Haute Couture
Molecules & Biocatalysts

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Dedication

This book is dedicated to the memory of my beloved mother,
HAJAH WAN SALIHA HAJI WAN YAHAYA
who passed away while I was finalising the draft of this book...
She was beside me all the way with her unconditional love and
endless prayers for my success in every highlight of my life...

With tears and prayers, I persevered to complete
my inaugural seminar

May Allah 'Azza Wajalla bless and grant you
Jannahtul Firdausi. Amin.

I LOVE YOU MAK...
Your Only Son
. Basyar .

Contents

ABSTRACT

For the next 50 years, chemistry will be just as fashionable as it has been in the past 50 years. In fact, the role of chemistry is bigger than ever with the help of bioinformatics that are riding the waves of genomic revolution. Bio-based processes and entities are becoming more organic with civilians requesting sustainable approaches to everything. Globally, the runway for the implementation of industrial biotechnology is nearly complete and is ready to overtake harsh chemical processes. However, major issues such as the high capital costs of advanced biocatalysts, organic solvent replacements and bioreactor technologies are the winding roads to progress. Research on enzymes and liquid engineering has brought us to new dimensions of understanding the unknown capabilities of unnatural enzyme systems at the molecular and atomic levels. The development of designer biocatalysts for industrial purposes to substitute traditional processes is gaining interest. Utilisation of local microbial enzymes and our tropical biodiversity for the discovery and identification of new biocatalysts through advanced structural and synthetic biology are the key focuses. In addition, bioinformatics or the *in silico* approach of designing novel single molecules to macromolecules is a powerful tool to model any mechanism and structure. By all accounts, the efficient use of bio-renewable resources requires blending different systems of chemistry and biology; heterogeneous and homogeneous, enzymes and metals, microbial and yeast, *etc*. This technology presents current and potential areas in which the use of a biocatalyst is a prerequisite for an economical application in green organic syntheses. Considering the industrial importance of the platform and fine chemicals, the optimal conditions for up scaling the process are evaluated by statistical methods while taking into consideration, all of the sustainable, environmental and economical evaluations.

It will be essential for chemists or biological engineers to design and model the biocatalysts to significantly guide and quicken the steps for synthesising new molecules and improve existing systems. We need many new revolutionary molecules and biocatalysts for a plethora of chemical reactions and industrial applications.

Keywords: biocatalyst, enzyme, metalloenzyme, immobilisation, ester, epoxide, ionic liquid, *in silico*, molecular dynamics, genome

OVERVIEW

Each biotechnological application is given its own colourful tag: white (industrial), red (pharmaceutical and medicine), grey (environmental), blue (marine) and green (agri-food) as presented in Figure 1. Red biotechnology enjoys wide support to combat diseases, meanwhile sustainable white and blue biotechnology focuses on bio-chemicals and bio-fuels. Encouraged by the historical successes of plant-based materials in competing for commodity industrial markets, many researchers have looked at the potential for further development of plant-derived chemicals (Keng *et al*., 2009; Törnvall and Hatti-Kaul, 2007).

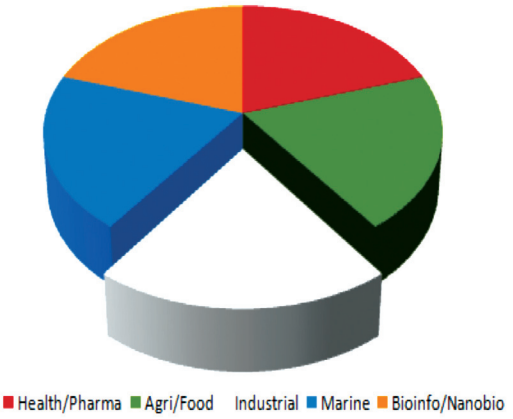


Figure 1 Colourful world of biotechnological applications

A study in the USA recommended that industry should set itself, the goal of increasing the percentage of chemicals and materials from plant resources fivefold, to 10%, by 2020, with another fivefold increase, to 50%, by 2050 (Black and Miller, 2006). More recently, the BREW project in Europe has estimated that using white

technology to convert plant feedstock could meet approximately one-third of Europe's total need for organic chemicals in 2050 (Patel, 2006).

Oil crops represent a considerable reservoir of useful and low-cost raw materials like fats (oils), proteins and carbohydrates. By selectively combining their molecular constituents (fatty acids, glycerol, amino acids, saccharides *etc*), a wide variety of specialty and fine chemicals (amides, esters, epoxides, surface-active materials *etc*) can be prepared. Each of these 'platform' chemicals stands at the apex of a cascade of transformations that will produce hundreds of commercially important materials (Figure 2). These renewable raw materials are going to play a vital role in the development of sustainable green chemistry. They offer a large number of possibilities for applications, which can rarely be met by nonrenewable sources (Abdul Rahman *et al*., 2008a).

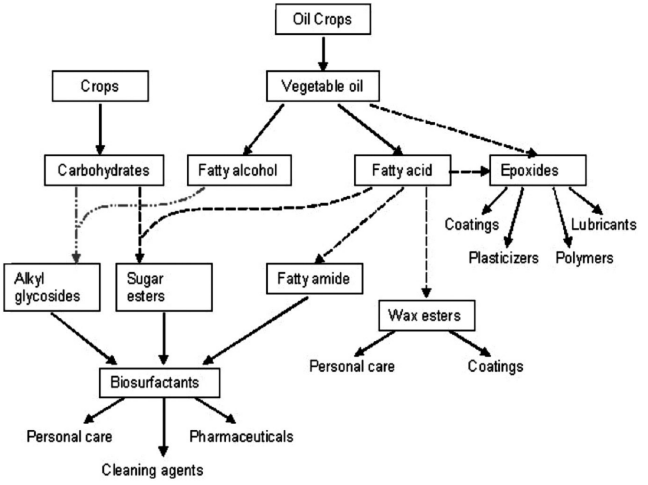


Figure 2 Flow of platform chemicals from oil crops

Green chemistry seeks to explore new technological options that can replace conventional technologies based on traditional chemical processes and fossil-based resources (Hatti-Kaul *et al*., 2007). All over the world, the focus is on development and application of clean green processes based on biocatalysis for the production of chemical products from renewable raw materials. Enzymes in synthesis is an exciting field that allows for the construction of complex and remarkable target molecules by exploiting the abundance of catalysts that nature has to offer (Bornscheuer and Kazlauskas, 2004). The last few decades has seen the discovery of highly selective enzymes and metalloenzymes with broad substrate specificities. Although often underappreciated, catalytic promiscuity has a natural role in evolution and occasionally in biosynthesis (Kazlauskas, 2005).

For decades, understanding the genesis of enzymatic functions was one of the challenges in protein design, mechanistic enzymology, and molecular evolution. Combined with protein engineering, the enzymes may extend their usefulness in organic synthesis (Abdul Rahman *et al*., 2003a). Initial applications of biocatalysis involved the scale-up of naturally occurring reactions, such as glucose isomerisation, protein digestion for laundry applications, or fermentation to produce natural amino acids. Later, biocatalysis extended to unnatural substrates, notably in the preparation of enantiopure pharmaceutical intermediates utilising hydrolases or lipases and bioconversions of renewable resources to platform chemicals (Abdul Rahman *et al*., 2009a). The challenge is to obtain platform chemicals from renewable resources directly in one enzymatic step using designer enzymes.

Bespoke Biocatalysts

Biocatalysis stands on the chemical transformation of man-made organic compounds via the employment of natural catalysts such protein enzymes or whole cells. We have witnessed substantial increases in the application of biocatalysis in the production of fine chemicals and therapeutics, especially in the pharmaceutical industry. A long-standing advantage of biocatalysts is in the sustainable production of bulk to fine chemicals, as they are environmentally acceptable and degradable, something that often plagues traditional methodologies. Enzymes are selective, chiral and prefer mild conditions, which minimises problems of undesired side-reactions and leaves highly pure desired products.

Enzymes are perfect as chiral catalysts for the bioorganic synthesis of chemoselectivity, regioselectivity and enantioselectivity molecules. However, the sensitive side of enzymes is its fragility in different environments, which leads to its denaturation (Abdul Rahman *et al*., 2009b). On the bright side, enzymes offer modification to its system via multidiscipline research opportunities. The key applications are the modification and improvement of existing industrial biocatalysts via artificial and rational design, mutation, chemical modification, synthetic approach and directed evolution of candidate enzymes as reflected in Figure 3.

Designer enzymes are crafted for a specific chemical reaction, but the interesting challenge is to design enzymes as a superbug or a powerhouse for multiple transformations. Debates rage around the globe about how this idea is to be implemented in the cells and laboratories. Thanks to the other bug, the super computer, the crazy ideas are now possible. Eventually, we will also be in the run for building variant libraries for hundreds of chemical reactions.

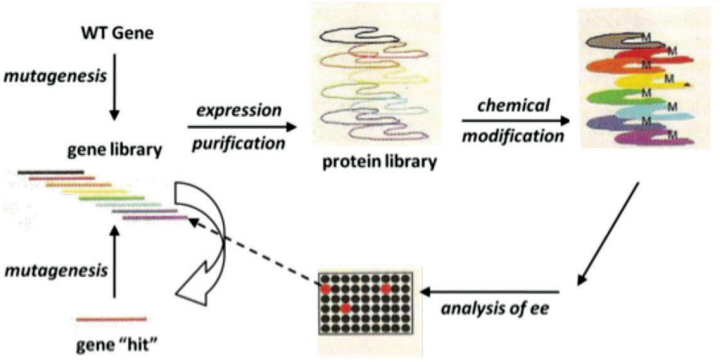


Figure 3 Rational design produces libraries of new biocatalysts

THE FASHION

Green is the New Gold

Green chemistry goes beyond the science, technology and design of chemical products that reduce and preferentially eliminate hazardous substances. A vision to demonstrate green-based chemicals from petrochemicals made using white biotechnology can reach the global market, thus contributing to a paradigm shift within the chemical industry. Modern society has become increasingly dependent on fossil resources, especially crude oil that is used not only as fuel but also as a raw material for a variety of chemicals and other commodities. We focused on the development and application of bio-based processes for effective transformations of renewable and non-renewable substrates into useful substances. These have led to patents as depicted in Table 1. New and improved product innovations for various domestic and industrial applications are presented in Figure 4.

Table 1 Patents deposit for enzymatic synthesis and their applications

	International Patents
	Patents Granted
1.	**Invention : Production of Wax Esters** United States Patent Number : 7557228
	Patents Filed
2.	**Invention : A Method for Epoxidation of Plant Oils** International Patent Application No. PCT/MY2009/000033 *Publication No. : WO/2010/098651*
3.	**Invention : A Method for Producing Adipate Ester** International Patent Application No. PCT/MY2008/000093 *Publication No. : WO/2009/066975*
4.	**Invention : Formulation for Coating Material** United States Patent Application No. 12/515,373 International Patent Application No. PCT/MY2008/000094 *Publication No. : WO/2009/066976*
5.	**Invention : Production of Wax Esters** Singapore Patent Application No. 200407514-9 Indonesia Patent Application No. ID 00200400665
6.	**Invention : Enantioselective Immobilised Lipase** United States Patent Application No. US 11/058,159
	Malaysian Patents
7.	**Invention : A Process for Producing Levulinate and Succinate Esters** Malaysian Patent Application No P1 2010xxxx (submitted)
8.	**Invention : Immobilised Enzyme Using a Mica Carrier** Malaysian Patent Application No P1 2010004391

9. **Invention : Antifreeze Peptides Derived From Fungal Protein**
   Malaysian Patent Application No P1 20095441

10. **Invention : A Herbicide Formulation**
    Malaysian Patent Application No P1 20093048

11. **Invention : An Emulsion System Derives from Engkabang Fat Esters**
    Malaysian Patent Application No P1 20092650

12. **Invention : A Topical Applied Formulation Containing Illipe Fat and/or Ester and A Method for Producing the Same**
    Malaysian Patent Application No P1 20091192

13. **Invention : Pharmaceutical Composition of Non-Steroidal Anti-Inflammatory Drugs**
    Malaysian Patent Application No P1 20090367

14. **Invention : Formulation for Coating Material**
    Malaysian Patent Application No P1 20072080

15. **Invention : A Method for Producing Adipate Ester**
    Malaysian Patent Application No P1 20072081

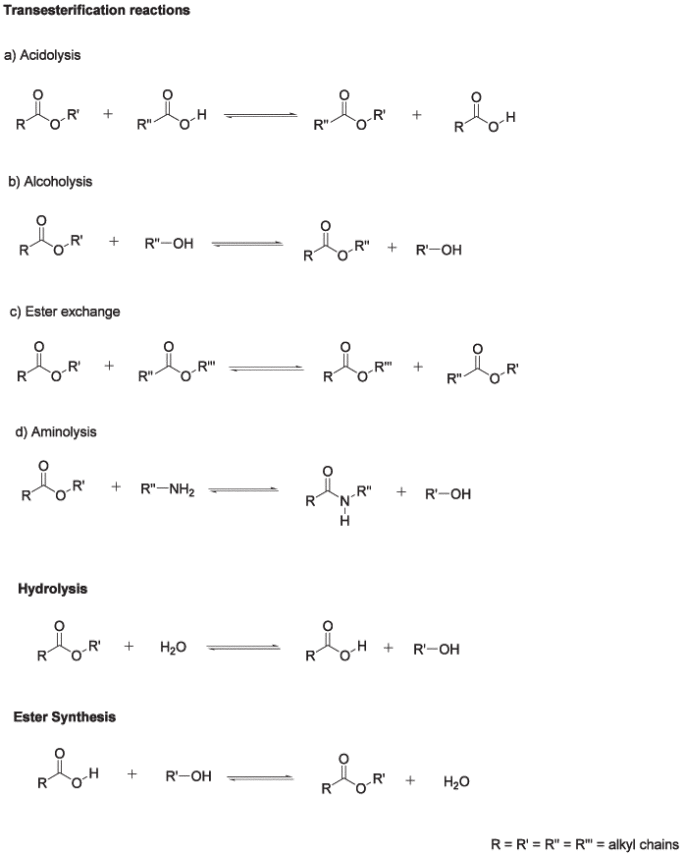
16. **Invention : Enantioselective Immobilised Lipase**
    Malaysian Patent Application No. PI 20040529

17. **Invention : Production of Wax Esters**
    Malaysian Patent Application No. PI 20033660

Figure 4 Novel and new products innovation for various applications

Fashionable Bio-chemical Entities

A savvy scientist hungers to recreate or create many new products. With strong beliefs supported by established data, many new bio-chemical entities were fashionably produced with the assistance of lipase biocatalysts. Our major products are high purity esters, epoxides, amines and imines as illustrated in Scheme 1. These novel and new derivatives of fatty acids, adipic acid, succinic acid, levulinic acid and betulinic acid were systematically studied and developed as summarised in Table 2. The enzymatic route of employing commercial lipases (e.g. *Candida rugosa* and *Candida antarctica*) and immobilised lipases (e.g. Novozym 435 and Lipozyme RMIM) at mild reaction conditions are our major contributions to green chemistry. We are also heavily involved in the exploration of novel enzymes from local strains and modifying them to suit industrial purposes.



Scheme 1 Biocatalysis routes of new potential derivatives

Table 2 Biocatalysed syntheses of novel and new high value-added products developed in our lab

Acids / Oils	Substrates	Products	References
Palm oil	Oleyl alcohol	Palm-based esters	Gunawan *et al*., 2004
Palm oil	Oleic acid Linoleic acid Ricinoleic acid	Epoxidised oleyl oleate Epoxidised oleyl linoleate Epoxidised oleyl ricinoleate Epoxidised pentyl diricinoleate	* research in progress
Palm olein	Oleyl alcohol	Palm olein-based esters Palm stearin-based esters Palm kernel-based esters	Basri *et al*., 2005a Keng *et al*., 2008
Palm stearin			
Palm kernel oil			
Palm kernel olein	Monoethanol-amine	Palm kernel alkanomide	Abdul Rahman *et al*., 2003
Palm kernel stearin			
Triolein	Oleyl alcohol	Oleyl oleate	Mat Hadzir *et al*., 2001

Caproic acid (C_6)	Oleyl alcohol	Oleyl caproate Oleyl caprate	* our work
Capric acid (C_8)			
Caprylic acid (C_{10})	Glycerol	MCGs of dicaprylin and tricaprylin	Basri *et al*., 2001
Lauric acid (C_{12})	Oleyl alcohol	Oleyl laureate	Basri *et al*., 2005b * our work
Myristic acid (C_{14})		Oleyl myristate	
Palmitic acid (C_{16})		Oleyl palmitate	
Stearic acid (C_{18})		Oleyl stearate	
Oleic acid ($C_{18:1}$)	Oleyl alcohol	Oleyl oleate	Abdul Rahman *et al*., 2001 Mat Radzi *et al*., 2005
Succinic acid (C_4) (amber acid)	Oleyl alcohol Chitosan	Dioleyl succinate Chitosan succinate	Abdul Rahman *et al*., 2010d
Adipic acid (C_6)	Methyl alcohol	Dimethyl adipate	Chaibakhsh *et al*., 2010a

Adipic acid (C_6)	n-butyl alcohol Lauryl alcohol Palmityl alcohol Stearyl alcohol Oleyl alcohol Arachidyl alcohol Chitosan	Dibutyl adipate Dilauryl adipate Dipalmityl adipate Distearyl adipate Dioleyl adipate Diarachidyl adipate Chitosan adipate	Abdul Rahman *et al*., 2009 Abdul Rahman *et al*., 2008 * research in progress
Levulinic acid (C_8) (keto acid)	Ethyl alcohol	Ethyl levulinate	Lee *et al*., 2010
Betulinic acid	Phthalic anhydride 3-methyl phthalic anhydride	3-O-phthalyl betulinic acid ester 3-(3-methylphthalyl) -betulinic acid ester	Moghaddam *et al*., 2010a Abdul Rahman *et al*., 2010h
Racemic (±)-menthol	Butyric anhydride	(-)-menthyl butyrate	Othman *et al*., 2008

Our history in green chemistry started with the establishment of high value-added products from the highly praised commodity of Malaysia, palm oil. The palm oil derivatives such as palm-based wax esters and palm-based medium chain glycerides (MCG) are becoming important components in major domestic and industrial applications (Basri *et al*., 2005a; Basri *et al*., 2005b). Previously, long chain esters (with chain lengths of 12 carbons or more) known as wax esters were harvested from animals (sperm whale oil), vegetables (jojoba seed) and minerals (petro-based). Since the sources are endangered, limited and expensive, biodegradable

wax esters derived from fatty acids and alcohols that resemble natural wax esters can meet the high demands from chemical-based industries as specialty oleochemicals (Table 3).

The high purity wax esters especially oleyl oleate are essential in cosmetics, pharmaceuticals and lubricants owing to their excellent wetting behaviour at interfaces but with a non-greasy feeling when applied on skin surfaces (Abdul Rahman *et al*., 2001; Gunawan *et al*., 2004). Physico-chemical properties of palm esters such as refractive index, density, surface tension, slip melting point, saponification value, iodine value and acid value were analysed following standard test methods modified from the American Oil Chemists' Society's standards. Simultaneous differential scanning calorimeter-thermal gravity analysis showed a high thermal stability profile of palm ester (Keng *et al*., 2009).

Two main routes of producing wax esters are esterification and alcoholysis, but the latter is simpler than direct esterification and the starting reactants are cheaper such as triacylglycerol oils and fats. Similar commercial values of wax esters also can be obtained from alcoholysis of triglycerides of long chain fatty acids and long chain fatty alcohols. Enzymatic alcoholysis of triolein and oleyl alcohol was studied by using Lipozyme RMIM and Novozym 435 as biocatalysts. The production of oleyl oleate catalysed by Lipozyme RMIM was found to be highest at 75.6% in nonpolar solvents with log P values greater than 2.5 at 50°C with molar ratio of oleyl alcohol to triolein, 6:1 (Mat Hadzir *et al*., 2001).

One of the abundant by-products in the oleochemical industry in Malaysia is glycerol. Increasing the commercial value of glycerol can be made by converting it to medium chain glycerides containing mono-, di- and triglycerides. This compound has been widely used as a carrier for materials such as perfume bases in toiletries and flavours in food industries.

Table 3 The percentage yield of palm-based esters at optimal enzymatic reaction conditions (Keng *et al.*, 2009)

Palm fraction	Ester oils composition (%)								
	Oleyl caproate (C24:1)	**Oleyl caprate (C26:1)**	**Oleyl caprylate (C28:1)**	**Oleyl laurate (C30:1)**	**Oleyl myristate (C32:1)**	**Oleyl palmitate (C34:1)**	**Oleyl stearate (C36:1)**	**Oleyl oleate (C36:2)**	**Oleyl linoleate (C36:3)**
Palm oil	n.d.	n.d.	n.d.	0.4	2.6	42.1	5.3	31.7	10.2
Palm olein	n.d.	n.d.	n.d.	1.1	2.5	35.8	2.4	30.2	11.8
Palm stearin	n.d.	n.d.	n.d.	1.0	3.6	63.2	5.1	12.9	4.0
Palm kernel oil	0.5	5.6	5.9	54.1	13.9	5.2	1.2	6.4	1.7
Palm kernel olein	0.6	6.1	5.1	49.3	11.9	5.5	1.7	9.7	2.6

n.d., not detected.

An enzymatic synthesis of medium chain glycerides by *Candida rugosa* lipase was investigated by utilising glycerol and caprylic acid. After parameter optimisation, a high purity of dicaprylin and tricaprylin was determined as potential commercial oleochemicals (Basri *et al*., 2001a).

Another high value-added palm derivative synthesis is palm kernel alkanolamide, normally used in baked goods, soup and as a cocoa butter substitute. The studies of fatty monoethanolamides proposed an easy *Candida rugosa* lipase-catalysed transamidation of palm kernel olein and palm kernel stearin in organic solvents at room temperature in mild conditions. Palm kernel olein had the higher conversion rate (77.0%) due to a greater number of shorter fatty acid chains if compared to palm kernel stearin (39.0%). Both fatty monoethanolamides are easily isolated and purified by extraction and crystallisation (Abdul Rahman *et al*., 2003a; Basri *et al*., 2001b).

With the strong bias towards the purity of enantiomers, sugar fatty acid esters program was refined in our research in the hunt of new functional esters (Abdul Rahman *et al*., 2010a). Several fatty acid gluocose-, fructose-, galactose-, lactose- and xylitol-based esters have been successfully synthesised using various lipases and optimised (Adnani *et al*., 2010). However, solubility of the substrates (sugar and fatty acids) and products remains the challenge to be defeated. These attractive sugar esters have a wide range of hydrophobic-lipophilic (HLB) values that suit many applications in food and flavours as well as high value surfactants with nutraceutical effects as summarised in Figure 5. Being tasteless, odourless and nontoxic, they are the best emulsifiers for foods and potential surfactants in drug delivery systems.

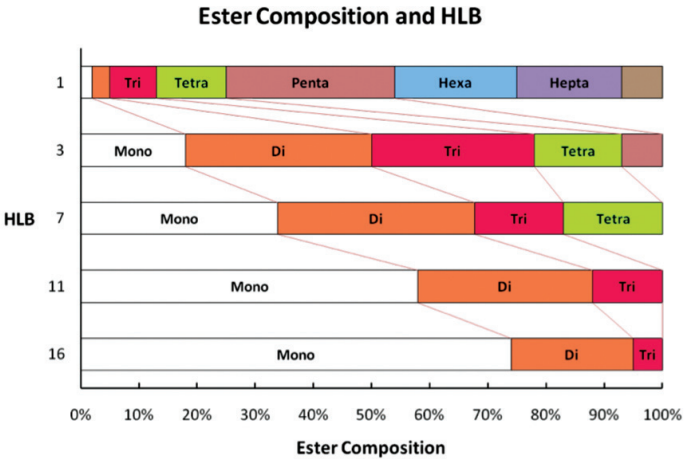


Figure 5 Sugar ester composition and its HLB value

Petro-based wax esters, which are derived from compounds of dicarboxylic acid and alcohol, are one of the most important classes of valuable raw materials. These specially formulated esters are synthesised due to their relatively low cost and good balance of properties using C_6 straight chain dicarboxylic acid, particularly adipic acid (Abdul Rahman *et al*., 2008a; Abdul Rahman *et al*., 2008b). The excellent properties of adipate esters such as its low toxicity, good thermal stability, low volatility and high biodegradability, make them very useful and significant for industrial and domestic purposes. Short chain adipate esters are most commonly used in manufacturing paint strippers, plasticisers, adhesives and in the coating industry. Medium chain adipate esters are frequently used as emollient esters in cosmetics, agrochemicals and pharmaceuticals. Long chain esters of adipic acid are used as food lubricants for the functions of stability, superior lubricity, corrosion protection and excellent performance at both high and low temperatures.

Syntheses of dicarboxylic acid esters including adipate and succinate esters were carried out through lipase-catalysed esterifications of dicarboxylic acid and monohydric alcohols with different chain lengths (C_1-C_{18}) and structures. The alcohol specificity of an enzyme is a determining parameter that affects the optimum conditions for synthesis of the esters. A high percentage of esterification (97%) was achieved for all the adipates in an organic solvent, at mild conditions and a short reaction time (Table 4) (Abdul Rahman *et al*., 2008a). The results imply that the maximum extent of esterification is independent of the alcohol chain length. Minimum reaction time for achieving maximum ester yield was obtained for butyl alcohol (C4). Methanol (C1) required an increased time and enzyme amount for attaining maximum yield due to inhibition of the enzyme. The maximum required temperature and time were obtained for octadecanol (C18) (Chaibakhsh *et al*., 2009a).

Table 4 The percentage conversion of petro-based adipate esters at optimal enzymatic reaction conditions

Product	Temperature (°C)	Time (min)	Enzyme amount (%w/w)	Alcohol to acid molar ratio	Conversion (%)
Dimethyl adipate	58.5	358	10.2	12.0	97.6
Di n-butyl adipate	63.2	215	2.5	8.8	98.2
Di iso-butyl adipate	54.0	220	1.5	6.0	97.0
Di sec-butyl adipate	55.0	264	2.5	6.4	96.3

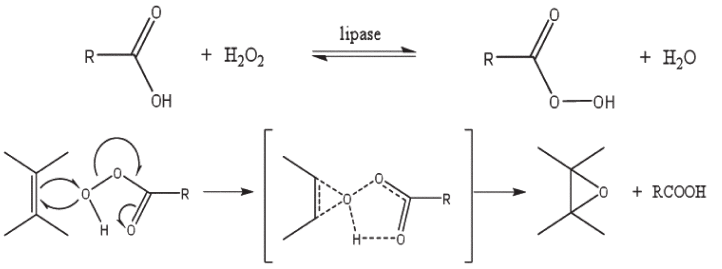
Dioctyl adipate	60.6	290	2.5	7.7	98.6
Didodecyl adipate	59.9	300	2.5	5.3	97.2
Dioctadecyl adipate	65.0	523	2.2	5.0	98.0

Succinates have potential applications as the starting material for producing bulk chemicals such as 1,4-butanediol (a precursor to biodegradable plastics), ethylene diamine disuccinate (a biodegradable chelator) and diethyl succinate (a "green" solvent replacement for dichloromethane) with a USD$15 billion market (McKinlay *et al*., 2007). The esterification percentage was 85.0% for the dioleyl succinate at the optimal conditions of 41.1°C, 272.8 min, 20 mg enzyme amount and 7.8:1 alcohol:acid molar ratio. The model can present a rapid means for estimating the conversion of succinic acid ester within the selected ranges (Abdul Rahman *et al*., 2010d). The results are useful for several industries especially lubricants, coatings and plastics that look for more environmentally acceptable and sustainable processes and products.

Recently, we developed a non-natural ester from a marine-based resource within 15 minutes of biocatalysis incubation, a chitosan adipate with a tunable property for veterinary applications. In recent studies, chitosan adipate was reported to be able to induce maturation and differentiation of thymocytes in mice. It also has been administrated into broiler chickens, which resulted in the reduction of *Salmonella galinarium* (Balicka-Ramisz *et al*. 2008).

Enzymes can also co-catalyse reactions in the presence of acid catalysts, known as chemo-enzymatic reactions. One example is a lipase-hydrogen peroxide system in epoxidation of vegetable oils through Prileshajev-epoxidation as illustrated in Scheme 2. If

the method is applied to an unsaturated plant oil, the peroxy acids generated will epoxidise the C=C-bonds, and the reaction product will be a mixture of epoxidised tri-, di- and monoglycerides, glycerols, and epoxy fatty acids (Klaas and Warwel, 1999). However, five mol % of fatty acids is adequate to suppress the formation of di- and monoglycerides completely.



Scheme 2 Chemo-enzymatic production of palm-based and fatty acid epoxides

We managed to maximise the epoxidation of palm-based oils (palm oil, palm kernel oil and palm olein) and free fatty acids (oleic acid, linoleic acid and ricinoleic acid) up by using Novozym 435/ H_2O_2 at mild conditions as summarised in Table 5. Monitoring the epoxidation of oils and fatty acids is mainly achieved by determining the oxirane number, which is a measure of the amount of epoxide's oxygen. The maximum oxirane number obtained is around 3.0 wt.%, which is very close to the theoretical maximum oxirane number calculated, indicating that the chemo-enzymatic epoxidation is an efficient way to produce palm-based epoxides, meanwhile up to 3.7 wt.% was achieved for epoxidised oleyl oleate followed by epoxidised oleyl linoleate and epoxidised oleyl ricinoleate.

Table 5 Determination of oxirane number from chemo-enzymatic epoxidation of palm oils and fatty acids

Epoxides	Palm Oil	Palm Olein	Oleic Acid	Linoleic Acid	Ricinoleic Acid
Oxirane Number (wt. %)	3.02	2.91	3.73	2.7	1.8
Conversion (%)	95.3	88.5	94.0	92.0	89.0

Modeling Optimisation

All enzymatic reactions are influenced by experimental conditions. Optimisation is an essential tool for improving the system and increasing the efficiency of the process without increasing the cost. Due to the nonlinear behaviour and complicated structures of biochemical processes, it is difficult to predict the effects of independent variables on the reaction yields. The sensitive nature of enzymes may potentially increase the complexity of the models. Response Surface Methodology (RSM) is an efficient statistical tool for optimising multiple variables to predict best performance conditions at the lowest cost and with the fewest experiments. Knowledge-based approaches such as Artificial Neural Network (ANN) are also useful tools for modeling and optimisation because of their adaptability, prediction ability and coping with nonlinearity. We have conducted several optimisation studies in various esterifications using these methods (Ahmad *et al*., 2010; Abdul Rahman *et al*., 2009a; Basri *et al*., 2007; Gunawan *et al*., 2005).

Fatty acid wax esters are fine chemicals, which are produced in low volumes but are highly priced with high profit margins. Many established synthetic ester routes are available but less suited to meet the stringent purity specifications. Large-scale enzymatic

productions of wax esters in bioreactors were conducted prior to commercialisation in order to fulfil the commercial demands of the products (Mat Radzi *et al*. 2005a). Oleyl oleate production in a 2 L stirred tank reactor with one multi-bladed impeller was optimised up to 97% in the presence of Novozyme435 (Mat Radzi *et al*. 2005b). Later, the larger scale-up bioreactor operation of up to 50 L successfully produced an excellent palm-based wax esters yield of more than 95% at optimum conditions and had a high percentage yield of more than 80% of wax esters even after 15 cycles of reaction (Keng *et al*., 2008).

Oleyl alcohol and adipic acid were mixed in the reactor equipped with a Rushton turbine impeller with a molar ratio of 5.3:1. Different amounts of Novozym 435, which were generated by central composite rotatable design (CCRD), were added to the mixture (Abdul Rahman *et al*., 2009a). To obtain a proper model for optimisation, the CCRD, which is generally the best design for response surface optimisation, was selected with four factors and five levels including temperature, reaction time, amount of enzyme, and impeller speed. By using RSM, the data was fitted to various models and their subsequent analysis of varience (ANOVA) showed that the reaction was most suitably described with a quadratic polynomial model according to the following equation:

$$y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i^{\,2} + \sum_{i=j}^{3} \sum_{j=i+1}^{4} b_{ij} x_{ij} + e$$

where y is the dependent variable to be modeled, x_i and x_j are the independent variables, b_0, b_i, b_{ii} and b_{ij} are the regression coefficients of model and e is the error of model.

The R^2 (0.99) implied that the model satisfactorily represented the real relationship of reaction parameters and the response. The normal probability plot also indicates that the residuals (difference

between actual and predicted values) follow a normal distribution and form an approximately straight line as shown in Figure 6.

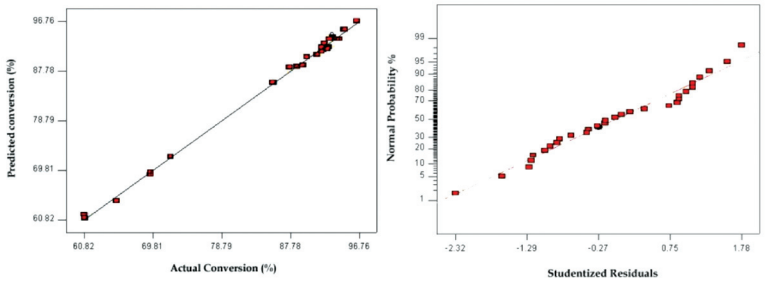


Figure 6 Plots showing the correlation between actual conversions and values predicted by the (a) model and (b) normal probability of residuals

Application of feedforward multilayer ANN trained by the Levenberg-Marquardt (LM) algorithm for modeling and optimisation of the operational conditions resulted in superior data fitting and prediction capability of ANN over RSM. The Levenberg-Marquardt training algorithm, which is a combination of gradient descent and Gauss-Newton iteration, is the fastest method for training moderate-sized feedforward neural networks (Al-Daoud, 2009). The algorithm for parameter updating (w) is presented by the following equation:

$$w_{k+1} = w_k - \left[J^T J + \mu I\right]^{-1} J^T e$$

where J is the Jacobian matrix, I is the identity matrix and μ is training parameter. The Jacobian matrix, J is obtained as:

$$J = \begin{bmatrix} \frac{\partial e_1}{\partial w_1} & \frac{\partial e_1}{\partial w_2} & \cdots & \frac{\partial e_1}{\partial w_N} \\ \frac{\partial e_2}{\partial w_1} & \frac{\partial e_2}{\partial w_2} & \cdots & \frac{\partial e_2}{\partial w_N} \\ \cdot & \cdot & \cdots & \cdot \\ \cdot & \cdot & \cdots & \cdot \\ \frac{\partial e_P}{\partial w_1} & \frac{\partial e_P}{\partial w_1} & \cdots & \frac{\partial e_P}{\partial w_N} \end{bmatrix}$$

The performance of ANN is dependent on the number and quality of data provided for training of the system. In addition, the performance of the trained network is estimated based on the accuracy of the network on a second set of data or test data. Finding the optimal number of neurons in a hidden layer is also an important factor for the accuracy of developed models. The optimum number of hidden neurons was found to be 7 for the developed network (Figure 7). The performance of the ANN model is measured by root mean squared error (RMSE) and coefficient of determination (R^2) between the predicted and experimental values. In this study, the values of R^2 and RMSE between the actual and predicted responses were determined as 1 and 0.0058178 for training and 0.99467 and 0.622540 for testing data sets (Abdul Rahman *et al*., 2009a).

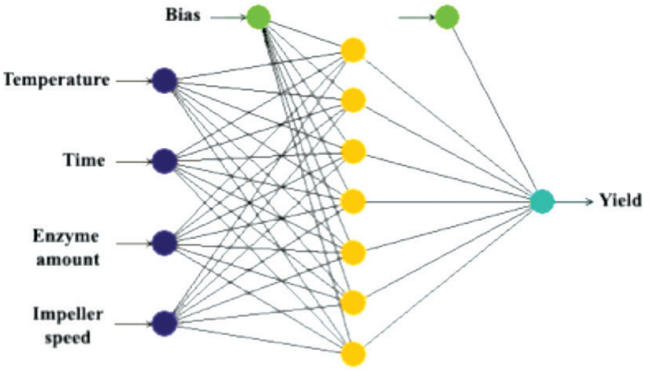


Figure 7 Structural organisation of the ANN used for the estimation of conversion in adipate ester synthesis

The operative variables affected the conversion with an order of contribution time > amount of enzyme > temperature > impeller speed. As shown in three-dimensional plots obtained by ANN analysis, all the selected variables had significant effects on the conversion of ester (Figure 8). Reactions with low temperature and low incubation time resulted in the lowest conversion. The conversion increased with increases in temperature up to around 55°C and thereafter decreased with further temperature increases to 75°C. The percentage conversion increased with increases in incubation time up to 346 min. The conversion of ester was found to increase gradually at higher impeller speeds above 100 rpm. Maximum conversion was obtained at 500 rpm. The conversion increased with increases in amount of enzyme up to 5.2% w/w. The results indicate that the volumetric productivity in solvent-free system is greater than that in organic solvent.

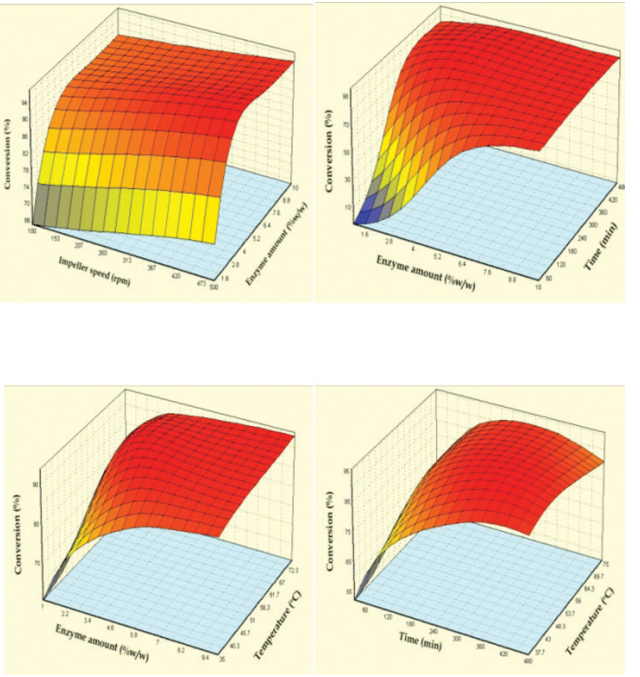


Figure 8 Three-dimensional plot showing the effect of impeller speeds, enzyme amount, reaction times and their mutual interaction on the synthesis of adipate ester

In an attempt to make the process more environmentally benign, synthesis of esters was performed in a solvent-free system. Although organic solvents provide several advantages in enzymatic reactions, their use in industrial processes is not desirable. They are a source of volatile organic compounds (VOCs) that affect the environment and human health, and their use requires costly post-treatment actions, larger and more expensive reactors and auxiliary equipment. High selectivity and volumetric productivity, improved substrate and product concentrations and fewer purification steps are some advantages to using solvent-free systems.

Enzymatic synthesis of gamma-keto esters, namely levulinate ester, was done in a solvent-free system resulting in a high conversion yield. The effect of main reaction parameters on the synthesis of ester was analysed. Under optimum conditions, 96% conversion of ethyl levulinate was obtained in a short reaction time, 42 min (Lee *et al*., 2010). Solvent-free Novozym 435-catalysed dioleyl adipate production in a 0.5 L stirred tank reactor was optimised to 96% using a low level of enzyme (2.5% w/w) by RSM (Chaibakhsh *et al*., 2009b).

Considering the advantages of using continuous bioreactors as opposed to the batch mode increased the potential for automating the process, reduced labour expenses due to automation, less non-productive time, consistent product quality and decreased toxicity risks to staff, the optimum conditions found in the batch stirred tank reactor was used in a 4 L continuous reactor (Chaibakhsh *et al*., 2010b). With a substrate flow rate of 5 ml/min, the conversion was nearly constant with an average of 93% throughout the process indicating the steady state operation of the reactor. The operational stability of Novozym 435 during the process was also examined where it retained high catalytic activity even after 28 hours and the percentage conversion decreased gradually from 90% to 70% after 70 hours.

Sustainable Product Design

A new eco-friendly process is paramount for advances in bioprocess technology. The use of biocatalysts for green organic synthesis, offering clean and mild reaction conditions provides opportunities to increase productivity, efficiency, purity and quality innovations. Furthermore, this green route may expand and diversify markets for food to agrochemicals.

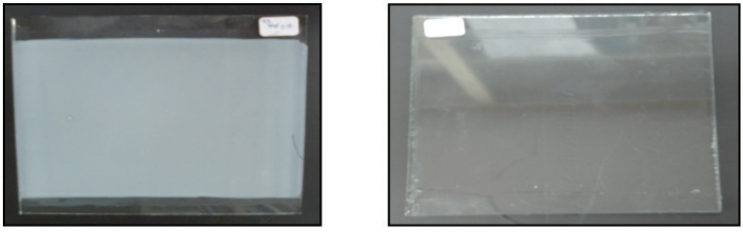
Cosmeceutical and drug nanodelivery systems have interesting and burgeoning applications for wax esters. A dermal irritation assay of palm oil esters hit a score below 0.9 of Human Irritancy Equivalent (HIE) shows the non-irritancy of the esters, meanwhile in acute moisturising tests, an increase in skin hydration of 40.7% after 90 min after application has proven the suitably of palm oil esters to be used in cosmetics (Keng *et al*., 2009). Recent advancements show both tocopherol acetate (vitamin E) and Pluronic F-68 to co-emulsify and co-stabilise the oil-in-water formulation for nanocosmeceuticals. The best formulation consists of 10% palm oil esters, 10% vitamin E, 24% Tween 80, 2.4% Pluronic F-68 and 53.6% deionised water. This composition has an average small particle size (94.21 nm), low occurrence of Ostwald ripening and stable at different storing temperatures (5, 25 and 45°C) for four weeks (Teo *et al*. 2010).

Nanoemulsions of palm-oil esters shows promise as a chemical penetration enhancer for trans-dermal drug and herbicide delivery in pharmaceuticals and agriculture. The long hydrocarbon chain of these fatty esters makes them ideal for producing oilier nanoemulsions. This highly hydrophobic system is a good carrier for hydrophobic drugs as it can hold the drugs until released by the drug-release mechanism (Abdul Rahman *et al*. 2010b). Economic and environmentally benign herbicide nanoemulsions were formulated in oil-in-water (O/W) by incorporating fatty acid methyl esters (FAMEs). The glyphosate formulations have highly soluble active ingredients and extend nanodelivery durations to control weeds such as *Asystasia gangetica*, *Diodia ocimifolia*, *Eleusine indica* and *Paspalum conjugatum* (Lim *et al*., 2010).

Developing waxes to serve as ingredients in coatings for wooden surfaces, with minimal pollutants, non-hazardous compounds and good biodegradability is seeing strong attention. The invention

of wax adipate-based esters to replace acrylic-based chemicals in nanocoating formulations for various surfaces has found new potential applications for commercialisation. Our recent study focused on the adhesion of epoxy acrylate oligomer and adipate ester (dioleyl adipate and dilauryl adipate) monomer (Abdul Rahman *et al*., 2010e). Initially, mixtures of epoxy acrylate and esters produced a whitish coating due to difficulties in achieving stable and fine droplet emulsions of epoxy acrylates through the physical emulsion process.

New formulations were obtained for radiation coatings with colourless mixtures using Brij 30, which has a 9.7 HLB value to solve adhesion problems (Parker *et al*., 2005). The final appearance of the formulation mixture is vital because it affects the film's coating. Figure 9 shows glass tiles after UV radiation with whitish and clear mixtures. Brij 30 has fewer polyoxyethylene chains and an average HLB value if compared to Tweens and Spans. Both factors might affect the solubilisation behaviour of this formulation. The materials of formulations need to be judiciously chosen, thus the characteristics of coatings should be further enhanced.

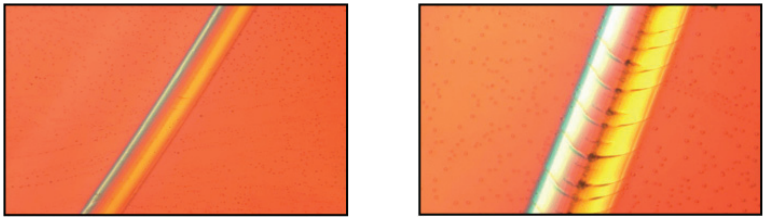


(a) (b)

Figure 9 Glass tiles surface after UV radiation (a) whitish film (b) clear coating

The adipate ester was mixed with polymer, surfactant and photoinitiator to undergo radiation-curing photopolymerisation. The coated film from this formulation gave good performance with gel content exhibiting more than 90% polymerisation (Kumar *et al*., 2006). The Köenig pendulum hardness test was found to be more than 50% hardness after a few cycles of radiation curing and infrared absorption bands correlated to the depletion of the C=C group. Both of these analyses were significant to determine the effect of irradiation passes and important mechanical properties.

Interestingly, the scratch resistance results of the surface coatings with a film thickness of 150 μm showed this formulation to be better than commercial or published results which are up to 4.5N weight loaded as depicted in Figure 10 (Salleh *et al*., 2002; Abdul Rahman *et al*., 2010e). The presence of dioleyl adipate improved properties of clear coatings on wood and glass surfaces. The replacement of acrylate by adipate esters, which are less toxic, is essential to meet a higher degree of safety and environmentally sustainable development.



(a) (b)

Figure 10 Scratch test on glass tiles surface showed (a) clear circle line (b) "fishbone" motif

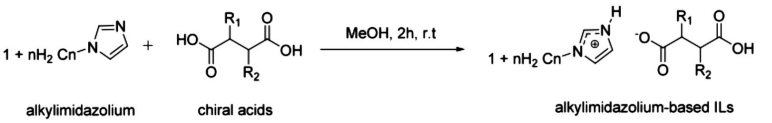
THE MOLECULES

Molten Marvels

Currently, molten organic salts below 100°C known as ionic liquids (ILs), have gained intensive attention in the science and industry communities as reaction media, extraction solvents and electrolytes. ILs have become popular and valuable substitutes for many volatile solvents, which can dissolve polar to non-polar substrates and almost anything including coal, plastics, woods, fibres and even rocks. It is not as flammable as classical volatile organic solvents due to negligible vapour pressure, making chemical processes safer and reducing environmental concerns. Thermodynamics and kinetics of reactions carried out in these liquids could be different than those in traditional solvents leading to a great interest in their potential use as solvents and co-solvents.

Chiral Ionic Liquids

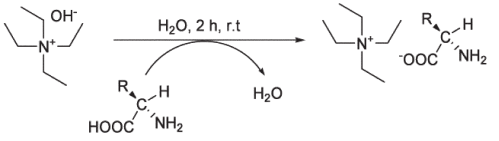
Chiral ionic liquids (CILs) can be synthesised from readily available starting materials containing chiral anions/cations or by modifying non-chiral materials to produce chiral products. However, the modification reactions are usually more complicated and many steps are required. By using starting materials such as lactates, sugars, sugar substitutes, plant acids and amino acids, a variety of CILs can be produced. Our expedition to synthesise CILs started early 2006 in collaboration with the famous Prof. Kenneth R. Seddon from Queen's University Ionic Liquids Laboratory (QUILL), Belfast. The first attempt to prepare CILs was by manipulating alkylimidazolium derivative cations with different chiral acids as counterions via ion exchange reactions as generalised in Scheme 3.



Scheme 3 General route to synthesis alkylated imidazolium-based chiral ionic liquids

Ionic liquids (ILs) can be divided into two categories: hydrophilic and hydrophobic ILs depending on the nature of anions, cations and the length of alkyl chains. Eighteen alkylimidazolium-based CILs showed hydrophilic-prone properties and were easily dissolved in water due to the presence of hydrogen bonding interactions between the cations-anions and molecules of water (Abdul Rahman *et al*., 2007a). Interestingly, the modified cation with increasing chain length of alkyl substituent on the alkylimidazolium ring influenced the physical properties of solids to viscous liquids. Among the alklyimidazolium cations synthesised were methylimidazolium, butylimidazolium and hexylimidazolium. Despite tremendous work with the alkylimidazolium-based CILs, the second generation ILs still pose some environmental problems.

The quaternary ammonium cation was selected together with life-based chiral acids for its low toxicity. Scheme 4 followed a simple and atom economic synthesis of new tetraethylammonium ($[N_{2222}]$) CILs involving a straightforward pathway of neutralisation reactions between tetraethylammonium hydroxide with selected chiral amino acids and plant acids (Abdul Rahman *et al*., 2009c).



Scheme 4 Synthetic pathway of tetraethylammonium-based CILs

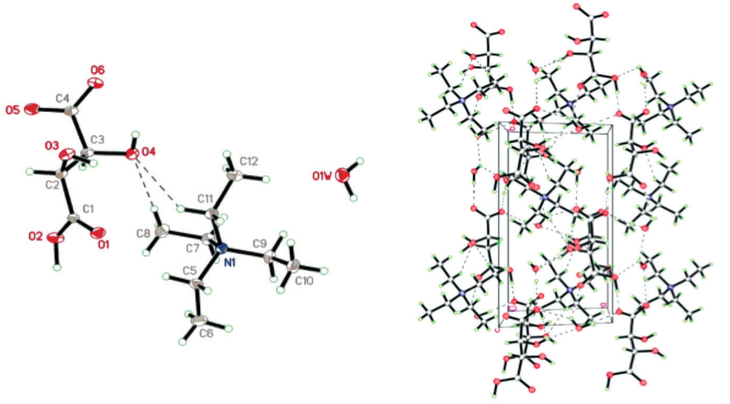
Since amino acids contain more than one functional group in a single molecule, this method has an advantage because the by-product is only water, thus avoiding further extensive purification treatment other than water removal. Based on this reaction, we have synthesised and characterised a new set of tetraethylammonium-based CILs for life-science purposes (Abdul Rahman *et al*., 2010c). Table 6 shows the physical properties of selected alkylimidazolium-based and tetraethylammonium-amino acids.

Table 6 Physical properties of selected synthesised CILs

No	Chiral Ionic Liquids	Abbreviation	Physical Properties	Melting Point (°C)	Yield (%)
1	Butylimidazolium L-lactate	[Bim][L-lac]	Yellowish liquid	- 68 ± 3.0	96
2	Hexylimidazolium L-lactate	[Bim][L-lac]	Yellowish liquid	Nd	98
3	Butylimidazolium L-tartarate	[Bim][L-tar]	Yellowish liquid	Nd	97
4	Hexylimidazolium L-tartarate	[Him][L-tar]	Yellowish solid	51 ± 2.0	98
5	Butylimidazolium champor-10-sulfonate	[Bim][sulf]	Colourless liquid	Nd	97
6	Hexylimidazolium champor-10-sulfonate	[Him][sulf]	Colourless liquid	nd	97
7	Methylimidazolium L-malate	[Mim][L-mal]	Colourless liquid	nd	98
8	Butylimidazolium L-malate	[Bim][L-mal]	Colourless liquid	nd	99
9	Hexylimidazolium L-malate	[Him][L-mal]	Semi solid	nd	96
10	Tetraethylammonium L-serinate	$[N_{2222}]$[ser]	Orange liquid	nd	96
11	Tetraethylammonium L-prolinate	$[N_{2222}]$[pro]	Yellowish liquid	nd	90
12	Tetraethylammonium L-threoninate	$[N_{2222}]$[thr]	Colourless liquid	nd	94
13	Tetraethylammonium L-isoleucinate	$[N_{2222}]$[ile]	Yellowish liquid	nd	87
14	Tetraethylammonium L-asparaginate	$[N_{2222}]$[asn]	White solid	58 ± 1.0	92
15	Tetraethylammonium L-histidinate	$[N_{2222}]$[his]	Orange solid	54 ± 1.0	87
16	Tetraethylammonium L-glutaminate	$[N_{2222}]$[gln]	Colourless liquid	nd	91
17	Tetraethylammonium L-glutamate	$[N_{2222}]$[glu]	Colourless liquid	nd	89
18	Tetraethylammonium L-methionine	$[N_{2222}]$[met]	Yellowish liquid	nd	95

nd : not detected

Tetraethylammonium L-tartarate was crystallised by slow evaporation for three days (Abdul Rahman *et al*., 2008c). The asymmetric unit of the title compound shows a monoclinic crystal system with space group P_{21}, containing a tartarate anion as a counterion, a tetraethylammonium cation and two water molecules of crystallisation in the structure (Figure 11). Two intermolecular C—H---O hydrogen bonds involving O4 as a bifurcated acceptor link anion and cation in the asymmetric unit to form a seven-membered ring, with R(7) ring motif. These formations of anion-cation pairs connected by two hydrogen bonds were found with C8—H8---O4 distance of 0.96 Å or O---O distance of 3.34 Å and C11—H11---O4 distance of 0.97 Å or O---O distance 3.26 Å, respectively.



(a) (b)

Figure 11 The molecular structure of tetraethylammonium L-tartarate dihydrate (a) showing 50% probability displacement ellipsoids and the atomic numbering (b) crystal packing as viewed down the *c*-axis. Hydrogen bonds are shown as dashed lines
(Abdul Rahman *et al*., 2008c)

Physico-chemical properties of CILs such as melting point, decomposition temperature, solubility, density, viscosity, conductivity, hydrophobicity and hydrophilicity can be fine-tuned by varying the cations and/or anions or both. We found that the charge, size of anion, hydrogen bonding and Coulombic interactions occurring in the structure of CILs also affects the melting point. As the size of the anion increases, the melting point of the salt decreases reflecting the weaker Coulombic interactions in the crystal lattice. Another factor that influences the melting point of CILs' salts is the effect of hydrogen-bonding interactions between cations-anions. We observed that, the lower decomposition temperature of these CILs is the result of Hoffman elimination reaction of tetraethylammonium salts, a good indication for the decomposition mechanism in nature (Abdul Rahman *et al*., 2010c).

The conductivity of an electrolyte is a measure of the available charge carriers and their mobility. Superficially, one would expect ILs to possess very high conductivity values because they are composed entirely of ions. Unfortunately, this is not the case. ILs possesses reasonable good ionic conductivities comparable to that of the non-aqueous solvent or electrolyte (generally up to 10 ms cm^{-1}). These new tetraethylammonium-amino acids CILs showed ionic conductivity values ranging from 0.16–0.54 ms cm^{-1}, much higher than those of other reported ILs. Meanwhile, their viscosity is closely related to ionic conductivity.

Generally, ILs are more viscous than common organic solvents. For comparative purposes, the viscosity of water, ethylene glycol and glycerol at room temperature are at 0.89, 16.10 and 934.00 cP respectively. Selected tetraethylammonium-amino acids CILs follows the linear relationship between ionic conductivity and viscosity as shown in Figure 12. This linear correlation indicated that at 25°C, the conductivity value is strongly affected by

viscosity, and thus the salts with low viscosity showed high ionic conductivities. The cation structure influences the viscosity, which is governed essentially by van der Waals interactions and hydrogen bonding (Abdul Rahman *et al*., 2010c).

The short and flexible alkyl chains of tetraethylammonium cation make the salts less viscous due to a decrease in van der Waals interactions and an increase in ion mobility of the salts, ensuring the high ionic conductivity of the new CILs. Higher viscosity than conventional ILs may due to the symmetry properties of the cation's structure, where symmetrical structures result in more viscous or solid ILs. Furthermore, the side chain of the corresponding amino acids and hydrogen bonding interactions in the structure of CILs would also affect the viscosity and ionic conductivity. In other words, the introduction of hydrogen bonding donor or acceptor increases the viscosity and decreases the ionic conductivity through intra/intermolecular interactions.

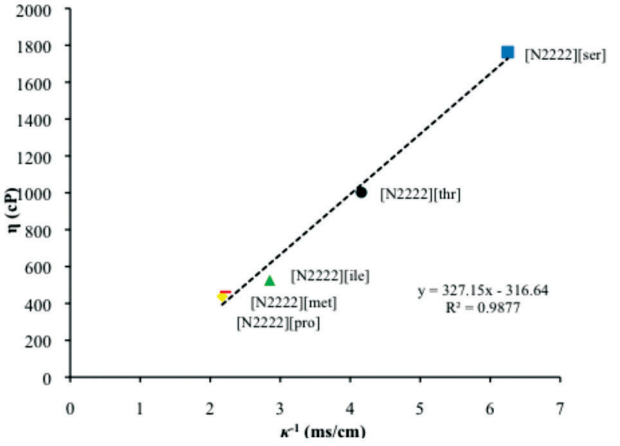


Figure 12 Relationship between ionic conductivity (κ^{-1}) and viscosity (η) for five tetraethylammonium-based chiral ionic liquids at 25 °C (Abdul Rahman *et al*., 2010c)

The experimental result of viscosity was supported by theoretical calculation by validation of the force field parameterisation for ILs (Abdul Rahman *et al*., 2010f). Table 7 reports the viscosity of amino acid ionic liquids (AAILs) obtained experimentally and by molecular dynamics (MD) simulations. Fukumoto *et al*. (2005) also reported that the side chains of the corresponding amino acids' anions and hydrogen bonding in the structure of chiral ionic liquids strongly affected the viscosity and ionic conductivity. The introduction of functional groups such as hydrogen bonding donors or acceptors at the side chains of the amino acid anion, i.e. $[N_{2222}]$ [L-Gln] increases the viscosity through intermolecular hydrogen bonds formed between the amino hydrogen and carbonyl oxygen atom (Figure 13a). This is usually due to increased molecular structure in hydrogen-bonded systems, which decreases the freedom of molecular motion and therefore tends to increase viscosity (Micaelo *et al*. 2006). Table 8 shows that the viscosity of $[N_{2222}]$ [L-Gln] depends on hydrogen bonding because it has a higher number of hydrogen bonds if compared to the other AAILs.

Table 7 Experimental and calculated viscosity values for the tetraethylammonium-based AAILs at 298.15 K

AAIL	**viscosity (cP)**	
	MD	Exp[a]
$[N_{2222}]$[L-Gln]	3476.52 (±735.58)	3450.0
$[N_{2222}]$[L-Ile]	521.25 (±171.97)	526.8
$[N_{2222}]$[L-Lys]	380.35 (±117.85)	352.3

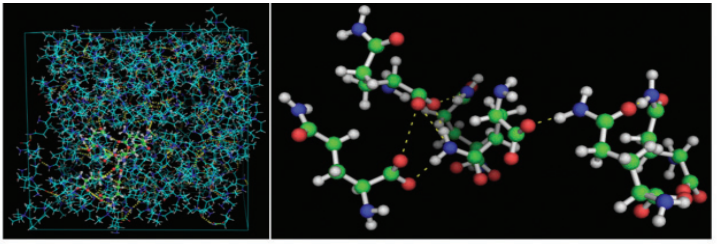
[a]Experimental values are from Abdul Rahman *et al*. 2010c

Table 8 Calculated hydrogen bonding values for tetraethylammonium-based AAILs

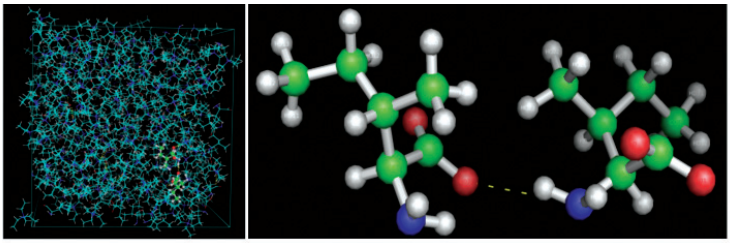
AAIL	number of hydrogen bonding	[a]r_{hb} (Å)	[b]α (°)
$[N_{2222}]$[L-Gln]	196.5	1.625	0.033(±0.009)
$[N_{2222}]$[L-Ile]	65.8	1.575	0.033(±0.036)
$[N_{2222}]$[L-Lys]	160.1	1.625	0.033(±0.012)

[a]r_{hb} stands for the length of hydrogen bonding; [b]α (°) stands for hydrogen bonding angle

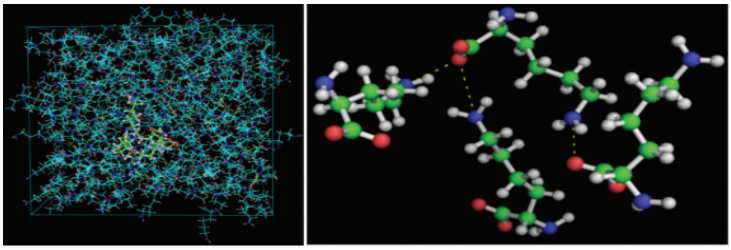
Additionally, the viscosities of liquids made up of molecules with a straight chain (normal aliphatic hydrocarbons) are low. We also found that the viscosity of AAILs with branched side chains of the amino acid anions, i.e. $[N_{2222}]$[L-Ile] is higher if compared to a straight alkyl chain i.e. $[N_{2222}]$[L-Lys] (see Figure 13b and 13c). The density (ρ) of AAILs can be directly obtained from the MD simulation results (Abdul Rahman *et al*., 2010f). We calculated the density of our model system based on the equilibration simulations of 2 ns. Liu *et al*. (2004) reported that the experimental density could also be used to confirm a proposed force field of room temperature ionic liquids rather than viscosity.



(a) Tetraethylammonium L-glutaminate $[N_{2222}]$[L-Gln]



(b) Tetraethylammonium L-isoleucinate $[N_{2222}]$[L-Ile], branched side chain on the amino acid anion



(c) Tetraethylammonium L-lysinate $[N_{2222}]$[L-Lys], straight alkyl side chain on the amino acid anion

Figure 13 Hydrogen bonding (shown as dashed yellow lines) for AAILs using molecular dynamics

From Figure 14, the densities for AAILs with tetraethylammonium cation, $[N_{2222}]^+$ follow this order: $\rho_{[L\text{-}Lys]} > \rho_{[L\text{-}Ile]} > \rho_{[L\text{-}Gln]}$, which are in agreement with the experimental densities for AAILs in the range of 0.9-1.0 g/cm^3 (Sirjoosingh *et al*., 2009). Theoretically, the densities for AAILs with tetraethylammonium cation, $[N_{2222}]^+$ should follow this order: $\rho_{[L\text{-}Gln]} > \rho_{[L\text{-}Lys]} > \rho_{[L\text{-}Ile]}$. ; this is because the presence of polar groups on the amino acid side chain increases the AAILs' density cm^3 (Gardas *et al*., 2010). However, the calculated density for $[N_{2222}]$[L-Gln] does not follow the theoretical order. Nevertheless, the calculated viscosities for these three AAILs are consistent with reported experimental results. Therefore, we propose

that the densities of our model AAILs are acceptable and consistent with reported experimental results.

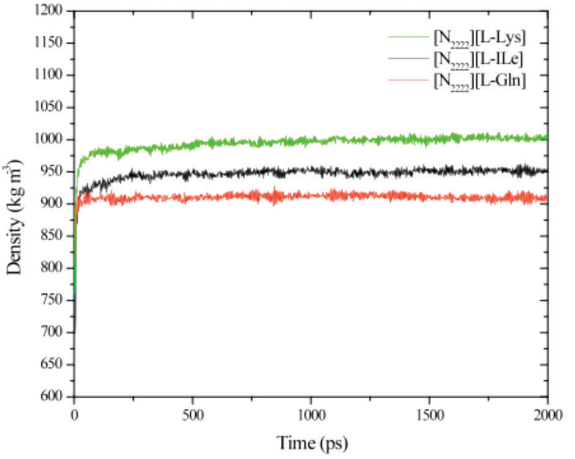


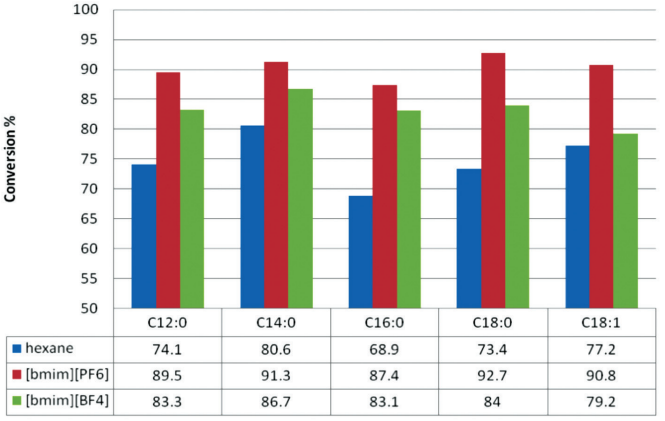
Figure 14 A representative plot of density against time. This graph clearly shows that the ionic liquid simulations require several hundred picoseconds to fully converge the system's density. The green line represent $[N_{2222}]$[L-Lys] , the black line $[N_{2222}]$[L-Ile] and, The red line represents $[N_{2222}]$[L-Gln]

Nonaqueous Biocatalysis

Nonaqueous biocatalysis provides a playful and innovative methodology in bioorganic syntheses. With little or no water, research in this area surged with the use of organic solvents (Salleh *et al*., 2002). However, it often suffers from reduced activity, selectivity or stability, and environmental concerns. Recently ILs have become a new class of alternative green media due to their unique properties and reusability. In biotransformation, one of the most exploited properties of ILs is the ability to enhance solubility of polar substrates and products without inactivating the enzymes or whole cell systems. The enhanced solubility of substrates usually

increases the conversion and often increases the selectivity of the products especially regioselectivity or enantioselectivity from a chiral pool. Ionic liquids also can be co-solvents in enzymatic reactions (Rantwijk and Sheldon, 2007).

A series of palm-based esters were synthesised namely oleyl laurate, oleyl myristate, oleyl palmitate, oleyl stearate and oleyl oleate in ionic liquids (Figure 15). A hydrophobic IL, 1-Butyl-3-methylimidazolium hexafluorophosphate, [bmim][PF_6] and a hydrophilic IL, 1-butyl-3-methylimidazolium tetrafluoroborate, [bmim][BF_4] were tested in the esterification of oleyl alcohol with fatty acids using *Candida rugosa* lipase. The best results were obtained with [bmim][BF_4] and [bmim][PF_6] media in the synthesis were 86.7 % and 92.7 % of conversion respectively (Abdul Rahman *et al*., 2010g). The hydrophobicity of [bmim][PF_6] was suitable for free lipase such as *Candida rugosa* as it does not strip the essential water layer on the enzyme, thus preventing denaturation (Lozano et. al., 2003) and reducing direct protein-ion interactions between *Candida rugosa* and [bmim][PF_6].



	C12:0	C14:0	C16:0	C18:0	C18:1
hexane	74.1	80.6	68.9	73.4	77.2
[bmim][PF6]	89.5	91.3	87.4	92.7	90.8
[bmim][BF4]	83.3	86.7	83.1	84	79.2

Figure 15 Esterification of fatty acids using *Candida rugosa* lipase

Lipase-catalysed esterification in ionic liquids was further investigated using fructose and fatty acids. It is important that the selected solvents dissolve enough substrate to allow the lipase-catalysed esterification. Esterification was performed in *tert*-butanol and different types of ionic liquids such as [bmim][BF_4], [bmim][PF_6] and 1-butyl-3-methylimidazolium trifluoromethanesulfonate, [bmim][TfO]. The effects of different solvents on lipase-catalysed synthesis of fructose oleate at 60°C were evaluated as in Figure 16. Even though, [bmim][TfO] is actually hydrophilic, it gave the highest conversion of 58% after 24 hours which is due to the high solubility of fructose. Moreover, some studies also revealed relatively high enzyme activities in hydrophilic ILs such as 1-ethyl-3-methylimidazolium tetrafluoroborate, [emim][BF_4] and 1,3-dimethylimidazolium methylsulphate [mmim][$MeSO_4$] (Park and Kazlauskas, 2003). The highest yield of sugar ester was obtained in ionic liquid ([bmim][TfO]) with 73.1% conversion after 48 hr with 2:1 ratio of fructose over oleic acid, at 60°C.

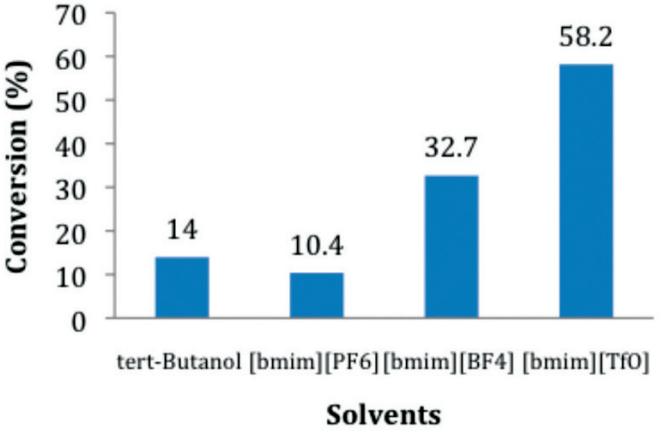


Figure 16 Comparison of different solvents in the lipase-catalysed synthesis of fructose oleate ester

Biocatalytic reactions in ionic liquids show high selectivity, great enzyme stability and fast rates to produce galactose oleate ester. Four different parameters; type of ionic liquids and lipases, rotational speeds and reaction times were studied to obtain optimum conditions. With ionic liquid as the reaction media, we have been able to reach a conversion rate of more than 80% whereas with organic solvents such as acetone and *tert*-butanol, the maximum conversion was only up to 40%. Highest conversion of up to 85% was achieved in 1-butyl-3-methylimidazolium bis(trifluoromethanesulfonyl) imide, [bmim][Tf_2N] with Lipozyme TLIM as biocatalyst at 60°C for 2 hours (Figure 17). Galactose has low solubility in all screened ionic liquids. Addition of dimethylsulfoxide (DMSO) as a co-solvent and solubilising agent helped to overcome this problem but the conversion decreased after 2 h for all lipases, which may due to lipase deactivation by DMSO.

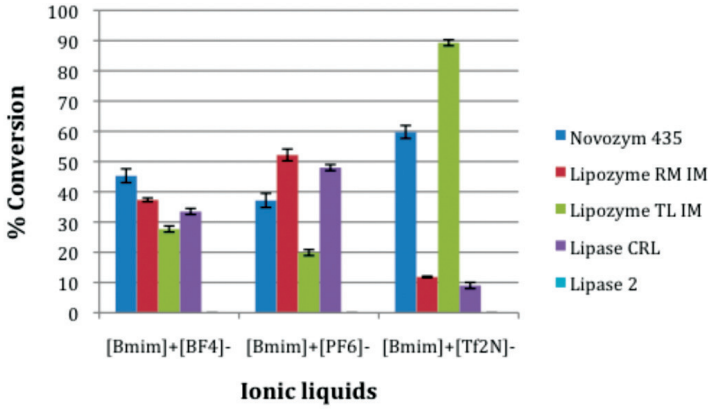


Figure 17 Screening of ionic liquids and biocatalysts for the production of galactose oleate ester

A biphasic solvent system comprising of [bmim][BF_4] and *tert*-butanol was examined for glucose oleate synthesis. The optimisation

of reaction parameters, in terms of temperature (40°C and 60°C), time (15 minutes and 30 minutes) and biocatalyst (*Candida rugosa* lipase and Novozym 435), were studied in a microwave reactor in comparison to other conventional methods with the aim of validating the efficiency of the microwave irradiation. Highest conversion of 90% was attained in microwave and the optimum conditions were found using Novozym 435, 60 °C, 30 minutes and 290 watts.

Our new approach in synthesising amino sugars, also tagged as revolutionary ester is meant for osteoarthritis therapeutic applications. Within 2 hours at 45°C using Novozym 435, we managed to achieve more than 70% ester conversion (7 fold better than in a single solvent) in a triphasic media of [bmim][PF_6], DMSO and *tert*-butanol. Optimum conditions reflect well on the low cost of production of amino sugars with high product conversion but with minimum enzyme usage.

Betulinic acid derivatives were successfully synthesised from reactions of betulinic acid with a series of anhydrides using an enzyme as a biocatalyst in ionic liquids. Initial screenings revealed that the ester produced from the reaction of betulinic acid with 3-methyl phthalic anhydride showed that the highest product conversion approximately 50% in ionic liquids, 1,3-dimethylimidazolium-bis(trifluromethanesulfonyl)imide, [mmim][NTf_2] catalysed by Novozym 435. High solubility of betulinic acid and anhydride in ionic liquids leads to a better yield and enzymes activities were maintained. [mmim][NTf_2] had the highest conversion due to its high hydrophobicity (large log P) of ILs, which is beneficial to enzyme stabilisation. Although high hydrophilicity would inactivate enzymes, it is also important as it allows for interactions and breaking of hydrogen bonds between substrates and enzymes.

RSM studies showed interactions between temperatures with a substrate molar ratio of 3-methyl phthalic anhydride has significant effects on the esterification of betulinic acid reaction (Figure 18). The optimum conditions derived via RSM were reaction time of 25.71 h, temperature of 49.48°C, enzyme amount of 99.52 mg (Novozyme 435) and substrate molar ratio of 2.78 in [mmim][NTf_2]. The actual experiment yielded 71.1% of betulinic acid ester conversion under optimum conditions, which are comparable to those predicted in RSM (71.68%) (Abdul Rahman *et al*., 2010h). Product conversion decreased at high molar ratio of substrate due to reduction of enzyme activity. Decrement of enzyme effectiveness may be caused by direct effects of the substrates on the enzymes giving what would be seen as substrate or product inhibition (Moghaddam *et al*., 2010a; Moghaddam *et al*., 2010b).

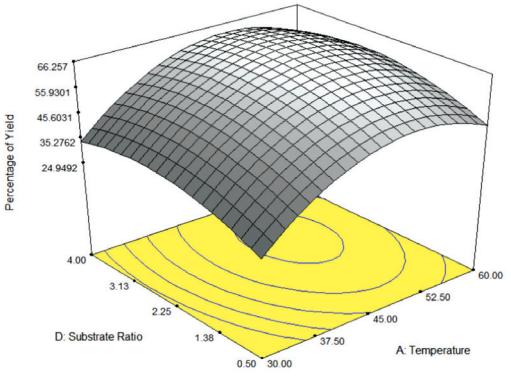


Figure 18 Response surface plot of temperature versus substrate molar ratio (X^2X^4) for quadratic model on the esterification of betulinic acid reaction

ILs can also be used as catalysts and co-catalysts especially in organic synthesis. In this section, we describe our work based

on chiral ionic liquid-coated enzyme (CILCE), a new type of immobilised enzyme as a biocatalyst in non-chiral and chiral esterification reactions. Native *Candida rugosa* lipase (CRL) was coated with four different CILs; hexylimidazolium L-tartarate, hexylimidazolium L-malate, tetraethylammonium L-asparaginate ($[N_{2222}]$[asp]) and tetraethylammonium L-histidinate since their melting points are below 60°C. Solid CILs were first melted before enzyme in powder form was added and the mixture was stirred. The heterogeneous solution was then allowed to cool until CIL-CRL mixture solidified. The small CILCE particles were then used without any further treatment (Abdul Rahman *et al*., 2007a).

Non-chiral esterification reactions between oleyl alcohol and various acids were carried out using tetraethylammonium L-asparaginate-coated enzyme (CILCE-$[N_{2222}]$[asp]) as a biocatalyst. The esterification using CILCE-$[N_{2222}]$[asp] showed a higher percentage of conversion of fatty acid esters compared to native CRL (Figure 19). Overall, it can be seen that CILCE in most cases are more effective than native CRL for short, medium and long alkyl chain acids. In addition, CILCE did not aggregate and the coating ILs are stable and were not removed from the enzyme surface during the reaction.

The presence of hydrogen-bonded nanostructures with polar and non-polar regions may be responsible for the stabilisation of enzymes coated in tetraethylammonium L-asparaginate that can maintain their functionality under extreme denaturative conditions. Thus, both the solvophobic interactions are essential to maintain the native structure and the water shell around the protein molecules, which are preserved by the 'inclusion' of the aqueous solution of free enzymes into the CILs' network. This will promote a clear enhancement of the enzyme stability.

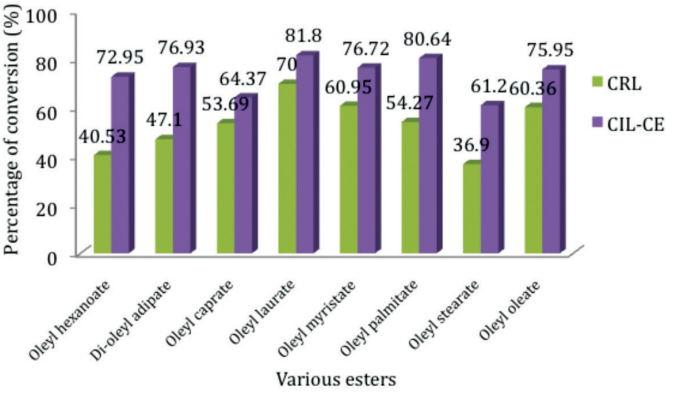
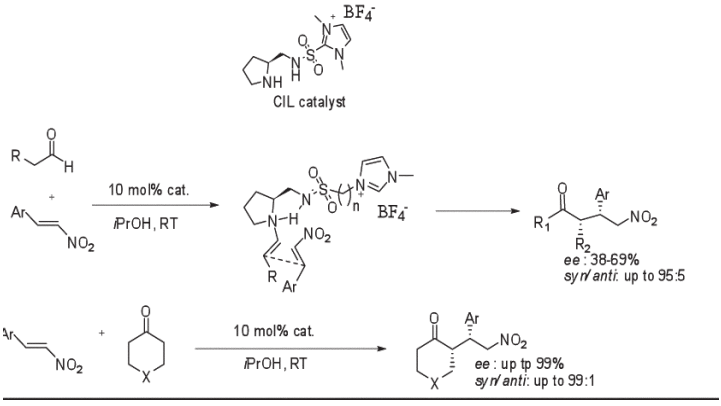


Figure 19 Esterification of fatty acid esters by using tetraethylammonium L-asparaginate-coated enzyme and *Candida rugosa* lipase in hexane at 40°C for 1 hour

The hexylimidazolium L-tartarate-coated enzyme was also described for enantioselective esterification of (±)-menthol with butyric anhydride as an acyl donor in organic solvent. High percentage of conversion up to 81.41 % of (±)-menthyl butyrate after 24 hrs incubation was achieved by CILCE, which is 3 times the percentage conversion of CRL (28.19 %). However, CILCE managed lower enantiomeric excess (*ee*) of 47.7 % and enantiomeric ratio of 1:1 with native CRL. This was due to different isoenzymes in commercial CRL and the use of a recombinant pure isoenzyme would exhibit significantly higher enantioselectivity. Thus, different CILs for enzyme coating may shed new light in the enzymatic resolution for chiral esterification reaction (Abdul Rahman *et al*., 2007a).

Development of chiral ionic liquid-based organocatalyst as an effective and novel organocatalyst for the asymmetric addition reactions is becoming a major focus recently in the organic

syntheses community especially with chiral catalysts associated with formation of C-C and C-O bonds. This chiral asymmetric catalyst is powerful in the catalytic synthesis of enantiomeric pure organic compounds that can be carried out under mild reaction conditions if compared to chiral homogenous catalysts, which normally pose problems related to the separation and recycling of expensive precious metals. Together with Prof. Allan D. Headley at Texas A&M University-Commerce, we developed proline-supported CILs that catalyse asymmetric Michael addition reactions of aldehyde and ketones to nitroolefins as proposed in Scheme 5.



Scheme 5 CIL organocatalyst catalysed asymmetric Michael additions of aldehydes and ketones to nitrostyrenes

The proline-based CILs' catalysts were found to be very effective organocatalysts for Michael additions, affording products with high yields (98%), excellent diastereoselectivities (syn/anti: up to 99:1), and enantioselectivities (up to 99% *ee*) at room temperature for ketones to nitroolefins. However, high yields (up to 98%), high diastereoselectivities (syn/anti: up to 95:5) and moderate *ee* (38-69%) were observed in aldehydes to nitroolefins. This indicated

that aldehyde was a reactive Michael donor if compared to ketone since it was found to exhibit a reaction time of less than 24 h. Most of the reaction times were 3-5 h without an acidic additive.

From the Michael addition reactions, it was believed that the enamine intermediate provides some stability and facilitates the reaction rate. The mechanism proposed that the N-H acidic from L-proline play a vital role by forming hydrogen bonds with the nitroolefins by stabilising the transition state. It will influence the catalytic activity and selectivity to the reaction. Moreover, most of these CILs are readily recovered and reusable several times without significant loss of catalytic activity and stereoselectivity. Further synthesis of these designer proline-based organocatalysts should also be expanded to other suitable natural and non-natural amino acids and for other asymmetric reactions (Ni *et al*., 2007). Even though the field of chiral ionic liquids is still in its infancy, researchers believe in its tremendous advantage and additional benefits to our future.

Dispelling Dissolution

Lignocellulosic biomass is composed mainly of three constituents, which are cellulose, lignin and hemicelluloses. Cellulose, as the most abundant renewable organic material exhibits outstanding properties and useful applications, also poses a tremendous challenge to economical and environmentally friendly chemical processing. ILs are alternative solvents for a large number of biomacromolecules, like cellulose (Zhu *et al*., 2006). Three selected ILs were chosen to dissolve oil palm biomasses (empty fruit bunch (EFB); oil palm frond (OPF); oil palm trunk (OPT)) based on their polarity, 1-butyl-3-methylimidazolium chloride ([bmim][Cl]), 1-ethyl-3-methylimidazolium chloride ([emim][Cl]) and 1-ethyl-3-methylimidazolium acetate ([emim][ac]).

The morphology changes of oil palm fibres dissolved in ILs via swelling and dissolution were observed by optical microscope. Oil palm fibres were systematically dissolved quickly in [emim][Cl] at 100°C, called mode 1 (fragmentation of fibres and dissolution) as can be seen in Figure 20. Mode 1 requires good solvents like an ionic liquid where cations and anions contained in ILs can interrupt the hydrogen bonds between cellulose chains (Swatloski *et al*., 2002). Five modes of interactions can be considered for dissolution of fibres in ionic liquids, which are:

Mode 1: fast dissolution by disintegration into fragments
Mode 2: large swelling by ballooning, and dissolution
Mode 3: large swelling by ballooning, and no dissolution
Mode 4: homogeneous swelling, and no dissolution
Mode 5: no swelling, and no dissolution

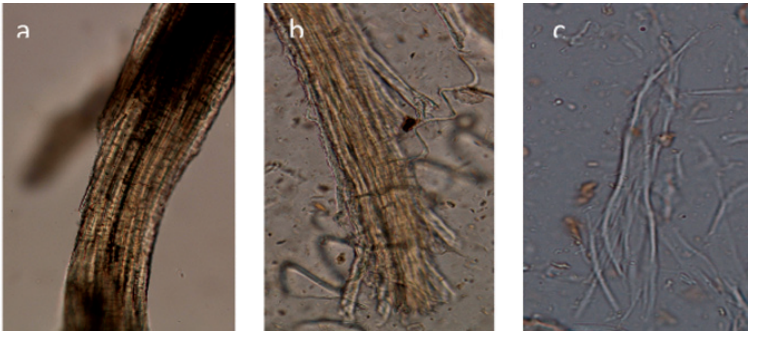


Figure 20 Dissolution by disintegration into fragments (mode 1). 5 wt% of EFB fibres dissolved in [emim][Cl] where (a) in IL at 0 hour, (b) 1 hour after heating and (c) after 4 hours of heating at 100°C

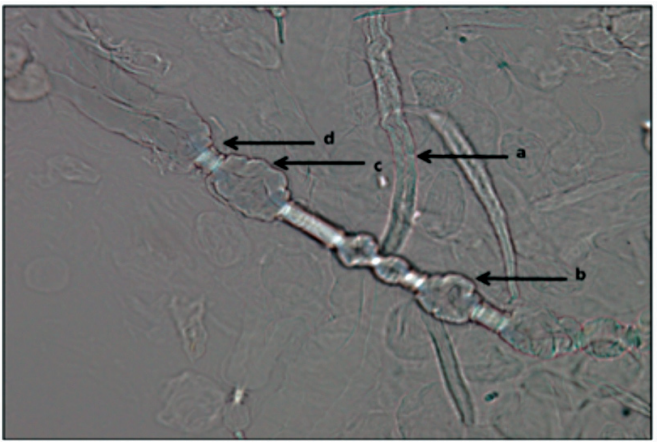


Figure 21 Several defined zones of cellulose of oil palm fibres, including EFB, OPF and OPT exhibited during the swollen and dissolution process

However, after the removal of non-cellulosic components, the structure of a swollen fibre consists as several well-defined zones: unswollen fibre (a), helical membrane (b), balloon (c) and unswollen fibre between two balloons (d), as seen in Figure 21. Each of these zones has a specific way of swelling and dissolving.

Phase 1: balloon formation

Phase 2: balloon dissolution

Phase 3: dissolution of the unswollen sections

Phase 4: dissolution of the balloon membrane scraps

Somehow, the mechanism does not occur all along the fibre at the same time, but at more or less regular intervals, which gives the shape of so-called balloons in the swelling zones (Phase 1) (Figure 22). However, the helical feature, which can be seen around the balloons, makes one turn along one balloon length (or between two adjacent unswollen sections). The unswollen sections dissolve

without swelling as can be seen in Figure 23 (Phase 3). Swollen and dissolution mechanisms for lignocellulosic oil palm fibres mainly EFB, OPF and OPT, cotton and wood fibres in ionic liquids are similar, as soon as non-cellulosic components are removed. This major finding may assist in the control of abundant biomass and enhance the capability of ILs, hence eliminating the harsh chemical conditions used in the treatment of lignocellulosic materials (Abdul Rahman *et al*., 2010i).

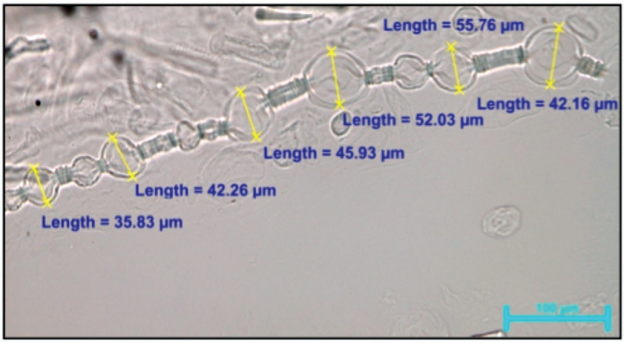


Figure 22 Measured length of the balloons, which formed all along the cellulose fibres after being dissolved in an ionic liquid

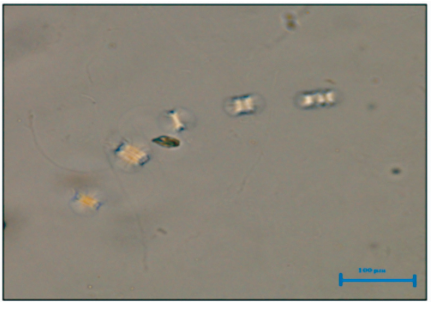


Figure 23 Third phase of Mode 2. The balloons have burst. Membrane scraps (very thin and not clear in the picture) and the unswollen sections that were between the balloons remain, seen as regularly spaced small cylinders

THE BIOCATALYSTS

Creative Catalysts

Industrial biotechnology is no longer a dream as it is expected to take over one third of all chemical processes by 2025 as depicted in the Lisboa Objectives for the European Union. A good biocatalyst is a vital player to propel white biotechnology. The major obstacles are still the engineering and the biocatalyst, where both are very costly for capital engagement. Finding a way to draw the cost down will not be achieved overnight, as we have met with many dead ends. As we trudged along with the interdisciplinary concepts, there were shards of lights in this runway to model the enzyme, and understand its structure and function at its molecular and atomic levels.

Art of Immobilisation

Immobilisation of an enzyme by means of its localisation or confinement in a certain defined region of space with retention of its catalytic activities will protect it against changes in reaction parameters. Pure enzymes are sensitive and not suitable in many catalytic reactions owing to their unstable nature, and are easily denatured under extreme conditions. This can be reflected by the enhanced activity and stability after the enzymes are immobilised. Physical methods such as adsorption of enzyme on a support are relatively easier and cheaper if compared to chemical methods such as covalent attachment of the enzyme and support. Primary considerations for the selection of supports applied are either hydrophilic or hydrophobic characteristics, high surface area, high porosity or surface chemical variety (Abdul Rahman *et al*., 2006a).

Earlier immobilisation work involved activated carbon (Abdul Rahman *et al*., 2003b) and modified zeolite-X13 (Abdul Rahman *et al*., 2004a) as the support matrix for lipase from *Candida rugosa*. We

then focused on layered double hydroxides (LDHs) owing to their potential applications as adsorbents, ion exchangers, detergents, polymer stabilisers, and catalysts. LDHs can be produced at a low temperature, which keeps the precipitated particles at the smallest size and their surface area and reactivity to a maximum (Abdul Rahman *et al*., 2005a; Abdul Rahman *et al*., 2004b). Ions or molecules such as enzymes can be introduced into the interlayer spaces, thereby generating a diverse group of materials with various applications in biotechnology.

Abdul Rahman *et al*. (2004b) reported the syntheses of Zn/Al-diocytyl sodium sulfosuccinate nanocomposite with a molar ratio of Zn/Al of 4:1 carried out by co-precipitation through continuous agitation. An expansion of layered structure of about 27.9 Å was observed to accommodate the surfactant anion between the interlayer. This phenomenon showed that the intercalation process took place between the LDH interlayer and the lipase molecules were immobilised by physical adsorption. Three types of LDHs ($Mg/Al–NO_3^-$, $Zn/Al–NO_3^-$ and $Ni/Al–NO_3^-$) were synthesised as supports for *Candida rugosa* lipase immobilisation for use in the enzymatic synthesis of methyl adipate (up to 80%) via green esterification of adipic acid and methanol in hexane (Abdul Rahman *et al*., 2008b). The percentages of protein loading on $Mg/Al–NO_3^-$, $Zn/Al–NO_3^-$ and $Ni/Al–NO_3^-$ were 71%, 67% and 58%, respectively, due to the larger surface area, porosity and basal spacing of the supports as summarised in Table 9. When compared to the native lipase, increases in optimisation conditions and enhanced stability properties were found after immobilisation.

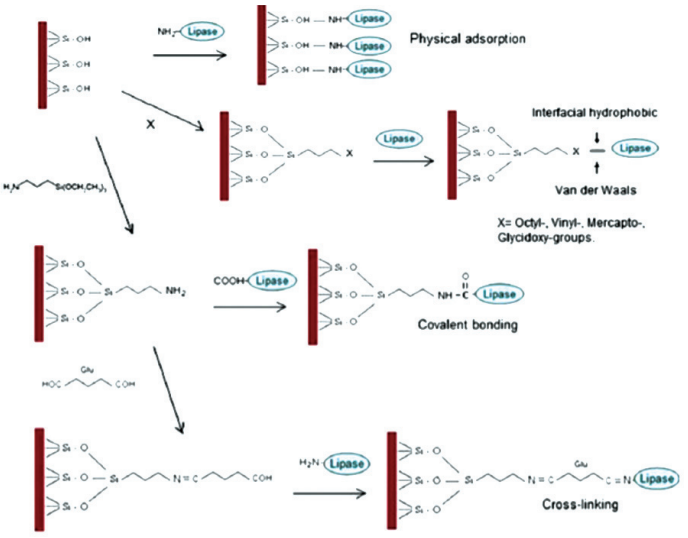
Table 9 Physico-chemical characterisations and activity properties of LDHs-lipases

Layered double hydroxides	**Mg/Al-NO_3^-**	**Zn/Al-NO_3^-**	**Ni/Al-NO_3^-**
BET surface area (m^2/g)	53	48	24
Pore volume (cm^3/g)	23 x 10^{-3}	9 x 10^{-3}	9 x 10^{-3}
Pore size (nm)	35	22	12
Protein adsorbed (%)	71	67	58
Activity (μmol min^{-1} mg^{-1})	3	2	2

Currently, strong interests in such natural supports are due to ecofriendly demands in many modern industrial applications. A natural support of clay-based kaolin is inexpensive and shows good potential as enzyme support material. In our previous study (Abdul Rahman *et al*., 2005b), 77% of protein content of lipase from *Candida rugosa* was immobilised onto kaolin by physical adsorption and applied in the esterification of 1-butanol and oleic acid in hexane. Kaolin-immobilised lipase exhibited activities higher by four fold than the native lipase after a thermal stability test at 70°C and was found to be stable in hexane at room temperature for up to 12 days. Leaching studies showed that the immobilised lipase remained fully active even after being washed with 20 ml of solvent. Natural kaolin has a promising future as a natural support for biocatalysts.

Mica (from the region of Gua Musang, Kelantan, Malaysia) was evaluated for its potential as a natural support for lipase immobilisation (Abdul Rahman *et al*., 2010a). Mica is from the phyllosilicate subclass having sheet silicate and forms parallel

sheets of silicate tetrahedra that shares its three oxygens with other silicate tetrahedra. In our recently reported study, mica was modified either by acid treatment, grafting with aminopropyl-, octyl-, vinyl-, mercapto- and glycidoxy-triethoxysilanes, and activation of pre-treated support with glutaraldehyde (Glu) as illustrated in Scheme 6. The derivatives were characterised by X-ray Diffraction (XRD), Fourier Transform Infra-red Spectroscopy (FTIR), Surface Area and Porosity Analysis, Scanning Electron Microscopy Coupled with Energy Dispersive X-Ray (SEM-EDX) (Figure 24) and Transmission Electron Microscopy (TEM) (Figure 25) techniques (Zaidan *et al*., 2010).



Scheme 6 Illustrations of mica modifications and lipase adsorption

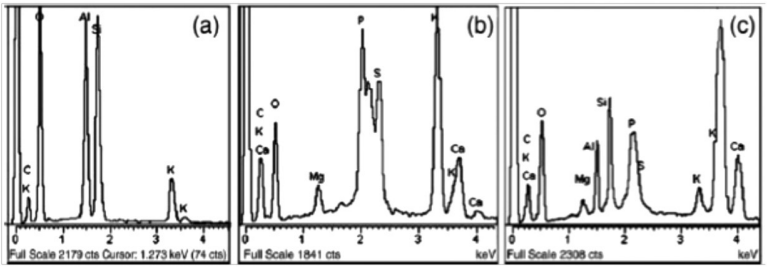


Figure 24 SEM-EDX spectra of (a) mica, (b) lipase and (c) mica-lipase (Mica-CRL)

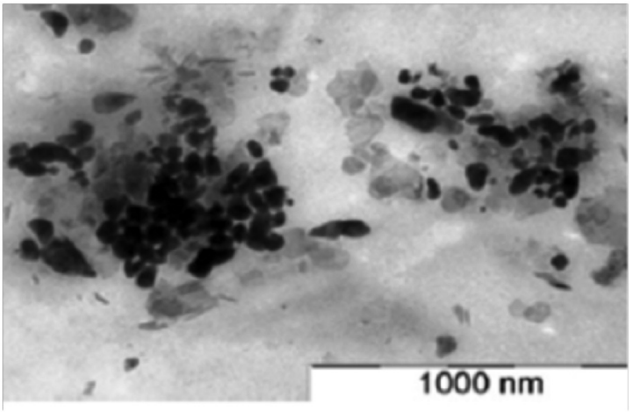


Figure 25 TEM micrograph of immobilised lipase on mica at 30,000X

The modified micas were used for immobilisation of CRL. Lipase activity was determined by lactose caproate esterification and exhibited improved activity if compared to the free enzyme following the order; Amino-CRL > Glu-Amino-CRL > Octyl-CRL > Vinyl- CRL > Glycidoxy-CRL > Mercapto-CRL > Mica-CRL (Table 10). Lipase-immobilised mica showed enhanced protein loading (up to 8.22 mg protein/g support) and immobilisation (up to 78%) if compared to the free lipase and unmodified mica.

Table 10 Lipase immobilisation and lactose caproate esterification activity of lipase-immobilised mica derivatives

Lipase derivatives	Protein loading (mg/g support)	Immo-bilisa-tion (%)	Activity (U/g enzyme)	Specific activity (U/mg protein)
Lipase (CRL)	_	_	_*	35.0
Mica-CRL	4.1	40.6	292.2	60.2
Amino-CRL	8.2	78.4	679.3	82.7
Octyl-CRL	7.7	71.4	593.5	77.2
Vinyl-CRL	6.5	66.4	457.3	70.1
Glycidoxy-CRL	5.4	63.9	342.7	63.0
Mercapto-CRL	4.1	41.4	260.6	63.2
Glu-Amino-CRL	7.7	75.4	598.8	77.3

*Activity of the free lipase was expressed for 0.15 g enzyme or containing 2.14 mg protein.

Instead of natural and synthetic supports, commercial supports such as polymer beads of Eupergit C and Eupergit C 250 L were also introduced to enhance enantioselectivity (Othman *et al*., 2008). Eupergit is an uncharged, spherical acrylic polymer, with a diameter of 150 μm, containing 0.8 mM epoxy groups per g dry weight as the reactive components. The oxirane group functions as a reactive component capable of covalently binding ligands containing mercapto, sulphydryl, amino or hydroxyl compounds, but does not introduce any alterations of electric charges into the matrix or the ligand. This interesting feature accommodates protein molecules as it allows for immobilisation of enzymes with high activity yields.

Optically active (-)-menthyl butyrate was synthesised by enantioselective esterification of racemic (±)-menthol and butyric anhydride using CRL immobilised onto epoxy-activated supports of Eupergit C and Eupergit C 250 L through physical adsorption as tabulated in Table 11 (Othman *et al*., 2008). The immobilised lipases retained high catalytic activity of up to 31% yield and 100% enantiomeric excess (*ee*) of the desired product, and showed better stability if compared to the native lipase. They also exhibited about 50% retained activity even after incubation at higher temperatures, storage at room temperature and after a long incubation in hexane. Immobilised lipases also showed considerably efficient reusability.

Table 11 Percent yield of (-)-menthyl butyrate and percent enantiomeric excess of (±)-menthyl butyrates on the thermal stability study

Temperature (°C)	Yield of (-)-menthyl butyrate (%)[a]			Enantiomeric excess, *ee* (%)[b]		
	NL	EC	EC250L	NL	EC	EC250L
30	6.77 ± 0.37	35.00 ± 3.91	43.37 ± 4.05	14.00 ± 0.73	90.38 ± 0.37	93.99 ± 0.55
40	6.41 ± 4.06	31.89 ± 0.44	41.80 ± 1.48	10.76 ± 1.50	94.48 ± 1.05	90.99 ± 0.73
50	7.00 ± 5.25	30.07 ± 0.02	39.94 ± 2.63	14.46 ± 0.41	90.89 ± 1.91	91.37 ± 0.06
60	6.32 ± 0.17	29.28 ± 2.20	39.11 ± 0.35	12.88 ± 0.81	91.67 ± 2.30	91.56 ± 0.45
70	3.29 ± 0.30	27.01 ± 0.02	30.70 ± 0.19	13.98 ± 0.66	93.08 ± 2.84	90.68 ± 0.15

[a] Yield is defined as (mmol ester/initial mmol alcohol present in the system) x 100 %

[b] Enantiomeric excess is defined as {[-] –[+]/[-] + [+]} x 100%. NL (native lipase); EC (Eupergit C-lipase); EC250L (Eupergit C 250 L-Lipase). The values are reported as mean ± SD.

Myths of Metalloenzymes

Approximately one-third of all known enzymes contain metal ions or metal-containing cofactors. Known as metalloenzymes, the metals play a significant role in a multitude of activities. In proteins such as hemoglobins and cytochromes, the metal ions are Fe^{2+} or Fe^{3+}, and are part of the heme prosthetic group. The metals are built into the structure of the enzyme. Therefore, the metal ion cannot to be removed without destroying the structure of the enzyme. In biocatalysis, metals are usually found in the active site or the domain of an enzyme. Their main function is in electron transfer where metals resemble protons (H^+) as electrophiles that are able to accept an electron pair to form a chemical bond. This versatility explains metals' frequent occurrence in many enzymes.

Although there has been an avalanche of recent progress in using organocatalysts, those involved in designer molecules are still excited by the evolution of artificial and synthetic enzymes because of the potential to combine the reactivity of traditional metal catalysts with the selectivity of enzymes (Kazlauskas, 2006). First attempts in making an artificial enzyme that relied on a metal complex wedged inside a protein host was well ahead of the curve as depicted by Wilson and Whitesides (1978). His pioneering concept and proof-of-principle of artificial metalloenzyme offer the best of both catalysis worlds, nature's own catalysts with tailor-made metal chemistry. In the two worlds of metal and enzyme catalysis, the chirality of a protein's surface influences the chirality of the environment around a metal atom. This is a concept that could be applied by designer biocatalysts to produce a perfect metalloenzyme to catalyse particularly difficult or nonnatural reactions.

Thermolysin (TLN) from *Bacillus thermoproteolyticus rokko* was modified as a semisynthetic metalloenzyme, which is comprised of the enzyme, a ligand and a metal ion. Ligands

selected were benzamidine (BEN), 1,10'-phenanthroline (PHN), *p*-aminobenzamidine (PBZ) and ethanolamine (ETA). The metal ions chosen were magnesium (Mg^{2+}), zinc (Zn^{2+}), calcium (Ca^{2+}) and nickel (Ni^{2+}). The semisynthetic metalloenzyme activities were employed in the hydrolysis of azocasein. Among the four ligands, the complex TLN-PBZ showed the highest specific activity (2,219.5 U/mg) at optimum concentration of PBZ of 0.6 mM. The study followed by attachment of Mg^{2+} to the TLN-PBZ complex (Figure 26) which performed the best specific activity among the metal ions (39,406.4 U/mg). The optimum concentration of Mg^{2+} was 0.08 mM. Several parameters were also investigated such as pH, temperature, time and thermostability. This semisynthetic metalloenzyme was best maintained at pH of about 7.0 in tris-HCl buffer and at 80°C for up to 3 hours (96.7% of relative activity). In the thermostability test, the semisynthetic metalloenzyme can maintain its activity up to 90% at a pre-heated temperature of 80°C.

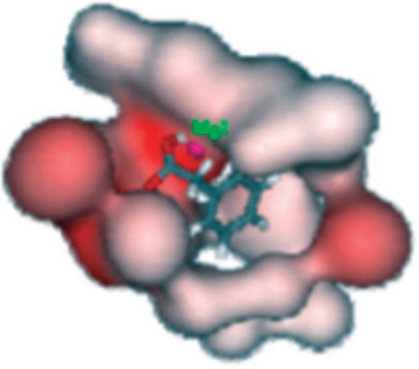


Figure 26 Visualisation on Mg^{2+} and PBZ docked to pocket number 45 in thermolysin

Electronic absorption using the UV/Visible (UV/Vis), UV/Fluorescence spectrophotometer and Circular Dichroism (CD) spectropolarimetric methods were used to characterise the optical properties of TLN-PBZ-Mg^{2+}. In UV/Vis spectrophotometer, the binding of PBZ to TLN curve caused a bathochromic shift (λ_{max} from 279 nm to 274 nm) and there was a hypsochromic shift (λ_{max} from 274 nm to 272 nm) with the addition of Mg^{2+}. Changes in UV/Vis were also supported by UV/Fluorescence, where changes were observed in the TLN-PBZ spectrum (373.2 nm) and the spectrum continued to shift (374.0 nm) for TLN-PBZ-Mg^{2+}. The CD spectropolarimetry suggested some changes in the α-helix and β-sheet at far-UV molar ellipticity readings with decreases of α-helix from 37% (TLN) to 20.6% (TLN-PBZ) and then to 19.8% (TLN-PBZ-Mg).

The development in metalloprotein research provides knowledge to increase enzyme performance, and creates and develops artificial catalysts into commercial practical catalysts for chemical processing. In essence, naturally occurring enzymes operate in mild conditions, reacts in aqueous media and associate with metal ions to perform efficient catalytic functions. To overcome this limitation we are trying to manipulate the structure by substituting the metal embedded in the enzyme with other transition metal ions such as rhodium, ruthenium and palladium. Metal substitution in enzymes is another strategy in enzyme design that may create or enhance catalytic promiscuity for biocatalysis (Kazlauskas, 2005; Bornscheuer and Kazlauskas, 2004). This strategy to insert transition metals in the active site of metalloenzymes opens opportunities to a wider range of enzyme-catalysed reactions (Jing *et al*., 2006).

The function of Zn^{2+} in the structure of lipase from *Geobacillus zalihae* (T1 lipase) was investigated by removing the native Zn^{2+}

ion and by comparing the properties of the native lipase to apo-T1 lipase. T1 lipase coordinates its Zn^{2+} with the side chain of two histidines (H81 and H87) and the two aspartates (D61 and D238) with high affinity, along with the Zn^{2+} ion being deeply buried, may be resistant to elimination (Leow *et al*., 2007). Although the facts of Zn^{2+} binding and its dissociation are available, the condition whether Zn^{2+} is subject to removal from T1 lipase is still unclear.

We conducted an experiment to study the effects of its removal using N,N,N',N'-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) or acidification and interpreted the experimental results by using *in silico* performance of stability into FoldX version 3.0 (Figure 27). TPEN is a hexadentate ligand and has the ability to eliminate *d*-block metals by complexing to form a distorted octahedral geometry (Blindauer et. al, 2006). Addition of TPEN resulted in the complete removal of Zn^{2+} based on the reduction of Zn^{2+} content in T1 lipase from 0.217 (ppb) to 0 (ppb) obtained by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) analysis.

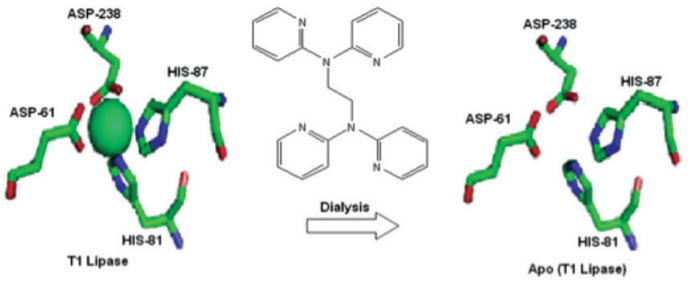


Figure 27 Dialysis of T1 lipase against TPEN removed the zinc ion to form apo (T1 lipase). The zinc binding site in the structural domain was generated using PyMOL program based on the X-ray crystallography structure (PDB:2DSN)

Removal of Zn^{2+} from T1 lipase resulted in a 27% reduction in enzymatic activity. The resulting apo-enzyme was less stable (-52.53 kcal/mol) when compared to native lipase (-64.52 kcal/mol). We hypothesised that the Zn^{2+} plays a role as a structural stabiliser and assists in the catalytic function of T1 lipase. In the near future, we will further the rational design by substituting Zn^{2+} ions with Ru^{2+} or Rh^{2+} ions while retaining the Ca^{2+} ion in the structure.

Enigmatic Enantioselective Enzymes

From foods to therapeutics, enantiopure molecules are at a crucial point. Furthermore, it gets more complicated when selecting a potential catalyst for chiral enantiomer. Generally, designer molecules have two options, either to use an enzyme or a transition metal complex. In nature, enzymes perform perfectly by enveloping a substrate in an active asymmetric site that chemists now routinely hijack for single enantiopure purposes. Non-naturally, transition metals with surrounding ligands act by ultimately controlling the way a reactant approaches the metal but are less precise than enzymes. Few approaches have been done to increase the enantioselectivity of lipases such as chemical modification, rational design, directed evolution and artificial evolution.

Chemical modification of *Candida rugosa* lipase was carried out via reductive alkylation to increase its hydrophobicity to work better in organic solvents (Rahman *et al*., 2004). The free amino group of lysines was alkylated using propionaldehyde with different degrees of modification obtained (49 and 86%). Far-UV CD spectropolarimetric of the lipase in aqueous solvent showed that such chemical modifications at the enzyme surface caused a loss in secondary and tertiary structure that is attributed to the unfolding of the enzyme. We hypothesised that in an aqueous environment, the loss in protein structure of the modified lipase is owing to the

disruption of stabilising salt bridges, particularly of surface lysines. Indeed, molecular modeling and simulation of a salt bridge formed by Lys-75 to Asp-79, in a nonpolar environment, suggests the adoption of a more flexible alkylated lysine that may explain higher lipase activity in organic solvents on alkylation.

Modifications via reductive alkylation were continued on T1 lipase using propionaldehyde. The targeted alkylation sites were lysine, in which T1 lipase possessed 11 residues. Four residues (Lys84, 102, 138 and 251) were exposed, four residues (Lys185, 329, 344 and 345) were moderately exposed and three were buried residues (Lys28, 207 and 229) as illustrated in Figure 28. Comparison of the far-UV CD spectropolarimetric between native and alkylated enzyme suggested the formation of a molten globule (MG)-like structure. This was further supported by 8-anilino-1-naphthalenesulfonic acid (ANS) probed fluorescence which indicated higher exposure of hydrophobic residues, a consequence of chemical modification and a small number of alkylated lysine residues were confirmed based on Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) analysis.

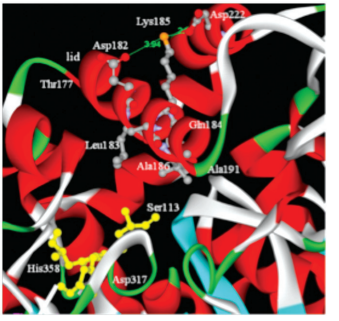
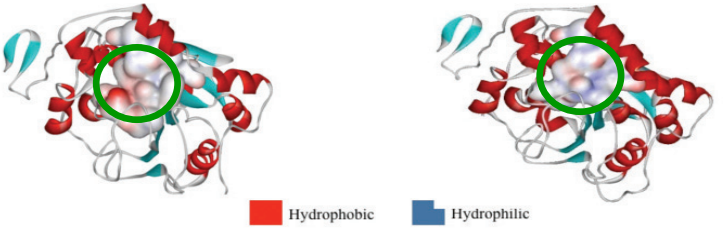


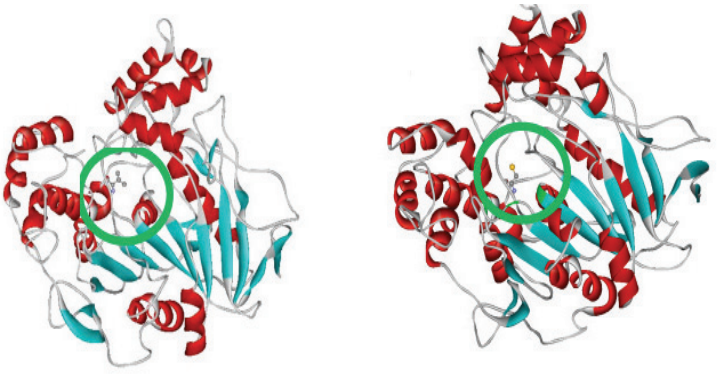
Figure 28 Local milieu surrounding Lys185 (orange) in T1 lipase. The lid stretching from Thr177 to Ala191 is located well above the catalytic triad (yellow)

Recently, rational design by metal ion introduction closer to the active site with a twist of inorganic approach in enzyme engineering was introduced. Lipases from *Candida antarctica* (CALB) and CRL are immaculate enzymes for this rational design. The more hydrophilic the protein, the higher is its ability to attract metal. Introduction of histidine and cysteine into an active site pocket could give a protein the ability to bind with transition metal ions. A set of more than 40 mutants of CALB and CRL were computationally designed with each structure modeled by using 30 replicates. For CALB, the site-directed mutations were done by changing the selected amino acid (e.g. Gly39, Thr40, Trp104, Gln106, Ala132 and Pro133) with isoleucine and combinations of all the selected amino acids with isoleucine (Figure 29). Whilst for CRL, the mutants were developed by addition of histidine in the C- or N- terminal such as V245H, V245C, F415H, F415C and combinations of both mutations (Figure 30). These potential mutant structures aid to screen or reduce number of experimental procedures and costs for enantioselective esterification of many chiral syntheses.



(a) CALB (wild) (b) Calb2dm3 (mutant)

Figure 29 Comparison between CALB (wild) and Calb2dm3 (mutant) using a surface look



(a) CRL (wild) (b) CRL 245C (mutant)

Figure 30 Comparison between (a) CRL1 (wild) and (b) CRL245C (mutant) using the ball and stick look

An artificial metal binding site for manganese (Mn^{2+}) was created in the T1 lipase active site based on a template of *Escherichia coli* Mn SOD structure (PDB:1vew) as reported by Edwards *et al*., (1998). This new manganese metal binding site consists of His16, Asp114, His164, His244 and a water molecule or hydroxide ion will be the fifth ligand coordinating the metal to form distorted trigonal bipyramidal geometry. This can be achieved by mutating only Ser113 into Ala113 but not another two catalytic triads as both play a critical role in lipase catalysis (Figure 31). Interestingly, preliminary molecular dynamics (MD) simulation showed that a water molecule begins to coordinate itself to the metal after 1.3 ns. These activities suggest that the coordinated water has a huge potential to replace the roles of Ser113 as the nucleophile.

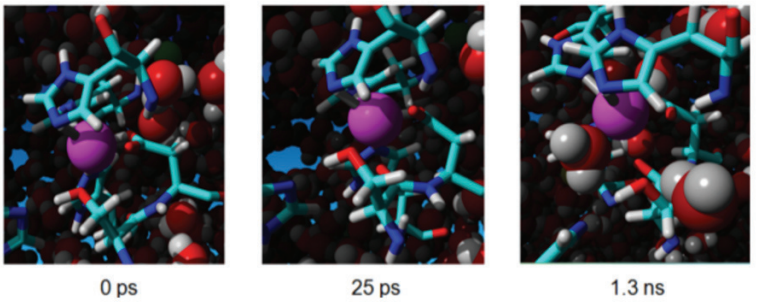


Figure 31 MD simulation of a newly designed T1-Mn lipase in water. After 1.3 ns, a water molecule began to coordinate itself to the Mn metal ion with approximately~ 2.8 Å between coordinated water and OH group of Ser113

Peptide Perspectives

The next approach is to design new artificial biocatalysts from natural peptide scaffolds for specific enzymatic reactions. We are embarking on this new adventure with mini proteins and peptides as effective biocatalysts. Many factors interfere with the design of a working sequence such as the folding, physical or chemical adaptability and stability of the structure. Our goal includes work with DNA by exploiting the double helix to make highly selective catalysts other than its usual function of transferring information to RNA, and then to protein synthesis and finally the catalysis of new chiral compounds. Roelfes *et al*. (2006) have achieved enantioselectivity of up to 99% *ee* with their DNA-Cu catalyst for Diels-Alder reactions, a key bond-forming reaction, where they thought that maybe the chiral information in DNA could be transferred directly in a catalytic reaction.

Malaysia Genome Institute sequenced the genome for antifreeze protein (Afp) from *Leucosporidium antarcticum* isolated from sea ice near the Casey Research Station, Antarctica. This

Leucosporidium antacticum Afp1 (Afp1) showed lack of similarity in the fold library with a percentage of sequence identity of below 30%. Hence, an I-TASSER *ab-initio* simulation was employed which lead to fundamental antifreeze peptide models from the Afp1 model (Figure 32). It contained four unique parts of alpha helix with the presence of serine, threonine, aspartic acid, asparagines and glutamine, which play significant roles in the ice binding mechanism and prevent the ice growth. The hydrophilic parts in the alpha helix attached to the ice surface while the hydrophobic parts inhibit any additional water molecules from adhering to the ice surface (Abdul Rahman *et al*., 2008d).

Molecular dynamics simulation of the Afp1 model in water was performed to investigate the stability of the structure when exposed to different temperatures (273K, 277K and 283K). Analysis of RMSD, RMSF and radius of gyration showed that the Afp1 structure was in the most stable 277K, which is in agreement with an optimal activity at this temperature in wet experiments. The structural characterisations and properties of Afp1 obtained by various computational techniques were used to aid the experimental approach and peptide design for commercial antifreeze proteins.

Antifreeze proteins have commercial potential in frozen food storage, artificial rain and cryopreservation (Kun and Mastai, 2007). However, the applications of AFPs are marred by high costs due to low yield. To solve this problem, we explored the possibility of using the most important peptidic fragment of the protein as the antifreeze agent. Our hypothesis is that the four helices in Afp1 are responsible for the antifreeze action of the protein and thus, the peptides derived from these four helical regions may show similar antifreeze activity as its parent protein. One obvious advantage of using antifreeze peptides over the use of antifreeze protein is that the smaller antifreeze molecules can act as “molecular tools”

in the study of the most important sequence in the mechanism of ice-binding by antifreeze proteins. Additionally, peptides in food have been known as a better alternative to antifreeze proteins in causing less severe adverse reactions in the body.

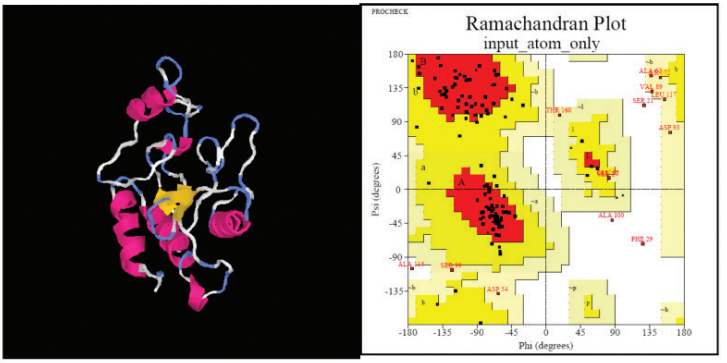
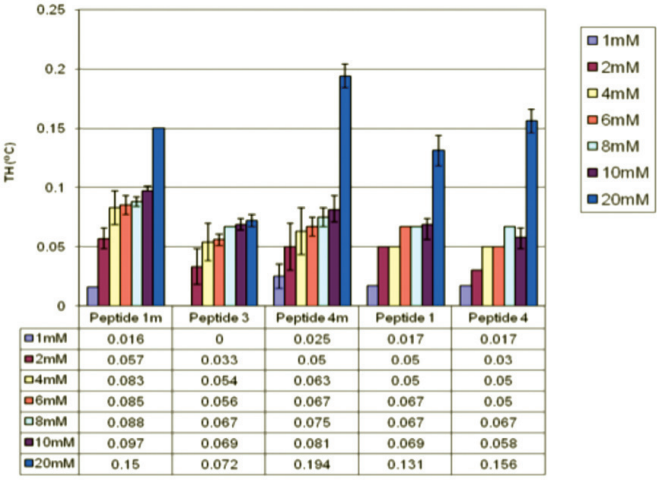


Figure 32 Predicted I-TASSER 3D model of *Leucosporidium antarcticum* Afp1 and its Ramachandran Plot. PROCHECK analyses showed 75.2% of the residues were located in the most favoured region (red) and only 4 residues (Ser21, Phe29, Ala100 and Ala114) were located in the disallowed region (white). The other residues were found to reside in the allowed (yellow) and generously allowed (light yellow) regions

We designed four peptides derived from the helical segments of Afp1 with various lengths of between 25 to 30 residues. Interestingly, three peptides possess non-zero thermal hysteresis as depicted in Figure 33. Two peptides were modified by Leu/Glu and Gln/Lys replacements in order to create helix-stabilising *i*, *i+4* salt bridges. We found that the peptides with additional salt bridges have increased thermal hysteresis, which suggests the role of the salt bridge in the stabilisation of the antifreeze peptide structure.



	Peptide 1m	Peptide 3	Peptide 4m	Peptide 1	Peptide 4
1mM	0.016	0	0.025	0.017	0.017
2mM	0.057	0.033	0.05	0.05	0.03
4mM	0.083	0.054	0.063	0.05	0.05
6mM	0.085	0.056	0.067	0.067	0.05
8mM	0.088	0.067	0.075	0.067	0.067
10mM	0.097	0.069	0.081	0.069	0.058
20mM	0.15	0.072	0.194	0.131	0.156

Figure 33 The antifreeze activity (thermal hysteresis, TH) of three synthesised peptides in different concentrations. Peptide 2 is not soluble in aqueous solution due to its hydrophobicity. Peptide 1m is Peptide 1 with Leu/Glu mutation and Peptide 4m is Peptide 4 with Gln/Lys mutation

Later, we conducted ice recystrallisation inhibition (IRI) experiments to observe physical changes on the ice crystal growth in the presence of the peptides. The peptides effectively inhibit ice crystal growth if compared to water alone as pictured in Figure 34. We rigorously tested the antifreeze activity of our peptides using two different methodologies and they show the effectiveness of our peptides in suppressing the growth of ice crystals.

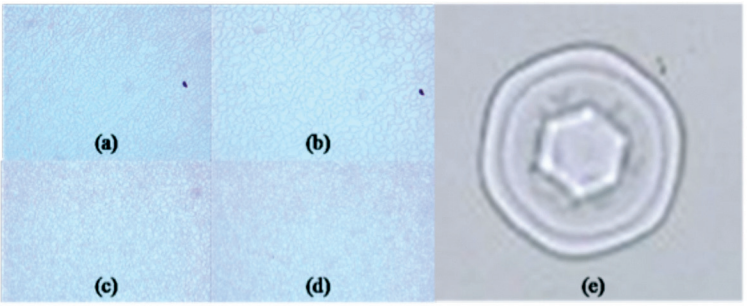


Figure 34 Physical changes on ice crystal growth with and without peptides. Both samples (water with and without peptides) are flash frozen to -6 °C and incubated for 3 h; (a) ice crystal without peptide, (b) shows larger crystals after 3 h, (c) ice crystal with 20 mM of Peptide 1m (Peptide 1 with Leu/Glu) modification, (d) shows similar crystals after 3 h, (e) observation of single ice crystal in Afp assays

Peptides containing 30 to 80 amino acid residues with a well-defined 3D structure and biological activity are particularly attractive scaffolds for protein design. The natural structure can be used as a scaffold and a new function will be transferred on an active site at a specific structural region, which is possible for sequence mutations and structurally compatible with the new function. Therefore, stabilising the peptide scaffold by structural constraints like disulfide bridges or metal ions is paramount. These small structures could be designed to be conformationally stable and compatible with rather different sequences and biological activities, such as the small architecture illustrated in Figure 35.

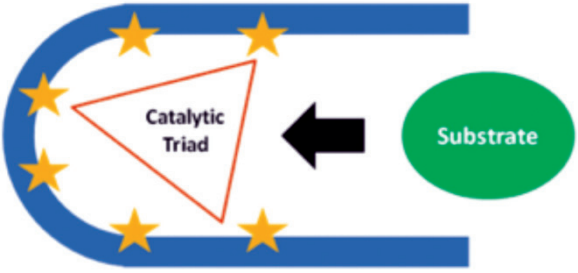
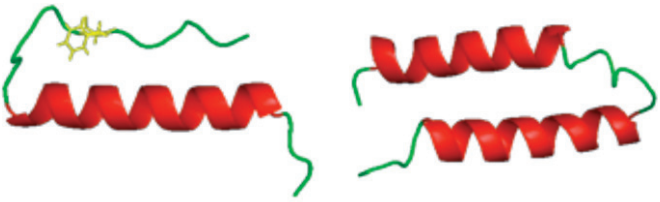


Figure 35 Overview of a peptide scaffold (in blue), and amino acids mutations (yellow stars) that could act as 'mini enzymes' for catalysis

In the first attempt, tyrosine (Tyr7) was substituted with phenylalanine (Phe7) residue (bPPY7_F7) with the aim to immobilise the random tail of the bovine pancreatic polypeptide (bPP). This bPP (PDB ID: 1LJV) scaffold has a flexible random secondary structure at one end and predominantly used for catalysis (Coquière *et al*., 2009). However, the substitution stirred instability for the active site design due to high flexibility at one end of the scaffold as modeled in Figure 36a. The flexibility problem could be solved by hybridising (bPP_HY1) with its own helix sequence to obtain a helix-loop-helix structure (Figure 36b). This model has a C-score of -1.20, a TM-score of 0.56± 0.15 with RMSD of 4.9 ± 3.2 Å. Trajectory results from MD simulations showed stability of both helices, indicating that this structure can be used as a scaffold. The 'active site' was planted onto the scaffold by replacing three original residues with His22, Ser24 and Asp30.



(a) bPPY7_F7 (b) bPP_HY1

Figure 36 Modified bovine pancreatic polypeptide scaffold structures

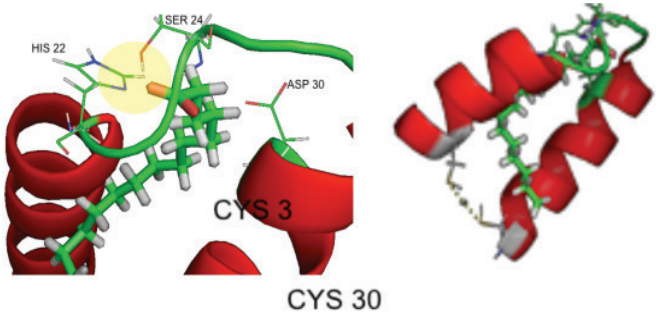


Figure 37 Prediction of oleic acid substrate entering into the hybrid bovine pancreatic polypeptide (bPP_HY1) scaffold

Later, the structure of oleic acid was appended to bPP_HY1 scaffold to determine the potential active site of the mini-enzyme as captured in Figure 37. Unnecessary residues were excised from the scaffold while Cys3 and Cys30 were introduced to maintain the shape of the scaffold by building a salt bridge between the two helices. With a better understanding of its mechanism, a new mini-enzyme structure will be synthesised for further investigation and opening up a new burgeoning field of biocatalysis.

THE MODELS

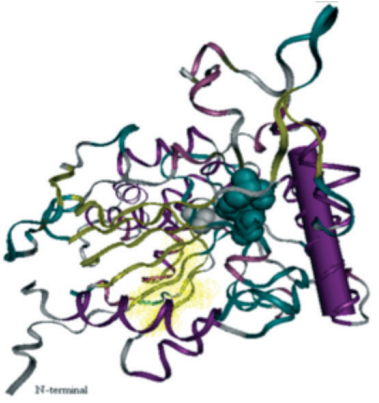
Insight Instinct

Computer simulation techniques such as molecular dynamics are important tools for atomic-detailed understanding of the physical basis of a molecule or protein. A simplified model of a real system will be created in order to reproduce the known structural features and dynamics of the model system. MD is used to investigate the dynamics of macromolecules and is rooted in the atomism of antiquity. It has been widely used to understand and explore the physical basis of numerous systems such as catalysis, biocatalysis, micellar and delivery. Insight and mechanism analysis from MD simulations would lead to significant discoveries and a better understanding of the structure and function of enzymes, designer molecules and biocatalysts.

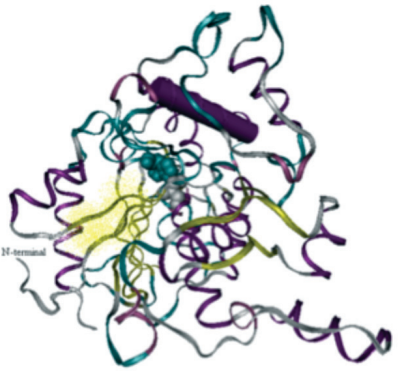
Proteins Unravelled

One of the most studied structural perturbation processes on proteins involves temperature induced unfolding. The thermostability and thermoactivity of thermoenzymes make them very important for many industrial applications. Any increase to enzyme thermostability would help to increase conversion rates and substrate solubility, which in turn increases the possibility of microbial growth and the reduction in media viscosity. The application of MD techniques in exploring the structural features and dynamic properties of proteins at different temperatures and the mechanisms of protein folding and unfolding have been studied for several years (Karplus & Sali, 1995; Colombo, 2004). Detailed comparisons of energetic, structural and dynamic properties of a protein at different temperatures will yield a large amount of data, which is not directly accessible from laboratory experiments (van Gunsteren *et al*., 2008).

To the best of our knowledge, our study is the first reported research on thermostabilty of thermoalkalophilic lipases using MD simulations (Abdul Rahman *et al*., 2009b; Abedi Karjiban *et al*., 2009; Abedi Karjiban *et al*., 2010). A comparative study of structure, flexibility and dynamics of both enzymes at high temperatures will help us gain the information required to stimulate the development of rational protein engineering and biotechnological use of these enzymes. Here, we selected the thermoalkalophilic lipase from *Bacillus stearothermophilus* L1 (L1 lipase) (Jeong *et al*., 2002) and *Geobacillus zalihae* T1 lipase isolated from palm oil mill effluent (POME) in Malaysia (Leow *et al*., 2007; Matsumura *et al*., 2007) as our model systems as depicted in Figure 37(a) and (b), respectively. Both enzymes are highly active at high temperatures (~70°C) and consist of 388 amino acids and one each of calcium (Ca^{2+}) and zinc (Zn^{2+}) ions. T1 lipase has the potential to catalyse the hydrolysis under distinct conditions such as in organic solvent and at high concentrations of alkali metals (Rahman *et al*., 2007).



(a) The catalytic triad residues of L1 lipase are Ser113, His358, and Asp317, which reside in a turn following strand β5, loop β9-α13, and loop α12-β8, respectively



(b) The overall structure of T1 lipase is globular in shape, with a central β-sheet consisting of seven strands surrounded by thirteen α-helices and ten 3_{10}-helices and loops, which results in an overall topology of a typical α/β hydrolase fold

Figure 37 Stereopictures of (a) L1 lipase and (b) T1 lipase with active site residues in van der Waals representation, lid highlighted in cartoon representation in purple and small domain dotted representation in yellow (α-helix in purple, β-sheet in yellow, turn in cyan, coil in white)

The temperature-induced unfolding MD simulations were performed to reveal the effects of high temperatures on the structure, flexibility and dynamics of the thermoalkalophilic L1 lipase and T1 lipase. Figure 38 shows the time evolution of radius of gyrations (R_g) of both model systems at 300 K, 400 K, and 500 K. This parameter has been widely used to show a measure of compactness of a protein structure and the tendency of our models to expand at different temperatures. The native state of both structures was partially lost at 500 K. However, both structures remained compact. A small increase in the radius of gyration can be a further indication of a slow change in the tertiary structure of the native state, which can eventually lead to unfolding (Colombo & Merz, 1999).

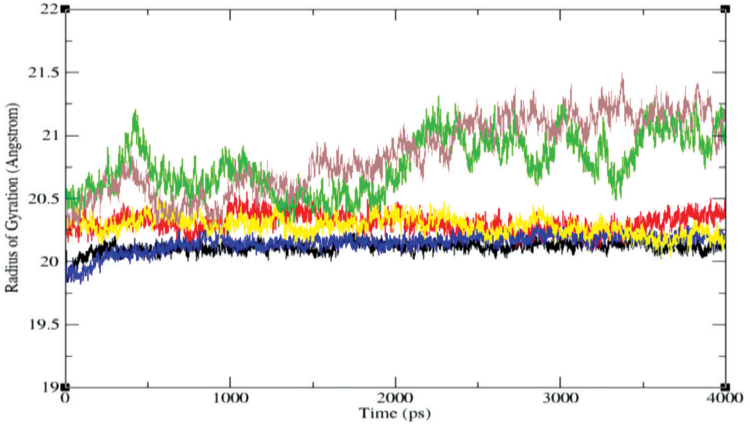


Figure 38 L1 lipase and T1 lipase radius of gyrations (Rg) as a function of time at 300 K (L1, black; T1 blue), 400 K (L1, red; T1, yellow), and 500 K (L1, green; T1, brown)

The hydrophobic effect is the dominant occurrence in protein folding which is usually measured by the solvent accessible surface area (SASA) of the protein, a part of the complex surface of a protein in direct contact with solvent (Leach, 1996). The all-atom SASA of both L1 and T1 lipases show significant increases at 500 K relative to 300 K and 400 K which indicate that a higher accessibility to solvent happened at 500 K (Figure 39). This implies a more open and mobile structure for both model systems at 500 K. The major deviations in SASA seem to be dominated by the side chain and non-polar subgroups as compared to polar and main chain subgroups for both systems with the side chains as the determinants of SASA changes during thermal unfolding MD simulations.

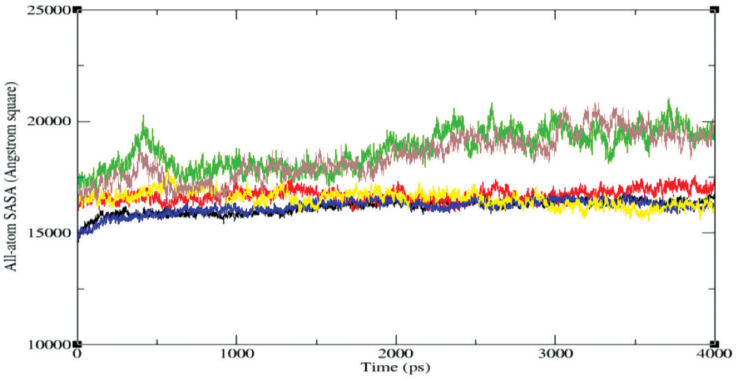


Figure 39 L1 lipase and T1 lipase all-atom SASA (Å2) as a function of time at 300 K (L1, black; T1 blue), 400 K (L1, red; T1, yellow), and 500 K (L1, green; T1, brown)

Proteins show a wide spectrum of flexibility. We studied the systemic flexibility in our proteins, which is referred to as the small-scale positional fluctuations in the backbone atoms in their native state and elevated temperatures. The backbone atoms of proteins and amino acid side chains are continuously moving due to thermal motion and the kinetic energy of the atoms. From the time-averaged RMS fluctuations (RMSf) over all residues including terminal residues, both structures average closer to the native state even at high temperatures while they experience greater fluctuations about their time-averaged structures (Abedi Karjiban *et al*., 2009; Abdul Rahman *et al*., 2009b).

The small and core domains of both lipases interact through the Zn^{2+}-binding coordination involving His-81 and His-87 of the small domain, and Asp-61 and Asp-238 from the core domain. There is a tight interaction between the characteristic cluster of helices (α8 and α9) and loops around the helical lid (helix α6). High temperatures make these interactions very weak. This idea was confirmed by

the highest amount of flexibility and dynamics of His-81 and His-87 residues among all residues at high temperatures, while the dynamic of Asp-61 and Asp-238 did not increase appreciably (Abedi Karjiban *et al*., 2009; Abdul Rahman *et al*., 2009b). Collectively, results presented here indicate that specific regions along the L1 lipase and T1 lipase polypeptide chain exhibit hypersensitivity to thermal stress.

Thermostability is correlated with higher flexibility rather than increased rigidity for both models. Our simulations propose that the N-terminal moiety of both enzymes and their small domains are critical regions of thermostability. These regions may represent some targets for amino acid replacement in the design of more stable mutants. Additional mutational studies are needed to delineate the full range of structural features that confer structural and functional integrity at high temperatures. Finally, this comparative study offers the possibility to study features responsible for the thermostability of large proteins.

Modeling Micelles

Problems associated with transdermal drug delivery are directly associated with the skin's lipid bilayer at the *stratum corneum*. Chemical penetration enhancers, such as swollen micelles that form through solubilisation of surfactants in nanoemulsion systems could provide an effective solution. Swollen micelles are known for their potential application in pharmaceuticals as a drug carrier for controlled-release systems. This vehicle was reported to have a momentous potential to increase the penetration of amphiphilic, lipophilic, and hydrophilic materials through the skin if compared to conventional transporters. The release mechanisms are dependent on physico-chemical properties of the components, internal structure of the swollen micelle and drug interactions.

We employed MD simulations to understand palm-based ester nanoemulsion self-assembly, specifically the aggregation pattern, formation pathway, and the shape and size of the micelle (Abdul Rahman *et al*., 2008e). The ability of palm-based nanoemulsion to self-assemble and the micelle sizes from our simulations suggest that palm-based nanoemulsion has a great potential in transdermal drug delivery due to its low energy micelle formation pathway (Abdul Rahman *et al*., 2009e). This process involves the uptake of oil droplets in the nanoemulsions by non-ionic surfactants to form oleyl oleate (OE) swollen micelles. Five series of 10 ns MD simulations were performed at different micelle compositions to determine the structural evolution of OE/Span20 (S20) swollen micelles. The hydrophobicity of OE enabled it to self-associate into colloidal systems like micelles (Abdul Rahman *et al*., 2010b).

From graphical snapshots of each simulation (Figure 40), micelles were formed at 20% and 30% where the latter clearly showed an infinite cylindrical micelle structure with a radius of ± 3.54 nm, which is in agreement with the experimental results, located in the isotropic region in the ternary phase diagram. At 40%, separated arrangements of OE and S20 molecules which were closely related to a cubical or hexagonal micellar system, were displayed while at 50%, a lamellar-like structure formed, displaying three layers of arrangements of molecules with OE located at the centre with an estimated *d*-spacing of ± 2.24 nm. These results were in agreement with predicted variation of shapes in a ternary phase system comprised of oil, surfactant and water by Davis (1994).

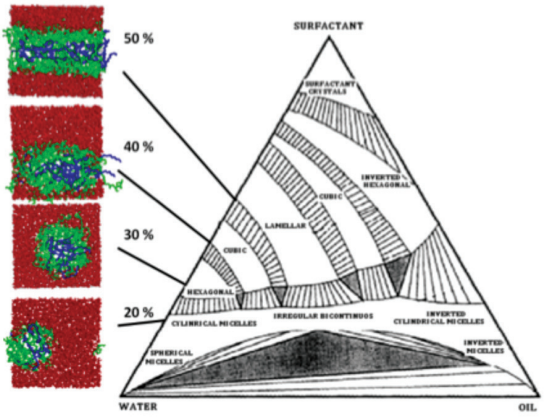


Figure 40 Graphical snapshots of each micelle simulation in agreement with structural properties based on the ternary phase diagram for emulsions

Four MD simulations of the structure of S20, OE/S20, Tween 80 (T80) and OE/T80 micelles revealed the effect of different non-ionic surfactants and different HLB values. Eccentricity values of 0.098 indicated that OE/T80 showed the highest tendency to form a spherical micelle. There is a considerable difference between the HLB values of T80 (15 ± 1.0) and S20 (8.6 ± 1.0) which may cause a stronger hydrophilic attraction between T80 and the surrounding water, producing smaller micelles if compared to S20 (Abdul Rahman *et al*., 2010b).

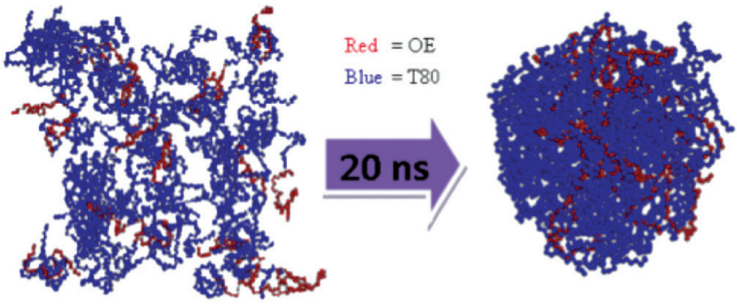


Figure 41 Left; initial arrangement of molecules and right; the final structure of OE/T80 swollen micelle

The self-assembly process between OE and T80 molecules was imitated by performing a 20 ns MD simulation of random initial arrangement of the molecules (Figure 41). The aggregation rate of nanoemulsions can be explained by the self-assembly of swollen micelles during the simulation. All of the OE and T80 molecules completely aggregated at 8.9 ns from smaller aggregates to form a bigger aggregate of the micelle. The phenomenon is related to Ostwald ripening, where bigger droplets grow at the expense of the smaller droplets. Eccentricity values calculated along the simulation showed that the structure was nearly a perfect sphere (average eccentricity of 0.17). However, no reverse-formation occurred which was expected, as the micelle is a highly dynamic system that continually breaks and forms. This micelle aggregate lasted until the end of 20 ns of the MD simulation without any dissociation.

The self-assembly study of the swollen micelles has lead to a chronological formation of mixed palm-oil esters (POEs) system up to 15 ns. The size of the swollen micelle (4.2 ± 0.05 nm consistent with the experimental nanoparticle size) suggests that palm-based

nanoemulsion has great potential to be used in transdermal drug delivery due to its low energy micelle formation pathway (Abdul Rahman *et al*., 2009e). Simulations of palm kernel-oil esters (PKOEs) with a pharmaceutical drug based on a stable experimental nanoformulation were run for 20 ns consisting of 2 oleyl oleate, 2 oleyl myristate, 10 oleyl laurate, 30 Tween 80 and 3 ibuprofen molecules.

Interactions among those molecules were followed at each step with various snapshots as pictured in Figure 42. At 20 ns, the ibuprofen molecules stayed in the outer layer of the micelle. Hence, we hypothesised that the drug release into the targeted part of the human body is an instant release. This is favourable because an anti-inflammatory drug like ibuprofen needs to react instantly after being topically applied.

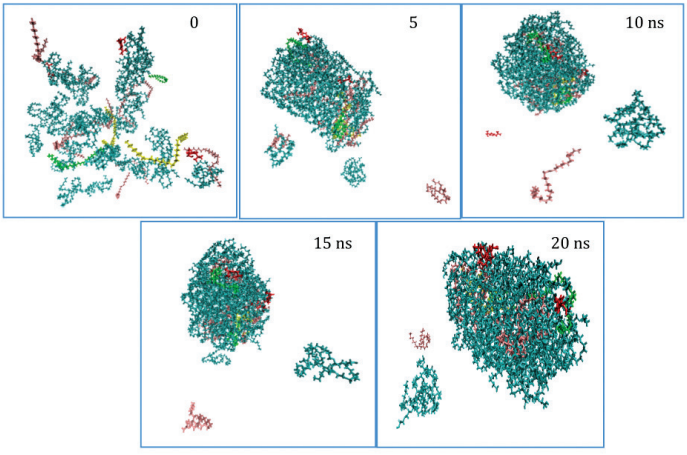


Figure 42 PKOEs with ibuprofen-swollen micelle formation snapshots trajectory at 0 ns, 5 ns, 10 ns, 15 ns and 20 ns. The number of molecules and their colour-codes: 30 Tween 80 (cyan), 3 ibuprofen (red), 10 oleyl laurate (pink), 2 oleyl oleate (yellow) and 2 oleyl myristate (green)

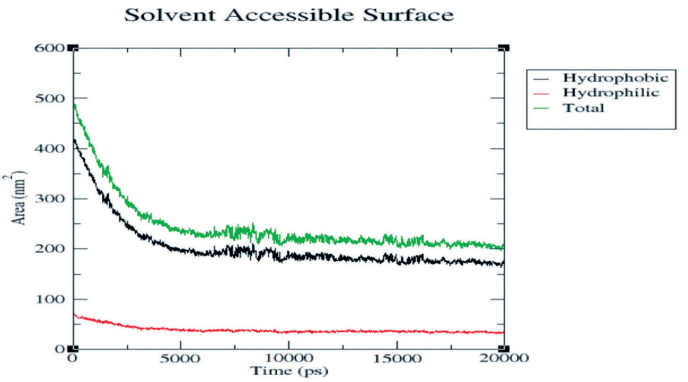


Figure 43 Hydrophobic and hydrophilic analysis of SASA versus time for PKOEs with ibuprofen

The hydrophobic effect plays a pivotal part in micelle formation in a nanoemulsion system where the hydrophobic tails of the molecules repel others to "escape" and push the water from the inner part of the micelle. As Figure 43 shows, the total of both hydrophobic and hydrophilic interactions decreased gradually from the beginning of the simulation until 7.5 ns and later stabilised from 10 ns until the end of the simulation. Both solvent accessible surface area analysis and radius of gyration data confirmed the vital role of hydrophobic interactions in the formation of micelles in a nanoemulsion system.

In conclusion, the aggregation process in the formation of micelles in our model system clearly shows that the palm-based wax esters potentially act as the carrier of ibuprofen due to the esters' amphiphilic properties. Ibuprofen molecules, which are located in the micelles' outer layer indicates that the interaction between ibuprofen and palm-based esters can result in an instant release of the drug to the human body. Our research shows that palm-based swollen micelles are like other theoretically studied micellar

systems. Given enough time with larger systems, MD simulations can provide comprehensive results regarding aggregation patterns as well as formation and reverse-formation of the micelle.

Computational Catalysis

Nowadays, protein structures are prone to modifications based on the fundamental rules of design and function. Calculations of free Gibbs binding energies (ΔG) of chemical molecules (effectors) that bind to proteins are important in molecular signaling processes and catalytic mechanisms of certain key enzymes. These calculations can be obtained via *in silico* modeling and theoretical approaches. Molecular docking is a basic tool in computer-assisted biocatalyst and drug design. The binding of putative ligands to protein binding sites or receptors can be thermodynamically predicted and interpreted as in the case of tyrosine inhibitor (Lam *et al*., 2010). Hence, this molecular docking procedure could be used to perform virtual screening on large libraries of compounds. The results can be ranked, structural hypotheses of novel biocatalysts can be proposed, inhibition of the target by ligands can be described, all of which are invaluable in lead optimisation (Morris and Lim-Wilby, 2008).

Previously, Abdul Rahman *et al*., (2005c) showed that the rigid ligand of PHN gives the most favourable value for final docked energy (-8.74 kcal/mol) at pocket 24 of trypsin (PDB:1AUJ) whereas PBZ preferentially binds to pocket 20 with a lowest final docked energy of -7.72 kcal/mol. The coordinated structure of Ser195 at the active site would attract PHN, with its highly electronegative nitrogen atom, into the pocket. As PHN contacts the pocket, it may induce conformational changes at the binding site to make the active site of trypsin (His57, Asp102, Ser195) capable of facilitating azocasein as the substrate. The results showed that pocket 24 provides the best site for interaction with PHN. Analysis

on docked complex of pocket 24-PHN showed that 9 residues were involved in hydrophobic contact with PHN. As depicted in Figure 44(a), those residues are Asp189, Ser190, Cys191, Gln192, Ser195, Trp215, Gly216, Gly219, and Gly226. Metal ions were later successfully docked to the PHN ligand at their lowest docked energy as illustrated in Figure 56(b).

Two semisynthetic complexes of trypsin-PHN and trypsin-PBZ were prepared and investigated for their roles in the hydrolysis of azocasein. Both bidentate ligands showed the ability to provide more sites for interactions with metal ions and contribute extensively to the development of a new generation of industrial biocatalysts. The trypsin-PHN complex showed an increment of 80% for the hydrolysis of azocasein as displayed in Figure 45. In the presence of 5 μM Ca^{2+}, the activity was higher than native but decreased in the presence of Mg^{2+}, Zn^{2+} and Fe^{2+}, thus, providing additional insight of potential inhibitors to the rational enzyme design.

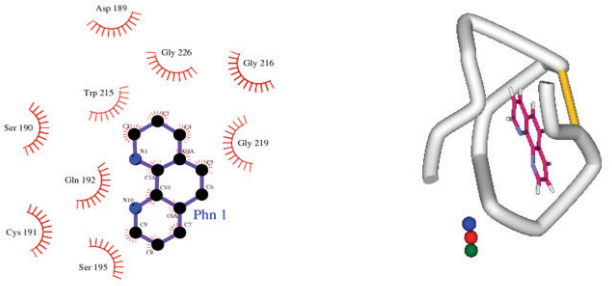


Figure 44 Left; hydrophobic interactions of the lowest docked energy of PHN with pocket 24 of pancreatic bovine trypsin. The comb-like arrangements indicate hydrophobic contacts. Right; orientation of trypsin24-PHN-metal complex at its lowest docked energy. The blue ball represents Ca^{2+} (-5.56 kcal/mol), the green ball represents Mg^{2+} (-6.99 kcal/mol), and the red ball represents Fe^{2+} (-9.33 kcal/mol)

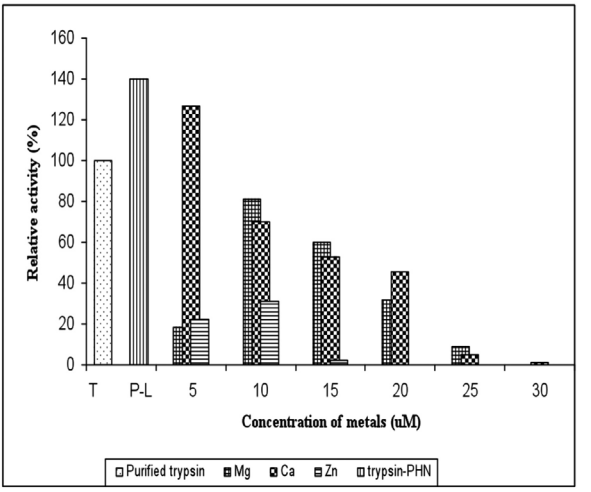


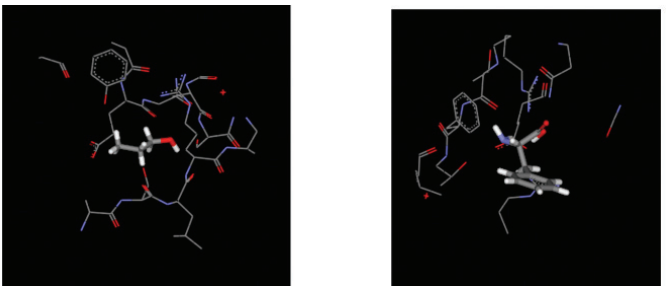
Figure 45 Effect of Mg^{2+}, Ca^{2+} and Zn^{2+} metal ions at different concentrations (5 to 30μM) on trypsin-PHN complex. Fe^{2+} inhibited activity at all concentrations

All these discoveries may provide something useful in the design of novel binding sites. A series of 48 pockets were identified in thermolysin (KEI) and the four biggest pockets were selected as they accommodated suitable modification sites. Application of molecular docking on phosphoethanolamine (PSE), which acts as intermediate ligand in the designated protein complex showed favourable final docked energy at different pockets (-8.49 to -4.80 kcal/mol). Following the procedure, Ca^{2+} ion was found to be most favorable with the lowest E_{docked} of -4.13 kcal/mol and within acceptable distance (1.5 Å ≤ M ≤ 3.0 Å). The semisynthetic metalloprotease of KEI-PSE-Ca^{2+} complex formed a distorted trigonal bipyramidal geometry by coordinating five neighbouring atoms (Abdul Rahman *et al*., 2007b). The geometry is similar to the reported trigonal bipyramidal geometry in rat annexin V protein complex coded as 1a8a in Metalloenzyme Data Base (Yu *et al*., 2001).

Characteristics of each binding site and chemical ligands enabled the determination of the protein-ligand interactions. The binding modes of a series of chemical ligands with aromatic rings, amine and hydroxyl groups were explored in depth for T1 lipase, which consists of 65 potential binding sites (pocket 65 being the largest binding site at 312.4 $Å^2$ and 581.1 $Å^3$). Differences of free binding energies from various ligands at the same pocket were related to the types of interaction involved in the selected pockets. Hydrogen bonds, van der Waals forces, electrostatic interactions, hydrophobic contacts and π-π stacking were identified as the interactions occurring between the docked ligands and surrounding pocket residues.

van der Waals interactions can be used to explain the differences in the final docked energies between two ligands in the same pocket. For ligand propanol (POL), it had eight van der Waals interactions with its surrounding pocket residues while ligand phenylacetic acid (PHE) had four van der Waals interactions as illustrated in Figure 46. The difference in the number of van der Waals interactions could led to the tendency of a ligand to be docked into the pocket and the lowering of the final docked energy of ligand POL (-3.39 kcal/mol) and ligand PHE (-2.14 kcal/mol).

A π-π stacking interaction is a type of binding mode occurring in molecules with aromatic rings. This interaction happens when two molecules with aromatic rings are close enough to interact through their *p* orbitals in π-conjugated systems. This event has been shown by the docked ligand PHE and pocket residue TRP60 at pocket 65 in Figure 47. The π bond from aromatic rings of ligand PHE and TRP60 were close enough to cross into each other's territory at a distance measured at 3.94 Å and 3.57 Å.



(a) (b)

Figure 46 Residues within distance 4.00 Å from ligands (a) POL and (b) PHE in van der Waals interactions at pocket 65 of T1 lipase

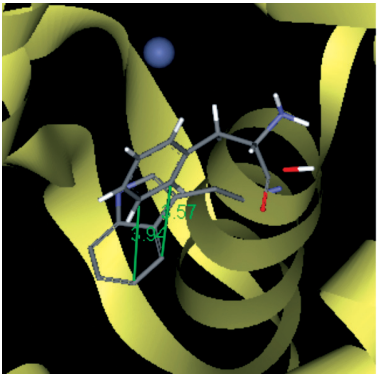


Figure 47 A π-π stacking interaction between ligands PHE and TRP60 in pocket 65 of T1 lipase

THE WAVES

Post Genomic Era

Genome-wide studies have the potential of providing researchers with a complete list of the molecular components present in living systems. Researchers have made great strides in the identification of the components of enzyme systems and of the relationships

between them in understanding the chemistry and physics of enzymatic catalysis. Such an unprecedented wealth of information has led to the renaissance of a long-standing goal of biological sciences, namely, the understanding of living organisms at the systems level, or systems biology. However, there is currently a lack of well-established experimental methods aimed at analysing the complete set of metalloproteins encoded by an organism (the metalloproteome), which is essential for comprehending processes in living systems.

Mechanistic Metallomics

Systems biology approaches require the combination of large-scale studies to catalogue genome-wide data sets and bioinformatics to obtain as detailed knowledge of the molecules and their interactions. Bioinformatics can give support to experimental methods in both of these efforts and are essential in gaining insight into metalloproteomes, given the fact that high-throughput experimental technology for their characterisation is not yet routinely available (Shi and Chance, 2008). For example, the comparative analysis of the content and usage of different metalloproteins across living organisms can be used to obtain hints on the evolution of metalloproteomes. The development of bioinformatics is a powerful predictive tool. With these methods, it is possible to scan entire proteomes for metalloproteins, such as transition metal proteins (e.g. Zn^{2+}, Mn^{2+} *etc*), which are identified by the presence of specific metal-binding sites, metal-binding domains, or both (Andreini *et al*., 2008). An analysis of enzyme mechanisms, restricted to enzymes with known structures, has shown that about 40% of enzyme-catalysed reactions involve metal ions.

Genome sequencing and postgenomic projects have predicted metalloproteins, which can be analysed to obtain information

on their function and evolution. Andreini *et al*. (2009) predicted the content of zinc, nonheme iron, and copper-proteins in a representative set of organisms taken from the three domains of life. The zinc proteome represents about 9% of the entire proteome in eukaryotes, but it ranges from 5% to 6% in prokaryotes, therefore indicating a substantial increase of the number of zinc proteins in higher organisms. In contrast, the number of nonheme iron proteins is relatively constant in eukaryotes and prokaryotes, and therefore their relative share diminishes in passing from archaea (about 7%), to bacteria (about 4%), to eukaryotes (about 1%). Copper proteins represent less than 1% of the proteomes in all the organisms studied.

Manipulation of proteins from genome data mining increases our knowledge of its structure and function and has paramount applications in biotechnology, pharmaceutical and agricultural industries. Designing any enzyme from scratch is a tall order; engineering a protein that can carry out a given function requires sophistication. It makes it clear that we can compute a structure that will catalyse a reaction where there was none before. Researchers can engineer never-before-seen catalysts using combinations of classical and new computational algorithms (Ghirlanda, 2008). In a major step forward for computational protein design, scientists have built from scratch, a handful of enzymes that successfully catalyse a specific chemical reaction. Jiang *et al*. (2008) developed a computational technique to build an enzyme called retro-aldolase to break carbon-carbon bonds in a non-natural chemical substrate, a computer based artificial enzyme from scratch, which is a relatively feeble catalyst if compared to natural enzymes. The geometry of the enzyme's active site was carefully crafted to hold the substrate in place and indicate components of the enzyme that are particularly important in prodding the reaction forward.

On top of it all, the relationship between protein fold and function is a fundamental question in enzymology. Studies of this type may advance our understanding of the potential function of protein folds, provide insight into natural evolution, and aid in the selection of scaffolds for future design (Bolon and Mayo, 2001). By using their minimally functional enzymes as evolutionary starting points, the researchers can use directed evolution to create catalysts that are more efficient. With modern analytical gadgets and sophisticated computers, bespoke enzymes might be designed for any chemical reaction from 20 natural amino acids and countless combinations of nonnatural amino acids. Interestingly, one of the key goals of the new science of computational enzymology is coming to fruition, and by combining computational design with a directed evolution approach, the catalyst's performance can be optimised through repeated rounds of random structural mutation.

CONCLUSION

Our fragile environment and scarce natural resources are being stretched to their limits. Behind these issues and challenges, there are key opportunities for chemical sciences to bloom and enhance basic science, meet industrial needs and even venture into personalised medicine. We have modeled and engineered a range of biocatalysts to be employed in a plethora of chemical reactions including non-natural reactions. Computational tools and simulations have facilitated a quantum leap in our endless exploration to understand chemical and biophysical behaviours of molecules and enzymes. Several novel molecules were carefully crafted to meet domestic and industrial demands, while still being compliant with new governmental acts and regulations regarding environmental concerns. Ultimately, our enzymatically synthesised fine and specialty chemicals can fuel many innovative and creative

ideas of new sustainable products and replace deleterious chemicals. New products for applications such as energy generation and storage, biomaterials and nanotechnology, will require science that crosses the boundaries of chemistry, physics, engineering and materials science. Many of the exciting technologies being developed rely on increasing interdisciplinary work. In the not too distant future, chemists will be at the forefront of synthetic biology, an emerging discipline at the interface of engineering and biology, the burgeoning field that could soon invent artificial life.

"Do or do not, there is NO TRY."
Yoda (*Star Wars Episode V: The Empire Strikes Back*)

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BIOGRAPHY

Mohd Basyaruddin Abdul Rahman was born in Penang (*Virgo*, 1972), started his early education at Methodist Primary School, and later attended Raja Tun Azlan Shah Science School (SERATAS) and graduated with flying colours in academics and sports (athletics, hockey and rugby from school to state level). He graduated with a *Double Major, B. Sc. (Hons.) in Chemistry and Computer Science with Education* from Universiti Teknologi Malaysia in 1995. A dynamic forward thinker, he then joined Professor John Evans' research group at the University of Southampton, England and received his PhD in Catalysis Chemistry. At the age of 26, he was one of the youngest Malaysian PhD recipients. His first appointment was as Quality Control Engineer (SONY), but developed his career as an academician at Universiti Putra Malaysia, beginning in August 1999. As a scientist, he gained experience working in world-renowned laboratories in England, Italy, France, Scotland, Japan and the USA, and he has travelled to more than 50 countries for the sake of science.

Dr. Mohd Basyaruddin is a true multidisciplinary scientist; his interests encompass broad areas from the single atom to complex biomacromolecules. He is among the pioneer chemists in this country who synergises experimental results with theoretical insights. Currently, his deep interests include designing novel metalloenzymes and nanobiomaterials as industrial biocatalysts for various specialty chemical reactions (with emphasis on pharmaceuticals, oleochemicals and petrochemicals). Additionally, he is investigating alternative solvent engineering for green processes, particularly chiral ionic liquids. Molecular interactions at the atomic level of protein-ligand-metal and transdermal nanodelivery systems are also being modeled. His long-term objective is to solve some fundamental problems related to

structural protein conformation and their functions. More than 10 research projects with grants from Malaysia and international bodies, including several 'top down' national projects funded under Priority Research and Strategic Research are under his leadership. A fundamental project based on novel metalloenzymes was sponsored by several research grants, namely the *Academy of Sciences for Developing World* (**TWAS Research Grant**), the *British Council* (**PMI 2 – Connect Award**), *Academy of Sciences Malaysia* (**SAGA Fund**), the *Ministry of Science, Technology and Innovation* (**Science Fund**), the *National Biotechnology Directorate* (**BIOTEK Fund**), the *Genetics and Molecular Biology Initiatives* (**GMBI Fund**) and the *Ministry of Higher Education* (**Fundamental Research**). Overall, he has secured a total of more than RM 15 million to conduct research. He has supervised and co-supervised more than 25 PhD and 30 MSc postgraduate students (including international students).

He received the **Japanese Society for the Promotion of Science Fellowship Award** (Osaka, 2000) to acquire knowledge in microbiology. He was conferred the **Young Scholar Award** from the *American Chemical Society* (Hawaii, 2005) for his work in immobilised enzymes and the **Young Chemists Award** by *International Union of Pure and Applied Chemistry* (Torino, 2007) for his work in protein flexibility and unfolding simulations. He received the prestigious **Engineering Conferences International Fellowship** to attend *Enzyme Engineering XIX 2007* in Canada. For his outstanding research, he was awarded the **Young Researcher Award** (2006), **Malaysia Excellent Scientist** (2005) and **Young Scientist Award** (2004). In June 2006, the Academy of Sciences Malaysia selected him as an **Outstanding Malaysian Young Scientist**. Lindau Nobel Council sponsored his attendance at the *56th Lindau Meeting of Nobel Laureates with Young Scientists*

in Germany. Later that year, he received the prestigious **Islamic Development Bank Merit Fellowship** for Post Doctoral research in Genetic Engineering at the University of Edinburgh, United Kingdom (September 2006), under the supervision of Professor Malcolm Walkinshaw.

On the international stage, he has made tremendous contributions to groundbreaking work and continues to collaborate with major world renowned laboratories such as University of Minnesota, USA (Professor Romas Kazlauskas – enzyme engineering); University of North Carolina at Charlotte, USA (Professor Donald Jacobs – biophysics); Texas A & M University at Commerce, Texas, USA (Professor Allan Headley – chiral catalysts); QUILL, Belfast (Professor Kenneth Seddon – ionic liquids) and GREENCHEM, Lund University, Sweden (Professor Rajni Hatti-Kaul – biocatalysis). His work in biocatalysis (liquid wax esters) and structural biology has been published (more than 80 cited and indexed papers and 10 chapters in books) in high impact journals including the *Journal of Molecular Catalysis B: Enzymatic, Catalysis Today, Biocatalysis and Biotransformation, Chemical Physics Letters, Molecular Simulation, The Protein Journals, etc.* These papers are regularly cited in ISI. He has presented his findings as the keynote speaker at international and local conferences (more than 200 proceedings). His expertise is recognised internationally and he is regularly invited as a reviewer. The mass media (radio, television and magazines) regularly interviews him for his contributions.

Prof. Mohd Basyaruddin's extensive research in enzyme technology for the production of immobilised enzymes, *Chirazim* and *MBzyme* (supports developed from natural materials and nanomaterials) aims to provide better alternatives to existing enzymes. The sustainable production of high-yield and high-purity palm-based esters and epoxides, adipate esters and sugar

esters products extend to bioreactors and statistical industry (in collaboration with Professor Mahiran Basri). Applications of liquid wax esters via environmentally benign processes have been extended to meet consumer needs such as in the production of *MBAdipate* (fine chemicals; lubricants), *MBiocoatings* (wood and surface coatings) and *MBSofax* (cosmecueticals; pharmaceuticals). These products have won numerous prestigious awards (more than 60, including 20 **Gold medals**) from international (Geneva, Pittsburgh, ITEX) and local exhibitions of product innovations including **National Patent Award 2009**. His research team is actively involved in protecting their method of production and research products. To date, 14 patent applications have been filed in Malaysia, 6 internationally (USA, Europe, Japan, Singapore and Indonesia) and 2 for trademarks.

In addition to successfully establishing a chemistry laboratory and turning it into one of the most productive biocatalysis laboratories in Malaysia, he has been instrumental in establishing theoretical and computational chemistry as a major field of study in Malaysia. He contributes to mathematical biology through collaboration with the Institute of Mathematical Research, UPM. His involvement in structural biology (in collaboration Professor Abu Bakar Salleh and Professor Raja Noor Zaliha) in the search for protein crystals has lead to national recognition for the first successful crystallisation and elucidation of a protein structure in Malaysia. His roles encompassed understanding the biophysical properties and design of the thermostable T1 lipase for application in industry.

With such a strong academic and technical background, he has shown his ability to contribute effectively. He played a pivotal role with utmost zeal as Lead Auditor and Head of the Implementation Committee in the development of ISO 9001:2000 procedures for

MS ISO certification in UPM. He was the **Head of Department of Chemistry** in the Faculty of Science, UPM. His promotion to Professorship at the age of 36 made him among the youngest in Malaysia. For his outstanding service at the university, he received numerous personal awards including **Excellent Service Award** (2004), **Certificates of Excellent Service** (more than 90% for 2003, 2005, 2006, 2007, 2008 and 2009) and the **Most Popular Lecturer (Chemistry)** (2007). He was **The Outstanding Young Malaysian 2008**; and named as one of the **Young Scientists of Asia 2009** and **Young Scientists of World Economic Forum 2009** for his extraordinary contribution to the scientific and technological development in the country. He energetically participates as a member of various committees for international and national conferences and workshops in the capacity of Chairman, Secretary and/or Chairperson of Sessions. He is the *Foundation Member* of the elite **Global Young Academy** under the umbrella of UNESCO and InterAcademy Panel (National Science Academies). Recently, his was appointed as an **Associate Fellow of ASM** and actively promotes the establishment of **National Young Academy** to cater to the needs of young talents. He is currently **Director of Structural and Synthetic Biology Research Centre**, Malaysia Genome Institute. This appointment enables him to play a more significant role in the country, contributing his ideas and knowledge in meetings and discussions, and exhibiting his organisational ability and leadership skills.

Supervising postgraduates and undergraduates and enhancing their laboratory skills, and his passion for teaching and educating them comes naturally. Being raised in a teacher's family, the young and vibrant Basyar enjoys being with students and students enjoy meeting him for counselling, motivation or just talking about life and activities. He teaches inorganic, physical chemistry, petroleum

chemistry and computational chemistry. His passion and vision for the youths has lead him to be active in outreach activities, especially as the Director for *MyBiotech@School* (promotion of biotechnology), *Back to Schools* (motivation and innovation in science), *National Science Challenges* and many other science motivational talks where he always emphasises the importance of chemistry, physics and mathematics in biotechnology-based knowledge and industry. He also volunteers in public schools by advising and mentoring many science and innovation project teams, especially with his previous school, SERATAS. He received **Outstanding Alumni Award 2010** for his contribution to the school and lifetime achievements. As a facilitator and motivator, he has interacted with more than 30,000 students (age 10-18) from more than 2,000 schools and matriculation centres since 2001.

Professor Dr. Mohd Basyaruddin has shown tremendous progress, breaking classical barriers and has become an energetic researcher who is fully committed to the advancement of science and technology in Malaysia, particularly multidisciplinary biotechnology. He is an individual who has not only proven his ability to "wear many hats," but he wears them equally well. His success has been inspirational to his peers and to the scientific community at large. His accomplishments make him a role model that should inspire many to follow the path he has set.

Science never gives up searching for truth, since it never claims to have achieved it.

John C. Polanyi, 1986 Nobel Laureate in Chemistry

ACKNOWLEDGEMENT

Above all, *Syukur* to Ar-Rahman for every blessing in my life and the faith in You that has brought me this far.

First and foremost, I'd like to thank my mentors, Professor Dato' Dr. Abu Bakar Salleh, Professor Dr. Mahiran Basri and Professor Dr. Raja Noor Zaliha Abdul Rahman for their kindness and welcoming my weird and fancy ideas throughout the establishment of my own *wing of research* in EMTECH Research Group (*established since 1986*). Thank you for introducing me to the eclectic and wonderful world of enzymology... and of course about research... I was very far from research initially...

A great thank you is definitely to be given to all MY STUDENTS who are also my *teachers*. My unimaginable and innovative ideas would never have been realised without your superb dedication and extra smart work. You guys are always there for helping and spending hours, days and nights with me on many occasions... exhibitions, birthdays and even at my wedding! You are truly the *wind beneath my wings*... My wish for you is that you will spread your wings in science and prepare to fly...

My regards to all EMTECH students and alumni (Bimo, Adam, Rosa, Salhah, Salina *etc*), staff members in Chemistry, FS and MGI; IBS, Inspem and ITMA; and to everyone who has ever helped me in any way, shape or form, and for their friendship. I also like to extend my regards to UPM, MGI, MOHE, MOSTI, MOE, ASM, JSPS, TWAS, ACS, IUPAC, ECI and IDB for all the funds and fellowships.

Not forgotten are my best buddies, Ibby, Zai, Kak Siti and SERATAS 89 for your '*nasty,*' '*scary*' and '*kewl*' advice. I can't believe you asked me to do the *doo-wop… those things*!!! Thank you for being so telepathic, understanding and listening to me rant and rave.

To my wife, Dr. Normi Mohd Yahaya, thanks love, for your tenderness, and welcome to UPM. In time, we could work as a pair like those famous scientist couples... *a dream is a wish your heart makes*...

Love and *terima kasih* to Mak (Allahyarhamah), Ba (Allahyarham), Aci, Kak Yah, Kak Dat and families (in-laws) for their support, encouragement, dedication and never ending love for me. I always need that. To all my nephews and nieces, I really miss your childhood time...

Finally, I must give thanks to myself for being ***myself but not too myself...*** You know you want this…

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6. Prof. Dr. Mohd. Ismail Ahmad
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7. Prof. Dr. Mohamed Mahyuddin Mohd. Dahan
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8. Prof. Dr. Ruth Kiew
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9. Prof. Ir. Dr. Mohd. Zohadie Bardaie
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13. Prof. Dr. Kapt. Mohd. Ibrahim Haji Mohamed
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