Biocatalysis techniques for the valorization of effluent and solid waste from the oils and fat industry to biofuels

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DOCTORAL DISSERTATION

Biocatalysis techniques for the valorization of effluent and solid waste from the oils and fat industry to biofuels

油脂産業由来の廃液および固形廃棄物からバイオ燃料へ変換するための生体触媒技術開発

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GRADUATE SCHOOL OF ENGINEERING

KOBE UNIVERSITY

QUAYSON EMMANUEL

PREFACE

This is a thesis submitted by the author to Kobe University in partial fulfilment of the requirements for the degree of Doctor of Engineering. The studies were carried out between the period of 2017 and 2021 under the supervisory of Professor Chiaki Ogino in the Laboratory of Biochemical Engineering, Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University.

I would like to express my gratitude to my Ph.D. Supervisor, Ogino Chiaki (Prof.) for the scholarship, support, and time-spent with me throughout this Ph.D. The decision to move to Japan would not have been possible without the support of my wife, Monica Skaten (Ph.D.). Her unwavering support, sacrifices, and encouraging me from her Ph.D. experience can only be found in a good wife. The trip to Japan for this Ph.D. journey would not have been possible without the recommendation by Jerome Amoah (Ph.D.). Together with Shinji Hama (Ph.D.) and Ayumi Yoshida (Ph.D.), our relationship yielded good publications that have been very important to the success of this Ph.D. I would also like to appreciate my lab colleagues' supportive relationship, Nova Rachmadona, Daniel Alfaro, Deddy T. N. Adi, Rendi Palar, Yusuke Harada, Roy Fong-Yi K., and others whose names cannot be listed here because it will be exhaustive. The hard work and support from all the research staff and lab technicians were also unparalleled in making it possible to conduct all the experiments necessary for this work. Several of the material analyses in this work were also conducted at Prof. Takashi Nishino's laboratory. There, Miyabi Yorifuji and Takuya Matsumoto (Ph.D.) were kind in offering me access and assistance to all the equipment I needed.

Lastly, my parents, siblings, nieces, and nephews can all be remembered in this journey for their support and prayers for a successful time in Japan. The arrival of my lovely son, Kwesi Skaten, concluded this Ph.D. journey with love and bliss.

The author would like to acknowledge support from Japan's Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan's Grants-in-Aid for the Strategic Innovation Promotion Program (SIP, Innovative Bioproduction, Kobe), and a New Energy and Industrial Technology Development Organization (NEDO) grant towards addressing energy and global environmental problems.

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CONTENTS

Introduction

Global energy demand: The role of biodiesel in the energy mix

Fuels, electricity, and various products made from hydrocarbons have been critical enablers of modern life. From large to the minuscule polymer used in every modern item today, it seems almost problematic to have a modern society without hydrocarbons. Hydrocarbons are merely organic compounds consisting of carbon and hydrogen atoms. They exist as liquids (e.g., crude oil), gases (e.g., methane), solids (e.g., bituminous coal), or polymers (e.g., polyethylene). Combustion is one of the most superficial reactions hydrocarbons undergo (Eqn. 1). The energy in the form of heat generated from the combustion of liquid hydrocarbons has been the most extensive product used to support the diverse modern human activities.

$$CH_4 + 2\ O_2 \rightarrow 2\ H_2O + CO_2 + \text{energy} \qquad [1]$$

The global demand for hydrocarbons for transport, power generation, and manufacturing has grown tremendously, with an ever-growing world population estimated to reach 9 billion by 2035. It is anticipated that global energy demand will parallel to 778 Etta Joules by 2035 from the current 580 Etta Joules (2020) [1]. However, this higher energy demand for the next decade will increase anthropogenic CO_2 from hydrocarbon combustion. Anthropogenic CO_2 and its associated greenhouse gas (GHG) emissions are significant drivers of climate change. The effect of climate change on life on earth has been elucidated in several recent works of literature [2,3]. The Paris Climate Agreement is one of several climate change actions taken to reduce global GHG emissions. Renewable energy sources are seen as a solution.

A global shift to renewable energy sources to replace fossil-derived hydrocarbons is set to grow by 50% in the next five years (2019 to 2024), and this will comprise chiefly of wind, photovoltaics (PV), hydro, biofuels, hydrogen, and nuclear energy into the electricity grid.

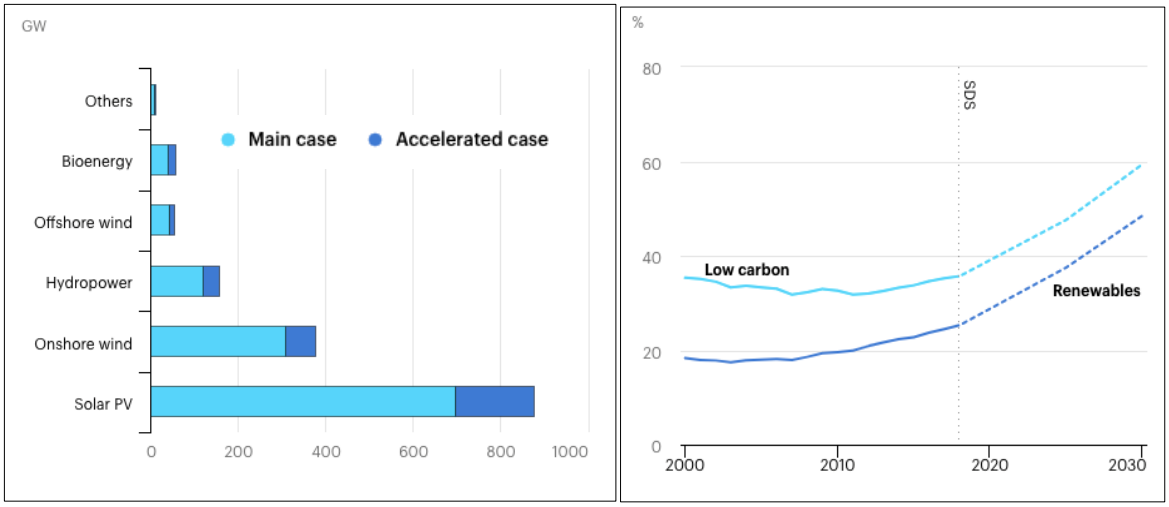

Figure 1-1 Forecast of Global renewable energy technology mix (2019- 2024) and (b) the share of renewables in power generation in a sustainable development scenario (SDS), 2000-2030 (IEA, 2019) [4].

The twin challenges of the ever-increasing energy demand and climate action calls suggest increasing biomass-derived energy sources (biofuels) [5]. Biofuels are cited to be indispensable in the transition process (Figure 1-1) [2]. Bioenergy use can augment electricity, heating, and transportation energy needs of the growing world population. For instance, with Biomass Energy and Carbon Capture and Storage (BECCS) as a model, biofuels have the potential to offset carbon emissions to possible net-negative [3]. Presently, bioenergy's contribution to global domestic and industrial energy demand is more than wind and photovoltaics combined (Figure 1-2). Increased political policy support has increased biofuels demand for heating, transportation, and electrification in the last decade [2,4]. Even though biofuels' combustion produces GHG emissions as other fossil fuels, carbon sequestration during biofuel crops cultivation places biofuels as a carbon-neutral fuel. The viability of utilizing methanogenic wastes (e.g., from agricultural and industrial sources) for biofuels production also adds to its carbon-neutrality essentials. For shipping, long-haul transport, and the aviation sectors where battery-powered engines have not been established, an immediate

solution is about using low-carbon biofuels such as biodiesel [6]. Besides its near-carbon neutrality, biodiesel has the advantages of sustaining the energy security of fossil-deprived economies and serving the future circular economy through waste utilization. The valorization of agricultural, industrial, and domestic wastes, such as waste oils/ fats into biodiesel, offers an opportunity to create a loop in the future circular economy.

Biofuels such as biodiesel can also serve to be precursors or building blocks to some of the fossil-derived fine chemicals such as in its application as green solvents [7]. Besides being able to serve a circular economy through waste utilization, biodiesel can support rural economic growth. For fossil-deprived countries, utilizing biodiesel wholly or in blends can save significantly on foreign exchange spending. Biodiesel's enormous benefits as an energy source have been mentioned in many good reviews and books on biodiesel production [5–7].

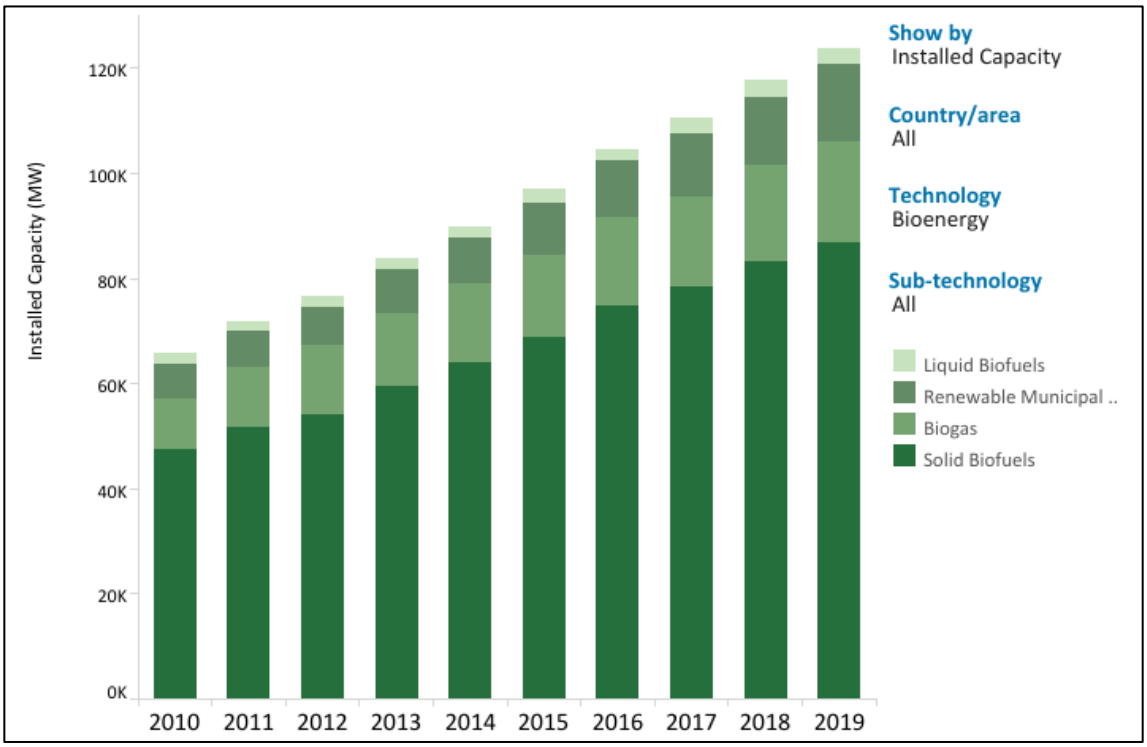

Figure 1-2 Global installed capacity (in megawatt, MW) of bioenergy from 2010 to 2020 [8].

An introduction to biodiesel

Biodiesel is produced via the catalysis of triglycerides or free fatty acids present in oils/fats to fatty acid alkyl ester. The synthesis of fatty acid alkyl ester (biodiesel) requires the substitution of acyl glycerides in the precursor oils/fats with alcohol. Methanol, ethanol, and *iso*-propanol are common alcohols used in the synthesis process. The synthesis process is referred to as transesterification when triglycerides are converted to other esters in the presence of alcohol. Figure 1-3 a illustrates the transesterification reaction.

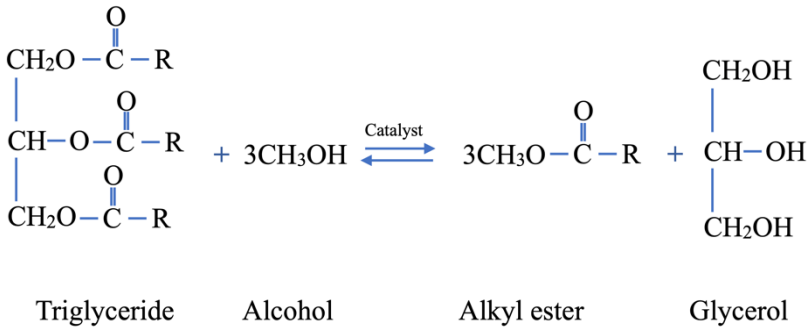

Figure 1-3 The transesterification reaction involves reacting triglyceride from oils/fats with alcohol (CH_3OH) to produce mono alkyl esters (biodiesel). The reaction is catalyzed by a chemical (e.g., acids or bases) or biological catalyst (e.g., enzymes).

Free fatty acids (unbounded to glycerides) can also be converted to fatty acid alkyl esters. This is referred to as esterification. However, transesterification of oils/fats yields glycerol as a byproduct, whereas esterification produces water as a byproduct. Like most organic reactions, catalysts are necessary to ensure efficient reactants utilization and faster product formation. Chemical (acids or bases) and biological catalysts (particularly lipases) are used (Figure 1-3). Commonly used chemical catalysts include H_2SO_4, HCl, KOH, and NaOH. Various lipases are continuously being researched to replace the corrosive and environmental damaging effects of using chemical catalysts.

Vegetable oils and animals' fats of varied origin are used as feedstocks. Soybean oil, palm oil, rapeseed, and sunflower are some of the common oils used. As liquid fuel biodiesel is miscible with petroleum-derived Diesel in ratios designated as BXX, where XX denotes the percentage of biodiesel present in the blend. For example, a blend of 30% biodiesel in diesel fuel is designated as B30.

The benefits of using biodiesel in blends or completely to replace petroleum diesel can be categorized but not limited as follows;

- Safety - biodiesel has a higher flash point compared to petroleum diesel;
- Development - supports rural livelihoods through agriculture;
- Economics - foreign exchange savings for fossil-deprived countries;
- Environmental -lower GHG emissions;
- Sustainability - agricultural and industrial waste utilization.

A brief historical account on biodiesel

The genesis of biodiesel as alternative diesel fuel is associated with many historical events such as the first and second world wars, the world energy crisis in the 1970s, and the American soybean agricultural boom in the 1980s [9]. The origin, inventors, and the first industrial-scale biodiesel production are inconsistently presented in the literature. Rudolf Diesel, the diesel engine's inventor, is often associated as the originator behind the fuel. However, in Diesel's book *Die Entstehung des Dieselmotors*, the inventor recognizes the use of peanut oil in a diesel engine at a Paris Expo by the French Otto company in 1900. The first patent accepted on palm methyl ester was by the Belgian scientist G. Chavanne (University of Brussels, Belgium). During the second world war, petroleum-deprived countries such as Japan are recorded to have used neat vegetable oils as liquid fuel for aircraft carriers and battleships.

A Chemical Abstract search by G. Knothe reports the first use of the term "Biodiesel" (regardless of capitalization or hyphenation) by a Chinese author in a 1988 scientific report. This is followed by a report in 1991 from Bailer and Hueber on the *Determination of Saponifiable Glycerol in "Bio-Diesel* [10]. Since then, the use of the term biodiesel has increased exponentially in literature.

Accounts of colonial expansions by the French, English, and Dutch in the 1800s also report the use of neat vegetable oils as liquid fuels to empower their colonization agenda in Africa, South America, and Southeast Asia. China and India later used petroleum cracking techniques to exploit vegetable oils during and after World War II. Although inefficient and hasty, China and India's cracking technique produced distillates of "gasoline" and "kerosene" from Tung and cottonseed oils.

In 1990, the United States enacted its Clean Air Amendment Act to mandate alternative fuels in trucks and bus fleets. This is reported to have made a significant increase in the use of biodiesel in the US, which is still the world's largest producer and user.

Walton, a pioneer in biodiesel research, reports the production of biodiesel from 20 vegetable oils that included beechnut, camelina, castor, chestnut, cottonseed, grapeseed, hemp, linseed, lupin, maize, palm, olive, pea, peanut, poppy seed, pumpkinseed, rapeseed, shea butter, soybean, and sunflower seed oils [10]. The higher kinematic viscosity of biodiesel drove Walton's interest in investigating the splitting of glycerol from triglycerides to use the remaining components as fuel. Preheating and later blending heated or chemically-treated vegetable oils took some aspects of later biodiesel research. The first report on biodiesel's cetane number as biodiesel's ignition quality compared to petroleum diesel is in a study by Abeele and Palme [11].

Thesis Objectives and Layout

This thesis is structured into six chapters. The structure is independently connected to publication dates but in a format that presents the overarching aim of this study and in order of research findings.

Chapter 1 provides a general introduction to the theme in relation to global energy demand. Statements on the role of biofuels in the future energy-mix presented in this chapter are linked to a brief historical record of biodiesel and the advances in technology that has occurred to date.

Chapter 2 is a published review of recent literature on the field of study. In this regard, fundamental biodiesel catalysis, the use of immobilized lipases to supplant chemical catalysts, the advantages of immobilized lipases over soluble lipases, and current and near-term future of immobilized lipases- sustainability, immobilization waste generation, applying green chemistry principles to immobilization and other related topics were thoroughly reviewed.

Chapter 3 deals with evaluating strategies that were relevant in achieving the viability of converting palm oil mill wastes to biodiesel. Sample preparation and material characterization techniques adopted for future work were developed in this chapter.

Chapter 4 presented an improved biodiesel production process that is tolerant of refined and crude vegetable oils. Mediating enzymatic transesterification with biodiesel had not been tested, thence was novel to be investigated.

Chapter 5 presents the actual viability of valorizing secondary and waste products from the palm oil mill to biodiesel. A cost-effective activated carbon production technique is developed in this chapter. The carbons were subsequently used for lipase immobilization for the

conversion of palm oil mill effluent to biodiesel. The feasibility of this thesis' proposal is emphasized in this chapter.

Chapter six, as the final chapter, presents general conclusions and recommendations for future work.

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Synopsis

Chapter I

Immobilized lipases for biodiesel production: Current and future greening opportunities.

As green catalysts, lipases' productivity, and wide substrate selectivity are preferable over chemical catalysts in biodiesel production. Lipases require milder reaction conditions and produce cleaner downstream products. To ensure practical recovery of lipases for repeated uses in biodiesel production, immobilizing lipases into solid forms has been suggested to complement its efficiency, utility, and sustainability. Immobilization, however, adds to the cost of the already economically inviable lipases. Most immobilization protocols also largely depend on fossil derivatives and produce an ever-increasing amount of waste. Therefore, it has been deemed necessary to delineate a scope for the fundamental success of using immobilized lipases for large-scale biodiesel production. Hence, this review presents a way forward to address immobilized lipase cost by using green chemistry strategies that have shown success in recent studies. A comparison of lipases with other biodiesel catalysts is presented in the early part of this review. Conventional and emerging immobilization protocols are also evaluated. The choice between synthetic and natural polymers for immobilization emphasizes the importance of using green chemistry metrics in addressing reusability, toxicity, resource efficiency, water, and carbon footprint of lipase immobilization. Therefore, this review advances immobilized lipase technology by identifying gaps that can be used by research and industry for its deployment to supplant chemical catalysts currently used in biodiesel production.

Chapter II

Valorization of activated carbon as a reusable matrix for the immobilization of Aspergillus oryzae whole-cells expressing Fusarium heterosporum lipase towards biodiesel synthesis.

The biomass-derived source, low-cost and hydrophobicity/oleophilic advantages of activated carbon (AC) was explored for the immobilization of *Aspergillus oryzae* whole cells expressing *Fusarium heterosporum* lipase. The adsorptive influence of AC favoured growth of the cells into its porous interfaces with paralleled exterior dense film formation. Increasing AC weights hindered extracellular lipase activity. Cell aggregation of 0.34 ± 0.02 mg/BSP was found to be effective in catalysing an industrially-challenging feedstock (68.77 % w/w free fatty acids, 20.48 % w/w triglycerides) to 98% fatty acid methyl esters (FAME). In a comparative investigation with polyurethane as matrix, higher trans/esterification facilitation was observed with AC. Benefiting from the oleophilicity of AC; denaturation effect from methanol on the lipases was reduced. Surface characterization with FE-SEM, XPS and FT-IR evidenced effective cell-matrix adhesion and a retention of the AC's intrinsic properties. The advantageous tribology of AC ensured recyclability of the matrix for fresh cells immobilization. Comparable FAME (98.08% w/w) was achieved with the recycled matrix in successive batches. The spent-matrix valorisation approach, thus, proposes sustainable biorefineries with immobilized lipase catalysed biodiesel production.

Chapter III

Biodiesel-mediated biodiesel production: A recombinant Fusarium heterosporum lipase-catalyzed transesterification of crude plant oils

Production of biodiesel from unrefined plant-derived oils has been considerably investigated but, to date, there appears to be a limit by phospholine gums and other contaminants in its conversion to biodiesel. The presence of such contaminants increases the melting point of the oils, thereby, making it unfavorable for lipase-catalyzed reactions at mild temperatures. Here, to circumvent this limiting effect, we explore the potential of biodiesel as a replacement for conventional organic solvents in solubilizing gum-formation contaminants in crude palm oil (CPO). A strategy of 1:1 CPO/biodiesel molar amount consolidates degumming and trans/esterification into one-step using immobilized *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase. The innocuous biodiesel solvent not only ensured a 98.8 wt % biodiesel yield but also improved the catalytic activity of the whole-cell lipase. This allowed repeated use of the recombinant lipase in nine consecutive batches. At room temperature, biodiesel as a solvent saves on post-separation and reduces environmental footprints of the biodiesel production process.

Chapter IV

Valorization of palm biomass waste into carbon matrices for the immobilization of recombinant Fusarium heterosporum lipase towards palm biodiesel synthesis

Continuous expansion of agriculture for the production of biofuels may be considered a potential source of greenhouse gas (GHG) emissions due to the ever-increasing amount of waste and fossil fuel-dependent materials involved. Palm oil production, for instance, produces palm kernel shells (PKS) and palm oil mill effluents (POME) as wastes in enormous amounts. PKS and POME account for >60% of solid and liquid waste generated from the mill. In this work, the feasibility of a circular palm bioeconomy is explored where waste PKS is directly converted to activated carbons (AC) in a cost-effective one-step technique (550 °C, 10 mL min−1 N2) that departs from the conventional two-step (carbonization and activation) technique. Characterization of the synthesized carbons, PKAC, using Fourier-transform infrared spectroscopy and scanning electron microscopy with energy-dispersive X-ray spectroscopy showed oxygen-rich morphological features that were 2-fold higher than in bituminous coal-derived AC. The <10 μm pore diameters of PKAC were relevant in the immobilization of Aspergillus oryzae whole-cells expressing Fusarium heterosporum lipase (PKAC-FHL). For biodiesel production, the 81.6 IU mg−1 specific activity of PKAC-FHL yielded ≥97.5 wt% fatty acid methyl ester (FAME) from POME. While offering the benefits of environmental remediation through effluent utilization, the produced FAME showed 48.8 Cetane Number and cold-flow properties that conform to ASTM and EN biodiesel requirements. This waste valorization approach, thus, proposes a green framework for the use of POME and PKS in immobilized lipase-catalyzed methanolysis.

Chapter I

Immobilized lipases for biodiesel production: Current and future greening opportunities

I.1. Introduction

To limit greenhouse gas emissions to pre-industrial 1.5 °C level, a global switch to renewable energy has become inescapable [1]. Renewables, such as wind and photovoltaics, for electricity generation and transportation, are expected to grow by 50% in the next five years (2019 to 2024) [2]. Along with the demand for renewables, the ever-more increasing global energy demand has identified biofuels as part of the future energy mix. In addition to its use as a transportation fuel, biofuels such as biodiesel can also serve as precursors to some of the fossil-derived fine chemicals such as surfactants, lubricants, plasticizers, fatty alcohols and solvents used in industry today [3]. Biodiesel has a significant role in the future circular bioeconomy through waste utilization.

For countries without natural sources of fossil fuels, utilizing biodiesel completely or in blends can save significantly on foreign exchange spending while supporting livelihoods in rural economies. The enormous benefits of biodiesel as a sustainable energy source have been chronicled in many good reviews and books on biodiesel production [4–6].

The genesis of biodiesel dates back to the 1890s and is associated with the first use of peanut oil in a diesel engine at a Paris Expo by the French Otto company [6]. Heat of combustion generated by early uses of neat vegetable oils in diesel engines proved similar to petroleum-derived diesel, which encouraged research towards lowering the cold-flow properties of neat vegetable oils to produce a more *petrodiesel*-like quality [6]. Crude oil

price uncertainties, state excise tax credits, and mandates have increased biodiesel blends from a minuscule 2% to as much as 30% currently in some countries.

Scientific research into biodiesel synthesis has also significantly supported petroleum diesel substitution with biodiesel [6]. Most of the scientific studies and patents filed on biodiesel have focused on improving its catalyst technology for efficient conversion. Other biodiesel research areas predominantly deal with feedstocks, reactor configuration, byproduct recovery, and combustion performance. A review of biodiesel's patent landscape showed that 1660 patents were registered on the fuel between the years of 1999 and 2018 [7]. Biodiesel's processing technology accounted for 64% of the share of registered patents (Figure 2-1) [7].

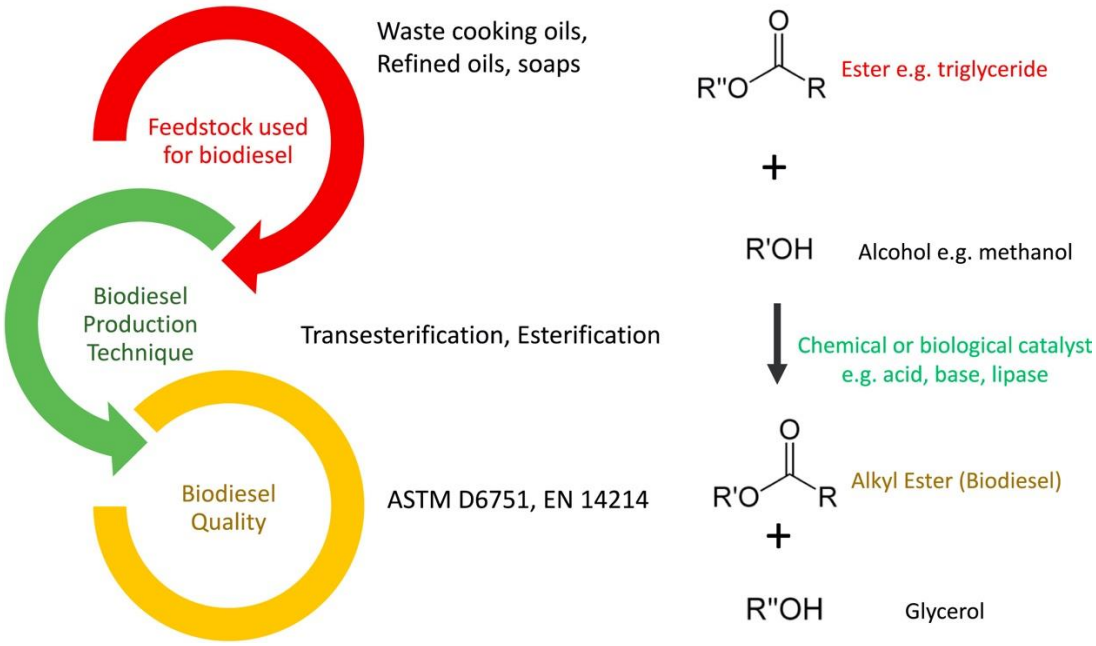

Figure 2-1 Biodiesel production technique is the main connecting link to ensure on/off-spec biodiesel irrespective of the feedstock used. The production technique accounted for 64% of the share of patents registered on biodiesel production from 1999 to 2018 [7].

Table 2-1 Biodiesel specifications by the world's largest producers and consumers of biodiesel. Dissimilar fatty acid methyl ester (FAME) specifications are adopted by different countries based on the geographical availability of feedstocks and seasonal weather variation.

		EU	USA	Malaysia	S. Korea	Japan
FAME Property	**Unit**					
Ester Content	**% (m/m)**	≥96.5	-	98.5	≥96.5	≥96.5
Acid Value	**Mg KOH/g**	≤0.5	≤0.8	≤0.5	-	≤0.5
Density @ 15°C	**kg/m^3**	860 - 900	800 - 900	870 - 900	860 - 900	860 - 900
Viscosity @ 40°C	**mm^2/s**	3.5 - 5.0	1.9 - 6.0	4 - 5	1.9 - 5.5	3.5 - 5.0
Iodine Value	-	≤120	-	≤110	-	≤120
Water Content	**mg/kg**	≤500	≤500	≤500	≤500	≤500
Cetane Number	-	≥51.0	≥43	≥51.0	-	≥51.0
Oxidative Stability	**h/110°C**	8	≥3	≥6	≥6	-
Triglycerides	**% (m/m)**	≤0.2	≤0.25	≤0.1	≤0.2	≤0.2
Diglycerides	**% (m/m)**	≤0.2	≤0.25	≤0.2	≤0.2	≤0.2
Monoglycerides	**% (m/m)**	≤0.7	≤1.0	≤0.4	≤0.8	≤0.8
Flash point	**°C**	>101	>130	>120	>120	>120
Polyunsaturated FA	**% (m/m)**	1	-	≤0.1	≤1	≤12
Sulfur content	**% (m/m)**	≤0.01	<0.05	≤0.001	≤0.001	≤0.001
Free Glycerin	**% (m/m)**	0.02	0.02	≤0.01	≤0.02	≤0.02

Irrespective of the feedstock or production techniques employed, market-ready biodiesel must meet specific standards (Table 2-1). The most widely adopted standards are the European EN 14214 and the American ASTM D6751 standards. In Malaysia, the Malaysian palm oil board (MPOB) suggests the MS 2008 as a national standard for palm biodiesel, whereas, in Japan, the Quality Assurance Law provides guidelines and specifications for blend-ready biodiesel (Table 2-1).

Biodiesel production via the established chemical transesterification process is energy-intensive [8]. Chemical catalysts such as acids and bases are typically used for the process. High temperatures are required, and wastewater is generated in large volumes.

The use of chemical catalysts also produces metal ions and salts that present disposal challenges. Efforts to green and minimize wastes in the biodiesel production lines have led to the introduction of biological conversion alternatives in the form of lipases to replace chemical catalysts [9]. Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are lipolytic enzymes that are being used to replace several chemical catalysts in modern organic chemistry due to its wide chemobiological activities, substrate specificity, functional group specificity, enantioselectivity, etc. Some known organic reactions with lipases include acidolysis, hydrolysis, epoxidation, esterification, alcoholysis, amination, and transesterification.

Earlier lipases isolated from prokaryotic microorganisms include the *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Pseudomonas fluorescens* species [10]. Protocols for production and utilization of any of the earlier isolated lipases can still not be generalized due in part to sequence complexity and molecular variety. The sequence of some earlier lipases of eukaryotic and prokaryotic-origin was described to consist of a Glycine (Gly)-X_1-Serine (Ser)-X_2-Glycine sequence, where X_1 is either a Histidine (His) or Tyrosine (Tyr) amino acid residue. Further characterization with X-ray and Fourier techniques later revealed the active

center of *Pseudomonas glumae* and *Chromobacterium viscosum* lipases as a catalytic triad configuration made of a Ser nucleophile, a His base, and an aspartic (Asp) acid residue [11,12]. *Pseudomonas cepacia*, now reclassified into the *Burkholderia* genus as *Burkholderia cepacia*, also revealed a similar active center containing Ser87, His286, and Asp264 residues of an α/β-hydrolase fold [13]. The active center participates in a hydrophobic-hydrophilic interface interaction that has been exploited in esterification and transesterification reactions. The active center (in most lipases) is covered by an amphiphilic polypeptide loop, referred to as the lid [14]. When in contact with hydrophobic substrates such as oil droplets, the lid assumes an open conformation for the active center to act. The lid's hydrophobic internal surface combines with the hydrophobic active center to create a large hydrophobic pocket. In the open conformation, lipase activity is significantly increased. This selectivity of lipases to interact with hydrophobic substrates is a beneficial phenomenon in oils and fats catalysis, and it is referred to as *interfacial activation* [15]. However, in aqueous media, selective interfacial activation of lipases can lead to lower lipase activity through the formation of dimeric and multimeric aggregates of lipases from the open form [16]. The aggregation of lipases can be controlled by the addition of detergents such as free fatty acids.

Over the last three decades, lipase's chemo, regio, and stereo-specificity have been exploited in esterification and transesterification of oils/fats to biodiesel. Notwithstanding, the instability of lipases under high temperature, pressure, solvents, and some substrates limits its use. To circumvent, novel biotechnological tools are being used to produce extreme-condition lipases from thermophilic and harsh-tolerant species [17]

In most industrial applications, lipases are used either in free liquid formulations or as aggregates or attached to solid carriers. The attachment of lipases onto solid carriers or modification into solids (aggregates) for possible recovery is referred to as lipase immobilization. Immobilized lipases have attracted attention over the years due to the ease of

practical recovery from product media. Lipase immobilization allows for repeated use of lipases. The technique of immobilization also offers the opportunity to chemically modify lipases to withstand inhibition effects from temperature, pressure, and organic solvents. However, it should be noted that assumptions have been made in the literature that the aggregation of lipases in anhydrous media is a form of immobilization. In this review, such aggregation is not considered a form of immobilization. The reason being that there is a lack of replicability of this lipase behavior, which is dependent on protein concentration, protein characteristics, protein purity, among others. [18]. The aggregation of lipases in anhydrous media also tends to affect lipase chemo-selectivity, regioselectivity, stereoselectivity, diffusional characteristics, and sometimes inverts them [19].

The high cost of lipases has stalled its commercialization for the production of bulk chemicals such as biodiesel [20]. Immobilization is proposed to lower the cost of lipases through the practicality of reuses. For liquid lipase production, the primary sources of costs are the initial biotechnological processes involved: lipase isolation, purification, and cultivation. The process of immobilization further adds new cost sources, including matrix selection, matrix stabilization, lipase immobilization, immobilized lipase stabilization, immobilization media handling, etc. In a study to promote the use of liquid lipases, Cesarini, and coworkers from Novozymes A/S (Bagsværd, Denmark) recommend a 30-50 times cost reduction between liquid and immobilized lipases from Novozymes A/S [21]. A 30-50 times price difference presents the immobilization process as a questionable method as to whether it is meant to reduce the cost of lipases or add to it. It also implies that if the high cost of liquid lipases can only be identified from its isolation and purification procedures, then combining modern biotechnological advances with inexpensive immobilization methods for the reuse of lipases should significantly lower lipase cost. Going forward, the sustainability of immobilization needs not be understated if biodiesel can be part of the future energy mix.

Therefore, this review summarizes opportunities in lowering lipase immobilization costs using green chemistry metrics and by presenting demonstrations that have shown viability in the literature. Unlike other reviews on lipase immobilization, this review mainly discusses the use of lipases as catalysts in biodiesel production, but, and more importantly, this is an examination into addressing lipase immobilization cost through green manufacturing. To ensure achieving reduced immobilized lipase cost, sustainability rationalization was used herein to address the challenges associated with the lipase immobilization process. An overview of conventional immobilization methods and materials is presented to outline advances needed to commercialize immobilized lipase technology on significant scales. Further, this review redefines lipase immobilization as it applies to the growing urgency of sustainability in every aspect of chemistry.

I.2. Background

I.2.1 Catalysis of oils and fats to biodiesel

Generally, via transesterification/esterification, biodiesel is produced by the conversion of acylglycerols and free fatty acids into fatty acid alkyl esters (FAAE) using short-chain alcohols (e.g., methanol) (Figure 2-1). The acyl migration with short-chain alcohols is a time-consuming process, and, therefore, catalysts or pre-conditions (e.g., super/subcritical) are required for quicker alkyl ester formation. The formed FAAE (biodiesel) has a lower viscosity and pour point than its precursor oil/fat.

The diverse composition of oils/fats necessitates investigations on the best catalysts for efficient biodiesel synthesis. Chemical catalysts, consisting mainly of acids (H_2SO_4, BF_3, H_3PO_4) and bases (KOH, NaOH), are industrially used in biodiesel production. However, chemical catalysts have distinct drawbacks in biodiesel catalysis. For instance, bases, though

the cheapest and fastest, are suitable for feedstocks with less than 0.5 wt.% free fatty acids (FFA) while acids require lower water content with mostly high FFA feedstocks such as waste cooking oils [22,23]. These drawbacks are coupled with high-temperature requirements, extensive product washing, and plant corrosion, which can be avoided when lipases are used. In a recent report, Tacias-Pascacio *et al.* used a response surface methodology to compare acids, alkaline, and lipases as catalysts in the conversion of waste cooking oil to biodiesel. Alkaline and lipase catalysts showed >85% biodiesel conversion in <120 mins. The *Thermomyces lanuginosus* lipase (TLL) - immobilized on octadecyl methacrylate - used for the study was recycled three times, unlike the alkaline catalyst [24].

This study supports the viability of using lipases as efficient catalysts to replace chemical catalysts.

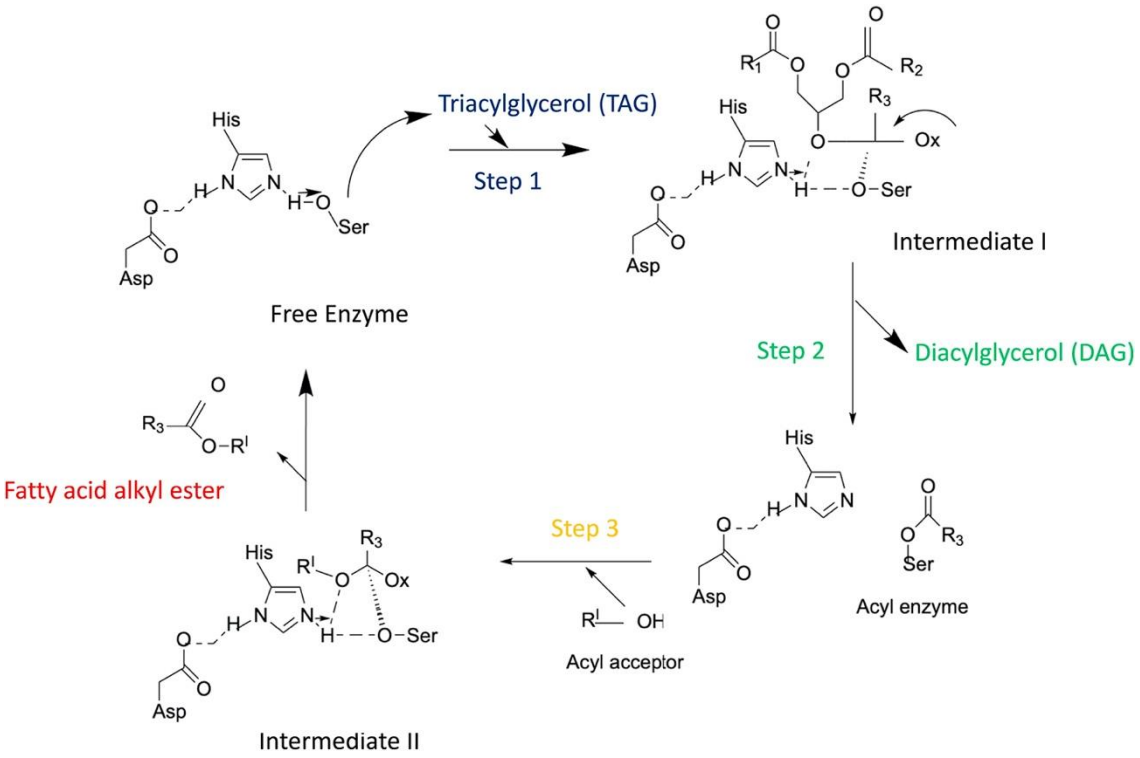

Figure 2-2 Lipase-catalyzed biodiesel synthesis illustration. Biodiesel synthesis via transesterification involves triglycerides' acyl migration to alkyl esters in the presence of a

catalyst. Intermediate diacylglycerides and monoacylglycerides are produced before the final release of the glycerol backbone on triglycerides.

As interfacial enzymes capable of catalyzing hydrolysis, lipases' natural substrates are glycerides, free fatty acids, monoglycerides, phospholipids, and other constituents in oils/fats [25]. Lipases can be classified into three specificity-based categories, namely, *sn*-1,3-specific, *sn*-2-specific, and nonspecific lipases. *sn*-1,3-specific lipases hydrolyze ester bonds at the R_1 and R_3 positions of triglycerides. *sn*-2-specific lipases catalyze intermediate position 2-ester bonds in triglycerides, while nonspecific lipases are promiscuous.

In non-aqueous media where triglycerides are substrates for transesterification, the Ser nucleophile in the active center of lipases participates in a charge relay system with His, glutamic acid (Glu), or Asp residues for a nucleophilic attack with alcohol on ester bonds (Figure 2-2). This produces an acylated enzyme and an intermediate product. Diacylglycerols are first released as a byproduct after alkyl ester formation with one carboxyl group in the triacylglycerol. Diacylglycerols are, in turn, converted into monoacylglycerols, and monoacylglycerol releases the glycerol backbone in triacylglycerols. In all stages of the catalysis, three mono fatty acid alkyl esters (biodiesel) are formed (Figure 2-2). The released mono alkyl esters are thermodynamically stabilized with excess alcohol.

Notwithstanding the excellent faculty of lipases, lipase deactivation easily occurs. Deactivation of lipases is characterized by aggregation, dissociation into subunits, and possible adoption of different pathways [26]. This is characterized by lower catalytic activities in hydrolysis and transesterification.

Although the application of lipases towards biodiesel synthesis remains at a nascent stage, its application in the food, detergent, drug, leather, textile, and cosmetic industries are well established [10,27]. As a bulk chemical, biodiesel must be produced inexpensively and in large volumes. Tufvesson and coworkers performed economic sensitivity analyses of

biocatalysts for drug, fine, specialty, and bulk chemical production. Tufvesson and coworkers proposed that the cost of biocatalysts (e.g., lipases) for bulk chemicals cannot exceed 0.05 €/kg to ensure economic viability. This was far lower than 10 €/kg allowed for drug production [20]. To compete with inexpensive chemical catalysts, biocatalysts must be improved to match the utility and cost of chemical catalysts.

I.2.2 Lipase technology versus other transesterification methods

Modern biotechnology tools such as directed evolution and recombinant DNA techniques have been hugely applied to evolve lipases from mild temperature-tolerant to harsh-thriving enzymes. The payoff, of course, has been that the use of lipases provides green downstream products. For instance, the most valuable byproduct of biodiesel production, glycerol, has a low market value, which necessitates inexpensive production. Glycerol produced from lipase-catalyzed transesterification approaches a purified form, and, thus, fewer pretreatments are required for its use in drug and food products.

Chemical catalysts, on the other hand, have also advanced to include heterogeneous types. Heterogeneous chemical catalysts from clays, zeolites, biochar, etc. have been synthesized to possess acidic and basic properties that can cater to different feedstocks (Table 2). Plant corrosion and easy leaching of heterogeneous chemical catalysts continue to define lipases as a greener alternative (Table 2). Energy consumption by heating in a typical hydrocarbon processing plant accounts for more than 70% of energy consumption [28]. Chemical-catalyzed biodiesel production requires 60–120 °C, while lipases can function adequately at room temperatures. As biodiesel's final sale price continues to be higher than that of petroleum diesel, cost input from energy consumption must be minimized to lower biodiesel

production overhead. Another vital advantage of lipases is its tolerance of diverse feedstocks. This eliminates the need for restrictive feedstock selection and pretreatment (Table 2).

Table 2-2 Catalytic strengths and weaknesses of the five main catalyst types reported in literature for transesterification of oils/fats to biodiesel. Heterogeneous chemical catalysts are synthesized by introducing acid/base active molecules onto/inside porous solid supports such as silica, alumina, zeolites, etc. In the table, O, indicates good/high performance, X indicates weak/low performance, and △, indicates intermediate performance

Catalyst	**Acid**	**Base**	**Heterogeneous Chemicals**	**Soluble lipase**	**IM lipase**
Parameter					
High FFA in feedstock	O	X	△	O	O
Partial glycerides in feedstock	△	O	△	O	O
High water content in feedstock	X	△	△	O	O
Alcohol tolerance	O	O	O	X	X
High temperature tolerance	O	△	△	X	X
Mild temperature tolerance	X	△	△	O	O
Cost of catalyst	O	O	X	△	X
Catalyst synthesis simplicity	O	O	△	△	△

Catalyst Reusability	X	X	O	X	O
Biodiesel productivity	△	O	O	O	O
Reaction rate (time)	△	O	O	O	△
Reactor simplicity	O	O	△	O	△
Glycerol purity	X	X	X	O	O
Glycerol tolerance of catalyst	O	O	△	X	X
Mass-transfer resistance	O	O	X	O	X
Downstream wastewater treatment	X	X	X	O	O
Plant equipment corrosion	X	X	△	O	O
Process simplicity	X	X	△	O	△

Super and subcritical alcoholysis, liquid-liquid film reaction, microchannel reaction, electrocatalysis, ultrasonication, and microwave-assisted alcoholysis are other transesterification methods that have been explored [29–35]. Amongst these, supercritical and subcritical alcoholysis is a well-studied non-catalytic method. It requires the use of alcohol at its super or subcritical state. Methanol's critical pressure and temperature are at 80.9 bar and 239.5 °C, while that of ethanol is at 63.0 bar and 240.8 °C. Although

super/subcritical alcoholysis proceeds faster and without the need for catalysts, its high temperature and pressure requirement raise energy efficiency, health, safety, and environmental (HSE) concerns. Modified reactor technologies such as ultrasound and microwave irradiation can also lower reaction time. Low-frequency ultrasound irradiation creates gas cavities in immiscible reactants for better collision. Gas cavities created increase oil-alcohol surface contact to offer catalysts the required interface to act. Using lipases, Quilles *et al*. reported the use of 15 sec 40 kHz ultrasonication, paused every 15 min, for the conversion of soybean oil to biodiesel under 24 h. Both the *Candida antarctica* lipase B and *Thermomyces lanuginosus* immobilized lipases tested recorded 189 and 40% biodiesel conversion increase, respectively, compared to without ultrasound [34].

I.3. Industry Expectations

Biofuels demand and the ever-more need for the chemicals industry to curb emissions is driving an industrial demand of lipases and other hydrolases (e.g., glycosidases, proteases, amylases, and cellulases) [36]. However, large-scale use of lipases for biodiesel production has not moved as fast as its application in other biotechnology areas. As cited throughout this review, the bottleneck has been the high cost of lipases compared with chemical catalysts.

Over the years, bench-scale experiments in literature have provided insights into the use of immobilization technology to reduce lipase costs. [37]. Exclusive experimental investigations on lipase carriers have seen the demand for industrial acrylic resins as novel immobilization carriers [38,39]. The identification of new immobilization carriers has both altered reactor configuration and product recovery techniques by the biodiesel industry [40,41].

On the industrial level, a few commercial projects on lipase-catalyzed biodiesel production have been reported. In 2014, a Novozymes A/S and Viesel Fuel LLC (Florida, USA)

collaboration reported a noteworthy scientific paper on the scale-up of Novozymes A/S's NS-40116 lipase (a liquid lipase from *T. lanuginosus*) for biodiesel production [42]. In China, two plants have tested the scalability of Novozym® 435 (immobilized lipase) and Lipase Candida sp. 99–125 (liquid lipase) for waste cooking oil conversion [36]. Sunho Biodiesel Corporation in Taiwan has also patented a combination of liquid and immobilized lipases in a technique termed as the ET® process. In Japan, a Kobe University and Kansai Chemical Engineering collaboration is piloting a 200 L/day biodiesel plant using immobilized lipases (IM lipases) [9].

For the current alkaline transesterification method used mainly by industry, waste oil feedstocks require intensive FFA pretreatments [43]. On the contrary, lipases require no pretreatments for high FFA feedstocks. This suggests that the market demand for lipases, particularly in immobilized form, by industry is expected to soar soon with the demand for sustainable biofuels. However, the industrial adoption of IM lipase technology will remain sluggish unless its high cost is curbed to meet the 10.3 billion liters of global biodiesel deficit projected for the next four years (2024) [2].

Table 2-3 Sources of isolation, immobilization techniques, and reusability of some widely-applied lipase strains used in biodiesel production.

Lipase	**Source**	**Immobilization method**	**Immobilization carrier**	**Reaction conditions**	**First-run Biodiesel yield (%)**	**Lipase Reusability (cycles)**	**Reference**
Burkholderia cepacia	Animal and plant pathogenic bacterium	Covalent binding	Magnetic nanoparticles grafted with 2,3-epoxypropyltrimethylammonium chloride and glutaraldehyde	In soybean oil, 4:1 MeOH/oil molar ratio, 0.3 wt.% *tert*-butanol, and 2.5 wt.% water at 45 °C for	96.8	15	[50]

				9 h per cycle			
Candida antarctica lipase B	Yeast (flora and mammalian hosts)	Adsorption	Reticulated polyurethane foams	Palm oil hydrolysate, 1.5:1 MeOH/oil molar ratio, and 0.5 g water at 30 °C for 48 h per cycle	≥ 90.0	20	[51]

Thermomyces lanuginosus	Compost-degrading fungi	Cross-linked enzyme aggregates and covalent binding	NKA, a commercial macroporous resin	10 g soybean oil deodorizer distillate, 4 mL MeOH, and 5 mL *tert*-butanol at 50 °C for 8 h per cycle	98.0	20	[52]
Candida rugosa	Yeast (flora and mammalian hosts)	Encapsulation	Zeolite imidazolate framework, ZIF-67	In soybean oil, 146 μL MeOH added 3	78.5	6	[53]

				times, 10 wt.% water at 40 °C for 60 h per cycle			
Rhizomucor miehei	A species of fungus	Encapsulation	Zeolitic imidazolate frameworks, ZIF-8	In soybean oil, 1:4 oil/ethanol molar ratio, 20 wt.% *iso*-octane, 9 wt.% water, at 45	>95.0	10	[54]

				°C for 24 h per cycle			
Rhizopus oryzae	Microfungus found in soil, dung and rotten vegetation	Covalent binding	Anion exchange resin, Amberlite IRA-93	In soybean oil, 1:4.8 oil/MeOH molar ratio, at 37 °C for 12 h per cycle	90.5	7	[55]
Aspergillus niger	'black mold'-causing fungi on onions, grapes, peanuts, etc.	Adsorption	Polydimethylsiloxane-coated metal organic framework, UiO-66	In soybean oil, 460 μL MeOH added 4	93.0	10	[56]

				times, at 45 °C for 12 h per cycle			
Pseudomonas fluorescens	A rod-shaped bacterium found in soil and water	Covalent binding	Amino-functionalized single-walled carbon nanotubes	In sunflower oil, 500 μL *iso*-octane, 1:5 oil/ethanol molar ratio, at 30 °C for 4 h per cycle	> 99.0	20	[57]

Fusarium heterosporum	Fungi, soil and plant origin	Adsorption	Reticulated polyurethane foams treated with glutaraldehyde	In soybean oil, 0.35 g MeOH added 4 times, 5 wt.% water, at 30 °C for 96 h per cycle	94.0	10	[58]

I.4. Source of Lipases

Lipases are ubiquitous in almost every living organism. For synthetic catalysis, lipases are isolated from plants, animals, microbes, etc. [25,27]. Table 2-3 summarizes sources of isolation for some commonly applied lipases in biodiesel synthesis (mostly in immobilized forms). Some recently identified lipase sources include oil mill effluents, oilseed cakes, paper mill wastewater, rhizosphere soil of plants, hot springs, shrimps, among others [44–47].

Salgado *et al.*, for instance, screened 32 yeast strains from an olive oil mill effluent to isolate the novel *Magnusiomyces capitatus* lipase. *M. capitatus* JT5 was identified by rapid plate detection and DNA sequencing. The *M. capitatus* JT5 lipase showed maximal lipase activity (0.4 U/mL) and biomass yield (15 g/L) when cultured in untreated olive oil mill effluent [44]. Lipases from Antarctic and Baltic sea shrimps are also some new sources of thermophilic lipases. Xin *et al*. used ammonium sulfate precipitation and a series of chromatographic separations to characterize the *Euphausia superba* lipase from Antarctic krill. The *E. superba* lipase showed stable lipase activity over a 0-50 °C temperature range. Some other marine sources yet to be explored include endemic crustaceans that exist in harsh environments (>400 °C, $pH_{25°C}$ 3, 1013 hPa) around hydrothermal vents on the seafloor (>4 km from seawater surface) [48,49]. Hydrothermal vents (HVs) are active hot fluid-producing chimneys found in the seafloor. HVs have received increasing deep-sea research interest due to its potential for massive metal deposits. Lipase-producing chemoautotrophic bacterium from active chimneys on the seafloor thrive in harsher conditions and can be used for efficient lipid catalysis.

Burkholderia lipases (formerly *Pseudomonas*) are among the pioneered group of lipases researched for biodiesel production. Species of *cepacia, mallei, pseudomallei, carryophylli, gladioli, pickecttii,* and *solanacearum* in the earlier group II of the *Pseudomonas* genera have

been tested in biodiesel synthesis [12]. *Burkholderia* lipases are widely used in other areas of biotechnology [50]. Amano Enzyme Inc., a specialty chemical company in Japan, supplies cloned *B. cepacia* lipase in dextrin-diluted liquid form. In liquid form, lipases are relatively easy to handle and require less retrofit for use in existing chemical reactors. However, the recovery of liquid lipases after use is a major hurdle. The processing cost and lack of cost-effectiveness of using lipases without recovery are huge, and, therefore, requires immobilization to circumvent.

I.5. Lipase Immobilization

The lack of long-term reusability of liquid lipases can be overcome by attachments onto solid carriers or into aggregates for convenient recovery. In solid form, lipases also show low contamination effect on products. Lipase immobilization can be used to improve lipase activities through chemical modification. Chemical modification approaches, such as coating lipases with surfactants, PEGylation, and conjugate ion additions, improve lipase activities [51]. Lipase resistance to inhibition (pH, temperature, alcohol, etc.) is also improved through immobilization. By immobilizing porcine pancreatic lipase onto ionic liquid-modified magnetic chitosan-graphene oxide composites, Suo *et al*. reported 92% residual activity after ten reuse cycles and a 6.7-fold lipase activity increase compared with a liquid form of the lipase [52]. Although liquid lipases promote hydroesterification by introducing excess water, liquid lipases settle at the glycerol phase of product formation. This easily denatures liquid lipases and thus presents cumbersome recovery challenges. Liquid lipase-catalyzed biodiesel may contain relatively high FFA due to the promotion of hydrolysis [53]. The advantages of immobilized lipases over liquid/soluble lipases are summarized and compared with other catalysts in Table 2-3 and some recent reviews [9,27,54].

Immobilization matrix selection, activating agent inclusion, and modifying immobilization protocols are popular strategies to minimize disruptive effects on lipases. Immobilization science has proposed conventional immobilization protocols. Popular among these protocols are Adsorption, Covalent Binding, Entrapment, and Cross-Linked Enzyme Aggregate (CLEAs) preparation (Figure 2-3).

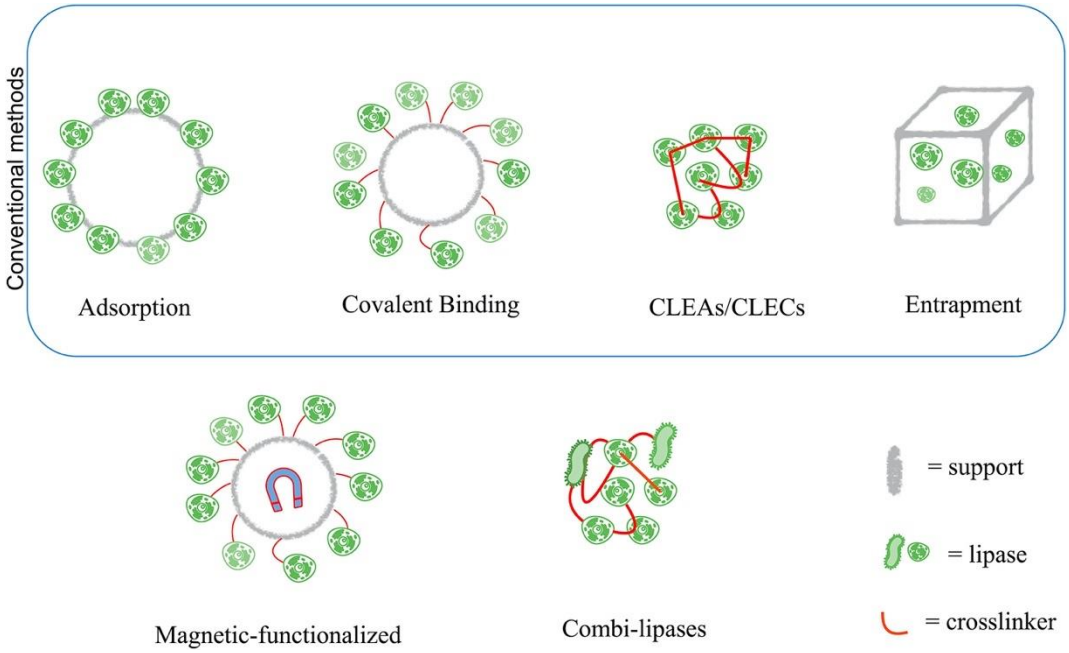

Figure 2-3 Conventional lipase immobilization methods are advancing with magnetization and combi-lipase strategies.

I.5.1 Adsorption

Immobilization of lipases by adsorption is primarily a physisorption process. It also simultaneously involves chemisorption through the utilization of biomolecules from matrices for lipase attachment. Adsorption immobilization is fundamentally understood as a weak-to-strong interaction between an adsorbate (the lipase) and an adsorbent (the matrix) via either hydrogen bonding, acid-base bonding, or van der Waals forces. The facile reversibility of

adsorption immobilization makes it the most widely used immobilization technique. Filho *et al*. summarized research related to lipase immobilization (from 2013 to 2018) and found adsorption to be the predominant method (42%) compared with other immobilization methods [27].

Porosity and surface area are crucial parameters in adsorption immobilization. Therefore, commonly used adsorption carriers include activated carbons, polyurethane foams, silica gels, acrylic resins, and biochar. Dhawane *et al*. prepared activated carbons (AC) using seed pods from the flamboyant ornamental plant and subsequently used it for immobilization [55]. The AC's surface area was reduced from 812 m^2g^{-1} to 35 m^2g^{-1} to signify the utilization of pore volume and surface area for immobilization.

Macro to mesoporosity is, however, preferable for immobilizing whole-cell lipases [56]. The Adsorption of whole-cell lipases from seven *Rhizopus* species onto nylon, polyester, stainless steel, cellulose, polyvinyl alcohol, and polyurethane was reported by Nakashima *et al*. [57]. The macroporosity of polyurethane (PU) foams (40 pores per linear inch) was relevant in attaining a 3.6 mg/PU cell concentration and 283 x 10^2 U/mL hydrolytic activity in *Rhizopus oryzae.*

Amongst industrial carriers used for adsorption immobilization, Lewatit® VP OC 1600 stands out as a successful macroporous resin. Lewatit® VP OC 1600 has been used for the immobilization of *C. antarctica* lipase B and marketed as Novozym® 435 [58]. The 315-1000 μm beads have 150 Å average pore diameters and >130 m^2 g^1 surface area [58]. The level of success using Lewatit® VP OC 1600 for immobilization has pushed the market for other acrylic resins such as Eupergit® C and Amberlite® [38,58].

As a physisorption process, the success of adsorption immobilization is dependent on pH, temperature, agitation rate, and ionic potential of the media. Theoretical electrostatic potential expressions of fine-tuned substrates could be used to improve adsorption

immobilization. The selection of relevant organic polymers for adsorption immobilization also supports chemical modifications [59]. For instance, the Lewatit® VP OC 1600 is prepared from methyl methacrylate cross-linked with divinylbenzene. This chemically modifies the temperature resistance of lipases immobilized onto the resin [58].

Leaching of lipases remains a drawback of adsorption immobilization. The lack of long-term attachments with adsorption immobilization lowers its reusability in rigorously stirred reactors [60]. The main advantages of adsorption immobilization are its simplicity and fewer reagent requirements. Improved adsorption immobilization would require excellent internal pore geometry of carriers that would allow buried attachment of lipases (e.g., in layered porous carbons).

I.5.2 Covalent Binding

Lipase immobilization via covalent binding relies on the amine moieties in proteins for chemical reaction with carriers. Reactive protein residues, such as ε-amino, α-amino, guanidinyl, and hydroxyl groups, in lipases, are utilized as target molecules [61]. Active groups in Asp and Glu amino acid residues play significant roles in covalent binding. Covalent binding is often combined with other immobilization techniques. Combining covalent binding with adsorption, for instance, creates long-term spacer links between matrices, lipases, and bifunctional reagents. Covalent binding involves introducing receptive functional groups on carriers, followed by activation of the functional groups for amine residue attachment. Silanization and grafting are some functionalization techniques used [62]. Functionalization affords an inert environment on carriers. Activation of the functionalized carrier is necessary for lipase binding. Aldehydes and epoxy are common activation reagents used [61–63]. Glutaraldehyde (GA) is the most widely applied activation reagent in covalent

binding. Under responsive conditions (pH, concentration, temperature, etc.), GA binds via different reaction mechanisms such as aldol condensation, cyclization, Michael-type addition, dehydration, and internal redox reaction of a Schiff base. Using magnetic nanoparticles as core shells, Li *et al*. reported the effect of GA in anion exchange to immobilize *B. cepacia* lipase. The controlled addition of GA not only offered >3500 U/ g protein specific activity but maintained >60 wt.% biodiesel yield from soybean oil over 16 reuse cycles [64].
The final cross-linked structure of covalently binded lipases is an intramolecular precipitate of proteins that have shown repeated uses in biodiesel synthesis [64–66].

I.5.3 Cross-linked Enzyme Aggregates and Crystals

Lipase immobilization into aggregates as Cross-Linked Enzyme Aggregates (CLEAs), Cross-Linked Enzymes (CLE), or as crystals (CLECs) is a form of chemical immobilization where monomeric enzymes are brought together to form multimeric aggregates without matrices. CLEAs/CLECs/CLEs are formed from aqueous buffer precipitations. The precipitates are cross-linked with bifunctional reagents such as GA. Salts aid the precipitation of the primary structures of proteins in the aqueous buffers. Nano spray drying techniques can be used for drying the lipases from aqueous buffers before cross-linking [67].
Lysine residues are essential in the formation of CLEAs/CLECs/CLEs. Lipase extracts containing less accessible lysine residues are supported with additional amino residues such as bovine serum albumin (BSA) and GA. This technique is called *proteic feeding* [68]. Proteic feeding with BSA is less toxic and is considered a greener approach compared with GA. Proteic feeding reduces direct conformational changes to the primary structures of lipases [68]. BSA-cross-linked CLEAs of *C. antarctica* lipase B (CALB) and *T. lanuginosus* lipase (TLL) were tested for the ethanolysis of rapeseed and fish oils. BSA addition increased

the activities of the CLEA-CALB and TLL by 24 and 8-fold, respectively [69]. For reusability tests, the CLEAs of TLL maintained ≥65 wt.% FAEE over six cycles [69]. The preparation of CLECs, however, requires highly purified lipases. CLEC synthesis requires multi-stage purification of lipases before crystallization [70–72]. CLEAs, on the other hand, can be prepared with crude lipases. CLECs are more robust than CLEAs. In biodiesel production, both CLEAs/CLECs have shown appreciable reusability and retention of lipase activities [73]. Without the need for matrices, CLEAs/CLECs can offer relatively cheaper immobilized lipases since matrices account for 90–99% of an immobilized lipase's mass.

Besides the fundamental advantages of practical recovery, the immobilization of different lipases with CLEAs are possible. Different catalytic activities from a single CLEA system can be realized in the new system referred to as Combi-lipases (Figure 2-3) [74]. As a multi-lipase cascade system combined into a one-pot system, Combi-lipases shorten reaction time, require fewer unit operations, and reduce waste. Combi-lipase immobilization is facile when prepared as CLEAs but can also be readily adsorbed onto carriers via covalent binding [75]. The synergetic effect of CALB and *Rhizomucor miehei* (RM) as a Combi-lipase has been reported by Banerjee *et al.,* where microcrystals of K_2SO_4 were used as a core-shell. Towards spent coffee oil biodiesel synthesis, the Combi-lipase ensures 83 wt.% conversion compared with the 60 wt.% and 40 wt.% yields of the individual CALB and RM lipases [76]. Demerits of CLEAs are the low mechanical robustness and substrate diffusion [75]. To improve the mechanical robustness of CLEAs, Zheng *et al.* combined adsorption and CLEA to immobilize Lipozyme TL 100L onto NKA, a macroporous resin [77]. The CLEA-TLL@NKA was prepared by pretreating 5 mL Lipozyme TL 100L with 20 mL *isopropanol* at 4 °C for 30 min. After centrifugation at 10,000 rpm, the bottom precipitate was mixed with 2 mL polyethyleneimine (2.5%, w/v), 2 mL GA (0.2%, w/v), and 2.3 g NKA. The resulting

CLEA-TLL@NKA vacuum drying was used for soybean oil deodorizer distillate (SODD) conversion to biodiesel. The CLEA-TLL@NKA showed a 10-fold activity increase compared with the pristine Lipozyme TL 100L. CLEA-TLL@NKA maintained >90 wt.% biodiesel conversion in 20 reuse cycles.

I.5.4 Entrapment and Encapsulation

Entrapment or encapsulation immobilization is simply a way of detaining lipases within a polymeric matrix (Figure 2-3). Although restricted by freedom of movement, semi-permeable matrices such as silica sol-gels and alginates allow for low molecular weight substrate and product diffusion. Unlike other immobilization techniques where the lipases are bound to the surfaces of carriers, entrapment allows free movement of lipases only in hollow microscopic networks inside a relatively larger polymer [78,79]. Alginate, agar, collagen, and other non-gel-like supports like diatomaceous earth, and zeolites, are often used for entrapment [80]. Entrapment protocols are relatively simple and can occur even under room temperature. Silica sol-gels prepared for lipase entrapment are cheap and room-temperature achievable. In the known-sol gel process, hydrolysis and polycondensation of Si $(OR)_4$ (e.g., tetraethyl orthosilicate (Si $(OC_2H_5)_4$) is used to trap lipases into macroporous Si polymers (Eqn. 1).

$$n\ Si(OR)_4 + 2n\ H_2O \xrightarrow{catalyst} (SiO_2)_n + 4n\ ROH \qquad \text{Eqn. 1}$$

Controlled drying and sintering of sol-gels produce porous matrices as lipase sites. However, sol-gels can exhibit less than 5% relative activities in immobilization [81]. Low lipase activity with pristine sol-gels is attributed to the low diffusion rate of substrates/products and the lack of proper dispersion of lipases in the matrix. To circumvent such drawbacks, a combination of interfacial activation with the technique has been proposed. By introducing

alkyl groups (e.g., $CH_3Si(OCH_3)_3$), simultaneous interfacial activation and lipophilic pore interaction for substrate flow can occur.

Entrapment can also be combined with covalent binding to produce stable formulations for long term storage. Towards the synthesis of isoamyl acetate, Gul and Esra combined entrapment and covalent binding for the immobilization of porcine pancreatic and *C. rugosa* lipases. The immobilization was performed by simply dropping a Na-alginate-lipase mixture into a $CaCl_2$-GA solution. The simple technique produced entrapped-lipase composites that retained >30% of lipase activity over 60 days (stored at 4 °C) [82].

Entrapped lipases used in biodiesel synthesis, have also shown considerable yields and reusability [83–86]. Towards biodiesel synthesis, Meunier *et al.* prepared diatomaceous earth-supported sol-gel composites of *B. cepacia* lipase [87]. The sol-gel was prepared by mixing 14 mL trimethoxypropylsilane with 2.95 mL tetramethyl orthosilicate in ultra-pure water (17.4 mL) and 0.1 M HCl (200 μL). The sol-gel-*B. cepacia* lipase composite showed 62% FAME yield in triolein after the first 1.5 M methanol addition.

However, precise microenvironment flow control remains a hurdle for entrapment or encapsulation immobilization [83,88].

I.5.5 Emerging immobilization methods

Lipase immobilization technology has advanced along with the increasing use of enzymes [89]. Not only lipases but fine-tuned, microenvironment-sensitive, *and in-situ* tunable matrices have been synthesized for biodiesel synthesis [90–92]. Chemical modification of conventional immobilization is an emerging technique that produces conformational changes-resistant lipases. Chemical modification uses microenvironment parameters such as pH, dissociation ions, isoelectric point, and thermal response of lipases in immobilization.

The isoelectric point (pI) of lipases in immobilization depends on the ionizable amino acids present in and around the catalytic triad. In immobilization, the surrounding solutes' pH plays a role in determining the extent of possible polymerization. Chemical modification of conjugated polymers through Atom Transfer Radical Polymerization (ATRP) and the Reversible Addition-Fragmentation Chain Transfer (RAFT) techniques have produced lipases with extended half-life and improved activities. This is also based on the careful selection of zwitterionic monomers for conformational change-resistant lipases. Applying the selection of zwitterionic monomers, Chen *et al*. compared the zwitterionic carboxyl betaine methacrylate (CBMA) and a hydrophobic tert-butyl methacrylate (TBMA) for modifying *C. rugosa* lipase [93]. Catalytic activity, thermostability, pH tolerance, and lipase-substrate affinity of *C. rugosa* were improved in both polymers and its equimolar versions. However, chemical modification with the hydrophobic TBMA showed a 77.3 U/mg protein specific activity compared with 39.3 U/mg protein for CBMA.

PEGylation is another form of chemical modification reported to improve lipase stability. PEGylation using polyethylene glycol (PEG) is generally regarded as safe (GRAS) in food applications and drug delivery. PEGylation of the macroporous resin (NKA) was studied by Wang *et al.* NKA-PEGylated CALB afforded >8000 U/g absolute activity in ethyl acetate esterification in six reuse cycles [94]. PEGylation introduces hydrophilic substrates to lower the active conformation exposure of lipases. For instance, a PEGylated CALB showed a two-fold activity increase compared with negligible activities in an unPEGylated TLL [95]. The increase in activity from PEGylated CALB is attributed to the smaller triad lid in CALB.

For scalable applications of adsorption immobilization, nanomaterials, and metal-organic frameworks (MOFs) are the new choice of candidate materials [96,97]. The coining of a new term, 'BioMOFs,' for MOF-lipase immobilization is used [98]. BioMOFs are porous, have a larger surface area, and possess functional activities that aid chemisorption of lipase

molecules. Pristine MOFs have thus far not shown high lipase activities, but modified forms have yielded exceptional activities [97].

Magnetic smart polymers are being explored for lipase immobilization [99]. Smart polymers can undergo reversible dramatic conformational changes in response to pH, temperature, and the ionic potential of a media [99]. Smart CALB and CRL were synthesized using Pluronic molecules known for temperature-responsiveness in aqueous media [100]. The Pluronic F-127 (POH) was oxidized with Dess–Martin periodinane to add aldehydes to its pristine hydroxyl group. The formulation was used for CALB immobilization. The Pluronic F-127 (POH)-CALB exhibited 6850% activity increase and simply recovered by precipitation at 4 °C in toluene.

For easy lipase recovery and reuse, magnetite (Fe_3O_4) addition and super paramagnetization hybridization are excellent ways efficiently used [101]. The addition of Fe_3O_4 to graphene, nanomaterials, chitosan, and alginate composites are reported. For biodiesel synthesis, magnetic CLEAs (mCLEAs) have been reported [99,102,103]. mCLEAs are suitable for smaller reactors with less pressure drop [104].

I.5.6 Improving immobilization via interfacial activation

Among earlier works utilizing the interfacial activation phenomenon, Bastida *et al.* reported octyl-agarose beads for the immobilization of *Pseudomonas fluorescens*, *C. antarctica*, and *R. miehei* [105]. Bastida *et al*. compared the need for ammonium sulfate in precipitation of proteins during immobilization. By decreasing ammonium sulfate concentration from 1 M to 10 mM, a 7-fold hyperactivation in *R. miehei* was achieved. Therefore, Bastida *et al*. presented interfacial activation as a one-pot purification and immobilization technique to increase lipase concentration and activity [105].

Interfacial activation as a catalytic mechanism is primarily an adsorption technique that explores the hydrophobic properties of lipase's catalytic triad. The triad lid is activated (opened) when in contact with hydrophobic substrates [58]. The hydrophobicity of the triad can also lead to dimeric or multimeric aggregate formation. Dimeric or multimeric aggregate formation decrease lipase accessibility, thereby lowering lipase activity. Dimeric or multimeric aggregate formation can also be used in lipase purification by altering the ionic strength of the media [106]. Detergents are used to control dimeric or multimeric aggregate formation. Fernández-Lorente *et al.* studied the addition of Triton X-100 as a detergent. In the immobilization of *C. antarctica* lipase A (CALA) on monoaminoethyl-*N*-aminoethyl agarose beads – an aminated hydrophobic support [107]. Both liquid and agarose-immobilized forms of CALA showed 100 and 300% activity increase, respectively, in Triton X-100.

Some known hydrophobic supports that have been used for interfacial activation immobilization include octyl sepharose, octyl-agarose, sol-gels, modified silica nanoparticles, and resins from propylene (e.g., Accurel MP), styrene (e.g., Lifetech™ ECR1090M), octadecyl methacrylate, etc. [108]. Tacias-Pascacio *et al.* reported on a battery of commercial hydrophobic supports for CALB immobilization [109]. Octadecyl methacrylate offered CALB a 0.42 U/g activity compared with 0.37 U/g for the commercial Novozym® 435. Towards waste cooking oil conversion to biodiesel, the octadecyl methacrylate-supported-CALB produced 71 wt.% FAME under 1.5 h compared with 60 wt.% for Novozym® 435. Insights on the role of pH in destabilizing interfacially activated lipases have been provided by Kornecki *et al.* [110]. The effect of phosphate anions at distinct pH (2, 7, 5, and 9) in destabilizing octyl agarose-immobilized lipases from RML, CRL, CALA, and CALB was reported. The study reports the negative effect of 100 mM sodium phosphate at pH 7. In GA-

activated supports, the effect is insignificant (e.g., in CALB) but significant in some cases (e.g., CALA).

Interfacial activation improved thermal stability and prolonged stability of lipases from TLL, CALB, *M. miehei*, *C. rugosa*, *B. cepacia*, etc. [106,111,112]. To study the reusability of interfacially activated-*R. miehei* lipase (RML), Zhang *et al.* added magnetic properties to surfactant-activated RML CLEAs [113]. Tween 80 and sodium bis-2-(Ethylhexyl) sulfosuccinate were compared in the open conformation of RML CLEAs. Interfacially activated- RML CLEAs showed a 2058% activity recovery and was reused for five cycles in jatropha oil biodiesel synthesis.

As a promising strategy, further studies on the role of ionic strength, pH, hydrophobic surface structures, detergents, and, most importantly, visualization of interfacial activation are needed.

I.6. Immobilization Materials

The universal interaction between matrices and lipases is complex. However, success parameters for immobilization can be measured with immobilization yield (%), immobilization efficiency (%), and activity recovery (%) [18].

The chemistry, thermal, mechanical, and even natural occurrences of matrices used for immobilization tend to eke out activities in lipases. Numerous natural and fine-tuned materials have been used in immobilization. The overriding objective has been to ensure reusability that enables lipases to replace chemical catalysts. The numerous materials used can be classified into two main groups: Synthetic Polymers or Natural Polymers.

I.6.1 Synthetic Polymers

In this review, we consider synthetic polymers to be the group of lipase carriers synthesized via chemical copolymerization of monomers to produce amine residue-responsive polymers for immobilization. Acrylic resins such as Lewatit® VP OC 1600, Amberlite XAD-7, and Eupergit® C are examples of synthetic polymers used for immobilization. Eupergit® C is an industrial acrylic resin competitor of Lewatit® VP OC 1600. Eupergit® C is produced via co-polymerization of N, N'-methylenebisacrylamide, glycidyl methacrylate, allyl glycidyl ether, and another methacrylamide [38,114]. Contrary to interfacial activation immobilization with Lewatit® VP OC 1600, Eupergit® C immobilizes lipases via 'multipoint attachment.' Eupergit® C binds covalently using its oxirane and sulfhydryl groups depending on the working pH. Comparing the unconnected success of the two synthetic polymers, we determined that immobilization on a battery of supports is necessary when assaying newly isolated lipases.

I.6.2 Natural Polymers

Biopolymers such as collagen, gelatin and albumin, and polysaccharides such as chitosan, chitin, starch, κ-carrageenan, cellulose, agarose, and dextran are excellent natural polymers immobilization. Natural polymers are biodegradable, biocompatible, non-toxic, and abundant in nature. Chitosan, a β-(1–4)-linked D-glucosamine (deacetylated) and N-acetyl-D-glucosamine (acetylated)-derivative, from chitin, is widely used in lipase immobilization [115,116]. Free protonable amino groups present in chitosan are used in ionic and chemical cross-linking of lipases [116,117]. Costa-Silva and co-workers prepared a chitosan-*Cercospora kikichii* IM lipase activated with GA. For a six-month storage test, 77.7% of its initial activity was retained [118].

Agarose is another conventional natural polymer used for lipase immobilization. Agarose produces inert short linkers in immobilization [119]. Agarose activated with GA eliminates non-ionized amino groups in immobilization. Agarose activation with GA yields long-term multi-interaction for lipases [119,120].

Cellulose, an abundant linear polysaccharide that consists of D-glucose residues linked with β– (1–4)-glycosidic bonds, is also a widely-used natural polymer. For the immobilization of CALB, grafting cellulose with diethylenetriamine (DETA), for instance, enabled five reuse cycles in the transesterification of *Yellow horn* seed oil [121].

Natural polymers require functionalization and activation for long-term use. The immobilization of *B. cepacia* lipase (BCL) onto κ-carrageenan reported by Raman Jegannathan *et al*. is a typical example. The free lipase of BCL showed better stability than the κ-carrageenan-immobilized BCL [122]. Therefore, activation of natural polymers with bifunctional reagents such as GA is required to produce improved activities.

I.6.3 Mechanical fragility of immobilization materials

Chemical-catalyzed industrial biodiesel production uses continuously stirred tank reactors (CSTRs), rotating drums, spinning disc, and microwave reactors as conventional reactors. For lipase-catalyzed biodiesel production on an industrial scale, reactors that have been tested include CSTRs, spinning tubes, packed-beds, trickle beds, membranes, and fluidized bed reactors. Through process intensification, emerging reactors that have been introduced for lipase-catalyzed biodiesel production include tubular micro-channels, ultrasonic, shockwave power, and reaction-separation reactors such as annular centrifugal contactors [5,123,124]. Rapid advancement of IM lipases to replace chemical catalysts would require using brownfield reactors before green-tailored reactors can be developed. Conventional CSTRs

use impellers or dynamic mixers. Since the efficiency of every catalysis reaction depends on efficient contact between reactants and catalysts, vigorous mixing using impellers or dynamic mixers favor homogeneity and fast product formation [42,125]. Therefore, to enable the reuse of IM lipases in CSTRs, mechanical robustness is desirable. Weakly bonded IM lipases cannot survive vigorous mixing as it easily detaches from matrices into the media. Matrix attrition from vigorously stirred reactors can also block reactor parts and cause the breakdown of process equipment. On a bench scale, a global mass loss of <10%, such as that reported by Ortiz and co-workers for Lewatit® VP OC 1600, may not be easily noticed [58]. However, this can accumulate to cause a reactor breakdown on an industrial scale. Therefore, it is essential to consider the fragility of immobilization materials. The fragility of matrices can be tested for in continuously stirred aqueous media (such as in ethanol, *n*-hexane) [58]. Detection of matrix attrition is accomplished using elemental and surface analysis tools such as FT-IR, SEM, particle size analyzers, and XRD.

I.7. Lipase Reusability

The fundamental concept behind using immobilized lipases is to enable practical recovery while capitalizing on its green advantages over chemical catalysts. This underlining principle has expanded research on lipase immobilization for biodiesel with more than 1,340 scientific reports since 2001 (Web of Science extract on 28th July 2020 using "immobiliz* lipase biodiesel" as terms). The chemistry of matrices, the immobilization protocol, and the engineering of lipases are the well-studied areas used to advance IM lipases for biodiesel catalysis. However, studies on matrix, lipase strains, and immobilization protocols can only produce IM lipases that would still have to deal with 'destabilizing conditions' in reaction

media. Therefore, it is also necessary to address IM lipases' reusability from the perspective of the destabilizing conditions while promoting the conditions that support lipase reusability. In a biodiesel synthesis media with lipases, the case of destabilizing conditions can be addressed from the denaturation effect of the reactants present; thus, from oils/fats, alcohol, water, co-solvents, glycerol, and biodiesel. The primary reactants, oils/fats, are heterogeneous and contain free fatty acid, glycerides phospholipids, lysolipids, metal traces, tocopherols, and other minor constituents. However, varying occurrence of phospholipids, free fatty acids, and partial glycerides in oils/fats are known to extend stress on lipases [58]. Free fatty acids and partial glycerides act as detergents in distorting lipase stability and favor lipase release. Eliminating free fatty acids and glycerides in biodiesel reactions is impracticable. Therefore, long-term affinity modifications such as covalent binding and PEGylation can lower the effects of hydrophobic detergents on IM lipases.

Table 2-4. Performance of functionalized magnetic supports used for lipase immobilization towards biodiesel production.

Magnetization method	**Lipase strain**	**Biodiesel feedstock**	**Biodiesel reaction conditions and yield**	**Number of reuses**	**Saturation Magnetization (emu/g)**	**Reference**
Coupling Fe_3O_4 nanoparticles with poly-glycidyl methacrylate-*co*-methacrylic acid for encapsulation and covalent binding	*Candida rugosa*	Soybean oil	4:1 MeOH/oil molar ratio at 40 °C for 60 h per cycle, 92.8% FAME	5	28.7	[136]

TEOS-treated and glutaraldehyde-crosslinked magnetic nanoparticles	*Candida rugosa*	Waste cooking oil and brown grease	1:3 oil/ethanol molar ratio, 0.04 wt.% water, 30 °C for 35 h, 100% FAME	5	41.0	[107]
Grafting magnetic nanoparticles with 2,3-epoxypropyltrimethylammonium chloride and glutaraldehyde for	*Burkholderia cepacia*	Soybean oil	4:1MeOH/oil molar ratio, 0.3 wt.% *tert*-butanol, and 2.5 wt.% water at 45 °C for 9 h per cycle, 96.8% FAME	15	55.0	[50]

lipase covalent binding						
Sol-gel prepared-core-shell magnetic nanoparticles coupled with glutaraldehyde for covalent binding	*Burkholderia cepacia*	Canola waste cooking oil	1:6.2 oil/MeOH ratio, at 34 °C for 25 h, 92% FAME	3	45.7	[137]
Surfactant imprinting on magnetic cross-linked enzyme aggregates (mCLEAs) with	*Burkholderia cepacia*	Waste cooking oil	1:7 oil/MeOH molar ratio, 0.04 wt.% *n*-hexane, at 40 °C for 24 h, 98% FAME	6	40.0	[138]

hydroxyapatite-coated magnetic nanoparticles						
Magnetic nanoparticles coupled with 3-glycidoxypropyltrimethoxysilane for Co^{2+}-chelated affinity immobilization	*Pseudomonas fluorescens*	Waste cooking oil	1:4 oil/MeOH molar ratio, 0.6 mL/ g oil *n*-hexane, 50 °C for 24 h, 95% FAME	10	77.1	[139]
Polyporous magnetic cellulose	*Candida antarctica* lipase B	*Yellow horn* seed oil	1.6 v/v MeOH/oil ratio, 4	5	-	[127]

beads modified with diethylenetriamine and glutaraldehyde for covalent binding			v/v *tert*-butanol, 150 μL/ mL oil water, 50 °C for ≥ 8 h per cycle, 92.3% FAME			
Tannic acid/polyamine binary surface coating of magnetic nanoparticles for covalent binding	*Candida antarctica* lipase B	Waste cooking oil	1:3 oil/MeOH molar ratio, 40 wt.% molecular sieves, 40 °C for 96 h, 63.8% FAME	9	44.0	[140]

Fe_3O_4 functionalized with 3-aminopropyl triethoxysilane and glutaraldehyde for covalent binding	*Rhizopus oryzae*	*Chlorella vulgaris*-derived micro algae oil	0.15 mL MeOH added 3 times, 5 mg/cm^3 *n*-hexane, 45 °C for 24 h, 71.19% FAME	5	16.1	[141]
Magnetic nanoparticles coated with polyacrylonitrile and BSA-proteic feeding for CLEA formation	*Aspergillus oryzae* ST11	Palm oil	1:3 oil/MeOH molar ratio, 30 wt.% water, 37 °C for 24 h, 95% FAME	5	-	[142]

Although phospholipids in feedstocks are controllable, utilizing lipases strains that are tolerant of phospholipids is more viable. To study phospholipids' effect on true lipases, Li *et al.* found an 8.5% decrease in lipase NS81006's efficiency when 10 wt.% phospholipid was present. Phospholipids' effect can be lowered using a Combi-lipase system of phospholipases (e.g., A1 Lecitase Ultra from the genetically modified *Aspergillus oryzae*) and phospholipid-tolerant true lipase strains such as *Fusarium heterosporum* lipase [56].

Organic solvents are also natural inhibitors of lipases. In alcoholysis, organic solvents improve media miscibility. The inhibition effect of organic solvents has been observed with lowered activity from increasing molar amounts. On a molecular level, protein unfolding and intra-protein interactions are distorted when high organic solvent amounts are present. Conventional organic solvents in biodiesel synthesis such as *n*-hexane and *tert*-butanol have found green replacements with deep eutectic solvents, supercritical fluids, and even biodiesel [126–128].

Glycerol's competitive inhibitory effect with alcohol in biodiesel catalysis is predominant in IM lipases. Hydrophilic lipase carriers readily absorb glycerol onto lipase surfaces. To study this effect, Xu *et al.* used Amaranth, a polar food-grade pigment, as a dye for comparing glycerol's affinity on hydrophilic and hydrophobic supports. Xu *et al.* observed increased glycerol affinity in silica than in polystyrene, but the least occurred in hydrophobic supports from polymethylmethacrylate and polypropylene [129].

The effect of water in lowering lipase reusability has not been established in the literature as some lipase strains are favored in excess water. However, excess water in transesterification promotes FFA formation, which are established detergents that negatively affect IM lipases. Where hydrophilic carriers are used, water is easily absorbed, exposing IM lipases to weak acid denaturation in low pH environments.

The separation technique and reactor configuration used in recovering lipases are also crucial for subsequent reuses. Centrifugation, gravity settling, filtration, water-washing, and post-treatment with solvents are widely adopted recovery methods. However, centrifugation is energy-intensive and has the potential of destabilizing fragile IM lipases. New recovery techniques that have shown practical success involve introducing magnetic properties onto matrix surfaces (Table 2-4). Fe's saturation magnetization can be up to 220 emu/g; therefore, when inculcated into lipases, it supports practical recovery and shortens recovery time (Table 2-4).

I.7.1 Towards immobilized lipase cost reduction

The cost of IM lipases, as outlined here, is dependent on three (3) main cost inputs illustrated with Figure 2-4:

- the cost of lipase (isolation, purification, etc.)
- the matrix (synthesis, fine-tuning, etc.)
- the immobilization protocol (reagents, etc.).

The current market price of the commercial immobilized CALB (Novozym® 435) is more than the liquid TL lipase (Callera Trans L) from the same company. The high price of Novozym® 435 is attributed to the high cost of the Lewatit® VP OC 1600 resin used. Lewatit® VP OC 1600 is used not only for lipase immobilization but also in numerous industrial wastewater treatments. According to Tufvesson and co-workers' study on biocatalyst costs, this high-value acrylic resin can account for ≥47% of IM lipase cost [20]. To offset the high cost of IM lipases, Cesarini and co-workers from Novozymes A/S recommend 100 reuse cycles of IM lipases [21]. However, 100 reuses have thus far not been reported in the literature. With all the kinds of immobilization techniques available today, the

few exceptional reuses reported in the literature have documented between 15-20 cycles only [56,64,98,127,130]. A way forward as it is proposed here is the use of waste-derived but inexpensive matrices. Waste-derived matrices can lower the selling price of lipase-catalyzed biodiesel, which can be roughly calculated using Eq. 2.

$$Cost\ of\ biodiesel\ per\ ton = \frac{kg\ catalyst\ per\ kg\ feedstock}{kg\ biodiesel\ per\ kg\ feedstock}\ (cost\ of\ catalyst\ per\ kg) \quad \text{Eq. 2}$$

Assuming the selling price of an IM lipase at US$10 per kg, a catalyst-loading amount of 10% per kg feedstock, and a product yield of 96.4 wt.%, the selling price per US gallon of biodiesel would be at US$ 3.93 while that of liquid lipase (assuming 47% less from immobilization cost) will sell at US$ 2.083. The price variation between liquid and IM lipase could be significantly lowered to nearly zero if cheaper and recyclable matrices are used. In an economic sensitivity analysis study, Budžaki *et al.* suggested Sepabeads EC-EP/M as cheaper carriers over Eupergit® C for a 10,000 kg/yr modular biodiesel plant. The cheaper cost of Sepabeads EC-EP/M, compared with Eupergit® C, was deduced from the number of reuses and the oxirane groups (immobilization protocol) of its IM lipases. Other recent studies have also supported how reusing lipases is significant in the economic viability of lipase-catalyzed biodiesel [37,131,132]. Therefore, it is imperative to offset the cost of IM lipases by using inexpensive matrices and continuous reuses.

I.8. Greening immobilization to reduce cost

The current large-scale production of several bioproducts, including IM lipases, relies on fossil derivatives. The way forward to ensuring sustainability in lipase immobilization is by complete reliance on bio-based derivatives. However, first, a few questions must be posed. Is it practical to rely 100% on biomaterials for lipase immobilization? What are the areas of

lipase immobilization that require greening? An attempt is made to answer these questions herein.

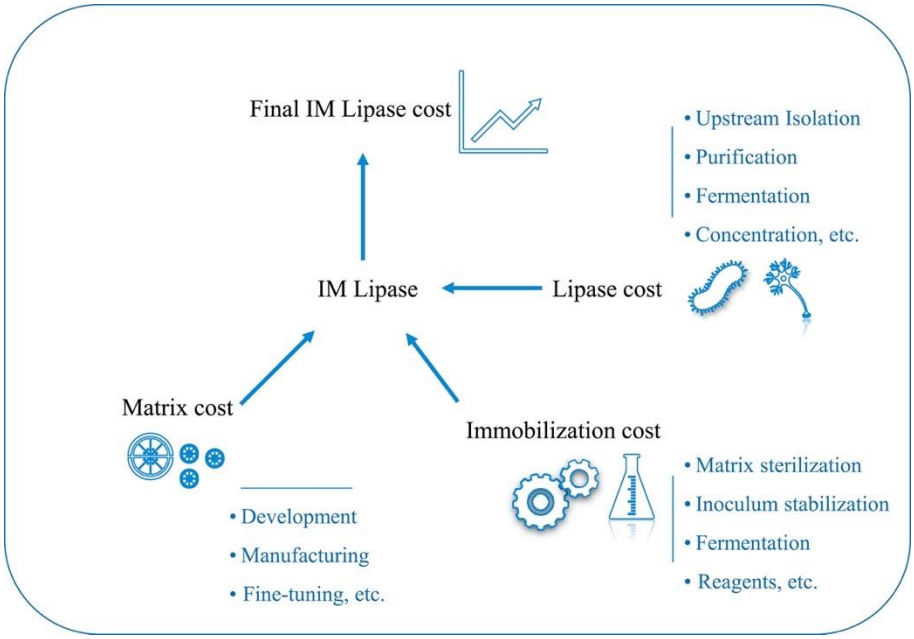

Figure 2-4. The final cost of immobilized lipases (IM lipase) depends on the cost of lipase processing, the matrix, and the immobilization technique employed.

Sustainability is measured with the so-called *E* (environmental) factors in green chemistry [89]. 'Green' lipase immobilization based on the *E* factors of green chemistry can be redefined as 'the design of immobilization processes and products that eliminate/reduce waste and avoid toxic/hazardous substances' [89]. Based on this redefinition, a criterion of success for greening lipase immobilization towards biodiesel synthesis would depend on six relevant factors, namely: environmental impact, safety, biocatalyst efficiency, resource efficiency, energy efficiency, and atom economy (illustrated with Figure 2-5). To assess the complexity but cost-effectiveness of using *E* factors in lipase immobilization, some reports in the literature have demonstrated the art, but have yet to address the 100-times reuse challenge (Table 2-5).

Mentioned throughout this review, IM lipases were initially introduced in biodiesel production to avert the detrimental environmental effects of using chemical catalysts.

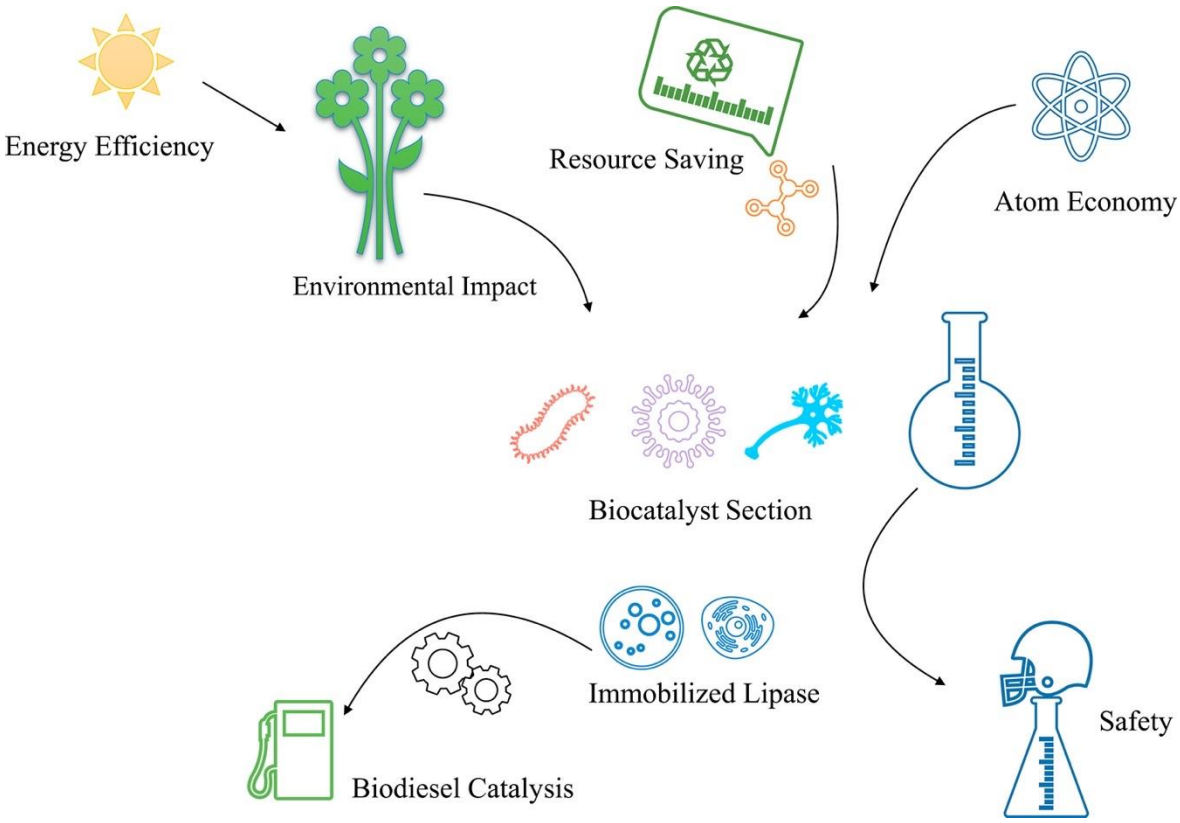

Figure 2-5. Criteria for green manufacturing of immobilized lipases. Lipase immobilization for sustainable biodiesel synthesis scale-ups should consider; Atom economy (e.g., re-immobilization), Energy efficiency (e.g., incubation time, temperature, pressure), Resource efficiency (e.g., lipase loading on minimum carriers), Biocatalyst selection (e.g., increasing substrate promiscuity), Environmental Impact (e.g., using biomaterials), and Safety (e.g., reagents toxicity and disposal).

However, this underlining objective has not been strictly followed. Most lipase immobilization and utilization yield adverse environmental effects similar to chemical catalysts. The immobilization of lipases where fossil-derived polyurethane (PU) foams are used is a pertinent example. After reuse of the IM lipases, the foams become unusable and are subsequently discarded [56].

Table 2-5. Performance of lipases immobilized using sustainable/green chemistry approaches towards biodiesel production. The use of biodegradable waste materials such as rice straw and the replacement of toxic reagents (e.g. glutaraldehyde) in lipase immobilization are considered promising sustainable chemistry approaches for lipase immobilization.

Lipase strain	**Immobilization technique**	**Green/Sustainability metric**	**Recovered / relative lipase activity**	**Product yield**	**IM lipase reuse cycles**	**Reference**
Thermomyces lanuginosus	Rice straw modified with Fe_2O_3 nanoparticles for lipase crosslinking with glutaraldehyde	Use of rice straw as lipase carriers	94.3%	83% FAME	10	[19]

Aspergillus niger MYA 135	Submerged fermentation of mycelium-bound lipase in cornmeal, molasses, waste cooking oil, milk serum, etc.	Fungi cells cultivated in agro-industrial waste	-	96% fatty acid butyl ester	Several [1]	[20]
Aspergillus oryzae ST11	Magnetic cross-linked enzyme aggregates (mCLEAs) co-fed with bovine serum albumin	Replaced glutaraldehyde with bovine serum albumin	81.7%	95% FAME	5	[18]

[1] **Continuous production in a packed-bed reactor**

Candida antarctica lipase B	Tannic acid-magnetic cross-linked enzyme aggregates with starch as crosslinker	Using starch as crosslinker	98.5%	85.9% fatty acid propyl ester	7	[21]
Candida antarctica lipase B	CLEA preparation onto octyl-modified mesocellular foams with oxidized sodium alginate	Oxidized sodium alginate was used to substitute glutaraldehyde	-	89.0% FAME	5	[22]

Candida rugosa	Bacterial cellulose from *Gluconobacter xylinus* impregnated with iron oxide nanoparticles for lipase cross-linking	Using bacterial cellulose as immobilization matrix	-	100% hydrolysis of *p*–NPP (0.1 M PBS, pH 7.0) at 37 °C for 5 min	5	[23]
Candida rugosa	Oil palm frond leaves- derived nanocellulose and montmorillonite in alginate for covalent binding	Oil palm frond leaves-derived nanocellulose as a matrix	111.5%	92.9% of ethyl levulinate	9	[24]

Burkholderia cepacia	Eggshell membrane stabilized with glutaraldehyde	Use of food waste (eggshell) as matrix	87%	-	10	[25]
Porcine pancreas lipase	Genipin for cross-linking chitosan beads towards immobilization	Use of genipin, a fruit extract as a glutaraldehyde substitute	-	92.3% FAME	10	[26]
Thermomyces lanuginosus	Adsorption immobilization in polyurethane foams obtained from bio polyol enzymatic glycerolysis of castor oil and glycerol	Use of glycerol, a biodiesel byproduct and castor oil for polyurethane synthesis	-	66% FAME	6	[27]

The foams are mainly produced from the polymerization of petroleum-derived polyester diol and a diisocyanate [133]. An effort to replace these fossil-derived, but excellent biomass support particles, with bio-based ones, has been attempted with vegetable oil-based polyol [134]. For the immobilization of *T. lanuginosus* lipase, Bresolin and coworkers synthesized PU foams using castor oil polyol and glycerol [134].

Nevertheless, the use of biowaste directly for immobilization is a more sustainable pathway. For instance, rice husks, waste biomass from rice mills, were used for the immobilization of *Cercospora kikichii* lipase towards coconut oil transesterification [135]. The GA-activated rice husk maintained >96% fatty acid ethyl ester (FAEE) yields in five reuse cycles [135]. Similar reports have utilized sugar cane bagasse, corn stover, coconut fiber, and eggshells [136] (Table 2-5). Agro-wastes, as these materials are referred to, are biodegradable and non-toxic. Agro-wastes can be used directly or with little modification as lipase carriers. A comparative study between eggshells and rice husks for *B. cepacia* lipase immobilization emphasized the need for crosslinkers (e.g., GA) to maximize its use activities over different temperatures (20–60 °C), acyl acceptors (ethanol, methanol, etc.), and pH range (6–10) [137]. The sustainability of crosslinkers and other reagents involved in lipase immobilization is also of paramount concern (Table 2-5). Cyanogen bromide (CNBr) is one of several classic cross-linkers used for its simplicity and mild pH [138]. However, CNBr is extremely toxic and volatile [139]. Even for small instances of skin or gastrointestinal contact, convulsions and death may result. This means health, safety, and environmental (HSE) barriers in CNBr use must be stringent to limit its discharge into the environment. Formaldehyde (FA) and glutaraldehyde (GA) are other popular but toxic protein cross-linkers used for lipase immobilization [61,65]. FA and GA are excellent at reinforcing adsorption and improving multipoint covalent attachment of lipases. Physico-chemical properties of FA and GA are compared in Table 6. The excellent chemical modification faculty of GA is cited throughout

this review. However, a 0.05 ppm GA exposure limit has been set by various public health organizations.

Table 2-6. Physico-chemical properties of glutaraldehyde and formaldehyde. Glutaraldehyde and formaldehyde are versatile cross-linking reagents widely used in lipase immobilization.

Property	**Glutaraldehyde**	**Formaldehyde**
CAS Registry Number	111-30-8	50-00-0
Chemical Formula	$C_5H_8O_2$	CH_2O
Melting point	-14 °C	-92 °C
Boiling point	187 °C	-19 °C
Vapor pressure	17 mmHg (20 °C)	< 1 atm
Solubility	Reactive in water	400 g/L water
Hazards Level	Corrosive, toxic, health hazard, environmental hazard	Flammable, corrosive, toxic, health hazard
Lethal Dose $(LD)_{50}$	134 mg/kg (oral, rat)	100 mg/kg (oral, rat)
Recommended Exposure Limit (REL)	0.2 ppm	0.1 ppm (15 min)

Symptoms found in humans exposed to GA include chronic bronchitis and nasal discharges [140]. A look into material safety data sheets of commercially-produced GA shows the availability of heavy metals such as arsenic and lead (Pb). Heavy metals increase the cost of wastewater treatment and raise the risk of environmental exposure. There is a need to substitute such hazardous reagents with non-toxic ones (Table 2-5). Mateo and co-workers propose using dextran polyaldehyde as a replacement for GA in the preparation of CLEAs [141]. Facile oxidation of dextran, a natural polysaccharide, yields a bulky polyaldehyde that excludes irrelevant amino residues in immobilization. The non-toxicity of natural cross-

linking reagents needs further exploration for the synthesis of inexpensive IM lipases (Table 5).

The energy efficiency of lipase immobilization also needs to be addressed in the green shift. In the area of lignocellulosic biomass conversion to bioethanol, growing attention on its energy inefficiency necessitated investigations into the use of photocatalysis [149–1 51]. Utilizing nature's abundant supply of photoelectrons for electrostatic immobilization, co-catalytic conversion, and electricity generation is an energy-positive pathway that can be explored.

The overlapping connection between sustainability and cost reduction is reached when waste-derived matrices, less-toxic reagents, recyclable matrices, and resource efficiency are used. In a recent report, biomass-derived activated carbons for the immobilization of whole-cell lipases were found to be recyclable in addition to the fundamental reusability. The mechanical robustness of activated carbons ensured recycled uses for lipase immobilization [56]. In a green framework, fundamental reusability and recycling of the matrices can significantly lower IM lipase cost in biodiesel production.

Nevertheless, using green techniques presents challenges that need investigations to promote industrial scale-ups. One of the significant challenges is the non-existence of standardized immobilization protocols. Characterization is required to sub-categorize lipases into protocol response groups. While for instance, CALB has shown success on hydrophobic supports, other lipase strains such as *Alcaligenes* sp have reported >58 IU g^{-1} lipase activity on hydrophilic supports [145].

Another challenge with promoting green lipase immobilization lies in the availability of inexpensive waste treatment techniques. Inexpensive effluent treatment can also lower IM lipase cost. For instance, Wijaya *et al.* reported upgrading lipase effluent from *F. heterosporum* lipase cultivation. Nanofiltration membrane separation was described as an

energy-saving technique to concentrate the culture effluent to attain 32.6 U/mL lipase activity and >94 wt.% biodiesel conversion in palm oil [146]. Life-cycle assessments and techno-economic investigations are also needed to quantify the viability of valorizing agro wastes. Modern biotechnology tools such as directed evolution and immobilization reagents that present long-term reuse cycles are also needed.

I.9. Conclusions

Large-scale application of immobilized lipases for biodiesel production is needed to guarantee biodiesel's credentials as a carbon-neutral fuel in the future energy mix. Currently, large-scale biodiesel productions are catalyzed by chemical catalysts, mainly acids, and bases. However, chemical catalysts generate large amounts of metal ions and salts into the products, byproduct, and waste streams. Chemical catalysts also require high temperature and pressure in the process. In this respect, in the last three decades, research has promoted the replacement of chemical catalysts with energy-efficient enzymes, particularly lipases. Using lipases in the immobilized form ensures practical recovery for repeated reuses.
Commercial deployment of immobilized lipases has yet to supplant chemical catalysts in biodiesel production. The delay in the deployment of immobilized lipases is due to its high cost. Immobilization carriers and protocols are suggested to be the active cost contributors. Using a green chemistry approach, the high cost of immobilized lipases can be addressed by considering energy efficiency, atom economy, environmental impact, resource efficiency, biocatalyst selectivity, and safety. These factors can be used to significantly lower the cost of immobilized lipases for economically viable scale-ups.

I.10. References

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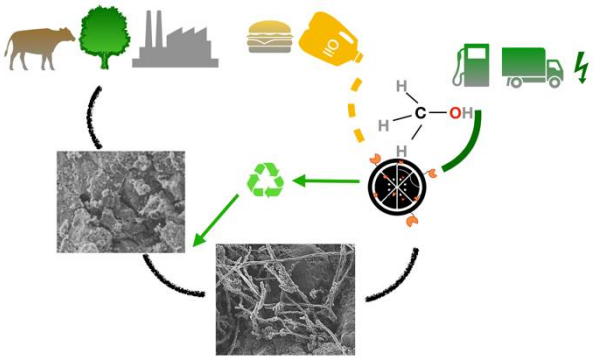
Chapter II:

Valorization of activated carbon as a reusable matrix for the immobilization of *Aspergillus oryzae* whole-cells expressing *Fusarium heterosporum* lipase towards biodiesel synthesis.

Graphical Abstract



II.1. Introduction

Depletion of fossil fuel reserves and increasing greenhouse emissions resulting from its use requires a sustained effort to introduce renewable fuels such as biodiesel. Biodiesel, together with other biomass-derived energy alternatives, can be developed to address the increasing global energy insecurity, mitigate climate change issues and contribute to rural economic development [1]. Biodiesel can generally be produced via transesterification/esterification of oils/fats with short-chain alcohols in the presence of a catalyst [2]. Although praised for its biodegradability, current industrial production employs chemical catalysts (acids or bases) which increases its downstream wastes and are limited by feedstock types [3]. The utilization of second and advanced generational feedstocks such as trap grease, inedible oils and lipids from oleaginous microorganisms presents a much sustainable approach for the production of biodiesel. Conversely, these feedstock types contain high free fatty acids, which in conventional biodiesel production, yields soaps and might require extensive pre-treatment steps [4–6]. Lipases (EC 3.1.1.3) as catalysts, on the contrary, pose less of these environmental concerns and are flexible to different feedstock types [7]. However, industrial application of lipase technology is limited by its cost and reusability. Genetic modification of native lipases into whole cell forms to produce more heterologous proteins for immobilization has been described as a better way to extend the reusability span of lipases but not its overall economic viability [7–9].

By utilizing environmentally-benign and cheaper lipase support matrices for immobilization, sustainable alternatives with lipase-catalysed biodiesel production can be achieved. Several inorganic to organic support carriers including polyurethane (PU), polyvinylic and metal-based oxides have been applied in the attempt of lipase immobilization [10]. The

extinction, toxicity, decomposition challenge and fossil sources of some of the applied matrices does not however conform with the green economy sought by biotech biodiesel production [11–13].

Readily available, activated carbon (AC) can be obtained from biomass sources including industrial and agricultural wastes. Qian *et al.* prepared activated carbon from cow dung; a cattle rearing nuisance due to its unpleasant smell and GHG emissions [14]. By exploiting its large surface area (600- 3800 $m^2g{-}1$) [15], bio-compatibility and tribology [16], a remarkable potential can be envisaged from activated carbon to reduce lipase immobilization cost [17]. Less explored, reported literature [18–20] (Table 3-S1) have covered its application in the immobilization of native lipases. We report for the first time, its utilization in the immobilization of recombinant whole cell lipases towards biodiesel synthesis.

To maximize matrix-to-lipase aggregation during immobilization, most studies have applied cross-linking reagents such as cyanogen bromide, glutaraldehyde, *N*-hydroxysuccinimides and others [21,22]. The most used protein cross-linker, glutaraldehyde, for instance can result in acute to sub-chronic respiratory effects on ingestion [23]. Commercially available glutaraldehyde also contains ≥2ppm Arsenic (As), Lead (Pb) and other heavy metals [24]. Increasing concerns on heavy metal micro-pollutants and microbial metabolites in industrial wastewater streams prompts greener manufacturing of biocatalysts [25,26].

In response to biocatalyst cost, toxicity of biocatalyst streams and challenge from second and advanced generational feedstocks, this study evaluates 'cross-linker free' aggregation between our engineered whole cell *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase and activated carbon for simultaneous transesterification and esterification. The recyclability of the spent matrix after repeated re-use reduces its carbon footprints and increases its value. This study also experimentally evidenced with surface characterization techniques from FE-

SEM, XPS and FT-IR that physical adsorption and deposition of the whole cell lipases occurred without cross-linking agents.

II.2. Materials and Method

Granular activated carbon of 8-30 mesh (0.595-2.38 mm) was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Soybean oil (188-195 KOH/mg saponification value) was obtained from Wako Pure Chemical Industries Ltd. (Kobe, Japan). All other reagents used were of analytical grade and were obtained from local suppliers (Hyogo, Japan).

II.2.1 Strain, Media and Immobilization

The mutant, *A. oryzae* niaD300, of the wild type, *A. oryzae* OSI1031, was used for the transformation and propagation of *A. oryzae* cells.[27] The improved promoter (P-No8142) for expression of *Fusarium heterosporum* lipase *(*FHL*)* was described in a previous study.[27] Formulations of dehydrated potato infusions and dextrose was used for mycelia cultivation at 30 °C. Transformant selections onto Czapek–Dox-$NaNO_2$ agar plates resulted in the *A. oryzae* - FHL mycelia, which was subsequently employed in whole forms.[28]

1 ml of inoculated spores, 100 ml of dextrin-peptone (DP) medium, with different weights of biomass support particles (BSPs)/without (0.85 g in the case of PU foams), was used for the immobilization (in a 500 ml Sakaguchi flask). The DP media mainly contained 2 g glucose, 2 g polypeptone, and nutrients solution (3 g KH_2PO_4, 0.6 g $NaNO_3$, and 0.3 g $MgSO_4.7H_2O$). Immobilization was done in a bioshaker (TAITEC GBR-300, TAITEC Corporation, Tokyo, Japan) at 30 °C and 150 rpm oscillations. The PU foams used were the 6

mm × 3 mm × 3 mm reticulated polyurethane foams (Bridgestone, Osaka, Japan) described in a previous study. [29] Different weights (5 g, 7.5 g, 10 g and 15 g) of the activated carbon (AC) were used for the immobilization. After 96 hours of cultivation, the culture broth of immobilized cells was filtered from the media, lyophilized and designated as IPU for the PU immobilized cells and IAC for the activated carbon type. IAC5, IAC7.5, IAC10 and IAC15 were designated as identification based on the weights of AC used.

II.2.2 Cell Aggregation Assay

The difference between the final weight of the immobilized BSPs and the initial weight of BSPs used was described as the total cell yield from the cultivation (Eqn. 1). Cell concentration measurement method described in a previous study [30] was used to measure cell accumulation per mg of BSP (Eqn. 2). Briefly, 10 immobilized BSPs was washed with acetone, dried under 80 ºC for 24 h and subsequently washed rigorously with NaOCl solution to remove attached cells before a 24 h oven-drying (80 ºC). Cell concentration measurement (Eqn. 2) was used in calculating the amount of lipase loading (wt. %) required in each trans/esterification (Eqn. 3). Total cell yield (Eqn. 1) considered both displaced pulp-like cells and BSP-attached cells from the culture broth. However, Eqn. 2 mainly measured cell accumulation per BSP but not the overall cell yield from the medium.

$$Total\ cell\ yield\ (mg) = final\ weight\ of\ BSPs - initial\ weight\ of\ BSPs\ used \quad (1)$$

$$q = W_i - W_f \quad (2)$$

$$\text{Lipase loading (wt. \%)} = \frac{(M_{cat})\text{q}}{Total\ weight\ of\ oil\ feedstock} \times\ 100\% \quad (3)$$

Where: q is cell concentration per mg of BSP, W_i is the weight of 10 immobilized BSPs, W_f is the final weight of the 10 BSPs after ripping-off the cells and M_{cat} is the weight of catalyst in each trans/esterification.

II.2.3 Catalytic Activity Assay

The hydrolysis of olive oil to produce fatty acids was used to measure the hydrolytic activity of the lipase. [31] One unit (U) of hydrolytic activity was defined as the amount of lipase (mg) that liberates 1 µmol of free fatty acid per min from olive oil at pH 10 and 37 °C. The reaction of 4 g refined soybean oil, 0.15 g methanol and 0.2 g water in a thermoblock rotator at 37 °C for 4 hours was used to measure the transesterification activity. One unit (IU) of transesterification activity was defined as the amount of lipase (mg) that produces 1 µmol FAME in an hour.

II.2.4 Extracellular Proteins Determination

The culture filtrate, after separating the immobilized components, was analysed for its remaining total proteins. The Pierce™ BCA Protein assay method was used. The quantification of total proteins in the culture was from supernatants of centrifugation and filtration (PDVF syringe filter, 0.45 µm pore size). Measurements were at A562 nm (PerkinElmer Plate Reader, Osaka, Japan) using a BCA standard calibration curve.

II.2.5 Transesterification, Esterification, and Characterization of FAME

To obtain high free fatty acid (FFA) feedstock of up to ~70% FFA, 100 g soybean oil, 50 g water and 4.5 g *Candida rugosa* lipase was used for hydrolysis in a bioshaker (TAITEC GBR-300, TAITEC Corporation, Tokyo, Japan) for 24 hours at 30 °C. The obtained partial hydrolysates were mixed with fresh soybean oil to account for the ~30% (w/w) triglycerides component of the feedstock. In one step, simultaneous transesterification and esterification of 4 g of the feedstock was conducted in the presence of 0.2 g water. 4 molar methanol equivalents of the feedstock were added stepwise at 0, 24, 48 and 72 hours (one molar per addition time). [32] Produced fatty acid methyl esters (FAME) were analysed using a GC-2010 (Shimadzu Corp., Tokyo, Japan) equipped with a Zebron™ ZB-5HT gas chromatography column (Phenomenex Inc., Torrance, CA, USA). Tricaprylin was used as internal standard for the detection of methyl esters and further characterization of the produced FAME. [33]

II.2.6 Reuse of the Biocatalyst and Recycling of the Matrix

At the end of each 96-hour trans/esterification reaction, the biocatalysts were separated from the produced FAME, washed with tap water, dried for 24 hours under room temperature and before reusing it in a fresh transesterification batch. After up to 5 batches (480 hours) of using the biocatalysts, the activated carbon was recycled as supports by washing with excess water and oven-dried (80 °C) before fresh cell immobilization under the same cultivation conditions (30 °C, 150 rpm in a bioshaker). However, in this subsequent cultivation, 7.5 g of recycled activated carbon (RAC) was used in a 100 ml DP medium and with 1 ml of inoculum spores. The presence of unremoved cells and unreacted oils on the surface of the RAC meant that a

higher weight of matrix was required to match up to the 5 g optimum weight. Cell yield, hydrolytic and transesterification activities of the recycled immobilized activated carbon (RAC-IAC) were also measured.

II.2.7 Surface Characterization by FE-SEM, XPS and FT-IR

Scanning Electron Microscopy (SEM) observation of the activated carbon, before and after the first immobilization, as well as the subsequent forms, was done under a JOEL JSM-7500F field-emission SEM (JOEL Ltd., Tokyo, Japan). The samples were oven dried, ion sputter-coated to inhibit cell charging and mounted on Aluminium stubs with carbon tapes for imaging at 7 kV accelerating voltage.

An X-ray photoelectron spectroscopy (XPS) survey to describe the surface elemental characteristics as well as the bonding configuration of IAC5 biocatalysts was done using an automated XPS microprobe (ULVAC-PHI Inc., Kanagawa, Japan). Samples were sputter-cleaned and measured on at least two acquisition points in 50 sweeps. Survey scans (1400- 0 eV) and N1s (411- 391 eV) binding state scans were performed using Al Kα X-ray source (15 kV, 51 W) at 45 degrees take-off angle to a 234-µm nominal acquisition area. PHI MultiPak™ version 9.4.1.2 was used for acquisition and evaluation of the data.

Fourier-transform infrared spectroscopy (FT-IR) analysis was performed using a Shimadzu AIM-9000 Infrared Microscope equipped with an IRTracer-100 (Shimadzu Corp., Tokyo, Japan). Transmission spectra acquired were the average of 20 scans (4000-400 cm^{-1} wavelength) with a KBr-only pellet background for correction of light scattering.

II.3. Results and Discussions

II.3.1 Adsorptive Influence and Biocompatibility of Activated Carbon Explored

The facile synthesis strategy ensured simultaneous physical adsorption and mushrooming of the *Aspergillus oryzae* whole cells onto the interior and exterior surfaces of the activated carbon (see Scheme S1). [18] The 1 – 2 μm spore diameters of *A. oryzae* cells means growth occurred at the porous interfaces of the moderately sized activated carbon.[34] Activated carbon have been explored as efficient adsorbents for the sorption of metal ions and organic molecules in both liquid and gaseous phase systems.[15,35,36] The media for the parallel cultivation and immobilization mainly contained the dextrin-peptone formulation in addition to nutrients (KH_2PO_4, $NaNO_3$ and $MgSO_4.7H_2O$). The adsorptive influence of activated carbon ensured an initial recovery of the mineral salts present in the medium to the active sites of the matrix, thereby, providing favourable conditions for cell growth to be actuated from the porous sites.[17,36–38] The networks of channels in AC (shown with SEM image in Fig. 3-4a), based on its complex structure, allowed the spacer arms of the cells to form rigid attachments to the uneven cracks, crevices, nooks and fissures between the carbon layers. The straightforward nutritional dependence of the *Aspergillus oryzae* fungi meant that the carbon substrate, source of nitrogen and salts of K, P, Na and Mg (in the medium) were readily adsorbed into the active sites of the carbon for growth commencement. [39,40]

II.3.2 Functional Activity Effects from Cell Aggregation

Immobilization efficiency was measured with the total cell yield and the remaining proteins in the culture medium (Figure 3-1). Optimization of carbon weights was used to investigate

achieving maximum cell accumulation and to ascertain the role of surface area availability in immobilization (Figure 3-1a). Higher cell aggregation (Total cell yield) was observed with activated carbon as matrix than with PU as matrix (Figure 3-1a). This is be based on the phenomenon [41] that constituents present in the AC matrix contributed to its higher cell growth. In a study where PU was compared to stainless steel and nylon, an unknown inherent element in PU made it a preferred choice. [42] The functional elements in AC or the contributing inherent elements were further investigated with surface characterization tools from XPS and FT-IR.

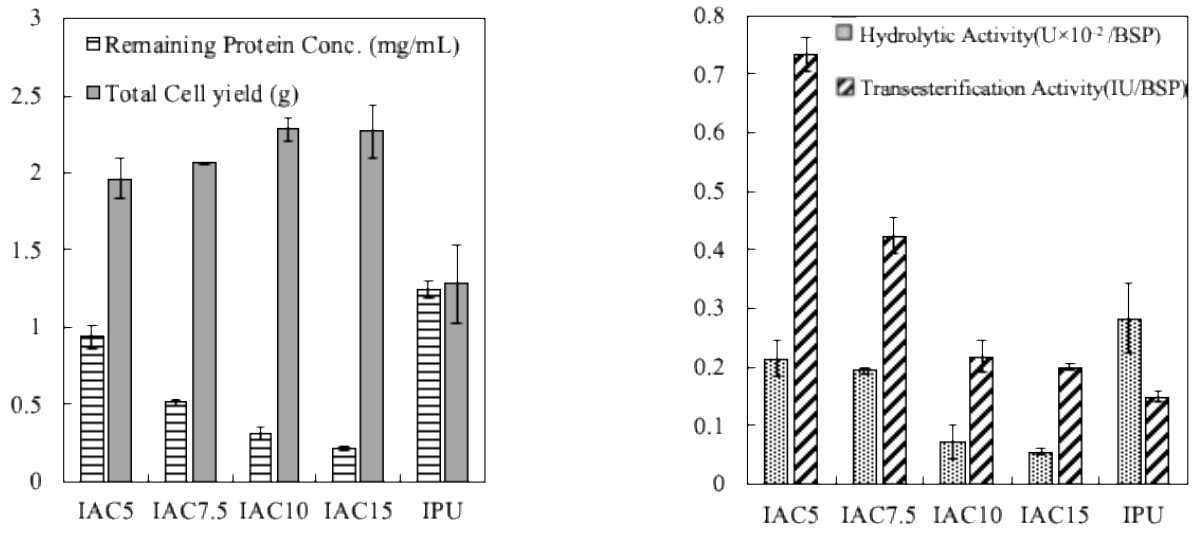


Figure 3-1. Effect of activated carbon (AC) weight on total cell yield and remaining proteins in the culture medium when immobilizing A. oryzae FH lipase (a) and its effect on catalytic activities (b). The weight optimization was compared to polyurethane (IPU) as matrix for FHL immobilization. Activated carbon weights for FHL immobilization (IAC) were 5 g, 7.5 g, 10 g and 15 g (designated as sample names).

With the same 1 ml of inoculated spores used for the cultivation, measuring total proteins remaining (Figure 3-1a) in the culture was vital in establishing the role of surface area

availability in immobilization. After initial adsorption of the nourishing medium, growth of the cells into the pores and the exterior surfaces ensured minimum cells (proteins) were displaced into the medium. The larger pores of the PU (40 pores per linear inch), on the contrary, displaced yielding proteins into the suspension with less deposition on the PU surfaces (remaining proteins concentration, Figure 3-1a). Remaining proteins in a culture with ACs was lower than with PU foams. Shown in Figure 3-1a, remaining proteins decreased with increasing AC weight. This however elucidates that activated carbon offered a larger surface area and voids for yielding cells to be absorbed.

The hydrolytic and transesterification activities of the produced biocatalysts were tested to understand the relationship of enzyme/carrier to product formation (Figure 3-1b). It was observed that IPU offered the highest hydrolytic activity of 28.35 $U \times 10^{-2}$/BSP. Also, similar higher hydrolytic activity was achieved with IAC5. However, the introduction of methanol in measuring transesterification activity made AC a preferred matrix. [43] The significantly high transesterification activity of the IACs compared to IPU is based on the hydrophobicity/oleophilicity characteristics of the matrix. [44,45] The increase in the hydrophobicity of the AC after cultivation (characterized with XPS) ensures that the non-polar components (oil) were the first to protect the surface films of the biocatalysts. Covered within the least dispersed component, the polar (water and methanol) and non-polar phase (oil) of the medium was maintained at the lipases' effective interface for catalysing effect. [46]

II.3.3 Simultaneous Conversions of Free Fatty Acids and Triglycerides to FAME

Preliminary lipase loading optimization was done to determine the minimum lipase weight required to achieve efficient conversion (Figure 3-S1). The industrially-challenging modelled feedstock of high FFA (68.77% w/w fatty acids, 20.48% w/w triglycerides) was comparable to

waste oils from palm oil mills and inedible oils (Table 3-S2).[5,6,47] Laborious pre-treatment steps have been suggested with such feedstock sources, increasing deficiencies of the already uneconomical biodiesel production.[4,5,48] A single-step production presents a viable solution.[32] Shown in Figure 3-2a, all the IAC biocatalysts were used for simultaneous conversion of the feedstock. IAC5 (0.34 ± 0.02 mg/BSP cell concentration, 4.25 wt. % lipase loading, Table 3-1) achieved 98% FAME. This meets EN 14214-ester requirement of 96.5% minimum FAME. Compared to other activated carbon immobilization studies, where native lipases were used, A. *oryzae* FHL whole cells applied in this study exhibited higher methanol tolerance towards FAME formation. [18–20,49]

Table 3-1. Cell concentration measurement (q) and lipase loading from 0.5g of IAC in a 4g feedstock. Each value was expressed as mean ± standard deviation from triplicate values (*n* = 3)

Sample	**Cell concentration, q (mg/BSP)** [a]	**Lipase loading (wt. %)**
IAC5	0.34 ± 0.02	4.25
IAC7.5	0.11 ± 0.03	1.38
IAC10	0.09 ±0.01	1.13
IAC15	0.03 ±0.02	0.38

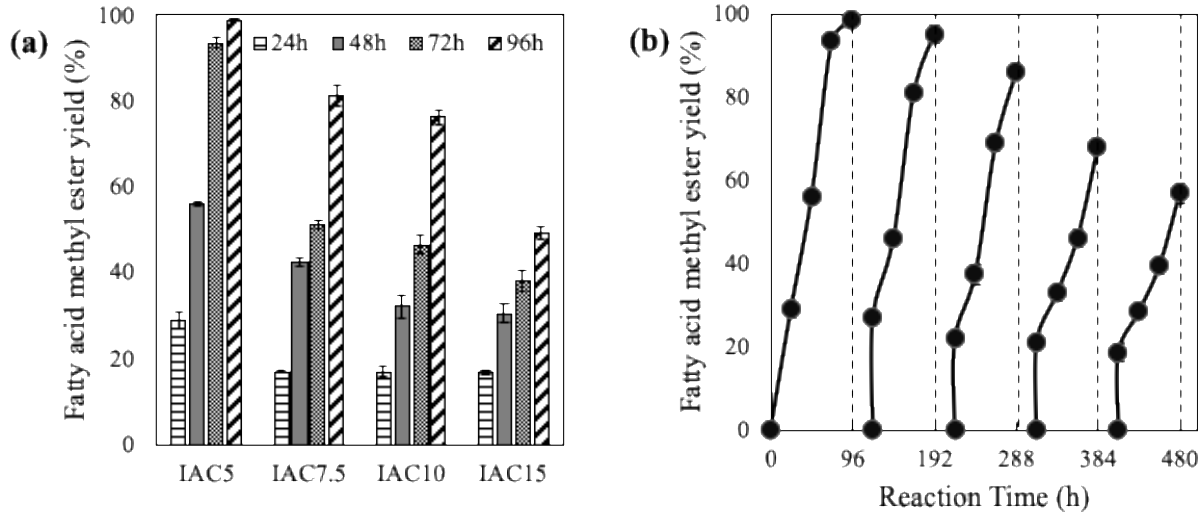


Figure 3-2. Fatty acid methyl ester (FAME) synthesis with the (a) all the different *A. oryzae* FH lipase biocatalysts (b) and the reuse of IAC5 in consecutive batch cycles. Methanolysis reaction conditions: 0.5 g immobilized biocatalysts (IAC5, IAC7.5, IAC10 and IAC15), 4 g substrate, 0.2 g water and 1:4 molar oil to methanol equivalent added stepwise at a 24-hour interval. (similar patterned bars in Fig. 3-2a indicate FAME yield at each 24 h interval).

II.3.4 Reusability and Matrix Valorization

After 480 hours of repeated batches with IAC5, 56.8% FAME was attained as the minimum FAME (Figure 3-2b). In terms of productivity, an average of 23.47% FAME was produced by the first molar addition of methanol in all batches. This suggests a consistently higher selectivity of the biocatalyst towards product formation. With methanol contact time predominantly vital to lipase survival, a continuous biodiesel production in a series of packed bed reactors evaluated in literature is also a viable option for this biocatalyst. [50]

In the quest for higher lipase reusability in biodiesel production, immobilization techniques applied in the area have focused on resolving the drawbacks of matrix-lipase adhesion, stability

and the long-term re-use of the lipase but not the matrix. [51,52] This study ventured into a new area of spent matrix valorisation.

The beneficial tribology of carbon after 480 hours of continuous shear and stress in this study ensured it was possible to re-use the spent matrix in fresh cell immobilization. Unlike polyurethane foams which loses its rigidity and form under continuous usage, the robustness of the carbon particles allowed the possibility of recycling the matrix. Even though biological to thermal regeneration systems are currently industrially applied in recovering spent activated carbon, a facile drying technique (80 °C oven drying for 24 hours) applied in this study was also suitable to maintain the value of the matrix for fresh cell immobilization. The immobilized recycled activated carbon, referred to as RAC-IAC in this study, showed higher cell concentration of 0.71± 0.05 mg/BSP, higher hydrolytic activity of 46.52 $U \times 10^{-2}$/BSP and transesterification activity of 4.59 IU/BSP. When compared to the activities of IAC5, this was due to the presence of unreacted triglycerides and fatty acids on the spent matrix when it was applied to fresh cell cultivation. The presence of fatty acids and triglycerides in immobilization media have been described as necessary for higher cell aggregation and lipase activity. [31,41,42] In a study where *Rhizopus oryzae* filamentous whole cells were cultured with oleic acid and olive oil, the inclusion of the fatty acids and triglycerides in the cultures enhanced its lipase activity. [53] In line with other studies, unreacted oils on the recycled carbon surfaces promoted higher cell yield and catalytic activity. When the immobilized spent matrix (RAC-IAC) was applied to similar batch conditions of trans/esterification, 98.08% (w/w) FAME could be achieved (Figure 3). RAC-IAC was further tested for its re-use stability and it demonstrated equivalent repetitive use in 5 batches (Figure 3). Establishing such matrix recycling routes reduces the carbon footprints of producing and shipping new matrices for immobilization.

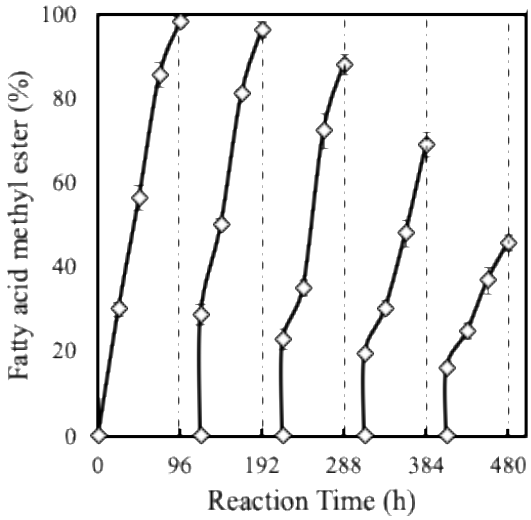


Figure 3-3. Repeated fatty acid methyl ester (FAME) synthesis with the A. oryzae FH lipase immobilized on recycled activated carbon matrices (RAC-IAC). Methanolysis reaction conditions: 0.5 g immobilized catalyst (RAC-IAC), 4 g substrate, 0.2 g water and 1:4 molar oil to methanol equivalent added stepwise at a 24-hour interval.

II.3.5 Biocompatibility Characterization of Activated Carbon

To confirm success in the setting of the cells within the matrix, scanning electron microscope (SEM) micrographs were used. A description of the distribution of the fresh AC's pore structure, the location of the cells after immobilization, the surface structure after 480 hours of reuse and after second batch of immobilization is presented in Figure 3-4. Micrographs of the activated carbon before immobilization (Figure 3-4a) shows a rough surface morphology and the presence of fissures as pores and even a well-developed micro-pore structure beneath. [17,54] Oscillations of immobilization rendered a slightly rough morphology to the surface (Figure 3-3b). Partly due to coating of the AC's surface with the medium, the rough morphology provided more rigid interfaces for cell attachment. [54] After 480 hours of reusing

the matrix, a finer morphology (partly coated with oil) is seen in Figure 3-4c. Figure 3-4d micrographs further provide visual indication of the presence of cells growing/penetrating from the crevices of the AC when it was reused for another cell immobilization. It is noteworthy that the attachment of the cells into the carbon surfaces (as indicated by SEM images 4b, 4c and 4d) emphasizes biocompatibility of the cells with the AC.

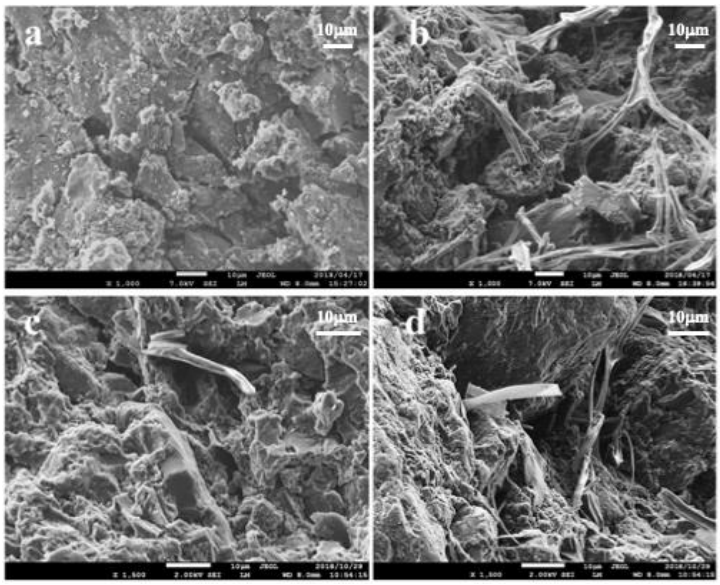


Figure 3-4. SEM micrographs of the fresh activated carbon (AC) (a), *A. oryzae* FH lipase immobilized onto the fresh AC (b), AC-immobilized A. *oryzae* FH lipase after 480 hours of use (c) and the *A. oryzae* FH lipase immobilized onto the recycled activated (RAC-IAC) (d).

An X-ray photoelectron spectroscopy survey was used to describe the functional elements present on the AC before and after immobilization (Figure 3-5). A survey scan of the blank activated carbon (Figure 3-5a) indicated an environment of C1s and O1s in the original AC as reported in other literature. [55] After immobilization, the emergence of the N1s peak was used to confirm the ammoxidation of amides onto AC. The C1s surface groups in the original AC were essential in the formation of the rigid peptide links of the cells.

[56] The N1s functional groups (~400.00 - 398.70eV binding energy range), thus, detected were consistent with amines to tertiary amides of cells when N1s was explored (Figure 3-5b). [57–59]

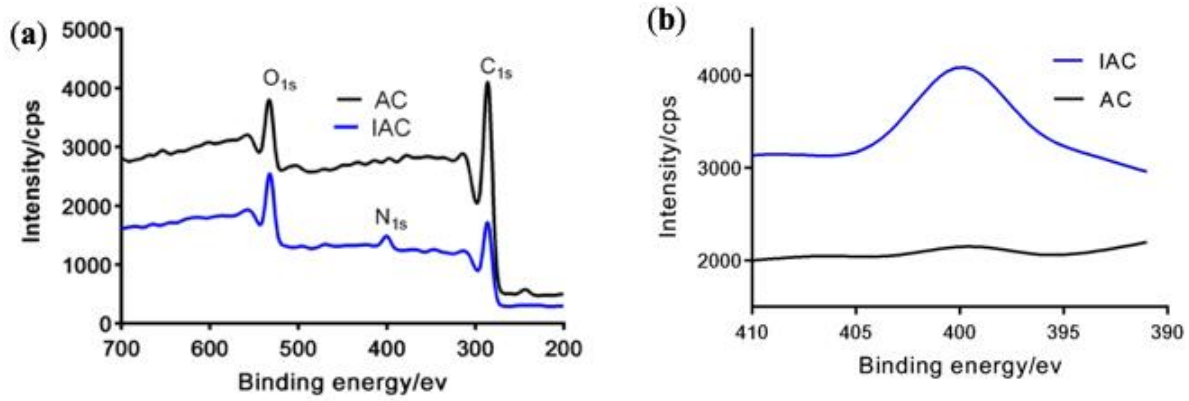


Figure 3-5. XPS spectra of the activated carbon (AC) and the *A. oryzae* FH lipase-immobilized activated carbon (IAC) (**a**); N1s spectra of the AC and IAC (**b**).

The detection of N1s on carbon surfaces have been used in other characterization studies where *Burkholderia cepacia* lipase (BCL) was used. [60]

A resultant alteration of the hydrophobicity of AC is explained with the amination of the surface chemistry and the considerable change in the oxygen-carbon (O/C) ratio (Table 3-2).[15,61] Oxidation and amination of carbon surfaces have both been explored for capacity and surface chemistry modification for improved utilization of activated carbon.[15,61] The increase in O1s was from the uptake of oxygen from the media during the formation of amides between the carboxylic group of the matrices/media and the amino acids present in the media. The uptake of oxygen to the reactive sites of the carbon paralleled with growth of the cells into the pores reduces its enthalpy of immersion in polar liquids, which renders it more hydrophobic even than before. [44] Based on the enhanced oleophilicity of the

IACs after immobilization, a higher transesterification facilitation was realized when compared to IPU.

Table 3-2. Elemental composition of the AC and the IAC by XPS.

Acquisition Sample	C1s (%)	O1s (%)	N1s (%)	O/C Ratio
AC	86.4	13.6	-	0.16
IAC	67.6	23.9	8.5	0.36

Fourier-transform infrared spectroscopy (FT-IR) was used in this study as well (Figure 3-6) to further characterize surface chemistry modification of the matrix. The stretching at 1066 cm^{-1} are attributed to C-O-C bonding from ethers that were present in the original carbon (Figure 3-6, AC and IAC). [62] The source of AC used may have provided an advantageous growth of proteins as ethers are prevalent in the build-up of carbohydrates and lignin in biocatalysts. [62,63]

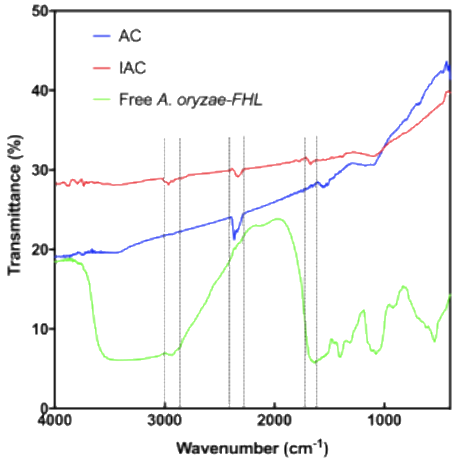


Figure 3-6. FT-IR spectra of activated carbon (AC), AC-immobilized *A. oryzae* FH lipase (IAC) and free cell form of A. *oryzae* expressing FH lipase.

Vibrational occurrence at 1732 cm^{-1} is attributed to a C=O carbonyl stretch. [62,64,65] The biocompatibility of activated carbon with the cells is confirmed with the characteristic interference at 2962 cm^{-1}. [66] However, significant interference peaks of organic groups on the carbon and a preservation of the carbon's intrinsic C≡O alkynes stretching at 2300 cm^{-1}[64] demonstrates biocompatibility and the extent of modification (summary on Table 3-S3).

This study emphasizes the utilization of whole cell lipases and explores the valorisation of a low-cost matrix for lipase immobilization in biodiesel production. Addressing immobilization cost, reducing toxicity of lipase streams, a greener route of 'cross-linker free' immobilization was achieved with activated carbon (AC) and *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase. Complementing its intrinsic hydrophobic/oleophilic properties, recyclability was added to the low-cost advantages of activated carbon as matrix in lipase immobilization. Characterization of the activated carbon before and after immobilization indicated a degree of adsorption that occurs on the exterior to porous surfaces of the AC. Efficient chemo-deposition and attachment of the cells' spacer arms, indicated by FE-SEM, XPS and FTIR spectra, were indicative of the chemo-biological advantages of the matrix. Compared to polyurethane foams as matrices, inherent properties of activated carbon facilitate higher cell growth and catalytic activity. 98% FAME was achieved with 4.25% loading of the biocatalyst when applied to an industrially-challenging feedstock of high free fatty acids (68.77%). The tribology of the matrix after 480 hours of continuous reuse ensured the possibility of recycling the matrix. This study, thence, presents a new approach of immobilization matrix recycling and its application to sustainable bio-refineries.

II.4. Supporting Information

Remarks for The Supporting Information

A literature survey on the use of activated carbon as lipase immobilization matrix in is presented in this supporting data (Table 3-S1). The survey supports the significance of this study, the first report on the use of activated carbon for the immobilization of recombinant whole cell lipases and its subsequent application to biodiesel synthesis. The filamentous fungi morphology of *Aspergillus oryzae* whole cells ensured growth of the cells at the porous crevices of the activated carbon before finally forming a dense film on the surface of the matrix in the immobilization process.

In Scheme S1, a schematic illustration indicating cell-matrix adhesion of *Aspergillus oryzae* whole-cells expressing *Fusarium heterosporum* lipase at the porous interfaces of activated carbon is presented.

In Figure 3-S1, an optimization of biocatalyst loading is presented. Preliminary lipase weight optimization was done to determine the minimum lipase weight required to achieve efficient conversion (Figure 3-S1). 0.4 g, 0.5 g and 0.6 g of IAC10 were used. By increasing the weight beyond these weights, miscibility of the fluid medium was reduced. 0.5 g IAC10 attained 75 % FAME, 1% less than 0.6 g IAC10 under optimum reaction conditions.

The industrially-challenging modelled feedstock used in this study was compared to feedstocks used in other biodiesel synthesis studies (Table 3-S2). The high free fatty acid composition of the feedstock was comparable to the listed feedstocks and such feedstocks have been described to be industrially challenging. A two-step transesterification and esterification has been suggested with such feedstocks. However, simultaneous transesterification and esterification was achieved with our engineered *Aspergillus oryzae* whole-cells expressing *Fusarium heterosporum* lipase.

Figure 3-6 in the main article demonstrates the IR spectra for the blank activated carbon, the immobilized form and the free cell form of the *Aspergillus oryzae* whole-cells expressing *Fusarium heterosporum* lipase. The functional groups identified by FTIR and its corresponding wavenumber positions are summarized in Table 3-S3.

Lipase Source	**Matrix**	**Feedstock**	**FAME[a] (wt. %)**	**Reference**
Candida rugosa lipase	Granular activated carbon	Palm oil	62.4	[20]
Candida Antarctica lipase	Activated carbon (granular and cloth)	Palm oil	28	[19]
Lipase from porcine pancreas, Type II	Granular activated carbon	-	Not applied	[18]
A. oryzae-FHL	Granular activated carbon	68.77 % FFA[b] and 20.48 % TAG[c] (Soybean oil)	98	This study

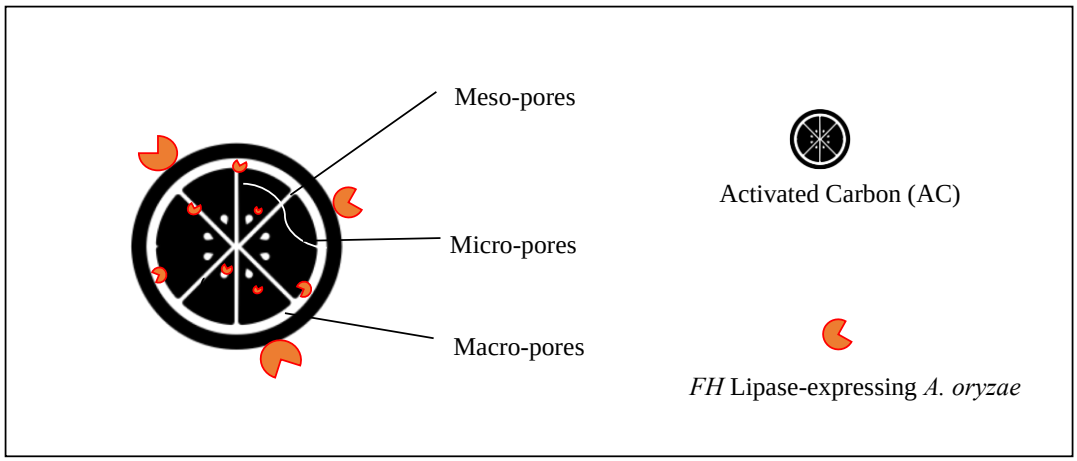
NB: Publications listed on Google Scholar database using "lipase+activated carbon+biodiesel" separated by document types.

[a] FAME = fatty acid methyl esters

[b] FFA = free fatty acid

[c] TAG = Triacylglycerides

Table 3-S1. A literature survey comparing *Aspergillus oryzae* whole-cells expressing *Fusarium heterosporum* lipase immobilized onto activated carbon for this study and other activated carbon-lipase immobilization studies.



Scheme 3-S1. Schematic illustration indicating cell-matrix adhesion of *Aspergillus oryzae* whole-cells expressing *Fusarium heterosporum* lipase at the porous interfaces of activated carbon.

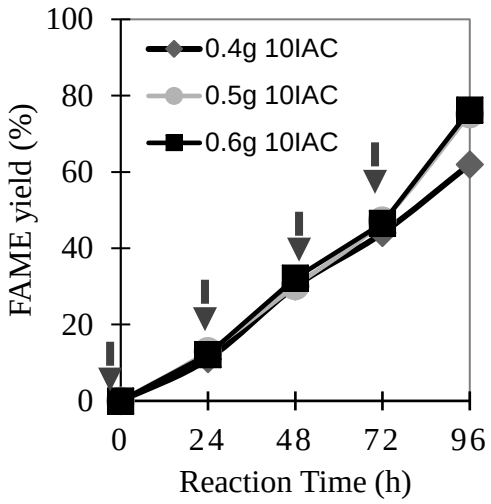


Figure 3-S1. Methanolysis optimization: lipase loading (0.4g, 0.5g, and 0.6g of IAC10) at 30°C with 1:4M stepwise methanol addition (indicated by arrows) at 0, 24, 48 and 72 hours.

Feedstock	FFA Content (Wt.%)	Reference
Palm fatty acid distillate (PFAD)	93	[47]
Soybean Hydrolysates	68.77	This study
Tobacco seed oil (TSO)	35	[67]
Waste cooking oil	11.93	[6]

Table 3-S2. Feedstocks containing high free fatty acids applied to biodiesel synthesis

Wavenumber (cm^{-1})	Assigned Functional group and Remarks	Reference
1066	antisymmetric stretching of C-O-C from ethers	[62,63]
1732	C=O carbonyl stretch due to the deformation of N-H in the build-up of peptides	[62,64,65]
2300	C≡O alkynes stretching	[64]

Table 3-S3. Summary of FT-IR Functional groups present on activated carbon and A. oryzae FHL-immobilized activated carb

II.5. References

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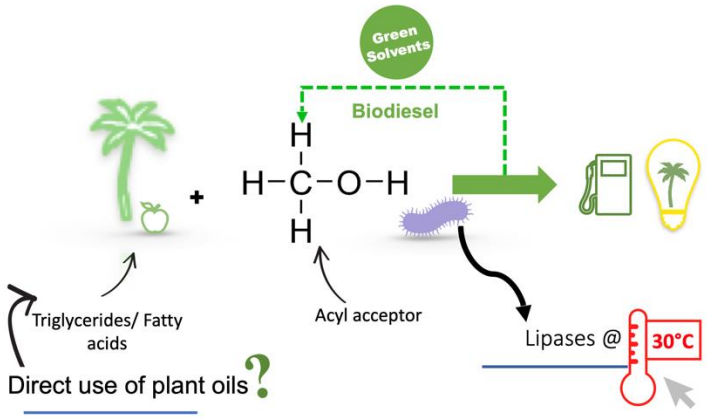
Chapter III:

Biodiesel-mediated biodiesel production: A recombinant *Fusarium heterosporum* lipase-catalyzed transesterification of crude plant oils

Graphical Abstract



III.1. Introduction

A sustainable biodiesel production industry based on second and advanced generation feedstocks from inedible oils and lipids from oleaginous microorganisms has been described as a necessary advancement for the renewable fuels industry [1–3]. Progress has been made in utilizing waste cooking oils and other inedible oils for biodiesel production but current techno-economic analyses continue to show that diverse feedstocks would be required to meet the demand [4–7].

The use of crude plant oils can offset the demand as well as reduce overall feedstock cost in biodiesel production [6]. Amongst plant oils, palm oil (*Elaeis guineensis)* is the most productive oil crop ($\geq$ 8-ton oil ha^{-1} $year^{-1}$) with an average 25-year lifespan comparable to the lifespan of a prolific oil and gas well [8,9]. The oil yield per hectare and the cheap cost of palm oil compared to other market-common oils, such as soybean oil, also makes it an economically viable choice. Like most oils, a sequence of extraction techniques is employed to obtain the easily processible and market-ready product of refined palm oil (RPO). The main processing steps involve sterilization, stripping, digestion, pressing, clarification, and drying to obtain the crude palm oil (CPO). Subsequently, the obtained CPO is degummed of phosphatides, entrained oils, meal particles and so-called gums [10]. Degumming reduces refining losses and increases the thermal stability of the oil [11]. The next step after degumming is the refining process. Whether via chemical or physical refining, the stages of refining generally involve the neutralization of free fatty acids, bleaching of color bodies, adsorption of trace metals, exclusion of oxidation products, and winterization of remaining waxes. Deodorization, the final step of refining, removes volatile compounds (mainly ketones and aldehydes) that contribute to oil odor and taste. After undergoing the numerous described processes, the oil may now be deemed edible or used easily as feedstock for biodiesel production. The processes

of degumming and refining result in extraction losses and increase feedstock cost. The cost of feedstock in biodiesel production accounts for up to 85% of the production unit cost [11]. The linear relationship between feedstock cost and biodiesel price means an economically viable option would be direct transesterification of the crude oil to biodiesel. More so, during the refining process, carotenoids and other essential antioxidant compounds are destroyed. Left-in-place antioxidant compounds in CPO biodiesel can be advantageous to the shelf life of biodiesel [12].

In literature, attempts have been made, however, high-heating components such as waxes and phospholine gums found in CPO hinder its mild-temperature conversion to biodiesel (Table 4-1). Unlike acid/base transesterifications which operate under relatively higher temperatures (60 – 120 °C), lipase-catalyzed transesterification thrives best under mild temperature conditions (30 – 40 °C). Lipase catalysis of CPO has shown to require up to 50 °C or the inclusion of organic solvents in the process (Table 4-1). Besides energy consumption from higher temperature plant operation, the additional cost, fossil source, health, safety and environmental (HSE) hazards of volatile organic solvents (such as *n*-hexane, *tert*-butanol, petroleum ether, etc.) maintain the question of sustainability to be answered [13,14]. The inclusion of organic solvents in methanolysis has shown to improve the solubility and miscibility of the hydrophobic(oil) and hydrophilic (alcohol) substrates.

Table 4-1. A review of literature on enzymatic biodiesel production using crude palm oil (CPO) as feedstock. Publications listed on Google Scholar database using "crude palm oil + enzymes + biodiesel" separated by document types.

Catalyst	Reaction Conditions	Esters Yield	References
Thermomyces lanuginosus lipase	3:1 ethanol/oil ratio at 50 °C in 4 h	25 % FAEE	[34]
Thermomyces lanuginosus lipase	45 °C, 30 wt % molecular sieves added	82 % FAEE	[35]
Thermomyces lanuginosus lipase	Centrifuged and upper surface used at 30 °C, *tert*-butanol as solvent, 30 °C and 150 rpm agitation	85 % FAME	[36]
Thermomyces lanuginosus lipase	Centrifuged at 32 °C and upper surface used at 40 °C in *tert*-butanol (150 rpm agitation)	87 % FAME	[37]
Thermomyces lanuginosus lipase	Centrifuged at 32 °C and upper surface used at 40 °C (150 rpm agitation)	96 % FAME	[38]
Recombinant *Fusarium heterosporum* lipase	30 °C, 35 rpm and 10 % biodiesel as solvent	97 % FAME	*This study*

In this attempt, recent literature proposes substituting organic solvents with deep eutectic solvents (DES); which are non-volatile and tunable alternatives [15]. However, their cumbersome synthesis technique and post-separation maintain the search for greener alternatives [13]. A promising alternative solvent can be envisaged in fatty acid acyl esters (biodiesel) [16,17]. Obtained from renewable sources, biodiesel is biodegradable, has low vapor pressure and it is compatible with other organic solvents. Hu *et al.* measured the solvent

power of some vegetable oil methyl esters to be up to 80 kauri-butanol value (KB). This is higher than 29-30 KB value for *n*-hexane [18]. Knothe and Steidley also characterized the solvent potential of fatty acid methyl esters and compared it as a substitute for common fossil-derived solvents such as toluene [19].

Therefore, in this study, we present the first report of the use of fatty acid methyl ester (biodiesel) as a green solvent in enzymatic biodiesel synthesis and evaluate its dissolution effect on high-melting components in CPO. While previous studies have attempted enzymatic catalysis of CPO in organic solvent media, an investigation into the denaturation effect of the solvents on the lipase is yet to be reported. Here, by using our engineered whole-cell *Aspergillus oryzae* expressing recombinant *Fusarium heterosporum* lipase (FHL), we explore the first use of biodiesel as a solvent in enzymatic transesterification and investigate how to maintain the reusability of the lipase in the presence of gums, phospholipids and other oil contaminants that have a negative effect on lipase activity.

III.2. Materials and Methods

Crude, degummed and refined palm oil were obtained through P.T Agricinal in Bengkulu and local market in South Sumatra, Indonesia. The difference in the physical appearance of the three (3) palm oil fractions at room temperature is shown in Figure 4-1. Methyl oleate, *tert*-butanol, *n*-hexane, petroleum ether and methanol (MeOH, ≥ 99.8% purity) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Activated carbon for immobilization of the lipase was procured through Nacalai Tesque Inc. (Kyoto, Japan). All other reagents used were of analytical grade and supplied by Sigma-Aldrich (Tokyo, Japan) and Nacalai Tesque Inc. (Kyoto, Japan).

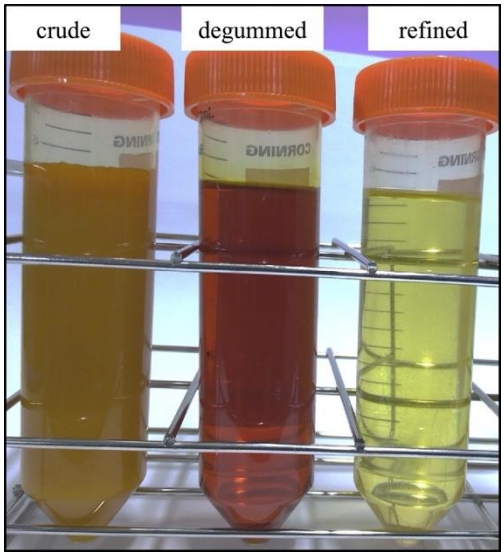


Figure 4-1. Crude Palm oil and its fractions (image obtained at room temperature after incubating the oils at 30 °C for 24 h). Crude palm oil is mechanically extracted from the mesocarp of oil palm fruits. Degummed oil is obtained after removing phospholine gums and other contaminants in the CPO. Refined oil is obtained after neutralization and bleaching of DPO to the desired consumable state.

III.2.1 Gene Expression and Transformation of a recombinant lipase into *A. oryzae* whole-cells

Gene expression and optimized promoter transformation of the *A. oryzae* wild-type fungi to express of *F. heterosporum* lipase recombinant strain used in this study was reported in a previous study [20]. Briefly, the *FHL*-encoding gene was amplified from pYGF2 by PCR using independent primers. FHL-F1 (5′-TCGCAAACATGATGCTCGTCCTATCTCTTC-3′) and FHL-R1 (5′-GCTCTAGACTAAATCATCTGCTTAACAAAT-3′) were used as primers. Construction of the modified pSENSU-FHL for expressing three copies of FHL was applied from a separate study [21].

III.2.2 Immobilization of *A. oryzae* whole-cells onto activated carbon

Immobilization of *A. oryzae* FHL whole-cells was performed under optimum conditions using 5 g granular activated carbon as the immobilization matrix [22]. A 500 mL Sakaguchi flask shake cultivation method was employed. [22] In a bioshaker (TAITEC GBR-300, TAITEC Corporation, Tokyo, Japan), the cultivation flask contained 100 mL dextrin-peptone (DP) medium and 1 mL inoculum spores of the fungi cells. For 96 h, cultivation was done at 30 °C and 150 rpm oscillations. After cultivation, the whole-cells were lyophilized and designated as the biocatalysts for subsequent transesterifications.

III.2.3 Methanolysis of Palm oil fractions to FAME

CPO, DPO, and RPO were used as feedstocks for methanolysis at 30, 35 and 40 °C. In a 10 mL borosilicate glass vial, the FHL-catalyzed methanolysis consisted of 4 g oil, 0.2 g immobilized lipase, 0.2 g water, and 0.15 g MeOH added stepwise at a 24 h interval (0, 24, 48, and 72 h). The reaction was conducted in a temperature programmable Thermo-Block Rotator (TBR) (Nissin, Tokyo, Japan) at a 35-rpm rotation speed. 80 μL samples were taken at each 24 h interval to analyze the produced FAME (fatty acid methyl esters).

III.2.4 Melting Profile Analysis of Palm Oil Fractions

Differential Scanning Calorimetry (DSC) was performed to evaluate the melting characteristics of the palm oil fractions. A computer-programmed, indium-calibrated DSC (Thermo plus Evo, Rigaku Corporation, Osaka, Japan) equipped with a liquid nitrogen refrigerant unit was used. Heat flow measurements were achieved with Aluminum sample pans and an empty Aluminum pan as reference. Nitrogen (99.98% purity) was used as purge gas at 50 mL min^{-1}. The melting profile was obtained by a three-step program of sample cooling to -70 °C, maintained at -70 °C for 10 min and raising the temperature steadily to 70 °C at 10 °C min^{-1}. Thermograms obtained were analyzed for its onset (T_{on}), peak (T_{peak}) and completion (T_{com}) temperatures, respectively using a Thermo plus EVO2 analysis software.

III.2.5 Solvent-Mediated Methanolysis of Crude Palm Oil

Petroleum ether, *n*-hexane, *tert*-butanol, and biodiesel (methyl oleate) were compared as solvents in the methanolysis of CPO to FAME. The reaction mixture contained 4 g CPO, 0.2 g water, 0.2 g *A. oryzae* FHL, 1:1 oil-to-solvent molar amount of solvent, and 0.15 g methanol added stepwise at 0, 24, 48 and 72 h.

III.2.6 Reusability test of *A. oryzae* FHL in methanolysis

For the reusability of the immobilized lipase, batch reactions were carried out using 10 mL glass tubes containing 4 g of the substrate (3.6 g CPO and 0.4 g methyl oleate), 0.2 g *A. oryzae* FHL, 0.2 g water, and 0.15 g methanol added stepwise. For the 480-h repeated batch reaction, FAME samples were taken at a 24 h reaction interval for chromatography analysis. At the end of each 96-h batch, the formed biodiesel/oil was decanted from the lipase. The collected immobilized lipase was washed with distilled water and transferred into a fresh substrate for another batch. To account for immobilized lipase losses in the process, 10 wt % fresh immobilized lipase was added to each new batch. The same technique of lipase treatment and transfer was used for the 24 h continuous re-use of the lipase in nine batches.

III.2.7 Lipase Hydrolytic Activity Assay

To evaluate the denaturation effect of the four (4) solvents on the activity of the lipase, 0.2 g immobilized lipase, 2.8 g Tris buffer solution (0. 1 M, pH 8) and 1.2 g solvent were incubated in 10 mL glass tubes in a TBR (35 rpm, 30 °C, 24 h). The Immobilized lipase was separated after 24 h and its hydrolytic activity measured using a method described previously [22]. One

unit of hydrolytic activity (U) was defined as the amount of enzyme (mg) required to liberate 1 µmol of free fatty acid from olive oil per minute under defined standard conditions. This was translated into relative activity as the ratio of the hydrolytic activity (U) of lipase incubated in a solvent-containing mixture to one without (Tris buffer only).

III.2.8 Analytical and Statistical Methods

FAME samples for gas chromatography analysis were centrifuged at 12,000 x g for 5 min at 6 °C. 10 mg of the supernatant (oil phase) was mixed with 3 mg of Tricaprylin (the internal standard). A 15 m x 0.25 µm x 0.10 µm high-temperature column (Zebron™ ZB-5HT, Phenomenex Inc., CA, USA) was used for the characterization of FAME. The injection volume was 1 µL of 1 mL hexane-diluted sample. The chamber vaporization temperature was set at 130 °C with Helium as a carrier gas. The initial 130 °C oven temperature was held for 30 s, increased to 300 °C at 10 °C min^{-1} and maintained at 300 °C for 10 min. By using the gradient of a standard FAME curve, FAME yield (wt %) was calculated based on the following equations;

$$FAME\ amount\ (mg) = \frac{Peak\ Area\ of\ FAME \times Weight\ of\ Internal\ Standard}{Standard\ Gradient\ (m) \times Peak\ Area\ of\ Internal\ Standard} \quad (1)$$

$$wt\ \%\ FAME = \frac{FAME\ amount\ (mg)}{Reaction\ sample\ (mg)} \times 100\% \quad (2)$$

Analysis of the acid value of the oils was performed following AOCS Method Cd 3d-63 for analysis of oils and fats. Unless otherwise indicated, all experimental values were average of triplicate values (*n*=3) with standard deviation indicated on the figures as error bars.

III.3. Results and Discussion

Crude palm oil is extracted from the mesocarp of the oil palm fruit. The mesocarp consists mainly of oil and palm fiber. The extraction process, referred to as digestion, is mostly done using hot water vapor. The steam liberates the oil from the fibrous mesocarp and via pressing, more of the crude oil can be released. The obtained liquid can then be separated from sand and debris to obtain CPO. These processes are energy-intensive but produce neither an edible grade nor an easily processible oil. The obtained CPO is thick semi-solid at room-temperature (shown in Figure 4-1). The thick semi-solid characteristics of CPO are attributed to the presence of phospholine gums, waxes, meal particles and mucilaginous substances in the oil [11]. The process of degumming removes these contaminants that give the semi-solid characteristics in CPO [11]. The degummed palm oil, DPO, is more fluid at room temperature but still contains phospholipids, ketones, and aldehydes that would require further processing (Figure 4-1). From the crude state, CPO can also be fractionated to obtain two distinct fractions of a solid palm stearin and a liquid palm olein. The hard palm stearin has a relatively high melting point (48 - 50 °C) compared to the liquid palm olein (25 °C) [23].

Refining of DPO removes remaining phosphoacylglycerols, free fatty acids (FFA), and other minor contaminants that affect the edibility of the oil. The refining process can either be via chemical refining where KOH is used to neutralize the remaining FFAs or physical refining where the FFAs and triacylglycerols (TAG) are separated by their boiling points. An analysis of the acidity and FFA composition of the palm oil fractions used in this study reveals up to 7.8 % FFA more in CPO when compared to RPO (Table 4-2). Using analytical grade refined soybean oil as a reference in this study (Table 4-2), refined palm oil may contain an edible FFA of around 0.34 % (Table 4-2) [24]. Residual

phospholipids in the form of non-hydratable phospholipids (NHPs) are also removed at the refining stage. Refining renders the oil appealing to taste and smell. However, for biodiesel production, an appeal to taste and smell is not of relevance to the fuel.

Parameter	**CPO**	**DPO**	**RPO**	**RSO**
Physical appearance (at 30 ºC)	Yellowish semi-solid	Reddish liquid	Pale yellow liquid	Colorless to pale yellow
Acid value (mg KOH)	16.22	12.75	0.68	0.41
Free Fatty Acid (%)	8.16	6.41	0.34	0.21

Table 4-2. Physico-chemical characteristics of the crude (CPO), degummed (DPO) and refined palm oil (RPO) used in this study, with refined soybean oil as a reference; triplicate values analyzed (n = 3).

III.3.1 *Aspergillus oryzae* FHL-catalyzed Methanolysis of CPO to FAME

At a 30 °C reaction temperature, *A. oryzae* FHL as a catalyst in the transesterification of CPO and DPO is limited by the effect of oil contaminants in the oil fractions (Figure 4-2a). Nevertheless, at the same 30 °C, *A. oryzae* FHL catalyzes the transesterification of RPO to a high yield of 95.2 wt % FAME (Figure 4-2a). With high-melting temperatures required for waxes and gums, an attempt to completely convert CPO at 35 and 40 °C was performed (Figure 4-2b). Increasing the reaction temperature to 35 °C ensured up to 53.2 wt % FAME compared to an earlier 16.9 wt % FAME at 30 °C. However, increasing the reaction temperature to 40 °C was unfavorable to the thermally mesophilic *A. oryzae* FHL. Besides, high energy consumption in plant operation, higher temperatures destabilizes the catalytic activity of most mesophilic lipases used in biodiesel production [25–27].

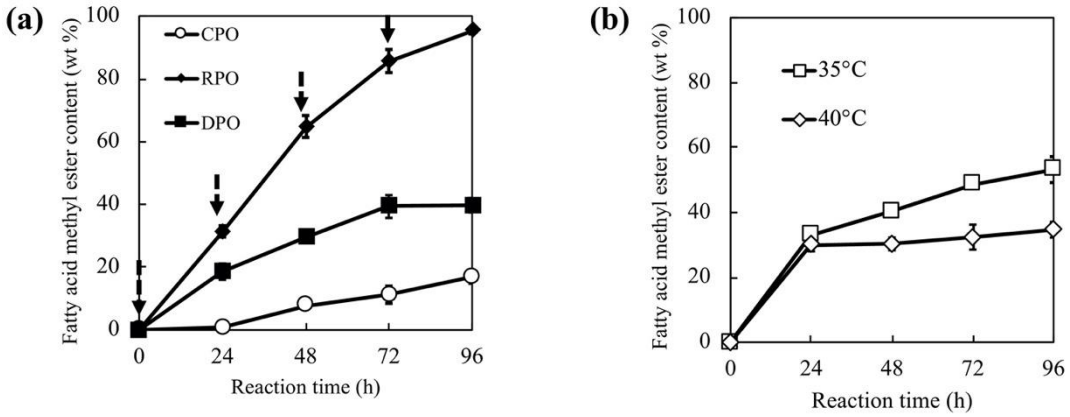


Figure 4-2. Fatty acid methyl ester yields for (a) crude palm oil (CPO), refined palm oil (RPO) and degummed palm oil (DPO) methanolysis at 30 °C and (b) CPO methanolysis at 35 °C and 40 °C. Methanolysis conditions: 0.2 g immobilized *A. oryzae* FHL, 35 rpm agitation, 0.15 g methanol added at a 24 h interval (arrows indicate the time for stepwise methanol addition).

Previous attempt in literature (Table 4-1) have demonstrated the need for centrifugation at 32 °C or a 50 °C reaction temperature for the direct use of CPO as feedstock (Table 4-1). *A. oryzae* FHL, like most mesophilic lipases, is functionally stable at a 30 °C optimum temperature [25]. The most commonly used lipase in biodiesel production, *Candida antarctica* lipase B (Novozym®435), functions optimally within a 30 to 40 °C temperature range [28]. To date, lipase instability and reusability remain a persisting hurdle in enzymatic biodiesel production. Utilizing lipases at mild temperatures not only extends the life span of lipases but also reduces the energy requirement for biodiesel production [29].

III.3.2 Differential Scanning Calorimetry of CPO, DPO, and RPO

To identify the melting point, as well as the thermal behavior difference of the palm oil fractions, differential scanning calorimetry (DSC) was used. Melting peak (T_{peak}) and melting

completion (T_{com}) of the oils' DSC scans were used in this study on the basis that, for a given transesterification reaction, a stable reaction temperature is necessary for interphase interaction of all hydrophilic and hydrophobic components in the reaction mixture. DSC scans of the oils showed a consistent correlation of their thermal behavior with the workability of the oils in transesterification at 30 °C (Figure 4-3). Amongst the 3 fractions, RPO indicated a lower melting peak (T_{peak}) at -6 °C (Figure 4-3a and 4-3b). This can be explained with its liquid state characteristics at room temperature [30]. Although DPO's T_{peak} was at 7.8 °C, melting completion (T_{com}) of the oil was not until 28.62 °C (Figure 4-3c). A DSC scan of CPO exhibited a complex double peak that corresponds to the polymorphic and easily separable two phases of low-melting olein and high-melting stearin in CPO (Figure 4-3d). Melting peak (T_{peak}) and melting completion (T_{com}) of CPO were at 33 and 40 °C respectively. This also, however, elucidates the low biodiesel conversion yield of the oil even at 35 °C.

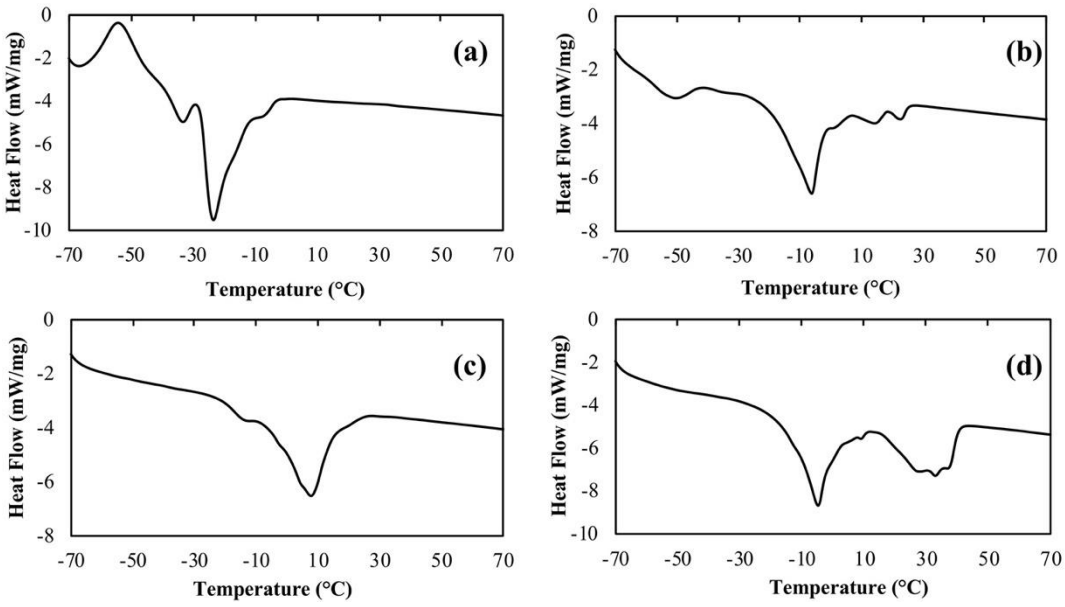


Figure 4-3. DSC heating scan of (a) refined soybean oil, (b) refined palm oil, (c) degummed palm and (d) crude palm oil.

III.3.3 Solvent-Mediated Methanolysis of CPO to FAME

The use of solvents in transesterification increases the phase mobility of the hydrophilic and hydrophobic reactants and lowers mass transfer limitations in the mixture. In this regard, petroleum ether, *n*-hexane, and *tert*-butanol have been widely applied as solvents. They are described to have low inhibition effect on lipase activity and have shown to positively alter methanolysis [31–33].

The effectiveness of using such organic solvents at a 1:1 oil/solvent molar ratio was tested with *A. oryzae* FHL and compared to a novel alternative from biodiesel (Figure 4-4a). Biodiesel yield with CPO increased from 16.9 wt % in a solvent-free medium reaction to 56.1 wt % when petroleum ether was used as a solvent (Figure 4-4a). However, the use of biodiesel as solvent ensured the conversion of CPO to 98.8 wt % FAME.

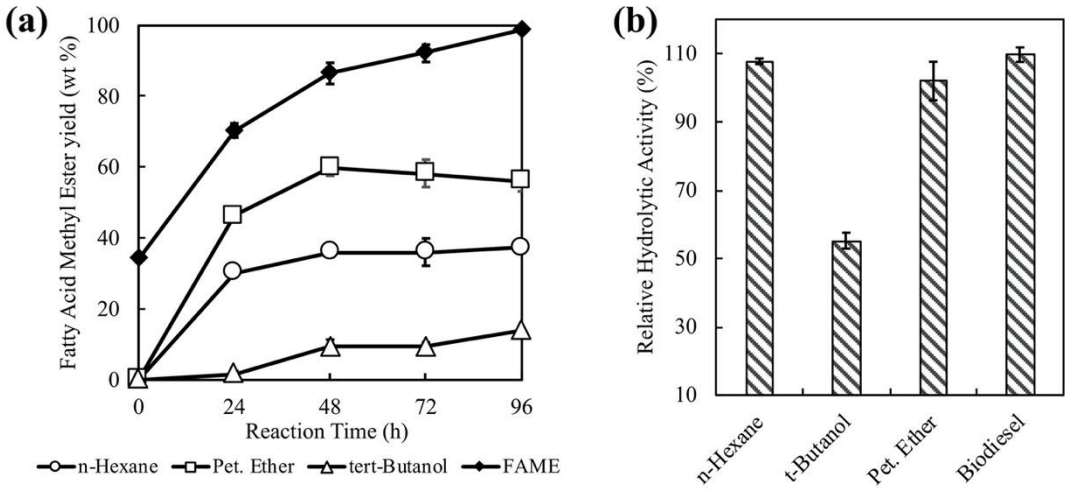


Figure 4-4. **(**a) Time course of FAME yield using *n*-Hexane, *tert*-Butanol, Petroleum ether, and FAME as solvents at a 1:1 oil to solvent molar ratio for methanolysis at 30 °C and (b) Effect of the four (4) solvents on *A. oryzae* FHL's hydrolytic activity.

In recent years, biodiesel is continuously being valorized as a greener substitute for many conventional solvents in fine chemicals synthesis (Table 4-3). For instance, in microalgae lipids extraction where chloroform or the conventional Soxhlet extraction are commonly applied, a replacement with biodiesel as solvent yielded more than a 100% relative lipid amount. Besides microalgae lipid extraction, biodiesel as a solvent has found green chemistry applications in solution polymerization of homopolymers, rare earth metals extraction, phenol extraction and polyester synthesis (Table 4-3). However, in biodiesel synthesis, the only attempt has been via chemical catalysis where KOH and H_2SO_4 were used. In that study, Hu et al. proposed a two-step reaction route where the FAME product of a first-step esterification of FFAs in rubber seed oil (RSO) was applied to a second stage transesterification. The inclusion of 30 wt % produced FAME facilitated the transesterification of RSO TAGs to FAME [34].

By using FHL in this study (instead of a chemical catalyst), a lower temperature (30 °C) and a lower agitation speed (35 rpm) were suitable for the conversion of CPO to FAME. The addition of biodiesel offered a solvation effect on the phospholine gums, as well as other high-heating components present in CPO. Unlike the cited conventional solvents which are mostly fossil-derived and raises high occupational HSE concerns, biodiesel is biodegradable and has a higher flash point (Table 4-S1). Besides the economics of not using a solvent from separate fine chemical industry, biodiesel as a solvent in biodiesel production saves on post-separation cost.

Table 4-3. A review of literature on the application of biodiesel as a solvent in various chemical reactions

Reaction Type	Product(s)	Conventional Solvents Used	References
Solution Polymerization	Homopolymers	Toluene, Benzene, Ethyl acetate, Xylene	[39]
Rare Earth Solvent Extraction	Rare Earth metals	Commercially, D2EHPA, HEHEHP, Versatic 10, TBP, and Aliquat 33 are used.	[40]
Microalgal Lipid Extraction	Lipids	Hexane, Chloroform	[41]
Polymerization of epoxidized methyl esters	Polyester	Hexane, Tetrahydrofuran, Acetone	[42]
Biodiesel production using KOH and H2SO4 as catalysts	Biodiesel	Hexane, t-butanol, Petroleum ether, Acetone	[32]
Phenol Extraction	Phenol	Octan-2-ol, Tributyl phosphate + kerosene, Cyanex923 + kerosene, Toluene	[43]
Biodiesel production via enzymatic catalysis	Biodiesel	Hexane, t-butanol, Petroleum ether, Acetone	*This study*

III.3.4 Conventional solvents versus biodiesel as a solvent on lipase functionality

The addition of certain organic solvents can be problematic as some solvents denature lipases rather than facilitate transesterification. Therefore, FHL was incubated in all four solvents (*n*-hexane, *tert*-butanol, petroleum ether, and biodiesel) and tested for its remaining lipase activity (Figure 4-6a). The incubation of the lipase in methyl oleate was found to enhance the activity of the lipase up to 109% rather than reducing it. Thus, an initial and prolonged contact time between the lipase and biodiesel rather has a positive effect on the activity of the lipase (Figure 4-6a). Samukawa *et al.* compared the incubation of *C. antarctica* lipase B in plant oil to that of methyl esters and found that the preincubation of lipase in methyl oleate for 0.5 h allows methanolysis to proceed faster than without incubation [35]. To compare the positive effect of methyl oleate not mainly on *A. oryzae* FH lipase, commercially applied Novozym 435 and another whole-cell fungus from *A. oryzae* expressing *C. antarctica* lipase B were also tested (Figure 4-S1). This further elucidates that biodiesel as a solvent in transesterification rather enhances the activity of the lipase other than decrease it.

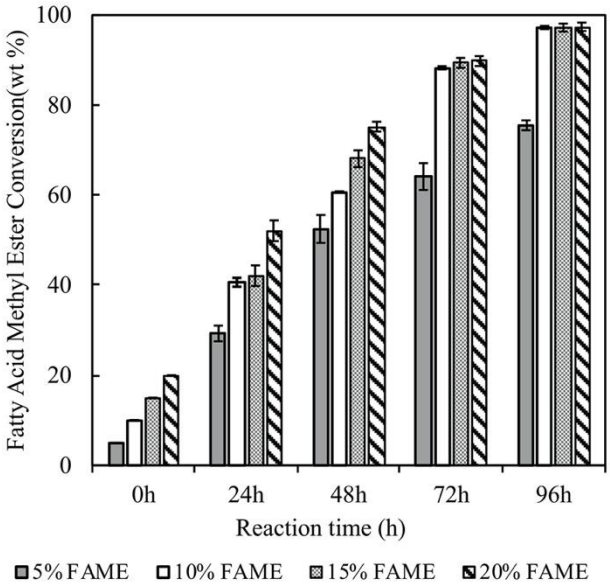


Figure 4-5. Optimization of FAME amounts (wt %) required for conversion of CPO to biodiesel at 30 °C. Methanolysis conditions: 0.2 g immobilized *A. oryzae* FHL, 35 rpm agitation, 0.15 g methanol added at a 24 h interval (each bar indicates FAME yield after 24 h).

III.3.5 Determination of optimum biodiesel amount for biodiesel synthesis

To optimize the required amount of biodiesel as a solvent in the reaction medium, the solvent was applied under various weight percentages for the transesterification of CPO to biodiesel (Figure 4-5). A variation of 5 wt % to 20 wt % of biodiesel (herein referred to as FAME) was used for the optimization studies (Figure 4-5). The addition of 10 wt % FAME or higher was considered enough to attain ≥ 97.24 wt % biodiesel. By increasing the weight percentage of FAME to 20 wt %, comparable 97.41 wt % FAME (P =.057; r = 0.9898) was attained. A 97.24 wt % FAME yield meets EN 14214 biodiesel specification where a minimum of 96.5 wt % ester is required.

III.3.6 Activation, Deactivation, and Poisoning of *A. oryzae* FHL in Repeated CPO methanolysis

The catalytic performance of *A. oryzae* FHL under continuous usage was further investigated for the methanolysis of the crude oil (Figure 4-6). 10 wt % of methyl oleate was maintained as the minimum reaction solvent. Over a 480-h reaction period, *A. oryzae* FHL maintained more than 82 wt % FAME yield in four (4) continuous batches but yielded 37.1 wt % FAME at the 480^{th} h (Figure 4-6a). Unlike a previous study with soybean oil and the same lipase which yielded more than 50 wt % FAME at the 480^{th} h, the presence of phospholine gums combined with prolonged methanol contact deactivated the biocatalyst over time [22].

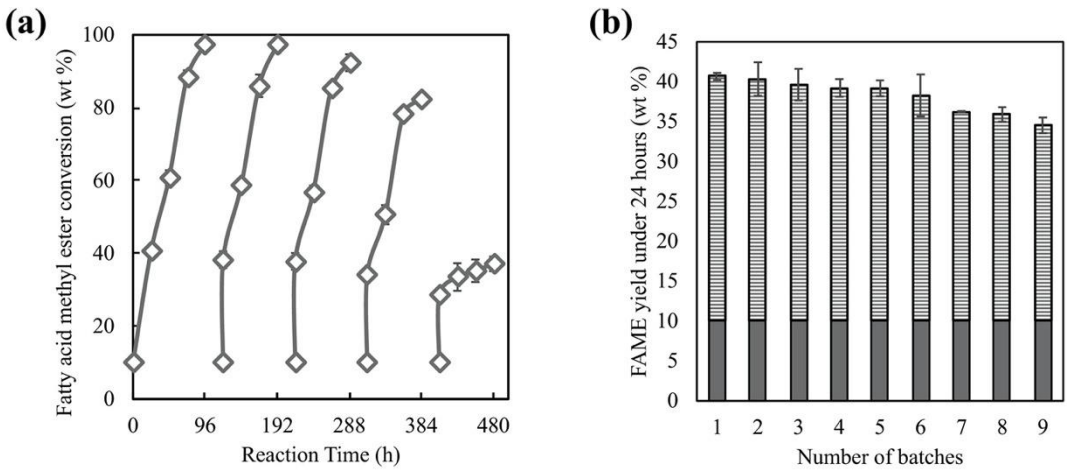


Figure 4-6. Repeated FAME synthesis using A. oryzae FH lipase in (a) 5 complete 96 h reaction batches and (b) 9 batches of a consecutive batch strategy with the first molar MeOH addition.

However, over the 480-h reaction period, FAME yield after the first MeOH addition was observed to be consistently maintained around an average of 35.9%. This is due to the inclusion of biodiesel at the start of each batch which enhances the activity of the lipase in the initial period. An average 35.9% FAME yield at that point is approximate to the expected theoretical 33% FAME yield after a first molar MeOH addition. The presence of oil contaminants and excess MeOH in the medium as the reaction progresses to 96 h lowers the activity of the biocatalyst. For this reason, a consecutive batch strategy with the first molar MeOH addition was tested with the lipase over nine batches (Figure 4-5b). A slight decrease in lipase activity was observed with this technique in terms of FAME yield but was around the theoretical average even over nine batches. This strategy suggests a continuous series of reactors to maintain the activity of the lipases. Yoshida *et.al* studied the continuous production of biodiesel using *A. oryzae* FHL and suggested a series of connected packed-bed reactors to maintain the activity of the lipase [21]. Thence, the process advantages of using immobilized biocatalyst in

biodiesel synthesis are maximized with this continuous batch approach which improves reusability and reduces lipase activity loss.

III.4. Conclusion

Biodiesel was successfully applied as a solvent in an enzymatic transesterification of unprocessed plant oil that negates the need for volatile organic solvents and high working temperatures. Fractionates of the most prolific vegetable oil crop, palm oil, was used in studying the effect of phospholine gums and high melting point contaminants in complete enzymatic transesterification at 30 °C. Degummed and refined fractionates of crude palm oil, as well as different working temperatures, were tested. The melting behavior of crude palm oil (CPO) investigated with differential scanning calorimetry revealed the need for working temperatures higher than 35 °C. However, the inclusion of 10 wt % biodiesel as solvent eased transesterification of CPO at 30 °C. Compared to conventional solvents (*tert*-butanol, *n*-hexane, and petroleum ether), the addition of biodiesel to the feedstock mixture not only increased the fluidity of the medium but also increased the activity of *A. oryzae* FH lipase to a relative 109%. A valorization study of biodiesel as a solvent in this study advances the potential of unrefined plant oils as direct feedstocks for biodiesel production and promises the utilization of biodiesel as a solvent in non-fuel applications.

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III.6. Supporting Information

Table 4-S1. Source, toxicity and safety information of biodiesel in comparison to conventional solvents used in biodiesel production.

Solvent	Source	Toxicity and Occupational HSE	Flash point
Hexane	Crude oil distillate	A high-risk neurotoxin. Highly volatility and explosion risk.	-26 °C
***tert*-butanol**	Crude oil refining	Moderately irritating to the skin. Vapor/air mixtures are explosive. Heating can cause rise in pressure with risk of explosion.	11 °C
Petroleum Ether	Petroleum fractionate	Inhalation overexposure can cause primarily central nervous system (CNS) damage.	< 0 °C
Acetone	Indirectly from propylene- a crude refining by-product	Inhalation or ingestion can cause primarily central nervous system (CNS) damage.	-20 °C
Biodiesel	Biomass (lipids/ fatty acids)	Biodegradable, lower acute toxicity but still have substantial impact on the environment if accidentally spilled in large quantities.	>130 °C

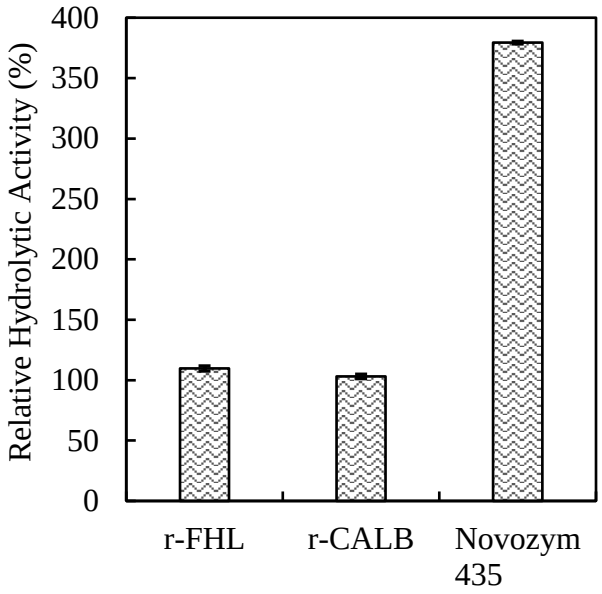

Fig. 4-S1. Relative hydrolytic activity Effect of A. *oryzae* r-*FHL*, *A. oryzae* r-*CALB* and Novozym 435 incubated in biodiesel.

Method Description for Biotransformation of *A. oryzae* Whole-Cells and Activity Tests

Gene expression and optimized promoter transformation of the wild-type *Aspergillus oryzae* fungi to express *Fusarium heterosporum* lipase and *Candida antarctica* lipase B recombinant strains used in this study is briefly described here from previous reports. [20,36–39] The *FHL* and *CALB*-encoding gene were amplified from pYGF2 and pNAN8142-PPM CALB by PCR respectively using independent primers. For *FHL*, FHL-F1 (5′-TCGCAAACATGATGCTCGTCCTATCTCTTC-3′) and FHL-R1 (5′-GCTCTAGACTAAATCATCTGCTTAACAAAT-3′) primers were used. Construction of the modified pSENSU-CALB for expressing *Candida antarctica* lipase was described separately by Adachi *et al.* [39]

Immobilization of *A. oryzae* whole-cell biocatalysts

Immobilization of the two *A. oryzae* whole-cells used in this study was performed under different optimum conditions reported. [22,40] For r-*FHL*, 5 g granular activated carbon was used as an immobilization matrix while for r-*CALB*, 300 pieces of $6 \times 3 \times 3$ mm cubic reticulated polyurethane foam was used as immobilization matrix. For both immobilizations, the shake flask cultivation method (500 ml Sakaguchi flask, 150 rpm, 30 °C, 96 h) was used [22]. The cultivated and lyophilized whole-cells were designated as r-*FHL* and r-*CALB* for subsequent enzymatic trans/esterification reactions.

Lipase Hydrolytic Activity Assay

To evaluate the effect of the four solvents on the activity of the lipase, 200 mg of immobilized lipase, 2.8 g Tris buffer solution (0.1 M, pH 8) and 1.2 g solvent were incubated in cylindrical glass tubes in a TBR (35 rpm, 30°C) for 24 h. The Immobilized lipase was separated after 24 h and its hydrolytic activity measured using a method described previously [22]. One unit of hydrolytic activity (U) was defined as the amount of enzyme (mg) required to liberate 1 µmol of fatty acid from olive oil substrate per minute under defined standard conditions. This was translated into relative activity as the ratio of the hydrolytic activity (U) of lipase incubated in a solvent-containing mixture to one without (Tris buffer only).

Reference for Supporting Information

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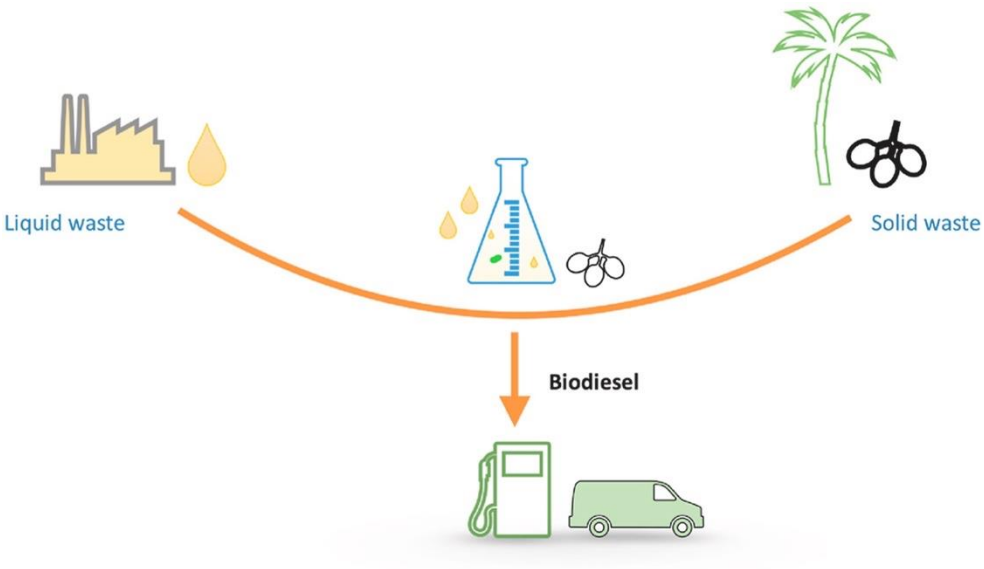
Chapter IV:

Valorization of palm biomass waste into carbon matrices for the immobilization of recombinant *Fusarium heterosporum* lipase towards palm biodiesel synthesis

Graphical Abstract



IV.1. Introduction

Palm oil (*Elaeis guineensis*), an essential source of dietary oils and fats, oleochemicals, and biofuels, has seen an overwhelming increase in production in the last decade that has raised environmental concerns [1]. It is the most prolific edible oil crop (≥8-ton oil ha^{-1} $year^{-1}$) but has come under intense scrutiny to ensure sustainability in its cultivation in Southeast Asia, Africa, and South America. Sustainable palm oil production would require achievable proper land usage and minimization of waste from its production [2]. A typical oil palm tree consists of 90-95% disposable solid lignocellulosic waste that includes the trunk, fronds, and fruit bunches [3]. Unlike other edible oil crops such as rapeseed and soybean oils with fewer uses of its biomass after harvesting, palm oil's solid biomass wastes such as empty fruit bunches (EFB) have several uses in the biorefinery [4–7]. At palm oil mills, post-oil extraction generates palm kernel shells (PKS) as solid wastes in large quantities (≥0.94-ton ha^{-1}) [3]. PKS is primarily used as solid fuel in biomass boilers. However, most biomass boilers' extremely low 10-20% electrical efficiency means alternative use of PKS needs to be considered in an integrated biorefinery scenario [8]. For the sustainable application of PKS as a solid fuel, bioenergy with carbon capture and storage (BECCS) is suggested to be in place [9].

Palm oil mill effluent, typically referred to as POME, is also generated in overwhelming amounts as liquid waste. The use of water for the sterilization of fresh fruits, oil stripping, fiber digestion, and oil clarification generates 2.5-3.5 tons of POME (consisting of wastewater, grease, and oil) per ton crude palm oil (CPO) produced. POME is typically left at landfill sites to decompose or anaerobically treated for disposal.

The disposal strategies mentioned above for both PKS and POME generate methanogenic emissions that increase net emissions from palm oil production. A sustainable means to

handle the mill's liquid and solid wastes would be its use in an integrated biorefinery for conversion to value-added products such as activated carbons and biodiesel. POME has found very few uses in biorefineries but can offset 80% of biodiesel's production cost when used directly as feedstock in place of refined vegetable oils [10]. The effectiveness of a biodiesel-focused integrated biorefinery could also be realized by supplanting chemical catalysts that are industrially used in biodiesel production with biological catalysts in the form of lipases [11].

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are suggested as greener catalysts to avert the corrosive and environmentally damaging effects of using chemical catalysts (mainly acids and bases) used [12]. Unlike chemical catalysts, lipases' wide substrate specificity is tolerant of high free fatty acids in waste oils such as POME and requires less complicated feedstock pretreatments [13]. Other added advantages of lipases over chemical catalysts include its tolerance for high levels of water in feedstocks and close-to-purified by-product (e.g., glycerol) production. However, the high cost of lipases (from isolation to purification) compared to chemical catalysts has stalled its large-scale application [12]. The economic viability of using lipases in biodiesel production requires practical recovery for repeated uses [12]. To this end, the immobilization of lipases onto solid carriers has been suggested as a feasible route to offset its high costs [14].

Immobilization, meanwhile, adds a 47% cost contribution from the lipase carriers needed [15]. Recent reports on lipase immobilization have proposed the substitution of high-priced synthetic carriers (e.g., Lewatit® VP OC 1600) with agro-waste or its derived-carbons to lower the final cost of immobilized lipases [16,17]. Besides serving as sustainable lipase carriers, agro-waste-derived activated carbons (e.g., from coconut shells) are industrially used as cheap and robust absorbents. For instance, activated carbons have recently found increased demand for hydrogen storage, compressed energy storage, and carbon capture, utilization,

and storage (CCUS) [18]. For water treatment, traditional activated carbons (ACs) are used for absorbing heavy metals and organic pollutants, whereas in gas treatment, ACs are used in Pressure Swing Adsorption (PSA) separators for gas species separation [19].

In this regard, porous-layered carbons synthesized via facile cost-effective techniques can be essential in the circular bioeconomy. The industrially-established AC production process involves carbonaceous matter generation via carbonization, torrefaction, or pyrolysis. Carbonaceous matter generation is subsequently followed by activation at a second higher temperature stage using activation agents such as KOH [20].

For the utilization of ACs towards biodiesel production, attempts reported in the literature have thus far applied a two-step AC production technique and mostly for chemical heterogeneous catalyst synthesis. Contrary to conventional carbon synthesis methods, this study attempts a cost-effective direct carbonization and activation technique. The one-step carbonization and activation technique produced porous PKS carbons (PKAC) possessing active functional groups similar in bituminous coal-derived activated carbons (BCAC). The textural and active groups in PKAC and BCAC were compared using Fourier transform infrared spectroscopy and field-emission scanning electron microscopy with energy dispersive x-ray spectroscopy. The PKAC and BCAC were also compared in the immobilization of *Aspergillus oryzae* whole-cells expressing *Fusarium heterosporum* lipase (FHL). Employing whole-cell lipases offers significant advantages over purified lipases [15]. Whole-cell lipases require facile processing steps, possess improved inhibitor resistance, and express promiscuous metabolites and higher activities than purified lipases [21,22].

Therefore, combining the advantages from facile AC synthesis, immobilization of whole-cell lipases onto waste-derived carbons, and FHL's high performance in high acid oils such as POME, this study demonstrates an integrated palm biorefinery with PKS and POME for sustainable biodiesel production.

IV.2. Materials and method

IV.2.1 Materials

Palm kernel shells (PKS) were obtained through a collaborative arrangement with KNUST's Chemical Engineering department (Kumasi, Ghana). Palm oil mill effluent (POME) was a kind gift from P.T Agricinal Palm Oil Mills (Bengkulu, Indonesia). Refined rapeseed and olive oils were purchased from Wako Pure Chemicals (Osaka, Japan). BCAC and methanol (MeOH, ≥99 %) were obtained through Nacalai Tesque Inc. (Kyoto, Japan). Novozym® 435 and Lipase OF was procured through Sigma-Aldrich (Tokyo, Japan) and Meito Sangyo (Nagoya, Japan), respectively. All other reagents used were of analytical grade and procured through local suppliers in Hyogo, Japan.

IV.2.2 Activated carbon synthesis from palm kernel shells (PKS)

The as-obtained PKS (1.5-3 cm averagely sized) was crushed to 0.3-0.5 cm using a Hammer crusher (Sansho Ind. Co. Ltd., Osaka, Japan). The PKS was water-washed and oven-dried (80 °C, 24 h) for carbonization and activation. One-step carbonization and activation was performed by mixing PKS with 1:4 wt.% aqueous KOH (0.5 mol L^{-1}) in a porcelain crucible. Under 10 mL min^{-1} N_2 gas flow, direct carbonization, and activation was performed in a KDF electric furnace (Denken-Highdental Co., Ltd, Kyoto, Japan) for 4 h under programmed heat flow (Table 5-S1). The produced PKAC was water-washed and oven-dried for subsequent uses.

IV.2.3 Strain, media, and immobilization of *A. oryzae* FHL

Upstream genetic manipulation of *Aspergillus oryzae* to express a recombinant *Fusarium heterosporum* lipase was adopted after Hama *et al.* [23]. The cells were maintained on potato dextrose agar plates at 30 ± 2 °C. Cell selection was performed on Czapek-Dox ($CDNO_2$) plates (5 - 6 days). The $CDNO_2$ medium contained 2% glucose, 10% Cz-Dox stock solution, 0.8 M NaCl, 1.5% purified agar and 0.01 wt.% trace elements (2% $CuSO_4 \cdot 5H_2O$, 1% $FeSO_4 \cdot 7H_2O$, 0.1% $ZnSO_4 \cdot 7H_2O$, 0.1% $MnSO_4 \cdot 7H_2O$, and 0.1% $AlCl_3$) adjusted to pH 5.5–6 with HCl [24]. Independent immobilization of *A. oryzae* FHL cells onto 5 g carbon matrices (PKAC or BCAC) was at 30 °C in a 100 mL dextrin-peptone media (2 % glucose, 2% polypeptone, 0.5% KH_2PO_4, 0.1% $NaNO_3$, and 0.05% $MgSO_4 \cdot 7H_2O$). The immobilized whole-cells were lyophilized using a Benchtop Freeze Dryer (Labconco Corp., Kansas, USA). *A. oryzae* FHL cells immobilized onto PKAC or BCAC were designated as PKAC-FHL or BCAC-FHL for subsequent experiments.

IV.2.4 Catalytic activity and cell loading assay

Hydrolytic activity assay of PKAC-FHL and BCAC-FHL was performed in an emulsion containing 2 g olive oil, 1 mL $CaCl_2$, and 9 mL acetic acid buffer (0.1 M, pH 5.6) at 40 °C. 10 mg PKAC-FHL or BCAC-FHL was added under 400 rpm magnetic stirring for 10 min. 50 mL anhydrous ethanol was used to terminate the reaction for NaOH (0.1 M) titration. Control-referenced hydrolytic activity (U x 10^6) of the immobilized lipase was defined as the amount of lipase (mg) that liberates 1 μmol of FFA per min from olive oil at pH 10. For transesterification activity and thermal stability tests, 4 g rapeseed oil, 0.15 g methanol, 0.2 g water, and 0.4 g lipase were used (30 - 45 °C, 4 h, 35 rpm agitation). One unit of transesterification activity (IU) was defined as the amount of lipase (mg) that produces 1

μmol FAME per h. Protein remaining in the supernatant of the immobilization culture was determined using the Pierce™ BCA protein assay.

IV.2.5 Sample characterization

Thermogravimetric analysis (TGA) of PKS and PKAC was recorded using a Thermo plus EVO2 balance (Rigaku Corp., Osaka, Japan). The TGA heating was at 10 °C min^{-1} from 25 to 500 °C under N_2. Gravimetric changes were recorded at mg s^{-1} with an empty aluminum pan as reference.

Microphotographs and elemental mappings of the samples were also recorded using a field-emission scanning electron microscope (FE-SEM) equipped with energy-dispersive x-ray spectroscopy (EDX) (JEOL JSM-7100F, JEOL Ltd., Tokyo, Japan).

Fourier transform infrared spectra (FTIR) of the solid samples were recorded at 4000 to 400 cm^{-1} using the KBr-pellet technique (Shimadzu IRTracer-100, Shimadzu, Kyoto, Japan). A 1000 x sample dilution with KBr was subjected to 50 KN pressure and vacuum-degassed at 4 °C. FTIR spectra of the biodiesel samples were analyzed using a NaCl aperture plate.

IV.2.6 Lipase-mediated methanolysis

PKAC-FHL's efficacy in transesterification and esterification was tested in an admixture of 70 wt. % oleic acid and 30 wt.% palm, rapeseed or soybean oil.

The primary feedstock used for this study was also an admixture of POME and rapeseed oil. POME and rapeseed oil were blended to lower the high melting point of POME and resolve the poor cold-flow behavior of palm biodiesel. Pretreatment of the POME and rapeseed oil admixture was performed using Lipase OF as biocatalyst to achieve 95% free fatty acids (35 °C, 12 h).

PKAC-FHL and Novozym® 435 were compared as immobilized lipase (IM lipase). Simultaneous esterification and transesterification with PKAC-FHL and Novozym® 435 were performed with 4 g oil, 0.4 g IM lipase, 0.4 g water (or without), and 0.15 g MeOH added stepwise at 30 °C in a Thermoblock rotator (Nissin, Tokyo, Japan) [24,25].

IV.2.6 Gas chromatography, statistics and biodiesel characterization

Compositional analysis of the produced biodiesel was performed using a gas chromatography instrument equipped with a flame ionization detector (GC-2010, Shimadzu, Tokyo, Japan). Species separation with helium as the carrier gas was in a 15 m × 0.25 μm × 0.10 μm Zebron™ ZB-5HT column (Phenomenex Inc., 2CA, USA). The fatty acid composition was separately analyzed using a Gas Chromatography Mass Spectrometry (GC-MS) instrument (GCMS-QP2010, Shimadzu, Tokyo, Japan). Unless otherwise stated, all represented values are the average of triplicates. Error bars indicated on the graphs are calculated based on Eq. 1 as follows;

$$\partial = \sqrt{\frac{\sum_{i=1}^{n}(x_s - \bar{x})^2}{n-1}} \qquad [1]$$

In Eq.1, ∂ represents the error values, n, the number of observations, x_s, the observed sample values, and $\bar{x}$, the average of observed values.

IV.3. Results and Discussion

IV.3.1 Biorefinery concept for palm biomass waste conversion to biofuels

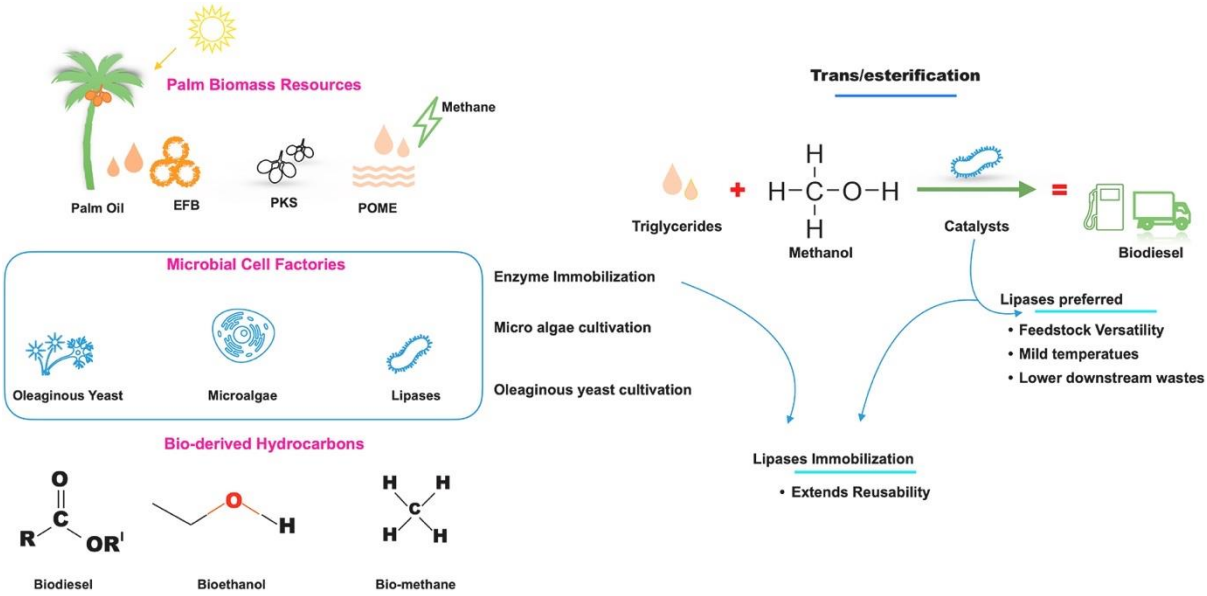
A sustainable integrated palm biorefinery would require exploiting available green pathways to efficiently use primary, secondary, and waste products in the palm value chain. Multi-products from the integrated palm biorefinery would include food, feed, bioproducts, and biofuels. Primary products from the palm oil mill are CPO and palm kernel oil, which are primarily refined for food and oleochemicals. To avoid interfering with primary food production, waste palm biomass such as PKS and POME needs exploitation using biological pathways to produce feed, bioproducts, and biofuels. Biofuels in the integrated biorefinery can be produced using microbial cell factories that promote the direct use of waste products from the palm oil mill (Scheme 1). For instance, in other studies, oxygen-rich sugars present in EFB have been used for biomethane and bioethanol production [4,5,26]. PKS, on the other hand, has found very few uses due to its high content of recalcitrant lignin (≥ 50.7 wt. %) [3]. Pyrolysis and torrefaction have previously been used for hydrothermal synthesis of bio-oils from PKS [27,28]. Pyrolysis and torrefaction, however, require high energy input, which renders them unsustainable.

Conversely, PKS was successfully converted to activated carbons in this study via a one-step technique that departs from the conventional two-step technique reported in the literature (Table 5-1). The one-step method used in this study is cost-effective for the valorization of PKS and other lignin-rich biomass to ACs. Further, the PKAC generated was used as a lipase immobilization carrier for the catalysis of palm oil mill effluent.

As a lipase immobilization carrier, the tribology of ACs allows for repeated reuses. Lipase carrier recycling has not been primarily demonstrated in the literature but can significantly reduce the cost of biocatalysts [15,24]. However, by skipping extensive pretreatment steps

required for lipid production, the fatty acid components in POME, were directly valorized to biodiesel in this study. Utilizing ACs as lipase immobilization carriers, a facile integrated biorefinery concept was developed in this study for the utilization of POME and PKS (Scheme 1, Scheme S1). This integrated approach expands the palm biorefinery concepts for the production of CO_2-neutral fuel solutions [32].

Effluent from the palm oil mill, POME, contains bio components that have been used for the cultivation of oleaginous yeast, bacteria, fungi, and microalgae [29]. In the literature, the oleaginous yeast *Rhodotorula glutinis* and microalgae strains such as *Chlorella sorokiniana* have shown high lipid productivity in POME [30,31]. Algal and oleaginous yeast oils can be subsequently converted to biodiesel using lipases as catalysts.



Scheme 5-1. Biorefinery concept for the utilization of waste from palm oil mills for biofuels production.

IV.3.2 Porosity and surface morphology effect in lipase immobilization

The one-step direct carbonization and activation method for AC synthesis has previously demonstrated success in the conversion of eucalyptus sawdust, *Sargassum fusiforme* seaweed, and the *Paeonia lactiflora* flowering plant to activated carbons [33]. Layered carbons with rudimentary porosity were successfully produced from PKS in this study. Carbons synthesized in this study showed similar porosity and surface morphology as bituminous coal-derived ACs (Figure 5-1a and 5-1b). Bituminous coal-derived activated carbons are highly ranked in the industry due to ready availability, structured porosity, and the presence of high levels of elemental carbon (60 – 89%), oxygen, nitrogen, and sulfur [34]. Similar morphological features were nevertheless produced with PKS. The PAKC in this study possessed 1-10 μm pore diameters and uneven structures (even beneath). The rough texture of PKAC and its associated porosity were essential in the immobilization of *A. oryzae* whole-cells (Figure 5-1c).

Table 5-1. Palm kernel shell-derived activated carbons (AC) used as catalyst carriers for biodiesel synthesis. The two-step AC synthesis technique involves carbonaceous matter generation via carbonization, torrefaction, or pyrolysis followed by activation at a second higher temperature stage using activation agents. Direct activation combines carbonization and activation into one step.

Method of AC synthesis	Catalysts Loaded	Feedstock	Biodiesel yield (%)	[2]Reference
Two-step physical activation	KOH	Refined palm oil	97.72	[45]
Two-step physical activation	KOH	Refined palm oil	94.0	[46]
Two-step physical activation	CaO	Waste cooking oil	94.0	[47]
Two-step physical activation	CaO	Palm stearin	96.0	[48]
Two-step pyrolysis and sulfonation	$-SO_3H$	Palm fatty acid distillate	95.0	[49]
Two-step carbonization and sulfonation	$Zr-SO_3H$	Palm fatty acid distillate	94.30	[50]
Direct activation	Lipase	Palm oil mill effluent (POME)	97.52	*This study*

[2] *Publications listed on Google Scholar database using "palm kernel shell + activated carbon + biodiesel" separated by document types.*

Shown with the SEM micrographs, the 1- 2 μm cells were found to penetrate the pores of PKAC while simultaneously mushrooming around it (Figure 5-1c). The adsorption immobilization method used in this study is facile and achievable without the need for toxic protein cross-linking reagents such as cyanogen bromide.

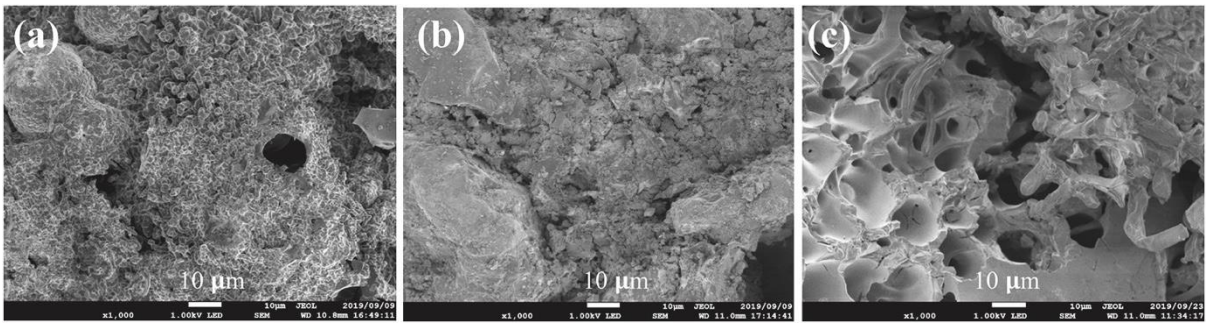


Figure 5-1. SEM images of (a) palm kernel shell-derived activated carbon (PKAC), (b) bituminous coal-derived activated carbon, and (c) PKAC used for the immobilization of *A. oryzae* FHL.

Other porous carriers that have demonstrated success in adsorption immobilization include metal-organic frameworks, polyurethane foams, and acrylic resins (Table 5-S2). The industrially-applied Novozym® 435 lipase is *Candida antarctica* lipase B immobilized onto Lewatit® VP OC 1600 beads via adsorption immobilization [35] (Table 5-S2). The replacement of synthetic polymers (e.g., Lewatit® VP OC 1600) with biodegradable and cheap carriers from biomass-derived ACs sustainably reduces the cost of IM lipases. Direct immobilization of *A. oryzae* whole-cells onto untreated PKS was attempted as a more sustainable approach but failed abruptly due to the lack of adequate porosity for multipoint attachment of cells (Figure 5-2a and 5-2b). For lipase immobilization, the relevance of porosity has inconsistent elucidation in literature as some non-porous materials such as untreated sugarcane bagasse and rice husk have demonstrated lipase immobilization

feasibility, albeit not without protein cross-linking reagents [16]. SEM images of the raw PKS showed a uniformly tight structure of negligible porosity. Immobilization attempts only coated the PKS surface with the media without cell attachments (Figure 5-2b).

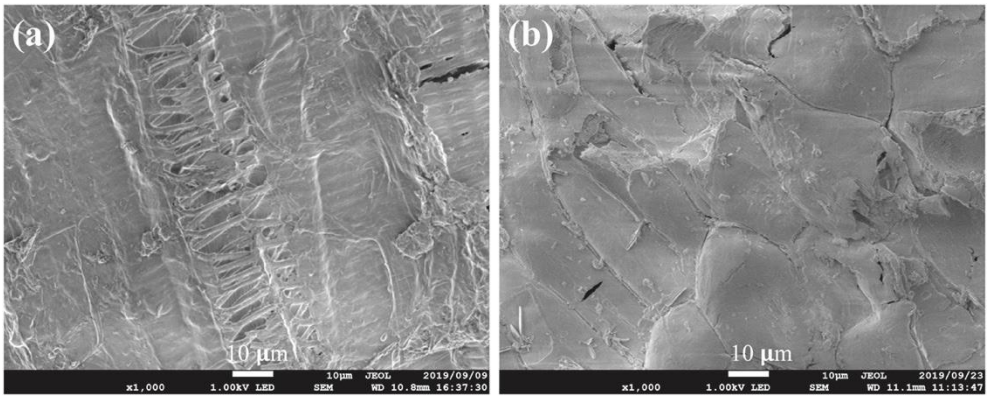


Figure 5-2. SEM images of (a) untreated palm kernel shell (PKS) and (b) PKS used for the immobilization of *A. oryzae* FHL.

IV.3.3 Thermochemical characteristics of PKS, PKAC, and BCAC

Thermogravimetric analysis (TGA) was used to evaluate the thermostability of PKS and the obtained-PKAC (Figure 5-3a and 5-3b). The TGA elucidated the distribution of cellulose, hemicellulose, and lignin as the major constituents in PKS. Under an inert-N_2 environment, the decomposition of PKS by TGA is categorized into three distinct stages (Figure 5-3a). The first stage is the ≤250 °C range, where <10% residual weight loss was observed. In this first stage, the loss in mass is attributed to the evaporation of moisture and volatile organic compounds present in PKS. The second stage is from 250 to 375 °C, where a substantial (≥50%) weight loss was observed. Derivative weight loss in the second stage showed two peaks associated with the considerable decomposition of hemicellulose and cellulose. The final decomposition stage occurs at ≥375°C, where there is steady but continuous degradation of lignin. TGA-monitored degradation of lignin extends beyond 800 °C and has been

reported in other studies [36]. 550 °C was nonetheless selected as the optimum activation temperature for the present study.

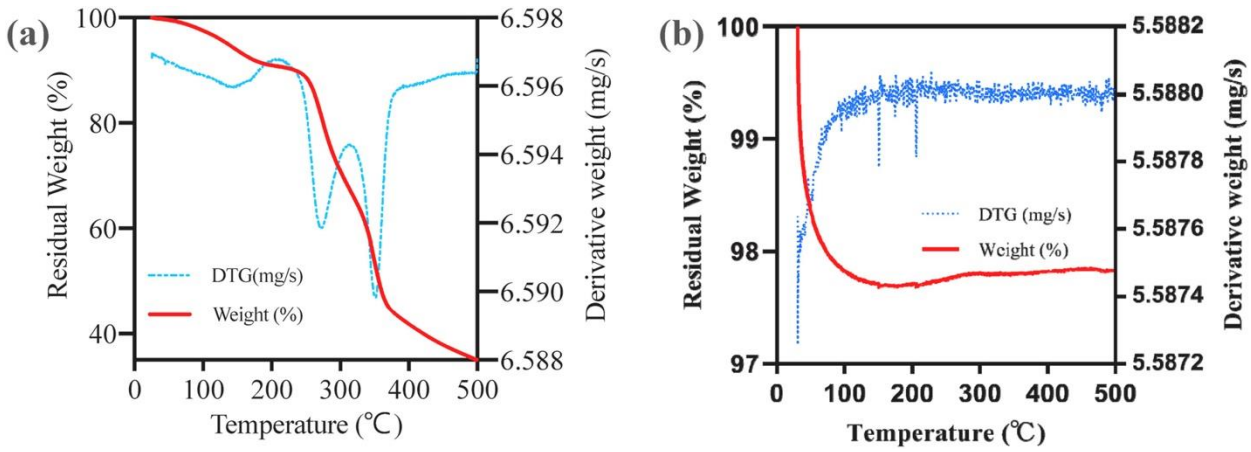


Figure 5-3. Mass and derivative thermogravimetric profile (TGA) of (a) palm kernel shell and (b) palm kernel shell-derived activated carbon.

A post-carbonization and activation-TGA profile of PKAC shows a stable activated carbon that volatilizes less than 3% of adsorbed moisture at 25 to 100 °C (Figure 5-3b). Table 5-2 presents the EDX elemental characteristics of PKS, the PKS-derived-AC, BCAC, and FHL-immobilized forms of the carbons. Elemental carbon in the precursor material, PKS, was 57.8 wt. %, but increased to 84.3 wt. % after carbonization - similar to 89.4 wt. % in BCAC (Table 5-2, Figures 5-S2 to 5-S4). Conversely, nitrogen content in PKS decreased insignificantly from 3.7 to 3.3 wt.% in PKAC but was only 3.2 wt.% in BCAC. Considerable oxygen reduction was observed in PKAC (11.5 wt. % compared to 38.1 wt.% in PKS). However, remaining oxygen content in PKAC was 2-fold higher than 5.5 wt.% in BCAC. The relevance of nitrogen and oxygen-containing compounds in supporting cell attachment and growth on carbon surfaces has been elucidated in a previous report [24]. Changes in carbon, oxygen, and nitrogen contents resulted in reductions of O/C and N/C atomic ratios in PKAC similar to that in BCAC (Table 5-2). Silicate formation was as low as that found in

PKS. Post-immobilization EDX showed significant increases in elemental nitrogen after the attachment of cells onto the carbons. The immobilization media also modified the elemental composition of the ACs, thereby suggesting favorable adsorption environments for cell growth (Table 5-2, EDX mappings in S4 to S7).

Table5-2. Elemental analysis of palm kernel shells (PKS), palm kernel-derived activated carbon (PKAC), bituminous coal-derived activated carbon (BCAC), and *A. oryzae* FHL-immobilized forms (PKAC-FHL and PKS-FHL).

Sample	C (%)	O (%)	N (%)	Si (%)	O/C Ratio	N/C Ratio
PKS	57.76	38.10	3.73	0.41	0.66	0.06
PKAC	84.29	11.45	3.27	0.99	0.14	0.04
BCAC	89.44	5.47	3.22	1.87	0.06	0.04
PKAC-FHL	78.22	5.96	15.62	0.20	0.08	0.20
PKS-FHL	60.79	9.45	29.71	0.06	0.16	0.49

Fourier transform infrared spectra (FTIR) was used to confirm the deterioration of weak bonds and the formation of active functional groups relevant in the immobilization of *A. oryzae* whole-cells (Figure 5-4). Raw PKS shows a broad O-H vibration at 3700 to 3100 cm^{-1}

(Figure 5-4). The broad O-H vibration is attributed to molecular hydrogen bonds characteristic of cellulose and phenol groups in PKS [37].

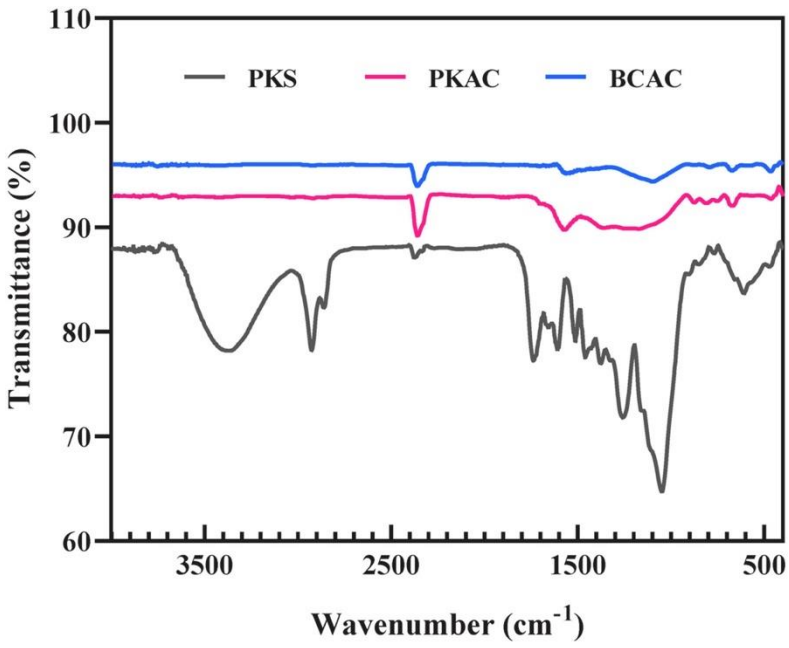


Figure 5-4. FTIR spectra of unprocessed palm kernel shell (PKS), palm kernel shell-derived activated carbon (PKAC), and bituminous coal-derived activated carbon (BCAC).

Decomposition of cellulose and hemicellulose after carbonization reduced hydrogen bonds in the AC samples. Peaks at 2915 and 2850 cm^{-1} are also assigned to other hydrogen-containing functional groups in the form of alkanes. C-H double peaks at 2915 and 2850 cm^{-1} are the aliphatic -CH_2 groups found in hydrogen-containing cellulose and hemicellulose [38]. Also, predominant in PKS is the C=O vibration of carboxylic acids (1730 cm^{-1}) from hemicellulose [27]. At 1600 to 1500 cm^{-1}, C=C and C=O show predominant stretching. In the torrefaction of PKS, Gan *et al*. attributed peaks at 1600-1500 cm^{-1} to ketones, carboxyl, ester, and aromatics from lignin found in PKS [27]. The success of the one-step carbonization and activation technique used in this study is explained with the decomposition of cellulose and hemicellulose (Figure 5-4). The PKAC formed at 550 °C is comparable to BCAC (Figure 5-4). Other lignin-related active groups are found at 1268 cm^{-1} in the raw biomass and the ACs.

Oxygen-rich peaks of aliphatic C-O-C and C-OH (1000 to 400 cm^{-1}) remaining in PKAC are identifiable in the biomass fingerprint region and can be further explained with the EDX data that revealed higher elemental oxygen content (Table 5-2).

Table 5-3. Catalytic activity, cell loading, and unrecovered proteins assay of *A. oryzae* FHL immobilized onto PKS-derived activated carbon (PKAC-FHL) and bituminous coal-derived activated carbon (BCAC-FHL).

Parameter	PKAC-FHL	BCAC-FHL
Hydrolytic Activity (U x 10^6 BSP^{-1})	31.48	8.31
Transesterification Activity (IU BSP^{-1})	81.56	19.72
Cell Loading /BSP (mg)	6.10	5.37
Unrecovered Proteins (mg mL^{-1})	0.82	0.74

IV.3.4 Functional activity of PKAC-FHL

In triglyceride transesterification to produce mono alkyl ester (biodiesel), the release of FFA is the rate-determining step. Therefore, the hydrolytic activity of the immobilized lipase was determined with the release of FFAs from olive oil (U x 10^6 BSP^{-1}). Hydrolytic activity of PKAC-FHL showed a 3.8-fold higher hydrolytic activity compared to BCAC-FHL (Table 5-3). As a biomass-derived carbon material, the oxygen-rich derivatives present in PKAC favored cell accumulation, and subsequent lipase activity increase. Transesterification activity of PKAC-FHL was also 4-fold higher than in BCAC-FHL (Table 5-3). Cell loading efficiency was also higher in PKAC than in BCAC. Higher cell loading resulted in higher specific activities in PKAC-FHL. However, unlike the fine-tuned adsorption efficiency of

BCAC, there was a higher level of unrecovered proteins in the PKAC immobilization media (0.82 mg mL^{-1}) compared to that of BCAC (0.74 mg mL^{-1}) (Table 5-3).

Thermostability of PKAC-FHL in transesterification was tested at 30 to 45 °C (Figure 5-5a). A 30 to 35 °C range was found to be favorable for the mesophilic *A. oryzae* whole-cells (≥942.07 IU x 10^{-3}). Above 35 °C, the activity of *A. oryzae* FHL decreases noticeably to less than 645.97 IU x 10^{-3} (Figure 5-5a).

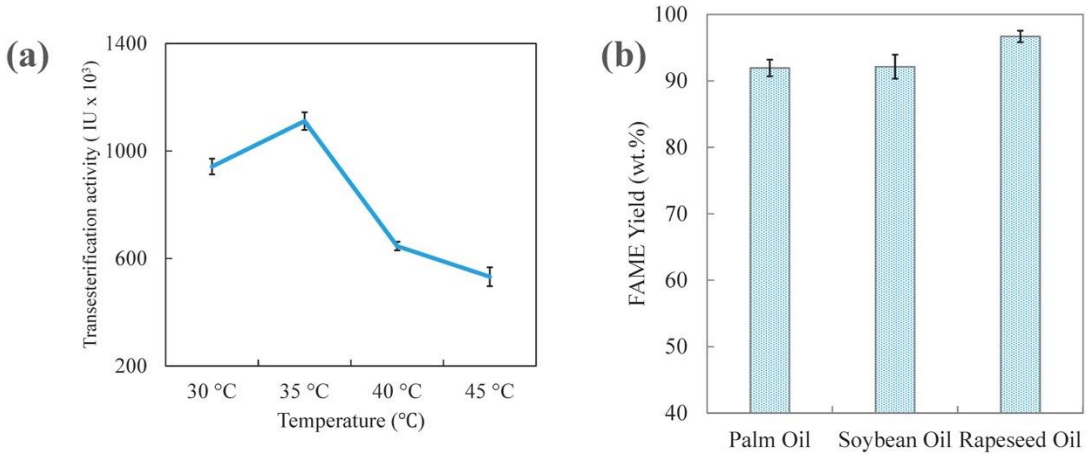


Figure 5-5. (a) Thermostability assay of PKAC-FHL and (b) Methanolysis with PKAC-FHL in palm, soybean, and rapeseed oils.

At 30 °C, PKAC-FHL was also tested for the simultaneous esterification and transesterification of oleic acid and refined oils (Figure 5-5b). Compared to the other oils, the lower melting point of rapeseed oil ensured 96.7 wt.% FAME conversion under 96 h, and the soybean and palm oil FAME yields achieved were 92.1 and 91.9 wt %, respectively (Figure 5-5b).

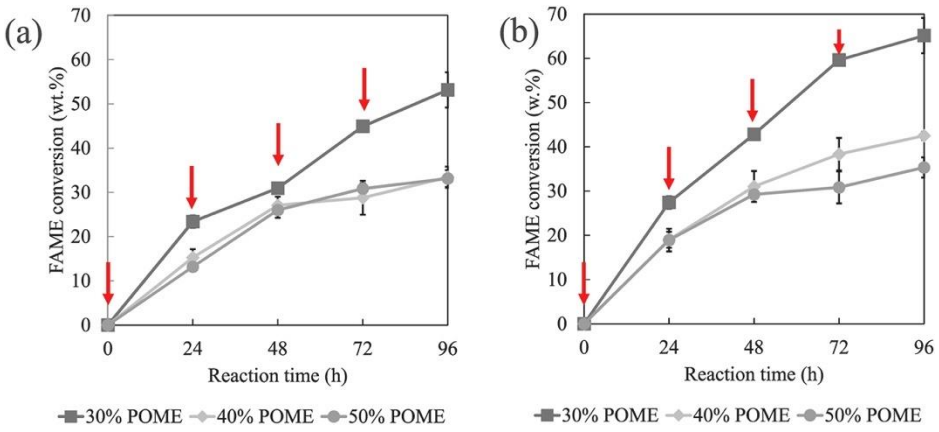


Figure 5-6. Transesterification and esterification of 30-50 wt.% POME mixed with Rapeseed oil (70–50 wt.%) at (a) 30 ℃ and (b) 35 ℃. Reaction conditions: 4 g oil, 0.4 g lipase, 0.4 g water, and 0.15 g methanol added at 0, 24, 48, and 72 h. Arrows indicate methanol addition times.

IV.3.5 *A. oryzae* FHL-catalyzed esterification of POME to biodiesel

The high melting point of POME (49.5 °C, Figure 5-S6) prompted deriving practical process alternatives for conversion under milder temperatures (30 - 35 °C). In addition to the instability of *A. oryzae* FHL at ≥35 °C (shown in Figure 5-5a), it is essential to limit energy consumption in lipase-catalyzed biodiesel production. Rapeseed oil was therefore blended with POME at a 30 to 50 wt.% ratio for reaction attempts at 30 and 35 °C (Figures 5-6a and 6b). Lower additions of POME (30 wt.%) were practical both at 30 and 35 °C but achieved only 53.2 and 65.1 wt.% FAME. The high melting point of POME limited conversion improvement even at 35 °C (Figure 5-6b). *C. rugosa* lipase from Lipase OF was therefore used for the hydrolysis of a 50:50 (wt. %) POME-rapeseed oil admixture. In contrast, the admixture of hydrolysates showed 97.5 wt.% FAME conversions with PKAC-FHL at 30 °C (Figure 5-7a). Thus, one-step esterification of the hydrolysate admixture was faster than the transesterification and esterification of the untreated oils (Figure 5-6).

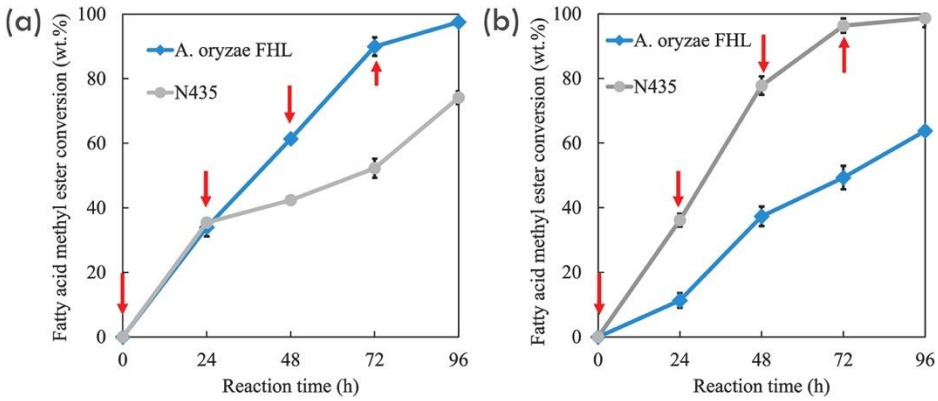


Figure 5-7. Methanolysis of POME-rapeseed oil hydrolysate admixture using *A. oryzae* FHL and Novozym 435 (N435) lipase in (a) a water-containing system and (b) an anhydrous system. Reaction conditions: 4 g oil, 0.4 g lipase, 0.4 g water (or without), 30 ℃ reaction temperature, 35 rpm agitation, and 0.15 g methanol added at 0, 24, 48, and 72 h. Arrows indicate methanol addition times.

Novozym® 435 was compared with PKAC-FHL in the conversion of the hydrolysates at 30 °C. Without water, Novozym® 435 adequately converts the feedstock to ≥97 wt.% FAME (Figure 5-7b). The exclusion of water, however, does not favor FAME conversion using PKAC-FHL (Figure 5-7b). The inclusion of 10 wt.% water to the reaction media significantly lowers Novozym® 435's conversion efficacy (Figure 5-7a). Water inclusion is more advantageous to PKAC-FHL since the conversion of FFA to FAME produces water as a byproduct. The water tolerance of PKAC-FHL is necessary for oil mill effluent (e.g., POME) conversion to biodiesel. Thus, precise water content control in biodiesel feedstocks is not necessary with PKAC-FHL - this is beneficial to the ever-increasing waste oil preference for advanced biodiesel production [39].

IV.3.6 Properties of a POME-rapeseed oil biodiesel

Biodiesel produced in this study was characterized using FTIR. The standard quality was also evaluated against ASTM and EN requirements. Refined palm FAME was used as a reference in the FTIR analysis (Figure 5-8). The FTIR spectra for both Novozym® 435 (N435) and PKAC-FHL-synthesized biodiesel showed similar characteristics as the reference biodiesel (Figure 5-8). Symmetric and antisymmetric stretching of C-H from FAME is indicated at 3090 to 2920 cm^{-1} [40]. A strong peak at 1750 cm^{-1} showed in all samples representing C=O stretching in ester groups [40,41]. The indicative methyl ester is shown at 1500-1300 cm^{-1} (C=O vibration). Double peaks at 1495 and 1400 cm^{-1} are also attributed to aliphatic CH_2 and CH_3 groups present in biodiesel. Peaks at 1300-1200 cm^{-1} represented C-O ester groups and were present in all samples. The fingerprint region (900-400 cm^{-1}) showed similar peaks and stretching from palm oil.

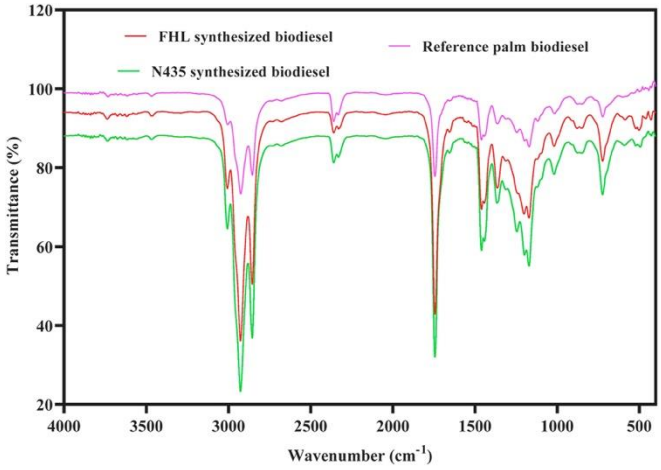


Figure 5-8. FTIR spectra of A. oryzae FHL-catalyzed and Novozym 435 (N435)-catalyzed biodiesel compared with standard palm biodiesel.

The POME-rapeseed oil biodiesel produced mainly consisted of C16:0 and C18:1 fatty acid chains. The admixture contained 20.3 wt.% saturated fatty acids and 79.7% unsaturated fatty acids. Saturated fatty acids are essential for a higher cetane number (CN) quality of biodiesel

[42]. On the other hand, unsaturated fatty acids contribute to a lower cold-filter plugging point (CFPP) in biodiesel for use in cold regions. Neat palm and rapeseed oil biodiesel's CFPP are at >9 °C and -12°C, respectively [43]. Combining both oils improved the CN quality and the cold flow properties of the palm biodiesel (Table 5-4). Like neat palm or rapeseed oil biodiesel, which has 48–56 CN values, the POME-rapeseed admixture biodiesel from this study showed a 48.8 CN value and -7.2 °C CFPP. The obtained CN and CFPP values, however, conform to ASTM D6751's requirements of a ≥ 43 CN value [44].

IV.4. Conclusions

The feasibility of optimum liquid and solid waste utilization for a biodiesel-focused biorefinery was evaluated in this study. Palm kernel shells, which account for ≥12% of solid waste generated at the palm oil mill, were valorized to activated carbons instead of being combusted in biomass boilers without CO_2 capture. The PKS-derived carbons were synthesized via a one-step carbonization and activation technique that produces inexpensive activated carbons applicable as lipase immobilization carriers. Compared with coal-derived-carbons, PKAC showed similar morphological properties that support lipase immobilization. FTIR and EDX analysis confirmed oxygen-rich functional groups (C=O, C-O-C, and C-OH) in PKAC. The unique oxygen-rich properties in PKAC supported the immobilization of a whole-cell-producing lipase. *Aspergillus oryzae* whole-cells expressing *Fusarium heterosporum* lipase was successfully immobilized onto PKAC. The immobilized lipase, PKAC-FHL, demonstrated excellent catalytic activities for converting liquid waste from the palm oil mill, POME, to biodiesel. At 30 °C and in a water-containing system, PKAC-FHL shows better conversion efficiency than the commercial Novozym® 435 lipase. Biodiesel produced in this study also showed a lower cold-filter plugging point and cetane quality that

meets EN and ASTM biodiesel requirements. The valorization of waste from palm oil mills to biodiesel shows promise for advancing waste-dependent palm biorefinery and its replicability to other areas in the future bioeconomy.

IV.5. References

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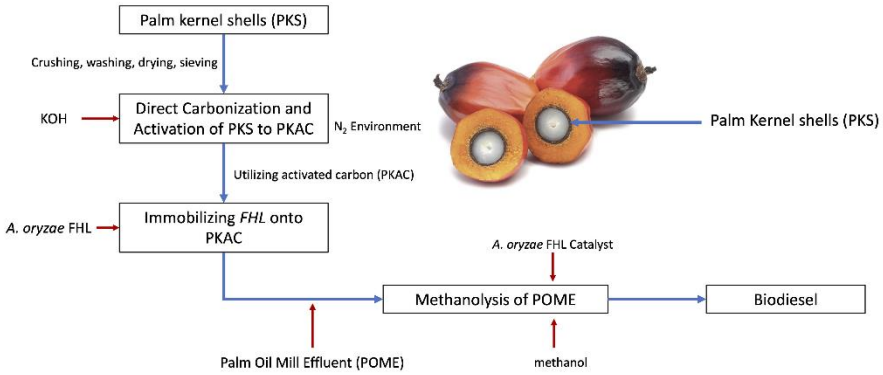
IV.6. Supplementary Information

Summary of Supplementary Information

Schematic representation of the methods used is presented. EDX mappings of PKS, PKAC and BCAC are shown. A literature review on the use of porous carriers for the immobilization of lipases are also tabulated.



Scheme S1. Process outline for the preparation of palm kernel shell derived- activated carbons for lipase immobilization and subsequent POME biodiesel catalysis.

Method for the direct carbonization and activation of PKS to AC

The furnace with the loaded PKS-KOH mixture in a crucible was initially purged with N_2 (20 mL min^{-1}) for 15 min to ensure an inert atmosphere. After activation, the furnace was turned off and allowed to cool under N_2 (20 mL min^{-1}). The obtained activated carbons were washed with excess water and HCl modified to a pH 7 filtrate solution.

Table 5-S1. Temperature program for direct activation of palm kernel shells to activated carbons under N_2 flow in a KDF electric furnace.

Temperature (°C)	**Holding Time (H:M)**
0 - 105	0:30
105	0:10
105 - 250	0:20
250	0:30
250 - 550	0:15
550 - 105	2:00
105 – room temperature	--

Table 5-S2. Some porous synthetic polymers/materials used for the immobilization of lipases towards biodiesel synthesis.

Immobilization Carrier	**Carrier Properties**	**Lipases Tested**	**References**
Lewatit VP OC 1600	Spherical synthetic acrylic resins, 150 Å average pore diameters, 130 m^2 g^{-1} average surface area, 315 -1000	*Candida parapsilosi*, *Candida antarctica* lipase B, *Rhizopus oryzae*, *Carica papaya*	[1–3]

	μm average particle sizes		
Metal organic Frameworks (MOFs)	≥1000 $m^2 g^{-1}$ surface area, 40 - 180 Å average pore diameters, nano to micro particle sizes.	*Aspergillus niger*, *Burkholderia cepacia*	[4,5]
Eupergit® C	Oxirane acrylic beads, 2000 Å average pore diameters, 100 – 250 μm macro particles	*B. cepacia*, *Candida rugosa*, *A. niger*	[6,7]
Polyurethane foams	6 × 6 × 3 mm reticulated foams, 20 pores per linear cm, 95 – 98% particle voidage	*Fusarium heterosporum*, *Rhizopus oryzae*, *C. antarctica* lipase B, *C. rugosa*, *Geobacillus thermocatenulatus*	[8–10]
Granular activated carbons	0.5 – 2.5 mm particles, ≥ 1000 $m^2 g^{-1}$ surface area, ≥400 Å pore diameters	*Fusarium heterosporum* lipase, *C. antarctica* lípase B, *C. rugosa*,	[11–13], *This study*

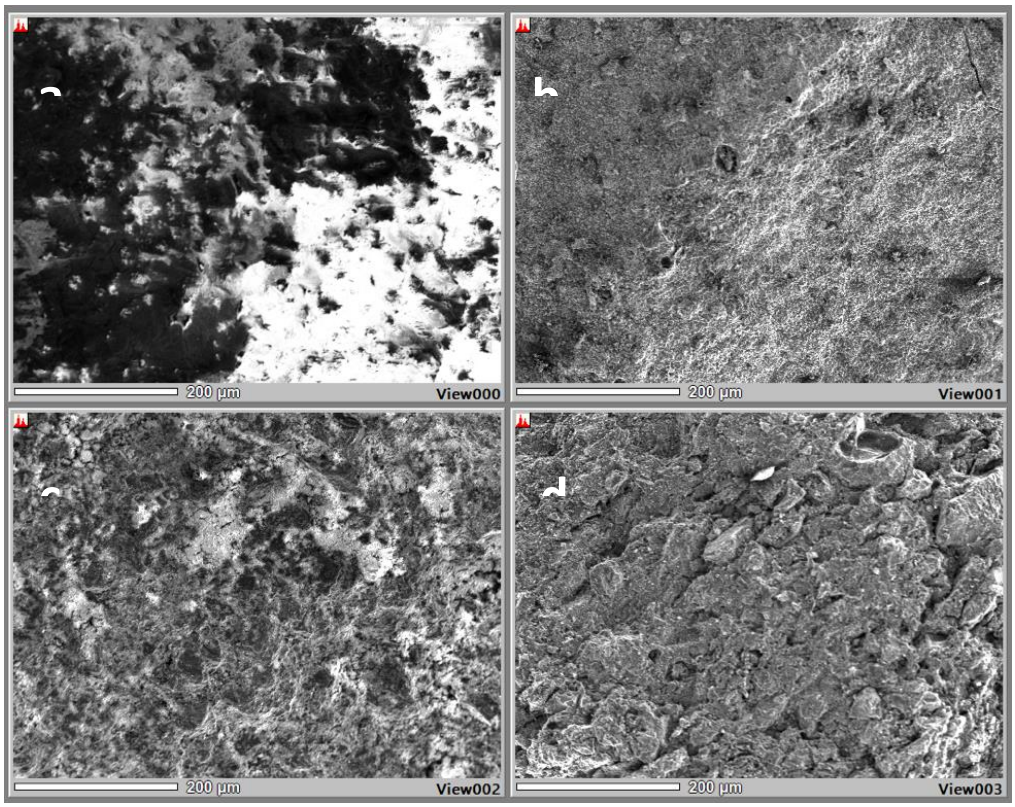


Figure 5-S1. SEM-EDX images of (a) untreated palm kernel shell, (b) palm kernel shell-derived activated carbon (PKAC), (c) bituminous coal-derived activated carbon, and (d) *A. oryzae FHL* immobilized onto PKAC – image acquired at 200µm

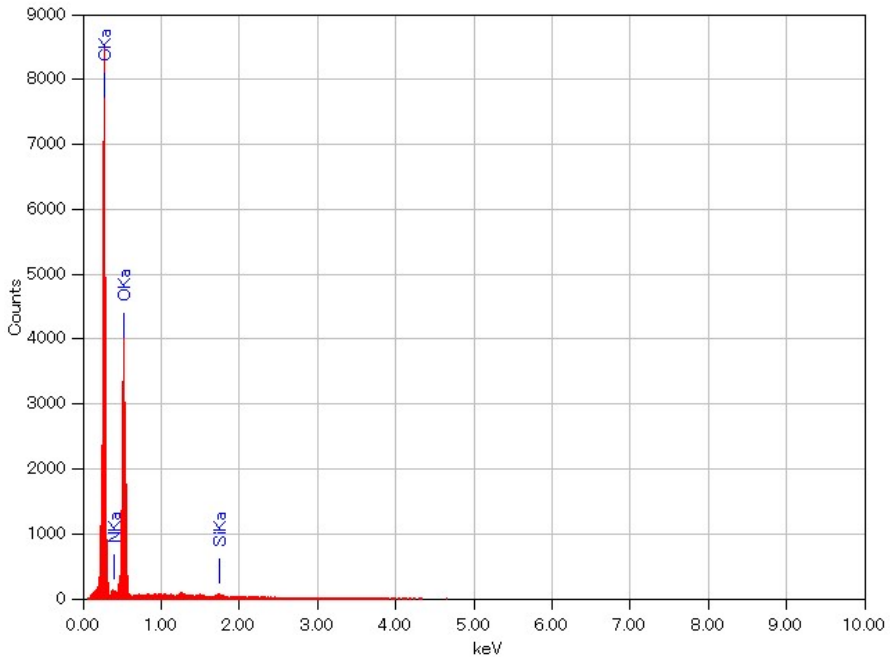


Figure 5-S2. EDX graph of Raw palm kernel shell

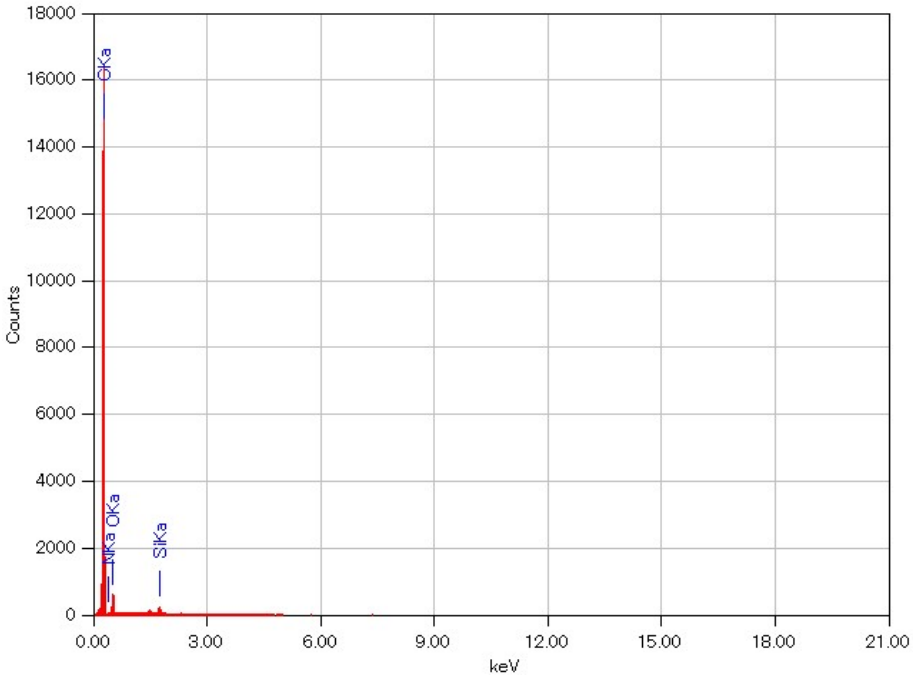


Figure 5-S3. EDX graph of bituminous coal derived activated carbon

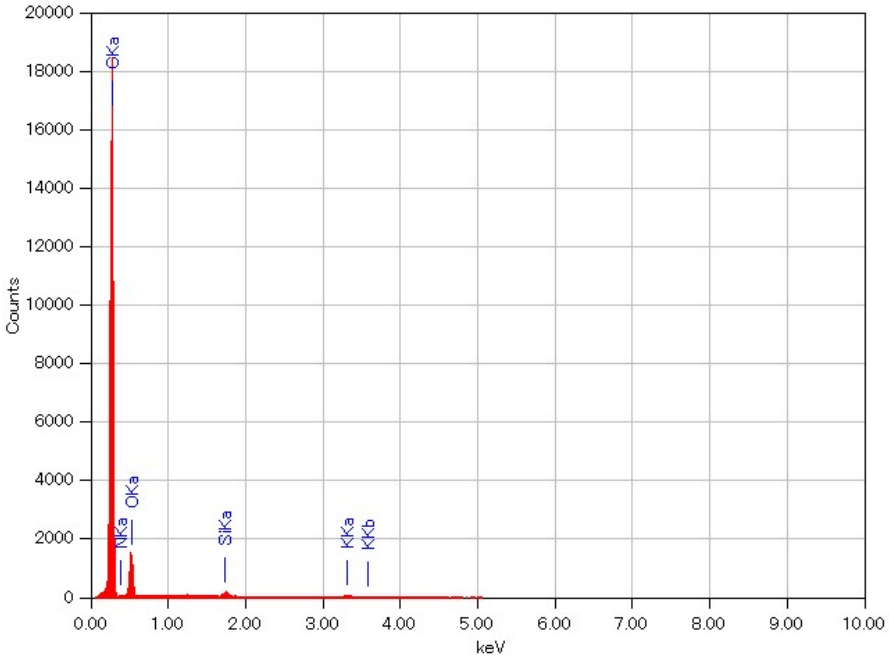


Figure 5-S4. EDX graph of Palm kernel shell derived activated carbon

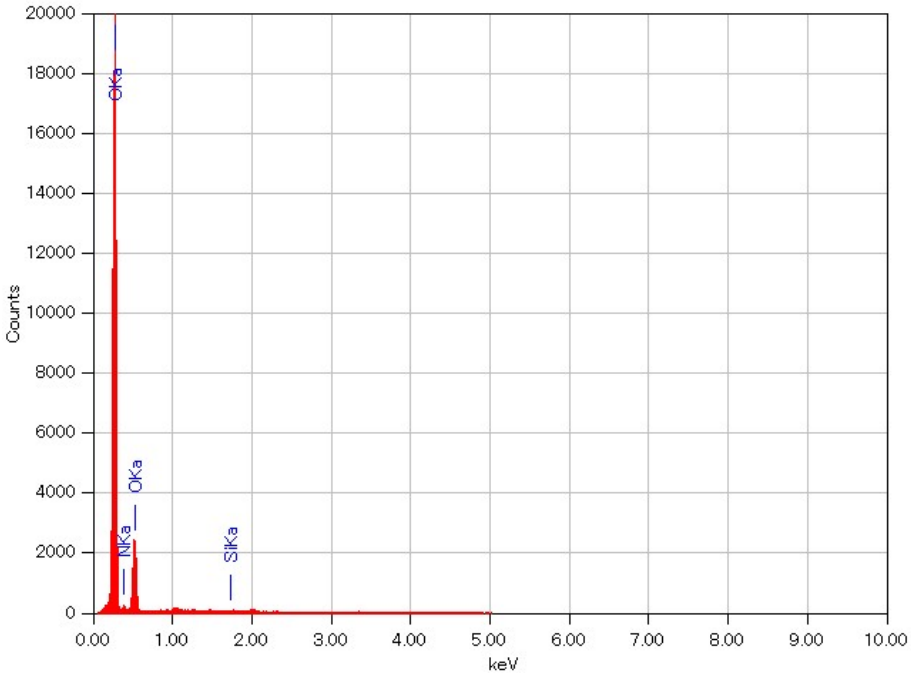


Figure 5-S5. EDX graph of Palm kernel shell derived activated carbon used for the immobilization of FH Lipase

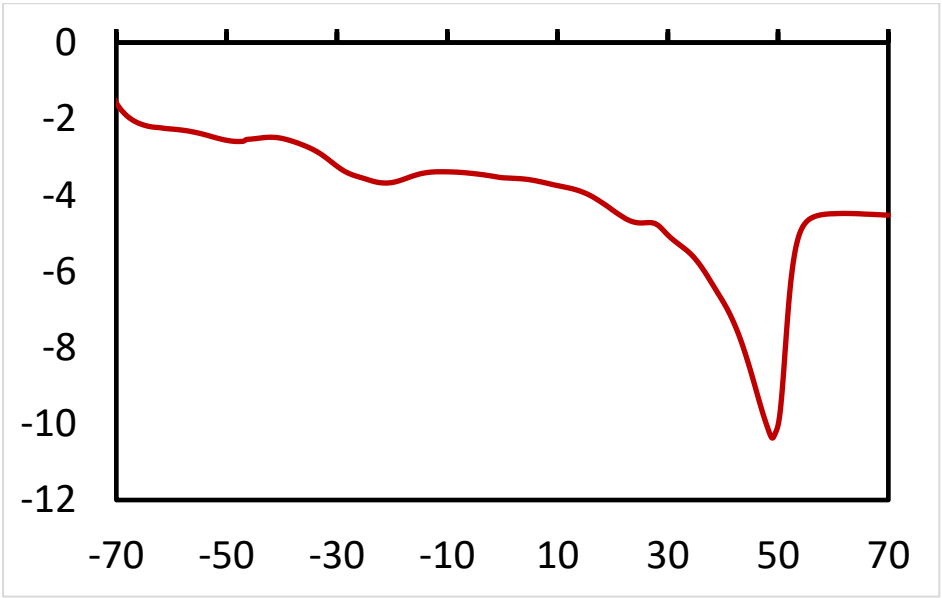


Figure 5-S6. DSC heating scan of Palm oil mill effluent (POME)

Table 5-S3. Fatty acid composition of feedstock used.

Parameter	POME	Rapeseed Oil	Admixture	Melting Point (°C)
C14:0 (wt.%)	0.76 ± 0.02 x = 49.48, y = -10.25	--	--	54.4
C16:0 (wt.%)	50.47 ± 0.63	3.48 ± 0.05	18.00 ± 0.26	62.9
C18:0 (wt.%)	5.06 ± 0.05	1.58 ± 0.02	2.29 ± 0.12	69.3
C18:1 (wt.%)	40.33 ± 0.54	63.90 ± 0.09	57.76 ± 0.34	13.4
C18:2 (wt.%)	3.38 ± 0.06	21.60 ± 0.05	15.96 ± 0.20	-5
C18:3 (wt.%)	--	9.45 ± 0.08	5.99 ± 0.05	-11.3
∑SFA (wt.%)	**56.29**	**5.06**	**20.29**	**N/A**

Biodiesel Properties Calculation

Fuel properties of the POME and Rapeseed oil admixture biodiesel was calculated using empirical equations [14]. Using the fatty acid composition and molecular weight of the oils used.

$$Saponification\ value\ (SV) = \frac{\sum(560 \times P)}{Molecular\ weight\ of\ each\ fatty\ acid} \quad [S2]$$

$$Iodine\ \ value\ (IV) = \frac{\sum(254 \times \text{no.of double bonds} \times \%\ \text{of each fatty acid}}{Molecular\ weight\ of\ each\ fatty\ acid} \quad [S3]$$

$$Oxidative\ Stability = \left(\frac{117.9295}{\sum(\%\ polyunsaturated\ fatty\ acids}\right) + 2.8905 \quad [S4]$$

$$Cetane\ number\ (CN) = 46.3 + \left(\frac{5458}{SV}\right) - (0.255 \times IV) \quad [S5]$$

$$Higher\ heating\ value = 49.43 - (0.041 \text{ x SV}) - (0.015 \text{ x IV}) \quad \text{[S6]}$$

$$Saturation\ factor = (0.1 \text{ x C16:0} + 0.5 \text{ x C18:0}) \quad \text{[S7]}$$

$$Cold\ filter\ plugging\ point\ (CFP) = (3.1417 \text{ x saturation factor}) - 16.477 \quad \text{[S8]}$$

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Conclusions and Recommendations for future work

Opportunities for Research and Development

Transesterification remains the predominant industrially-applied technology for the conversion of palm oil to petroleum diesel substitute. However, the reaction is relatively slow, therefore, the industry continuously faces the challenge of having a versatile transesterification technique. The other drivers for change from transesterification include feedstock recalcitrance, alcohol availability, and poor cold-flow properties of palm biodiesel. Cited throughout this thesis, palm oil's milling and refining process produce various primary, secondary, and waste lipid products that can be used as viable biodiesel feedstocks. However, inconsistent FFA levels in the feedstocks make it cumbersome to adopt a single transesterification method. Two-step transesterification with acids and bases increases material handling cost and CAPEX (accounting stainless steel reactors for acid catalyst). Versatile lipases as catalysts also come with high costs (that can be mitigated only by reusing the lipases). The other challenge with secondary and waste palm oil feedstocks are the presence of water that would require pretreatment with most catalysts. Refined palm oil, although it can easily be converted without FFA and water hurdles, its high cost (70 - 85% of production overhead) makes it economically unviable as biodiesel feedstock. PFAD, PAO, POME, DPO, and other secondary products are half to ¼ of the cost of refined oils. The inherent high degree of saturation in palm oil is another feedstock challenge. Saturated fatty acids such as C14:0, C16:0, and C18:0 in palm oil accounts for higher pour point and cloud point of its biodiesel. The poor cold flow behavior of palm oil has been approached with cold-flow improvers and winterization technologies that add cost to the competitively priced commodity.

Although methanol is the predominant alcohol used in transesterification, ethanol and other long-chain alcohols have shown promise for faster reaction. The cost of methanol and ethanol can to compared on the availability of feedstock and process. technology. In Brazil where ethanol can be obtained from large production of fermentable corn sugars, ethyl esters are cheaper to produce than methyl esters. In predominant palm oil-producing areas like Indonesia and Malaysia, a biorefinery concept where EFB and trunk are used for fermentation can be proposed (see the section below).

Based on the slow reaction, feedstock variation, alcohol selection, and poor cold-flow behavior of palm biodiesel, other transesterification methods such as in-situ transesterification and super/subcritical technologies are being explored for palm biodiesel conversion. In-situ transesterification avoids the extensive lipid extraction steps. Under optimum conditions, in-situ transesterification can be established from the fruits digestion step in palm oil extraction for instance. Biodiesel produced at this step will skip numerous energy-intensive extraction steps such as decantation, centrifugation, fatty acids distillation, bleaching, and deodorization. In-situ transesterification serves as a direct transesterification that can reduce large oil extraction equipment costs. However, the demerit of this technique is the high volume of solvents and catalyst required. Extra process equipment is also required for recovery of solvents for reuse besides the associated health and safety problems. For microalgae biodiesel synthesis, in-situ transesterification has been largely adopted and aided with microwave irradiation, ultrasonication, supercritical conditions, and excess co-solvent addition techniques. Further studies are also required to evaluate the oil extraction rate (OER) with in-situ transesterification compared with the conventional post-oil extraction procedure.

Supercritical and subcritical alcoholysis have recently been progressively studied to produce biodiesel in shorter reaction times. Supercritical transesterification is non-catalytic but requires

the supercritical state use of alcohol at higher temperatures and pressure. The critical pressure and temperature of methanol are at 80.9 bar and 239.5 °C while that of ethanol is at 63.0 bar and 240.8 °C. Supercritical trans/esterification proceeds faster than conventional transesterification due to increased miscibility between methanol and oil beyond methanol's critical point. At the supercritical fluid state, alcohol exhibits compressible properties of a gas but also possesses the density of a liquid, becomes an excellent solvent, and mixes well with nonpolar organic molecules. For palm oil transesterification, supercritical alcoholysis has been largely used for transesterification of refined palm oil but not its secondary and waste products A high molar amount of methanol (42:1 methanol to oil molar ratio, >38% of the reaction volume) is required and that poses solvent cost and safety concerns. Also, high energy consumption from high temperature and pressure of the process is yet to be quantified to justify its shorter reaction time advantage over conventional transesterification.

Besides transesterification, medium-chain fatty acids like palm oil can be converted via alternative methods to petrol-diesel like fuels for combustion engines. Direct blending, pyrolysis, microemulsion, hydrocracking, hydrogenation, and other hydrothermal treatment techniques produce diesel alternative that can be used directly or blended for use in diesel engines without modification. Industrially-adopted terminologies specifically refer to biodiesel as transesterified oils/fats. The alternative methods to transesterification only produce alternative-diesel that have earned descriptors such as renewable diesel, green diesel, SVO, HVO, etc. Straight vegetable oil (SVO) or pure plant oil (PPO) are directly-derived, unmodified vegetable oils that be used directly as alternative diesel fuels. To use SVO efficiently as fuel, modifications of the diesel engine would be required to overcome its higher viscosity, poor fuel atomization, and incomplete combustion. To lower CPO SVO's viscosity

to a diesel engines' operating fuel viscosity (3 -8 mPa s), preheating the oil to > 80 °C is suggested. Although preheating offers smooth fuel flow and minimal effect on the engine's injection system, CO and NO emissions from CPO-SVO can be higher than fossil diesel. Other methods such as blending palm oil with diesel and biodiesel have been studied to measure the heating value of the blend, HC, CO, NO, and other emissions. In all, although direct use of palm oil as SVO or blending offers the advantages of overcoming logistics burden for conversion plants, engine health problems and air pollution from its use negate its climate change mitigation benefits.

Conclusion

Reaction limitations from FFA, water, high temperatures, and the inflexibility of acids and bases as catalysts for transesterification led to advances with enzymes as alternative catalysts. Lipases, a subgroup of enzymes, were first reported to be FFA and water-tolerant in the transesterification of sunflower oil to alkyl esters. This has supported advances over the last three decades on investigating the complex role of lipases in catalysis of oils/fats to fatty acid alkyl esters. Besides varied feedstock applicability, lipase-catalyzed transesterification produces higher quality glycerol that can be used for food, pharmaceuticals, etc. easily without extensive pretreatment. The alcoholysis of oils/fats to fatty acid alkyl ester with lipases is dependent on the active center of most true lipases. The active center of true lipases such as the human pancreas lipases are described to be a catalytic triad consisting of a center serine residue (Ser) which participates in a charge relay system with histidine (His) and aspartic acid (Asp) residues. Activation of lipase for access to the catalytic triad is achieved by the movement of a 'covering lid' at an aqueous-organic interface where water/MeOH and triglycerides exist in transesterification. The orientation of the lid for the catalytic triad access

can be funnel-shaped, tunnel-like, or placed on an external surface of the enzyme. Such peculiar orientation differs in various lipase strains and it is described to be responsible for different interfacial activation in different lipases.

Strains of lipases that have been tested in the transesterification of palm oil include *Candida antarctica lipase* B, *Burkholderia cepacia*, *Thermomyces lanuginosus, Rhizomucor miehei*, *Rhizopus oryzae,* etc. Notwithstanding the many advantages of using lipases to replace chemical catalysts, lipases denature under harsh conditions. Alcohol, temperature, solvents, and oil contaminants are the major denaturation factors in the use of lipases. Most lipases such as *Candida antarctica lipase* B that have been applied widely in alcoholysis thrive optimally between 30 and 45 °C. This becomes a demerit of lipases when used in palm oil alcoholysis. Crude palm oil's slip melting point is > 35 °C compared to -25 °C for soybean oil. Higher temperatures are therefore necessary for homogenous transesterification mixture. High water content in feedstocks is also found not to be problematic in the use of lipases. In a co-authored study, high-water tolerance of *Thermomyces lanuginosus* lipase in the conversion of POME was observed. 45% Aqueous ethanolysis of POME achieved >96% biodiesel compared to 80 wt.% biodiesel in anhydrous methanol. The high cost of lipases for industrial scale-ups is possible to reduce when used in immobilized form. Cheaper matrices from agricultural waste residues have been described to also help lower the cost of immobilization.

Recommendations for future work

A Biorefinery concept for alcoholysis of palm oil to biodiesel

The goal of a palm biorefinery concept in this context is to convert all available secondary and waste palm biomass residues into biodiesel and its related bioproducts through biochemical pathways that eliminates waste. Using lipase-mediated methods, we assume the feasibility of

the ethanolysis of liquid waste from the palm oil mill. Ethanol can be derived from the lignocellulosic biomass residues from the mill. Lignocellulosic biomass from the palm industry such as palm trunks and empty fruit bunches have been studied for their compositional distribution of cellulose and hemicellulose as fermentable sugar sources. Besides their use as precursors for activated carbon, palm kernel shell carbon have been used as excellent immobilization carriers for lipases towards biodiesel production. Other lignocellulosic biomass that can find significant value chains in alcoholysis includes mesocarp fiber, kernel cake, kernel fiber, etc.

CPO and palm kernel oil, meant for food and oleochemicals, are the main high-value commodities from the palm oil industry. However, to produce these two primary products, various liquid byproducts and wastes are generated in large amounts that can be more in tonnage than the primary products. For instance, 2.5 - 3.5 tons of POME is produced per ton CPO produced. Other liquid byproducts and wastes generated include PAO, PFAD, Soap stock, POME, etc.

A sustainable alcoholysis biorefinery concept should explore all available opportunities of maximizing value from all byproduct streams from cultivation, processing, and conversion of palm biomass to high-value bioproducts. The actualization of a palm biorefinery should yield a bioenergy dependent processing facility that caters to food as the primary products but with essential products for agriculture, pharmaceutical, chemicals, materials, and other related industries.

Publications List

Chapter I:

E. Quayson, J. Amoah, S. Hama, A. Kondo, C. Ogino, Immobilized lipases for biodiesel production: Current and future greening opportunities, Renew. Sustain. Energy Rev. 134 (2020) 110355. doi:https://doi.org/10.1016/j.rser.2020.110355.

Chapter II:

E. Quayson, J. Amoah, S. Hama, A. Yoshida, K. Morita, A. Kondo, C. Ogino, Valorization of Activated Carbon as a Reusable Matrix for the Immobilization of Aspergillus oryzae Whole-Cells Expressing Fusarium heterosporum Lipase toward Biodiesel Synthesis, ACS Sustain. Chem. Eng. 7 (2019) 5010–5017. doi:10.1021/acssuschemeng.8b05649.

Chapter III:

E. Quayson, J. Amoah, N. Rachmadona, S. Hama, A. Yoshida, A. Kondo, C. Ogino, Biodiesel-mediated biodiesel production: A recombinant Fusarium heterosporum lipase-catalyzed transesterification of crude plant oils, Fuel Process. Technol. 199 (2020). doi:10.1016/j.fuproc.2019.106278.

Chapter IV:

E. Quayson, J. Amoah, N. Rachmadona, K. Morita, L. Darkwah, S. Hama, A. Yoshida, A. Kondo, C. Ogino, Valorization of palm biomass waste into carbon matrices for the immobilization of recombinant Fusarium heterosporum lipase towards palm biodiesel synthesis, Biomass and Bioenergy. 142 (2020) 105768. doi:https://doi.org/10.1016/j.biombioe.2020.105768.

Co-authored Publications

[I] H. Wijaya, K. Sasaki, P. Kahar, **E. Quayson**, N. Rachmadona, J. Amoah, S. Hama, C. Ogino, A. Kondo, Concentration of lipase from Aspergillus oryzae expressing Fusarium heterosporum by nanofiltration to enhance transesterification, Processes. 8 (2020). doi:10.3390/PR8040450.

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I. **E. Quayson**, et al. Palm Methyl Ester Synthesis: A recombinant *Fusarium heterosporum* lipase-catalyzed transesterification with biodiesel as a novel solvent, 25th Symposium of Young Asian Biological Engineers' Community, Nov. 2019, Ewha Women's University, Seoul, South Korea.

II. **E. Quayson**, et. al, Valorization of activated carbon as a reusable matrix for the immobilization of *Aspergillus oryzae* whole-cells expressing Fusarium heterosporum lipase towards biodiesel synthesis, 9th International Symposium of Innovative Bioproduction Kobe, Feb. 2018, Kobe University, Kobe, Japan.

III. **E. Quayson**, et. al, Preparation and characterization of robust whole cell biocatalyst from *A. oryzae* expressing *Fusarium heterosporum* lipase immobilized onto activated carbon for biodiesel synthesis,70th Annual Meeting of the Society of Biotechnology of Japan (SBJ), Sept. 2018, Kansai University, Osaka, Japan.

Doctoral Dissertation, Kobe University

"Biocatalysis techniques for the valorization of effluent and solid waste from the oils and fat industry to biofuels", (192 pages)

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