SUSTAINABLE WATER TREATMENT

Innovative Technologies

edited by
Zainura Zainon Noor
Noor Salehan Mohammad Sabli

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Preface

This book focuses on wastewater treatment with green and innovative technologies that promote sustainability. It talks about studies conducted on innovation from existing biological, chemical, and physical processes in wastewater treatment. It aims to help researchers or related parties that are interested in implementing wastewater treatment with greener technologies. Since this book covers all the fundamental processes (biological, physical, and chemical) in wastewater treatment, it will bring great benefits to readers as they would gain better understanding of green technologies in wastewater treatments.

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First and foremost, all praise be to God for his blessings and guidance for giving us the inspiration to embark on this project and instilling in all of us the strength to see that this book becomes a reality. Many people have contributed to the creation and completion of this book. We would like to acknowledge with appreciation numerous valuable comments, suggestions, constructive criticisms, and praise from evaluators and reviewers. Their suggestions have greatly helped us to improve the quality of this book. We also would like to express our gratitude to all who have helped in one way or another in the planning, brainstorming, writing, and editing stages of this book especially our Green Technology Research Group. We would also like to extend our appreciation to Universiti Teknologi Malaysia for providing us with the facilities vital to the completion of this book. Finally, we would like to express our appreciation to our families for their continuous patience, understanding, and support throughout the preparation of this book.

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Zainura Zainon Noor is an associate professor of chemical engineering at Universiti Teknologi Malaysia. She embarked on her career in UTM in 1999 as a research officer in a chemical engineering pilot plant prior to joining the Faculty of Chemical Engineering and Natural Resources 2 years later. A well-trained chemical engineer specializing in environmental engineering, Dr. Zainura is an intrinsically passionate individual driven toward finding greener and eco-friendly solutions. Through her unremitting interest, years of academic study, as well as conducive research and consultation activities, Dr. Zainura has established and strengthened her expertise in green technology, including cleaner production, life cycle assessment (LCA), water and carbon footprints, greenhouse gas inventory and projection as well as sustainable development. She is an accomplished project manager and is currently leading the Green Technology Research Group (Green Tech RG) at one of UTM's prominent centers of excellence, the Institute of Water and Environmental Management (IPASA). Recognizing her expertise in green technology, in 2009, the Department of Environment (DOE) Malaysia appointed her to develop the Cleaner Production Module, which was later used as the training module for the department's officers from all over Malaysia. Recently, she was selected by the Malaysian Government (under the Ministry of Natural Resources and Environment) as a consultant for the development of the Malaysia Environmental Performance Index (EPI). She is also an appointed committee member of the Green Technology Focus Group (sustainable solid waste) under the Ministry of Green Technology, Energy and Water. Dr. Zainura is also a renowned speaker and has given talks at numerous seminars, workshops, and short courses at both national and international levels. Dr. Zainura earned her PhD and MS degrees from Newcastle University, UK, and BS degree from Vanderbilt University.

Noor Salehan Mohammad Sabli is currently a PhD student in environmental engineering at the Faculty of Chemical Engineering, Universiti Teknologi Malaysia (UTM) under the supervision of an associate professor Dr. Zainura Zainon Noor. Her study is in the field of environmental engineering specializing in water footprint. Her research focuses on developing water footprint framework for calculating water usage through crude palm oil production from nursery until the mill, together with adapting the life cycle assessment approach in the framework.

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Section I

Innovative Biological Processes for the Recovery of Value-Added Products from Wastewater

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1

Enzymatic Hydrolysis of Waste Cooking Palm Oil by PVA–Alginate–Sulfate Immobilized Lipase

Nor Badzilah Hasan, Tan Wei Yie, and Nor Azimah Mohd Zain

CONTENTS

1.1 Introduction

Fatty acids exist in nature as carboxylic acids with long hydrocarbon chains, which are either saturated or unsaturated. They consist of carbon (C), hydrogen (H), and oxygen (O) and are arranged as a carbon chain skeleton with a carboxyl group (–COOH) at one end. The hydrocarbon chain length may vary from 10 to 30 carbons but it is usually from 12 to 18 carbons. Fatty acids are usually derived from triglycerides and are the main component of vegetable oil and animal fats. Fatty acids are widely used as raw materials in food, cosmetics, the pharmaceutical and dairy industries, and skin care products. Today, the production of fatty acid and glycerol from cooking palm oil is vital especially in oleochemical industries (Serri et al., 2008). Many researchers have used enzyme-catalyzed hydrolysis in order to reduce energy consumption and minimize thermal degradation of the products. However, studies using immobilized lipase which has the ability to hydrolyze cooking palm oil into fatty acid and glycerol have not been widely explored.

Waste cooking oil (WCO) is known for its high acid value of free fatty acids (FFAs; Araujo, 1995). FFAs are value-added products because of their wide industrial applications such as soap production, surfactants manufacturing, biomedical uses, and biodiesel production (Hill, 2000; Habulin and Knez, 2002).

1.2 Waste Cooking Oil

WCO is the residue from the kitchen, restaurants, and food factories. WCOs are basically generated from vegetable oils used at high temperature in food frying. As a result, this process causes hydrolysis, polymerization, and oxidation reactions which change the physical and chemical properties of the oil.

1.2.1 Environmental Pollution Cause by WCO

Increasing production of WCO from household and industrial sources is a growing issue all around the world. Table 1.1 shows the quantity of WCO produced in selected countries. This residue usually contains large amounts of FFAs, polymers, and decomposition products besides triglyceride and

TABLE 1.1

Quantity of WCO Produced in Selected Countries

Country	Quantity (million tonnes/year)
United States	10.0
China	4.5
European Union	0.7–1.0
Japan	0.45–0.57
Malaysia	0.5
Canada	0.12
Taiwan	0.07

some diglyceride due to the reaction of oxidation and hydrogenation (Lam et al., 2010). This residue is regularly being poured down the drain resulting in a wastewater treatment problem. Besides this, the residue can be integrated into the food chain via animal feed, thus resulting in a potential human health risk (Costa Neto et al., 2000). Recently, WCO rich in fatty acids has gained great interest due to its use in biodiesel production.

1.2.2 Composition of WCO

WCOs consist of saturated and unsaturated fatty acid, for instance; waste cooking palm oil is rich in palmitic acid, oleic acid, linoleic acid, and stearic acid. The fatty acids that do not have double bonds are termed "saturated," such as stearic acid and palmitic acid. These chains contain the maximum number of possible hydrogen atoms per atom carbon. Fatty acids that have double bonds are termed "unsaturated," such as linoleic acid and oleic acid. These chains do not contain the maximum number of hydrogen atoms due to the presence of double bond(s) on some carbon atoms (Lam et al., 2010).

WCO and fatty acid compositions are summarized in Table 1.2 (Hingu et al., 2010) and Table 1.3 (Wan Omar et al., 2009). The analysis in Table 1.3 shows that

TABLE 1.2

WCO Profile

WCO Composition	Percentage
Fatty acid	32.13
Ester	42.55
Methyl ester	2.14
Ketone	2.54
Aldehyde	4.69
Alkane	2.16
Alkene	0.68
Alcohol	0.29
Other	11.41

TABLE 1.3

FFA Profile in WCO

Fatty Acid Composition	Percentage (%)
Palmitic acid C16:0	9.08
Stearic acid C18:0	2.16
Oleic acid C18:1	35.34
Linoleic acid C18:2	53.4

they are mainly composed of 90% of unsaturated fatty acids (linoleic and oleic acids) and 10% saturated fatty acids (palmitic and stearic acid).

1.3 Lipases

Lipase enzymes are important in biological systems which belong to the group of serine hydrolyses (E.C. 3.1.1.3) (Jaeger and Eggert, 2002). They are mostly found built on an alpha and beta hydrolase fold with beta sheet containing the catalytic residues. Their catalytic site is composed of serine, aspartic acid, and histidine. The interior topology of alpha and beta hydrolase fold protein is mainly composed of parallel beta pleated strands separated by an alpha helix. Figure 1.1 shows the 3D structure of lipase from *Candida rugosa*. In Figure 1.1, "a" represents the helices which are packed against the central L-sheet. "b" represents the central L-sheet while "c" represents the smaller

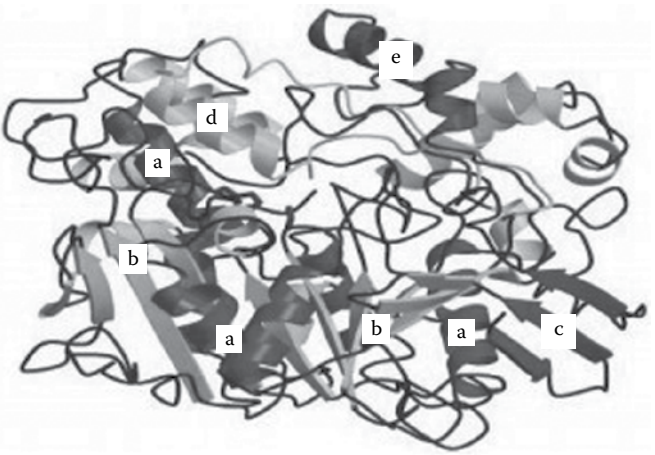

FIGURE 1.1
CRL enzyme 3D structure (Adapted from Cygler, M. and Schrag, J. D. 1999. *Biochimica et Biophysica Acta*. 1441: 205–214.).

N-terminal L-sheet. The closed conformation of the lid is represented by "d" and the open conformation is represented by "e". The residues forming the catalytic triad are indicated by "e".

Lipases are like esterases. They catalyze the hydrolysis and the transesterification of ester groups. However, the difference between esterases and lipase is that esterases act on soluble substrates while lipases catalyze reactions on water insoluble substrates and the presence of the water or lipid interface is a usual prerequisite for efficient catalysis (Cygler and Schrag, 1999). Their natural substrate is triglycerides. Lipases catalyze the hydrolysis of triglycerides to glycerol and fatty acids (Vaidya et al., 2008).

Lipases can also catalyze a wide range of enantio- and regioselective reactions such as hydrolysis, esterification, transesterification, aminolysis, and ammonolysis depending on the nature of substrate and reaction conditions (Vaidya et al., 2008). The versatility of lipase-catalyzed reactions made them greatly applied in numerous industrial processes including oils and fats, detergents, baking, cheese making, hard-surface cleaning as well as leather and paper processing (Schmidt and Verger, 1998; Jaeger et al., 1999; Villeneuve et al., 2000).

1.3.1 Lipase Sources

Various enzymes that are isolated from 2% of the world's microorganisms have been used as enzyme sources (Hasan et al., 2006). However, microbial enzymes are often more widely used compared to enzymes that are derived from plants or animals because of the great variety of catalytic activities available, high yield possibility, ease of genetic manipulation, and rapid growth of microorganisms on inexpensive media. In addition, microbial enzymes production is more convenient, safer, and exhibits higher stability (Wiseman, 1995).

In recent years, research on microbial productions of lipase has increased because of their wide application in industry such as the hydrolysis of fats, production of fatty acids, food additives, detergent additives, cosmetics, and care products (Bjorkling et al., 1991; Malcata, 1996). Lipases have been isolated from a wide number of plant, animal, and microbial sources (Sangeetha et al., 2010). Lipase producing microorganisms includes bacteria, fungi, and yeasts (Rapp, 1995). The ease of isolation of microbes' lipase has made both bacteria and fungi predominant sources of lipase. Lipases obtained from fungal sources were thought to be the best source for commercial application until bacterial lipases were discovered. Table 1.4 (Hasan et al., 2006) shows a lipase producing microorganism.

1.3.2 Lipase from *C. rugosa*

Lipases from *C. rugosa* were firstly described early in the 1960s by isolating the yeast from natural soils. It is also known due to its great lipase production (Yamada et al., 1963; Tomizuka et al., 1966). In addition, the lipases from yeast were nonpathogenic. Therefore, there were a great number of reviews about

TABLE 1.4

Isolation of Lipase from Various Microorganisms

Type of Microorganism	Lipase-Producing Microorganisms	Reference
Bacteria	*Bacillus* sp.	Imamura and Kitaura (2000)
	Bacillus subtilis	Ruiz et al. (2005)
	Pseudomonas sp.	Sarkar et al. (1998)
	Pseudomonas aeruginosa	Chartrain et al. (1993)
	Staphylococcus aureus	Gotz et al. (1998)
	Penicillium cyclopium	Chahinian et al. (2000)
	Lactobacillus plantarum	Lopes et al. (1999)
	Chromobacterium viscosum	Taipa et al. (1995)
Fungus	*Candida cylindracea*	Muralidhar et al. (2001)
	Rhizomucor miehei	Herrgard et al. (2000)
	Acinetobacter sp.	Snellman et al. (2002)
	Fusarium solani	Knight et al. (2000)

C. rugosa, highlighting its different aspects like biochemical, fermentation technology, or some biocatalytical applications (Benjamin and Pandey, 1998; Cygler and Schrag, 1999; Akoh et al., 2004). Furthermore, the lipase produced by *C. rugosa* is one of the most commonly used enzymes in organic solvents due to its high activity of hydrolysis, esterification, transesterification, and aminolysis (Villeneuve et al., 2000). The findings were also proven in the studies by Winayanuwattikun et al. (2011). In this study, *C. rugosa* lipase (CRL) also showed a broader range of substrate specificity with high activity for the substrates from 4 to 16 carbon chain length (Winayanuwattikun et al., 2011). Formally, *C. rugosa* is known as *Candida cylindracea* (Khor et al., 1985).

1.3.3 Hydrolysis Reaction of Lipase

A lipase reaction system usually consists of two immiscible phases where the water phase contains dissolved lipase while the organic phase contains dissolved substrates (triglycerides). The water phase of lipase is contacted with the triglycerides in organic phase forming liquid–liquid dispersion. Lipases catalyze the hydrolysis of triglyceride into FFAs and glycerol at the interface between the two liquids (Murty et al., 2002). Triglycerides do not dissolve in the water phase, thus the reaction has to be placed at the interface of the lipid and water phase (Pronk et al., 1988). The reaction often starts with reversible adsorption of the enzyme at the interface which then binds to the substrate to initiate catalysis (Tsai and Liching, 1990). Since the hydrolysis activity occurs at the lipid–water phase, the presence of an organic solvent is necessary to solubilize the lipid in water in order to achieve their hydrolysis catalyzed by lipases (Torres and Otero, 1996). On the other hand, emulsifiers can be added to increase the interface of the lipid–water emulsion. However,

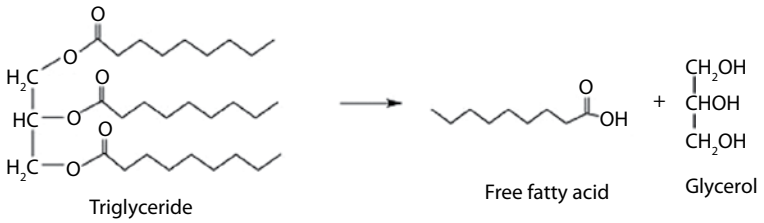

FIGURE 1.2
Hydrolysis reaction of lipase (Adapted from Gupta et al., 2008. *International Journal of Biological Macromolecules.* 42: 145–151.) The hydrolysis reaction yields 1 mole of glycerol and 3 moles of fatty acid per mole of triglycerides (Adapted from Murty, V. R., Bhat, J. and Muniswaran, P. K. A. 2002. *Biotechnology and Bioprocess Engineering.* 7: 57–66.)

according to Chew et al. (2008), the addition of emulsifiers is not preferred to avoid additional separation processes which are not favorable in industry. Figure 1.2 explains the hydrolysis reaction by lipase (Gupta et al., 2008; Villeneuve et al., 2000).

1.4 Immobilization Method

Immobilization can be defined as the technique used for physical or chemical fixation of cells, organelles, enzymes, or other proteins onto a solid support, into a solid matrix, or retained by a membrane, in order to increase their repeated or continuous use (IUPAC). There are three main types of methods used for immobilizing enzymes, such as entrapment, cross-linking, and carrier binding as shown in Figure 1.3. The selection of the immobilization method is based on several factors, such as overall enzymatic activity, cost of immobilization method, toxicity of immobilization reagents, and the effectiveness of enzyme utilization (Ozturk, 2001). Among various techniques employed for the immobilization of enzymes, entrapment methods are mostly used in cell immobilization procedures. The inert characteristics of the matrix result in relatively little damage to the native enzyme's structure. Besides that, the polymer used was inert to enzymes. Therefore, enzyme denaturation can be avoided. Furthermore, entrapped enzymes are more suitable for use with smaller size of substrates as larger enzymes will not be able to pass through the membrane and reach the active site of the biocatalyst (Villeneuve et al., 2000).

1.4.1 Advantages of Enzyme Immobilization

Enzymes are widely used as catalysts in many industrial, biomedical, and analytical processes. There has been considerable interest in enzyme

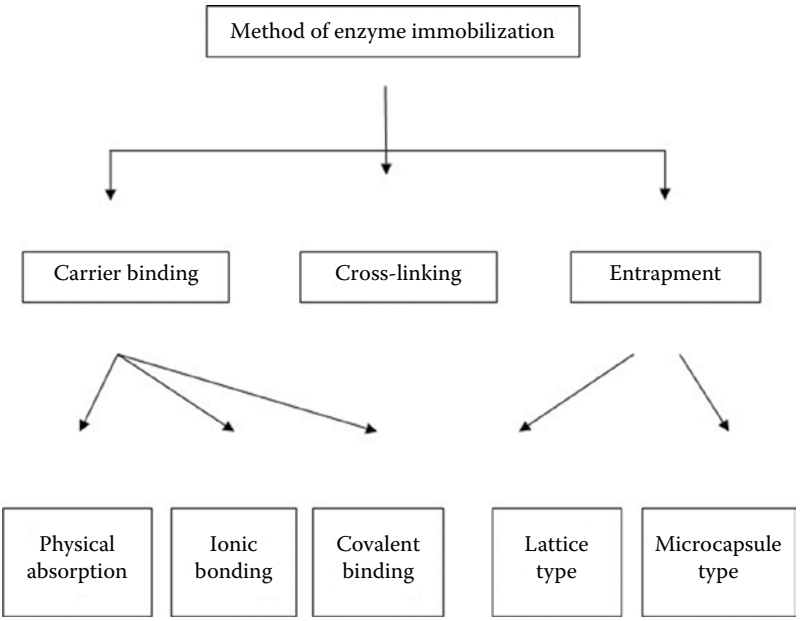

FIGURE 1.3
Methods for enzyme immobilization.

immobilization due to the simple preparation. The use of immobilized enzymes offers several advantages compared to free enzymes. The first advantage of immobilization permits the repeated use of enzymes. Although the immobilization procedure further increases the manufacturing expense, the multiple use of immobilized enzymes provides cost advantages. An ideal immobilization model is one which permits a high turnover rate of the enzyme yet retains its high catalytic activity over time. Secondly, the repeated use of enzymes allows continuous processes in industry as immobilized enzymes can be used immediately after a reaction. Thirdly, many experiments have shown that immobilized enzymes significantly enhanced stability in terms of thermal and pH aspects. Immobilization increased the thermal stability of enzymes that conferred their use for longer periods at a higher temperature compared to free enzymes. Besides that, immobilization broadened the working pH of an enzyme. This could allow enzyme activity over a wider pH (Twyman, 2005). Other than that, it enables the enzyme to be easily separated from the product. This would simplify enzyme applications; support a reliable and efficient technology, as well as provide cost advantages (Tischer and Wedekind, 1999).

1.4.2 Polyvinyl Alcohol

Polyvinyl alcohol (PVA) has been used as immobilization matrix since about 14 years ago (Hassan and Peppas, 2000). It is also the largest hydrophilic

synthetic polymer produced in the world (Ramaraj, 2000). PVA is rubber elastic-like in nature, nontoxic, and odorless. PVA is a polymer of great interest due to its desirable application in the pharmaceutical and biomedical fields (Hassan and Peppas, 2000). In the biomedical field, PVA has been proposed as a promising biomaterial that is suitable for tissue mimicking, vascular cell culturing, and vascular implanting (Cygler and Schrag, 1999). For instance, PVA has been applied in tissue engineering for regenerating a wide variety of tissue and organs, including arterial phantoms, heart valves, corneal implants, and cartilage tissue substitutes.

1.4.3 PVA–Alginate as Supporting Material

Cell entrapment in polymeric matrices is widely used for cell immobilization (Wu and Wisecarver, 1992; Zhang et al., 2005). It also proves that the immobilization enzyme on polymer matrices lead to better reusability (Idris et al., 2008). The living cells are enclosed in a polymeric matrix which is porous enough to allow the diffusion of substrates into the cells and permit products of enzymatic reaction to move away from the cells (Wu and Wisecarver, 1992). A wide variety of materials have been successfully used for cell entrapment such as agar, agarose, kappa carrageenan, collagen, alginates, chitosan, polyacrylamide, polyurethane, and cellulose (Ariga et al., 1987). Recently, the use of PVA for immobilization purpose has been investigated (Wu and Wisecarver, 1992; Hashimoto and Furukawa, 1987). Ariga et al. (1987) used the freezing and thawing of the PVA method to form a gel suitable for cell immobilization. They found that this technique produced a low cost material and exhibited high strength with rubber-like elasticity (Ariga et al., 1987). PVA cross-linked with boric acid has been developed by Hashimoto and Furukawa (1987). They have used a new inexpensive and less energy-intensive immobilization method to immobilize activated sludge using PVA. They cross-linked the PVA using boric acid solution and a monodiol type of PVA–boric acid gel lattice was produced. The activated sludge was successfully immobilized in PVA–alginate beads without loss of biological activity (Hashimoto and Furukawa, 1987). A modified PVA–alginate bead was developed and reported by Idris et al. (2008) by introducing sodium sulfate. The beads produced by this technique were found to be more stable in terms of chemical and mechanical strength. The beads also displayed superior enzyme activity and showed relative good diffusivities (Zain et al., 2010). Similar findings were also reported by Takei et al. (2011).

1.4.4 Advantages of PVA–Alginate Matrix

In recent years, the use of PVA for cell immobilization has attracted wide attention due to several advantages that it offers. PVA is a cheap and nontoxic synthetic polymer as well as being easy to process. Besides this, enzymes immobilized in a PVA matrix showed high activity and high stability with

repetitive use, thus increasing economic viability of biosynthetic processing (Zain et al., 2010). On the other hand, PVA is a hydrophilic supporting material. Generally, the use of hydrophilic support in immobilization enhances enzyme stability while the use of hydrophobic support material appears to have its disadvantages. PVA can cause protein stabilization by its attachment to the polymer chains (Kozhukharova et al., 1988). It is suitable for use for immobilization purposes because it can be easily modified through its hydroxyl groups. PVA also offers assorted advantages over the conventional alginate matrix including lower production cost, higher robustness, and nontoxicity to viable cells. PVA beads exhibit rubber-like elasticity in nature (Hassan and Peppas, 2000). Thus, PVA beads provide stronger mechanical strength compared to alginate beads. Moreover, PVA beads display high stability within a wide range of pH that is from pH 1 to pH 13, while alginate beads are relatively stable in the range of 6–9 (Khoo and Ting, 2001). Furthermore, alginate beads encountered a weight loss up to 20% and 24% at low and high pH. By being aware of these advantages, PVA has been used widely in cell immobilization (Idris et al., 2008).

1.4.5 Drawbacks of PVA and Its Solution

The PVA–boric acid technique provides an easy and low cost method in enzyme immobilization. However, there are some problems when using the PVA–boric acid method for immobilization. PVA is a sticky material, thus PVA beads have the tendency to agglomerate (Wu and Wisecarver, 1992). Nevertheless, this matrix is still used by many researchers. In order to eliminate the agglomeration, calcium alginate has been introduced. It also serves to improve the surface properties of the beads (Wu and Wisecarver, 1992; Yujian et al., 2006). The application of calcium alginate in a mixture with PVA for enzyme immobilization has been reported by Wu and Wisecarver (1992). The PVA–alginates beads produced were proven to be very strong and durable with no biological loss for 2 weeks of continuous operation in a fluidized bed reactor.

The introduction of sodium alginate in the PVA–boric acid method was also suggested by Slokoska et al. (1999). The finding of Slokoska et al. (1999) demonstrated that the photo-cross-linked PVA and calcium alginates beads are suitable for the entrapment of fungal cells (Slokoska et al., 1999). PVA exhibits a high degree of swelling in water (Hassan and Peppas, 2000). It will readily dissolve in aqueous solution causing the enzyme to leak out from the matrix (Zain et al., 2011). Therefore, the PVA must cross-link either chemically or physically to make it soluble. The most popular cross-linking reagent for immobilization is glutaraldehyde (Villeneuve et al., 2000). Besides that, cross-linking the PVA using boric acid solution to produce a monodiol type PVA–boric acid gel lattice has also been reported by Hashimoto and Furukawa and Wu and Wisecarver. The other drawback of this polymer is that the saturated boric acid solution is highly acidic (pH < 4) and it causes a drastic

decrease in the viability of immobilized cells. This obstacle can be overcome by adding sodium sulfate which acts as an inducer for cross-linkage of PVA to avoid the drastic decrease in cell viability caused by saturated boric acid solution (Idris et al., 2008; Takei et al., 2011).

1.5 Case Study

1.5.1 Materials

CRL (3.1.1.3) (Type 1176 U/mg) was purchased from Sigma Aldrich (Japan). PVA 60,000 MW and boric acid were purchased from Merck Schuchardt OHG, Darmstadt, Germany. Sodium alginate was obtained from FlukaChemie GmbH, Buchs, sodium sulfate from GCE Laboratory Chemicals, and calcium chloride from R&M Marketing, Essex, UK. Iso-octane with 99.84% assay was purchased from Fisher Chemicals (UK). Other reagents used were analytical reagent grades and used without further purification including phosphate buffer solution pH 7.5 or otherwise stated.

1.5.2 Pretreatment of WCO

WCO was obtained from a food stall near Universiti Teknologi Malaysia (UTM). For a successful reaction, the oil must be free from water and other impurities. Initially the samples of waste cooking palm oil were filtered to remove any suspended food particles. Then, the waste cooking palm oil was heated at 105°C for 1 h to remove its water content. After that, the titrimetry method with NaOH was used to determine the FFA content in the WCO (Patil et al., 2010).

1.5.3 Preparation of Lipase Enzyme Solution

The pH of the phosphate buffer solution was adjusted to pH 7.5. CRL (5 g) was dissolved in 100 mL of phosphate buffer solution. Then, the enzyme solution was filtered using a 0.45 µm nylon syringe filter to sterilize the enzyme. The sterilized enzyme solution was stored at 4°C until further used.

1.5.4 Immobilization of CRL

CRL solution with the volume of 10 mL, 5% (v/v) was mixed with 90 mL PVA–alginate solution. The mixture was mixed comprehensively and introduced as drops by using a rotary pump into a 100 mL mixed solution of saturated boric acid 5% (w/v) and calcium chloride 2% (w/v). The beads were stirred gently for 30–50 min to complete the solidification. Then, the PVA–alginate

beads were stored at 4°C for 24 h. After 24 h, the mix solution of boric acid and calcium chloride was discarded and replaced with a 7% (v/v) boric acid solution. The beads were stirred in the boric acid solution for 30 min and the solution was then replaced with 0.5 M of sodium sulfate solution and stirred for another 30 min. Then, the beads were kept at 4°C until further used (Idris et al., 2008).

1.5.5 Waste Cooking Palm Oil Hydrolysis

A conical flask of 250 mL was initially filled with 3 g of cooking palm oil and 30 mL of iso-octane solvent. Phosphate buffer solution (30 mL, pH 7.0; unless otherwise stated) was added into the conical flask so that the ratio of oil to aqueous (buffer solution) is 1. The mixture formed two layers. Three other identical mixtures as above were prepared. To start the reaction, 0.3 g of CRL was added to three flasks of reaction mixtures and one was left without the CRL for control measurement. The mixtures were agitated in the orbital shaker at 45°C at 200 rpm. Samples were withdrawn from the oil every 30 min. The same procedure was carried out using immobilized lipase (Serri et al., 2008). To determine the effect of parameters on the hydrolysis of WCO, three variables were taken into consideration which is pH, temperature, and enzyme concentration. The pH varied from 7 to 8. The temperature varied from 30°C to 50°C and enzyme loading varied from 2 to 8 g of beads which correspond to 96.43 to 385.73 U/mL enzyme.

1.5.6 Determination of Degree of Hydrolysis (Conversion) and Rate of Hydrolysis

The degree of hydrolysis was determined by titration of the oil phase samples with 0.1 M sodium hydroxide (NaOH). To each sample, 5 mL of the oil phase was dissolved in 5 mL ethanol:diethyl ether (1:1% v/v). The amount of 0.1 M NaOH required to neutralize the acid was noted. A blank titration was done as a control sample. Phenolphthalein was used as an indicator. The degree of hydrolysis, X is calculated as below (Serri et al., 2008).

$$X\% = \frac{\text{(ml NaOH used) (molarity of NaOH) (average molecular weight of fatty acid)}}{\text{10 (weight of sample)}} \tag{1.1}$$

1.5.7 Fatty Acid Concentration Determination

All samples collected were analyzed using Perkin Elmer Autosystem XL equipped with a flame-ionization and a Nukol™ 15 m × 0.53 mm i.d. column coated with 0.5 μm (25326) column. Helium served as a carrier gas at a flow rate of 20 mL/min. The column temperature was programmed from 110°C to 220°C with the increase of 8°C/min. The injector and detector

were monitored at 250°C and the amount of sample injection was 0.2 µL with direct injection. The presence of fatty acid was based on the comparison of retention time and peak area of the sample with oleic acid as standard.

1.6 Results and Discussions

1.6.1 Effects of Temperature on Immobilized CRL

The reaction temperature is an important parameter in enzyme catalysis. The effect of temperature effect is significant because in order to increase the reaction rate, the temperature has also to be increased. This fact is also supported by Kumari et al. (2009) where an increase in temperature will speed up enzyme-mediated reaction. In this study, the reaction mixture was incubated at temperatures varying in the range of 30–50°C with the immobilized lipase. As shown in Figure 1.4, the highest fatty acid concentration and hydrolysis conversion were achieved at 50°C.

If the enzyme is immobilized on a suitable support, thermal stability is usually improved. Therefore, determination of the optimum temperature at which the lipase does not lose its activity is very important in finding the optimum operating conditions for immobilization. Previous study by Dave and Madamwar (2006) also reported that the optimum temperature was found at 50°C when *C. rugosa* lipase was immobilized in PVA–alginate matrix for the esterification reaction. Similarly, an optimum temperature of 50°C was reported by Santos et al. (2008) when using *C. rugosa* lipase immobilized by covalent attached on polysiloxane–polyvinyl alcohol for the hydrolysis of olive oil.

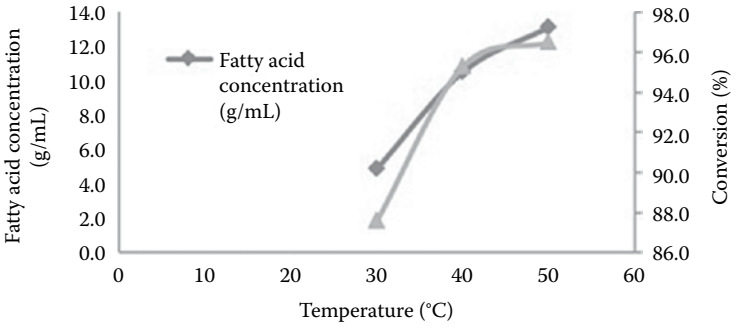

FIGURE 1.4
Effect of temperature on fatty acid production and hydrolysis conversion by using immobilized CRL (pH = 7.0, enzyme loading = 8 g of immobilized beads [385.73 U/mL], 200 rpm).

1.6.2 Effects of pH on Immobilized CRL

As enzyme activity also changes with pH, choice of the working pH also depends on the optimum working pH of the enzyme. Therefore, in order to maximize the immobilization yield, to work in a suitable pH range is essential. The effect of pH of the reaction medium on hydrolytic activity of the immobilized lipase was evaluated by adjusting the pH in the range of 7–8 at 50°C. A pH of 7 for the immobilized lipase was found to be optimum for achieving efficient hydrolysis with highest fatty acid production and highest hydrolysis conversion (Figure 1.5).

Similarly to this study, an optimum pH of 7 was reported by Garcia et al. (1992) when a lipase from *C. rugosa* was immobilized by adsorption on flat sheets made of microporous polypropylene for the hydrolysis of milk fat triglycerides. Kang and Rhee also obtained an optimum pH of 7 when using *C. rugosa* lipase immobilized by adsorption on swollen Sephadex for the hydrolysis of olive oil. However, Santos et al. obtained an optimum pH of 8 when a lipase from *C. rugosa* was immobilized on poly(N-methylolacrylamide) by physical adsorption. Lipases undergo structural changes in some pH values and this leads to inactivation of the enzyme or change in its activity due to perturbation in the vicinity of the active site. Similarly, these reactions might have occurred in CRL proteins, causing low activity at pH > 8.0 (Akova and Üstűn, 2000). Therefore, the working pH depends mainly on the method of immobilization and the interaction between enzyme and support (Ting et al., 2006).

1.6.3 Effect of Enzyme Loading on Immobilized CRL

The effect of enzyme loading on the hydrolysis reaction was also investigated. The enzyme loading varied from 96.43 to 385.73 U/mL which corresponds to 2–8 g. Figure 1.6 shows that the maximal fatty acid production increases with the biocatalyst loading.

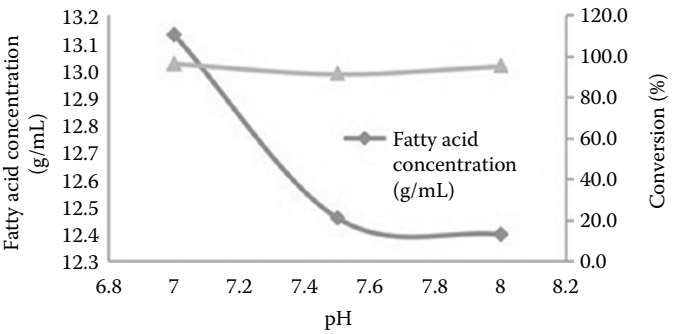

FIGURE 1.5
Effect of pH on fatty acid production and hydrolysis conversion by using immobilized CRL (temperature = 50°C, enzyme loading = 8 g of immobilized beads (385.73 U/mL); 200 rpm).

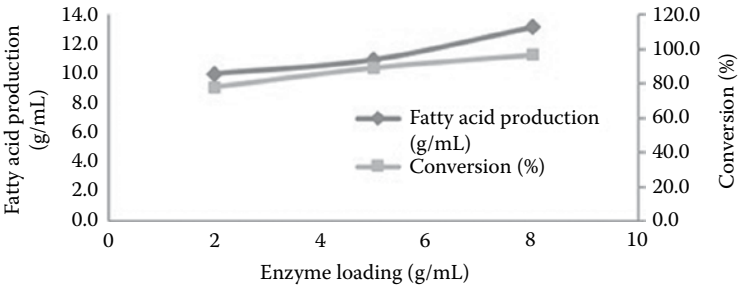

FIGURE 1.6
Effect of enzyme loading on fatty acid production and hydrolysis conversion by using immobilized CRL (temperature = 50°C, enzyme loading = 8 g of immobilized beads, 385.73 U/mL; 200 rpm).

The optimum enzyme loading was found to be 385.73 U/mL which is equal to 8 g of immobilized beads. A study by Akova and Üstűn (2000) suggested that at low loadings, there is a large excess of surface area that the enzyme can occupy and the lipase attempts to maximize its contact with the surface, which results in a loss of conformation and consequently in a reduction of activity. As the loading increases, less area is available for the lipase to spread itself, more of its active conformation is retained, and the loss in activity is reduced. However, in the presence of an excessive amount of lipase, the active site of the enzyme cannot be exposed to the substrate and many molecules in the enzyme tend to aggregate together (Liou et al., 1998; Foresti and Ferreira, 2005). On the other hand, Salis et al. (2008) obtained the highest enzyme activity which was 600 mg/g (8.35 kLU/g) when CRL was immobilized on macroporous polypropylene. It is suggested that the optimum enzyme loading needed for reaction also depends on the interaction between support and enzyme.

1.6.4 Comparative Study of Free and Immobilized CRL

The maximum yield of fatty acid was 13.13 g/L (of which 96.5% hydrolysis conversion was achieved) with 8 g of immobilized beads (385.73 U/mL), working pH 7 at 50°C. The same condition was also performed on free enzymes to study the hydrolytic activity. The results obtained were compared with immobilized CRL.

Based on Figures 1.7 and 1.8, PVA–alginate immobilized CRL showed the highest production of fatty acid and conversion of hydrolysis with 13.13 g/mL and 96.5%, respectively. While that, the production of fatty acid and hydrolysis conversion for free lipase was 2.9 g/mL and 68.75%, respectively. Immobilized enzymes offer a lot of advantages ranging from increasing enzyme activity to withstanding environmental stress. Immobilization reduces contamination risks, allows enzyme reuse, increases stability, and rapidly gives positive results compared to free enzymes (Idris et al., 2008; Zain et al., 2011). According

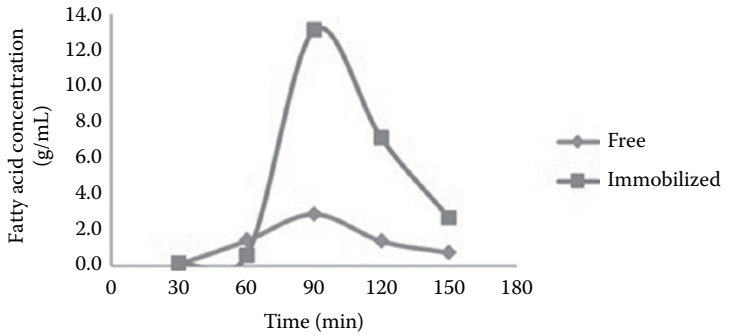

FIGURE 1.7
The fatty acid production for WCO by using free and immobilized enzyme (temperature = 50°C; pH = 7.0; enzyme loading = 8 g of immobilized beads, 385.73 U/mL; 200 rpm).

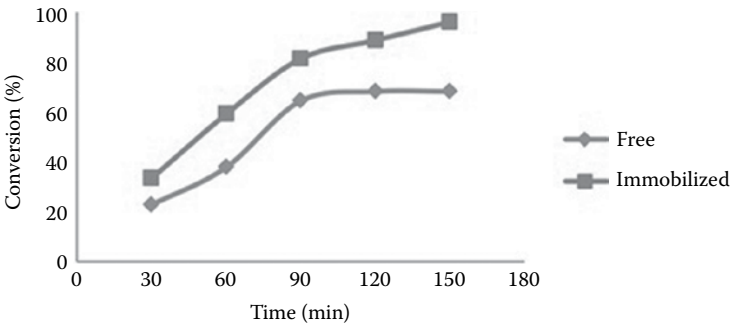

FIGURE 1.8
The hydrolysis conversion for WCO by using free and immobilized enzyme (temperature = 50°C; pH = 7.0; enzyme loading = 8 g of immobilized beads, 385.73 U/mL; 200 rpm).

to Saraiva Silva et al. (2004) PVA/alginate immobilized lipase appears to be more positive to hydrolysis of oil compared to free lipase due to the better prevailing interface condition between the PVA and the alginate. Another study was conducted by Shah and Gupta (2007) whereby the immobilized lipase gave higher biodiesel production compared to the free enzyme as it provides larger surface area of the biocatalyst preparation. Besides, the powder form of the enzyme tends to aggregate in low water media creating problems in mass transfer resulting in the low activity of lipase.

1.7 Conclusion

The enzyme acts as a biocatalyst and it is much more expensive than other catalysts. This is the main reason for using PVA–alginate beads to immobilize

lipase. By immobilizing the enzyme, it is expected that it could be reused several times. Immobilization also helps in enhancing enzyme catalytic activity, enzyme stability, reusability, and recovery of enzymes. Based on the case study, immobilized lipase successfully produced more fatty acid and hydrolysis conversion compared to free lipase. This is the first time such modified PVA–alginate matrix is used to immobilize lipase to treat WCO. Lipase from *C. rugosa* was immobilized in modified PVA–alginate matrix using entrapment and the cross-linking method. PVA–alginate beads were chosen because they are a nontoxic synthetic polymer and also low cost.

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