hydroxyls at 74.47 ppm and 74.66 ppm, -O-CH_2 at 64.48 ppm, -CH_2- from 24.96 ppm to 34.37 ppm, two –CH_2CH_3 groups at 22.68 ppm and 22.65 ppm and –CH_3 at 14.12 ppm.

Optimisation of reaction condition. Five commercial immobilised enzymes for DHSA ester synthesis were screened to identify the most suitable enzyme for subsequent esterification reactions. All the immobilised enzymes tested were able to catalyse the desired esterification reaction. However, lipases from Mucor miehei (Lipozyme IM) and *Candida antarctica* (Novozyme 435) showed higher conversions of about 83% while lipolase (lipase from *Thermomyces lanuginosus*) only exhibited a low conversion of 7.99%. Lipases from different sources exhibit different specificity towards different substrates.

Changes in the reaction temperature affect the activity and stability of the enzymes and thus the rate of reaction. The influence of temperature on the esterification of DHSA and 1-octanol within the temperature range of 25°C to 70°C was studied (Awang *et al.*, 2004; 2006). Lipozyme IM and Novozyme 435 performed most efficiently at 50°C. The conversions were slightly decreased or unchanged when the temperatures were higher than 50°C.

In this esterification reaction system, the percentage conversion increased with increasing amounts of 1-octanol up to 2.0 mole, after which a plateau was obtained. This result indicated that Lipozyme IM and Novozyme 435 were not denatured by excess alcohol although significant substrate inhibition of these enzymes by alcohols have been previously documented (Basri *et al.*, 1997). The different observation in this study is due to the non-polar nature of octanol (log P= 2.9), which strongly favours its solvation in the organic phase while at the same time reducing inhibitory interaction with the enzyme.

The rate of the esterification reaction was also seen to increase with increasing amounts of the enzyme. When the enzyme load reached a certain level (more than 10%), the reaction rate reached a steady state within which the degree

of esterification was only slightly increased with increasing enzyme load. The overall water content in the system increased with increases in enzyme load for the same amounts of reactants. Studies on the effect of organic solvents have shown that the percentage conversion of ester was much higher in organic solvents with log P values from 2.0 to 4.0 (Awang *et al.*, 2004).

MEDIUM CHAIN TRIGLYCERIDES

Medium chain glycerides (MCG) are mono-, di- and tri-glycerides of caprylic acid ($C_{8:0}$, octanoic acid) and capric acid ($C_{10:0}$, decanoic acid). They are derived from a glycerol esterified to one, two and three caprylic (or capric) acid molecule(s), respectively. The medium chain triglycerides (MCT) contain predominantly caprylic (50%-80%) and capric (20%-50%) acids with lesser amounts of caproic (1%-2%) and lauric (1%-2%) acids (Traul *et al.*, 2000). The chemical structure and composition of MCT confer to the compounds functions that are similar to regular liquid vegetable oils, the primary content of which is long-chain triglycerides (LCT).

The MCT are ester oils that have a low viscosity (25-31 mPa.s at 20°C) which render them stable to high and low temperature extremes. Consequently, not only are they liquid at room temperature, they are also able to remain clear and non-viscous at 0°C. This obviates the need for warming prior to use of MCT even at low temperatures. Their high content of saturated fatty acids furthermore confers to them high stability against oxidation. As the compounds are flavourless and colourless, they do not mask or interfere with flavours they act as carriers for. Collectively, these characteristics have led to their wide usage as solvents, carriers and diluents for concentrated essential oils, flavours and colours in confectionary products, reduced calorie foods, and specialty nutritional products such as vitamins.

MCT are able to spread easily on most surfaces and adhere well to metal surfaces. They therefore form good lubricants for moving parts in food processing/packaging machinery as well as edible lubricants for use as ingredients in foodstuffs. In contrast to LCT in vegetable oils, MCT have smaller molecular sizes and are derived from shorter chain fatty acids that have greater solubility in water. These in turn lead to the MCT having different physiological routes of transport and metabolism from the LCT. LCT are absorbed and metabolised slowly but they also require energy for their oxidation and utilisation as fuel and building blocks. Conversely, MCT are metabolised as rapidly as glucose and provide over twice the energy of carbohydrates (*i.e.*, MCT provides 8.3 calories per gramme compared to 4 calories per gramme for carbohydrates) (Traul *et al.*, 2000).

Such a quick high energy source from MCT has led to their extensive use as energy sources by athletes, for individuals with fat malabsorption syndromes, in parenteral nutrition for individuals requiring supplemental nutrition, and in infant formulae. They can furthermore be directly substituted for vegetable oils in product formulation to lessen absorption of conventional fats and oils in the body. MCT also have a cholesterol lowering effect in that they reduce absorption of cholesterol in the intestine and limit the deposition of cholesterol in all body tissues.

Enzymatic Synthesis

Despite Ghosh and Bhattacharya's (1997) study showing better incorporation of medium chain fatty acids (MCFA) into coconut oil via the chemical pathway, the enzymatic route of synthesis for MCT have many advantages such as lower energy consumption due to possibility of reaction at ambient temperature and pressure and higher product purity due to better reaction selectivity and consequent lesser by-products. Higher selectivity also enables incorporation of specific fatty acids at specific positions on the glycerol molecule to give specific glyceride products.

In order to perform the non-selective reaction under the moderate reaction conditions achievable via enzymatic routes, a non-selective lipase was used in place of the chemical catalyst in our esterification of glycerol with mid-chain fatty acids (Basri *et al.*, 2001b; Wong *et al.*, 2000). The objective of both studies was to systematically optimise the conditions for the lipase-catalysed reaction with both MCFA. The two esterification reactions were carried out at 37°C under atmospheric pressure.

In both reactions, the fatty acid acts as the acyl donor which reacts with the lipase to form an acyl-enzyme intermediate. The acyl-enzyme complex is subsequently attacked by the alcoholic nucleophile, in this case, the triol glycerol. The non-specific native lipase used was from *Candida rugosa*, which was able to incorporate a MCFA onto the *sn*-2 position of *sn*-1,3 dicaprylin (or *sn*-1,3 dicaprin) to synthesise tricaprylin (or tricaprin). Quantitatively, the remaining fatty acids in the reaction mixtures were determined by autotitration with 0.15 M NaOH to an end point of pH 9.5. Reactions performed under optimal conditions were also quantitatively analysed using GC (internal standard method).

Optimisation of reaction condition. The optimum conditions based on percent conversion of both caprylic and capric acids are summarised in *Table 4*.

Reactions of both MCFA with glycerol were seen to reach equilibrium after 24 hr, probably as a result of the production of water which then acted as a substrate in the hydrolysis of the acyl-

TABLE 4. OPTIMISED CONDITIONS FOR THE SYNTHESIS OF MEDIUM CHAIN GLYCERIDES (MCG) USING *C. rugosa* LIPASE

	MCFA used	
	Caprylic acid (Basri *et al.*, 2001b)	**Capric acid (Wong *et al.*, 2000)**
Time (hr)	24	24
Organic solvent	Isooctane or n-Heptane	Isooctane
Molar ratio (MCFA/glycerol)	2.5	2.5
Enzyme amount (mg)	100	100
Water activity (a_w)	0.328	0.328

Note: MCFA – medium chain fatty acids.

enzyme intermediate. Reaction in solvent was better than reaction under solvent free conditions due to the requirement for solvent to solubilise the MCFA, decrease substrate viscosity and improve substrate mixing. The non-polar solvents (hexane, Log P = 3.50; heptane, Log P = 4.00; isooctane, Log P = 4.20; *n*-octane, Log P = 4.50) appeared to be the best solvents for the two reactions since the polar solvents (acetone, Log P = -0.23; ethyl acetate, Log P = 0.68; chloroform, Log P = 2.00; toluene, Log P = 2.50; carbon tetrachloride, Log P = 3.00) stripped the layer of essential water off the enzyme molecule while the highly non-polar solvents (*n*-decane, Log P = 5.60; hexadecane, Log P = 8.00) hindered interaction between the catalyst and substrates with their high viscosity (Basri *et al.*, 1997).

In both reactions, the MCG synthesis increased when the amount of lipase was increased up to 100 mg, after which very little improvement was observed possibly due to substrate limitation. The two reactions were also best carried out with an excess of the fatty acid substrate (caprylic or capric acid) as implied by the optimal MCFA/glycerol ratio of 2.5. Inhibition by excess glycerol was caused by high solubilisation of the enzyme in high volumes of glycerol, decreasing the lipase concentration at the interphase which in turn decreased the reaction rate.

Under these optimal conditions, 20.99% and 22.12% of caprylic acid were converted to dicaprylin and tricaprylin, respectively, while 0.99%, 14.97% and 17.28% of capric acid were converted to monocaprin, dicaprin and tricaprin, respectively. The non-specificity of the lipase used enabled synthesis of tricaprylin and tricaprin from their respective fatty acids via all possible routes. Namely, the non-specific lipase first synthesised the α-monoglyceride (*sn*-1- and *sn*-3-monoglyceride) and *sn*-2-monoglyceride from the fatty acid and glycerol at the same rate, and then they esterify not only the α-monoglyceride to α,β-diglyceride (*sn*-1,2-diglyceride and *sn*-2,3-diglyceride) and *sn*-1,3-diglyceride but also *sn*-2-monoglyceride to α,β-diglyceride. Finally, both α,β-diglyceride and *sn*-1,3-diglyceride were esterified to triglycerides.

CONCLUSION

The synthesis of specialty oleochemicals was successfully accomplished with lipases as biocatalysts and palm oil as novel acyl donors in organic solvent systems. Each reaction has a set of reaction conditions that is unique to the particular system. The successful use of lipases as biocatalysts in reactions provides a potential alternative to conventional chemical routes disadvantaged by high reaction temperatures and product contamination. The enzymatic route was able to proceed under milder reaction conditions (50°C) in common organic solvents (like hexane and heptane) using immobilised lipases that can easily be separated from the reaction mixture to yield products of higher purity.

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