Immobilization of Lipase and Biodiesel Production from Fishery and Animal Processing Waste

by

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ABSTRACT

Biodiesel (BD) or alkyl esters is conventionally produced by the transesterification of vegetable oils or animal fats with a monohydric alcohol and base catalyst, and known for its many technical and environmental advantages over petrodiesel. However, these oils are not economically viable BD feedstocks due to their value as edible oils. Alternative, inexpensive feedstocks with minimal to no food value such as the large quantities of fishery and animal processing by-products which are often discarded as waste were explored in this project. Lipase was investigated as an alternative to chemical catalyst due to the tolerance of the former to a wide variety of feedstocks and simpler post-production processes among other advantages. Approximately 23.32-61.53% (on dry weight basis) of salmon skin oil (SSO) was recovered by the various solvent systems evaluated. Salmon skin subjected to Soxtec-hexane, and the Bligh and Dyer method, respectively gave the highest and lowest oil yield. While a study on the effects of temperature (25, 4, -18, and -80^{o}C) and time (1, 5, 10, 15, 20, 25, 30, 35, 40, and 45 d) on the quality of SSO during storage revealed small but non-significant changes in the fatty acid profile between day 1 and 45, and 25 to -80^{o}C, the free fatty acid (FFA) content increased from 7.32, 7.09, 7.14, and 6.58% to 8.50, 8.29, 8.19, and 7.86%, peroxide value increased from 18.09, 6.61, 17.16, and 17.80 to 88.19, 64.53, 59.38, and 34.47 mequiv peroxide/kg oil, and thiobarbituric acid reactive substances increased from 4.32, 4.13, 2.86, and 3.89 to 32.43, 26.33, 25.80, and 17.04 μg MDA eq/g oil, respectively. A Fourier transform infrared (FTIR) spectroscopic method was assessed as an alternative to the conventional AOCS titrimetric method for the determination of FFA content. With modifications, the new method was found to be capable of responding linearly to oleic acid (0-6.5%) addition, producing a FFA calibration equation with a S.D. of ±0.014% FFA. Based on the results from the initial assessment of the effects of reaction temperature (25-65^{o}C), oil:alcohol molar ratio (1:1-1:6), alcohol type (ethanol or methanol), and reaction time (8-120 h) on Lipozyme®-IM-catalyzed transesterification of the recovered SSO, a commercial blend of yellow grease and rendered animal fat (RC), and olive oil (OO) to fatty acid ethyl ester (FAEE) for use as BD, the process was considered for optimization. In three experiments, the linear, quadratic, and bilinear effects of the reaction variables on FAEE yield were assessed with response surface

methodology (RSM) based on central composite rotatable design (CCRD). In each experiment, second-order polynomial models fitted to FAEE yield provided response surfaces at the various reaction times (8-48 h). These models were generally significant and produced reliable and stable predictions. The optimum conditions were found to be close to the centre point of the reaction variables (50^{o}C, enzyme load of 39.06 U, and oil:alcohol molar ratio of 1:2). A high performance liquid chromatography (HPLC) unit equipped with a size exclusion column, and a refractive index (RI) detector separated, and simultaneously identified, and quantitated the reaction components; FAEE, unreacted triacylglycerol (TAG), residual diacyl- and monoacyl- glycerol (DAG and MAG), and alcohol as well as FFA. The elution profile in order of increasing time was: TAG < DAG < FAEE < FFA < MAG < ethanol. Fuel testing of the transesterified oils revealed various proportions of total and bound glycerol, acid number, TAG, DAG, MAG, and moisture. To expand the uses of lipase recovered from fish processing discards to include catalyst for BD production, lipase from crude preparations of delipidated grey mullet (*Mugil cephalus*) viscera were isolated on *para*-aminobenzamidine agarose (*p*-ABA) and immobilized on octyl Sepharose CL-4B (o-Sep). A signal in the amide I absorption region of the FTIR spectrum was attributed to the protein layer on o-Sep. Immobilized grey mullet lipase (GML*i*) had a 10°C higher optimum temperature compared to the free enzyme (GML) for the hydrolysis of *p*ara-nitrophenyl palmitate (*p*-NPP). Immobilization lowered the enthalpy of activation (ΔH*), and free energy of activation (ΔG*) by more than 313 and 1315 cal/mol, respectively, while it enhanced the reusability, thermal, storage, and organic solvent stabilities of GML.

RÉSUMÉ

Le biodiesel (BD), ou des esters d'alkyle, est classiquement produit par la transestérification d'huiles végétales ou de graisses animales avec un monoalcool et un catalyseur de base, et est également connu pour ses nombreux avantages techniques et environnementaux par rapport au pétrodiesel. Toutefois, ces huiles BD ne sont pas des matières premières économiquement viables en raison de leur valeur principale en tant qu'huiles comestibles. Ce projet a exploré des matières premières de substitution, peu coûteuses avec peu ou pas de valeur alimentaire telle que les grandes quantités de sous-produits detransformation du poisson et de sous-produits animaux qui sont souvent jetés avec les déchets. La lipase a été étudiée comme une alternative aux catalyseurs chimiques en raison de la tolérance de la lipase à une grande variété de matières premières ainsi que son processus de post-production beaucoup plus simple entre autres avantages. Environ 23,32 et 61,53% (sur la base du poids sec) de l'huile de peau de saumon (SSO) a été récupéré selon les différents systèmes de solvants évalués. La peau de saumon soumis à des méthodes de extraction par Soxtec-hexane et de Bligh et Dyer, a donné respectivement un rendement en huile le plus élevée et le plus faible. Au cours de l'entreposage, les effets de la température (25, 4, -18, et -80°C) et du temps (1, 5, 10, 15, 20, 25, 30, 35, 40, et 45 d) sur la qualité de SSO ont été étudiés et ont révélé de petits changements, mais significatif, dans le profil des acides gras entre le premier jour et le jour 45, ainsi qu' entre 25 à -80°C, la teneur en acides gras libres (AGL) a augmenté de 7,32, 7,09, 7,14, et 6,58% à 8,50, 8,29, 8,19, et 7,86%, respectivement, l'indice de peroxyde est passé de 18,09, 6,61, 17,16, et 17,80 à 88,19, 64,53, 59,38, et 34,47 mequiv peroxyde/kg d'huile, et les réactives de l'acide thiobarbiturique ont augmenté de 4,32, 4,13, 2,86, et 3,89 à 32,43, 26,33, 25,80, et 17,04 µg MDA éq/g d'huile, respectivement. Pour la détermination de la teneur en FFA, une méthode de spectroscopie infrarouge à transformée de Fourier (FTIR) a été évaluée comme une alternative au procédé AOCS conventionnel. Avec des modifications, la nouvelle méthode a été jugée capable de répondre de façon linéaire à l'addition d'acide oléique (0 à 6,5%), avec la production d'une équation d'étalonnage FFA avec une SD de ±0,014% FFA. Sur la base des résultats de l'évaluation initiale des effets de la température de réaction (25-65°C), un rapport l'huile:alcool molaire (1:1-1:6), le type d'alcool (éthanol ou méthanol), et le temps de réaction (8-120 h) sur du la transestérification catalysée Lipozyme®-IM, un mélange

commercial de graisse animale jaune et de graisses fondues (RC), et d'huile d'olive (OO) à ester éthylique d'acide gras (EEAG) pour une utilisation comme BD, le procédé a été considéré pour optimisation. Dans trois expériences, les effets linéaires, quadratiques et bilinéaires des variables de la réaction sur le rendement EEAG ont été évalués avec la méthode de réponse de surface (RSM) basée sur la conception centrale composite rotative (CCRD). Dans chaque expérience, des modèles polynomiaux du second ordre équipés d'EEAG ont modelé le rendement des surfaces de réponse fournis aux divers temps de réaction (8-48 h). Ces modèles sont généralement importants et produisent des prévisions fiables et stables. Les conditions optimales ont été trouvées être proche du point de centre des variables de réaction (50°C, charge de l'enzyme 39.06 U, et l'huile:rapport molaire de l'alcool 1:2), et simultanément identifiés, et quantifiés. Les différents composants de la réaction (par exemple: EEAG, triacylglycérol n'ayant pas réagi (TAG), diacyle et résiduelle monoacyle-glycérol (DAG et MAG), et l'alcool ainsi que la FFA), ont été séparés, identifiés et quantifiés en utilisant la chromatographie liquide à haute performance (HPLC) équipé d'unité de colonne d'exclusion de taille, et un détecteur par indice de réfraction (RI). Le profil chromatographique d'élution en augmentant le temps de rétention était: TAG < DAG < EEAG < FFA < MAG < éthanol. Le test de carburant des huiles transestérifiées a révélé des proportions diverses de glycérol total et lié, indice d'acide, TAG, DAG, MAG, et humidité. Pour élargir les usages de la lipase récupérée à partir de rejets de transformation du poisson pour inclure un catalyseur pour la production de BD, de la lipase à partir de préparations brutes de délipidé mulet (*Mugil cephalus*) les viscères ont été isolées sur le para-aminobenzamidine agarose (*p*-ABA) et immobilisées sur Sepharose CL-octyle 4B (o-Sep). Un signal dans la région d'absorption amide I du spectre FTIR a été attribué à la couche de protéine sur o-Sep. La lipase de mulet immobilisée (GML*i*) a eu une température optimale de 10°C plus élevée par rapport à l'enzyme libre (GML) pour l'hydrolyse de *para*-nitrophényl palmitate (*p*-NPP). L'immobilisation a abaissé l'enthalpie d'activation (AH*), et l'énergie libre d'activation (AG*) de plus de 313 et 1315 cal/mol, respectivement, alors qu'alla améliorer la capacité thermique, la, réutilisabilité, et la stabilité des solvants des GML.

CONTRIBUTION OF AUTHORS

This thesis is presented in manuscript format and consists of ten chapters. A general introduction in Chapter I opens the concept of converting processing waste into value-added products. It also provides the outline of the proposed research, rationale and objectives. Chapter II provides a detailed review of the literature on biofuels, feedstocks, lipid extraction, catalysis, biodiesel production technologies, the interplay between process parameters and biodiesel yield, and fuel properties. Chapters III, IV, V, VI, VII, VIII, and IX constitute the main body of the thesis. Chapters III to VII are published manuscripts and Chapters VIII and IX have been submitted for publication. Connecting statements provide bridges between chapters. A general conclusion is drawn in Chapter X. This dissertation format is in accordance with the guidelines for thesis preparation provided by the Faculty of Graduate and Postdoctoral Studies.

The present author designed the study, conducted the experiments, collected and analyzed the data, was responsible for the statistical concept and analyses, and prepared and submitted all the manuscripts. Dr. Benjamin K. Simpson proposed the thesis hypothesis, provided laboratory space, and supplies for the experiments, advice on direction, content of the research, and edited the manuscripts. Dr. Leroy E. Phillip provided advisory input and editorial assistance for Chapters IV, VI, and VII. Dr. Roger I. Cue provided editorial assistance and technical advice on the statistical analyses for Chapters IV, VI, and VII. Dr. Fredrick R. van de Voort provided laboratory space, and supplies, and his expertise for Chapter V. Dr. Pierre R.L. Dutilleul provided editorial assistance and technical advice on the statistical analyses for Chapter VIII. Mr. Michael Paszti performed the fuel quality test experiments, provided technical advice and editorial assistance for Chapter VIII.

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4. **Aryee, A.N.A.**, Simpson, B.K., Cue, R.I. and Phillip, L.E. (2011). Enzymatic transesterification of oils and fats from animal discards to fatty acid ethyl esters for potential fuel use. Biomass Bioenerg. 35(10): 4149-4157.
5. **Aryee, A.N.A.**, Phillip, L.E., Cue, R.I. and Simpson, B.K. (2011). Identification and quantitation of reaction intermediates and residuals in lipase-catalyzed transesterified oils by HPLC. Appl. Biochem. Biotechnol. 165(1): 155-77.
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2. **Aryee, A.N.A.** and Simpson, B.K. (2011). High-value resources from residual biomass. 8th Interdisciplinary Graduate Student Research Symposium, McGill University, May 17-18, Montreal, Québec, Canada.
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by HPLC. 102nd AOCS Annual Meeting and Expo, May 1-4, Cincinnati, Ohio, USA.

4. **Aryee, A.N.A.** and Simpson, B.K. (2011). Changes in the quality of salmon skin oil during storage. 102nd AOCS Annual Meeting and Expo, May 1-4, Cincinnati, Ohio, USA.
5. **Aryee, A.N.A.**, Fournier, V., Phillip, L.E. and Simpson, B.K. (2009). Immobilized lipase as catalyst for biodiesel production from recovered processing waste oil and fat. IFT09, Annual Meeting and Food Expo, June 6-9, Anaheim, California, USA.
6. **Aryee, A.N.A.**, Phillip, L.E., Simpson, B.K. and Ngadi, M.O. (2008). Evaluation of solvent extraction techniques for the recovery of oil from salmon processing discards. Annual CIFST-AAFC Conf. May 25-27, Charlottetown, Prince Edward Island, Canada.
7. **Aryee, A.N.A.**, van de Voort, F.R., Ngadi, M.O. and Simpson, B.K. (2007). An inexpensive feedstock for biodiesel production from fishery processing discards and by-products. International Congress on Biodiesel: The Science and the Technologies. AOCS conf. November 5-7, Vienna, Austria.
8. **Aryee, A.N.A.**, van de Voort, F.R. and Simpson, B.K. (2007). A modified method for the determination of free fatty acids in oils using Fourier Transform Infrared (FTIR) spectroscopy. "RELATENZ 2007", June 20-23, Varadero, Cuba.

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ABBREVIATIONS AND ACRONYMS

AN.....Acid number
ATR.....Attenuated total reflectance
B100 ..... 100% biodiesel/pure biodiesel
B20 .....20% biodiesel, 80% petrodiesel
BD .....Biodiesel
CP..... Cloud point
DAG.....Diacylglycerol
FAAE .....Fatty acid alkyl esters
FAEE.....Fatty acid ethyl esters
FAME.....Fatty acid methyl esters
FAO.....Food and Agriculture Organization
FFA .....Free fatty acid
FP ..... Flash point
FTIR.....Fourier transform infrared
GC ..... Gas chromatography
GHG.....Greenhouse gas
GML.....Grey mullet lipase
GML*i*..... Immobilized grey mullet lipase
HPLC .....High performance liquid chromatography
IEO .....International Energy Outlook
MAG .....Monoacylglycerol
MUFA .....Monounsaturated fatty acid
NOx.....Nitrogen oxides
OECD.....Organization for Economic Co-operation and Development
o-Sep .....octyl-Sepharose CL-4B
PUFA ..... Polyunsaturated fatty acid
SDTC .....Sustainable Development Technology Canada
SFA .....Saturated fatty acid
TAG .....Triacylglycerol
p-NPP.....*Para*-nitrophenyl palmitate

CHAPTER I

INTRODUCTION

Energy is essential to meet our most basic needs, such as; cooking, lighting, and heating. Energy is traditionally sourced from fossil, hydro, and natural gas among others. In fact for years, fossil fuel has provided mankind with the most reliable source of energy. It has however been reported that energy to drive industries and provide for the necessities of life is increasingly becoming scarce and out-stretched. This is due to depleting natural resources, population and economic growth, and soaring prices of fossil fuel. The combustion of fossil fuels has also been partly linked to increased greenhouse gas (GHG) emissions and climate change. These among other factors are serving as catalyst for change, and driving the engines of research for alternative, renewable, reliable, secure, and environmentally sustainable energy sources.

Biofuels such as biodiesel (BD) have been recognized as a viable alternative fuel in the quest for renewable energy sources. Majority of the current feedstocks for BD production however are from vegetable oils. These feedstocks are expensive and have food value. To reduce the cost of BD production and to diversify feedstock resources to include non-food stocks, there is an upsurge in the search for alternative and broader feedstock base. This is expected to include forestry and agricultural residues (biomass) from food crops and animals, such as the large portions of production and processing by-products from the fishery and animal industries which are often dumped or end up in landfills. Interestingly, current stricter environmental laws make this disposal tactic uneconomical and legally contentious. A more sustainable approach involving incentives to explore other alternative uses of these by-products is warranted.

BD is conventionally produced by base-catalyzed transesterification reactions. However, lipase in immobilized forms is rising to meet the challenges of using chemical catalysts. It is against this backdrop that this project was hypothesized. The overarching goal of this project is twofold; namely, to enhance and maximize the value of fishery and animal processing by-products through the recovery and use of their oils as cheaper and alternative feedstock for BD production, and secondly to exploit on the numerous advantages offered by immobilized lipases in transesterification reactions.

The overall aim of this research is to delve into the possibility of using fish oil and

animal fats recovered from fishery and animal processing discards, respectively in BD production by lipase-catalyzed transesterification reactions. The specific objectives include;

1. To extract and characterize oil from salmon skin and evaluate its stability during storage.
    a. Evaluate the effects of solvent type and extraction time on oil yield, and free fatty acid (FFA) content.
    b. Investigate the effect of storage time and temperature on BD-related quality indices.
2. To study the suitability and applicability of the Fourier Transform Infrared (FTIR) spectroscopic method as an alternative to the conventional titrimetric method for the assessment of FFA content.
    a. Develop a rapid method by modifying the sodium hydrogen cyanamide (NaHNCN)-based FTIR FFA determination method to suit the wider range of FFA in crude fish oil.
3. To investigate the production of BD with the recovered oils from fishery and animal processing waste via lipase catalysis.
    a. Evaluate the suitability of an immobilized lipase as catalyst for the transesterification reaction.
    b. Study the effects of reaction parameters on BD production and yield.
4. To optimize the lipase-catalyzed transesterification reaction of BD production with statistical methodologies and designs.
    a. To evaluate the relationships and interactions between process parameters and their effect on BD yield.
    b. To establish the optimum conditions for BD production for the recovered oils.
5. To immobilize and characterize lipase recovered from fishery processing waste and asses its potential as catalyst for BD production.

CHAPTER II

LITERATURE REVIEW

2.1. Biofuels: Alternative Energy, and Classifications

According to the projections of the energy information administration (EIA), global energy (marketed) consumption will grow by nearly 50% between 2007 and 2035 (IEO 2010). As such in the face of increasing world population, emerging economies, depleting natural resources, the perceived effects of fossil fuel combustion on climate change, the perpetual unrest in some oil producing countries, and the skyrocketing price of crude oil, immense debate is fuelling around the future of our energy sources (Meher et al., 2006; Pousa et al., 2007; Hammond et al., 2008). The inevitable consequences of our ever growing demand on dwindling supplies for finite petroleum resources have ignited renewed interest in alternative, renewable energy, and biomaterials (Knothe, 2005b; Hammond et al., 2008; Steubing et al., 2011). As discussed by Knothe (2005b) and other authors (Meher et al., 2006; Hammond et al., 2008) new energy sources are being explored and technologies to make them usable and more reliable are emerging.

One of such energy source is biofuel; a renewable and biodegradable fuel derived from living and recently living biological materials (Knothe, 2005b). Biofuels is grouped in three main categories namely; solid, liquid, and gaseous fuels (SDTC 2006). Solid biofuels release their stored energy from the combustion of agricultural by-products. The feedstocks for solid biofuels are sourced from the pulp and paper industry, agricultural processing by-products such as cereal grain straw, stalks, leaves, husks, chaff, shells, and peels (SDTC 2006; Tilman et al., 2009). Due partly to the absence of significant technological advancement, solid fuels from mostly wood and charcoal are the primary source of energy and continue to provide much of the energy mainly used for cooking, heating, and lighting in many developing countries (OECD/IEA 2010). Although solid biofuels are a huge source of energy, some researchers have questioned their sustainability and impact on the environment (Stein, 2007; Murphy and Power, 2008).

The gaseous form of biofuel is commonly called biogas. Biogas is produced by the anaerobic fermentation of organic matter (Amigun et al., 2008; Murphy and Power, 2008; Ryckebosch et al., 2011). The impact of biogas on air quality has also been questioned. Feedstocks for biogas production include livestock manure, sewage, sludge,

and municipal waste (Arthur et al., 2011; Ryckebosch et al., 2011). The process of biogas production generates mainly methane (40-75%), and carbon dioxide (CO_2, 15-60%) (Cantrell et al., 2008; Ryckebosch et al., 2011). Biogas is currently supported and used in several developing countries such as Senegal, Ethiopia, and Kenya, and some parts of Asia, mainly for cooking (SNV 2010). Biogas has been compressed and used to power vehicles in Sweden (Svenskbiogas 2005).

Liquid fuels account for the largest source of energy, and are important for transportation and industrial applications worldwide (IEO 2010). The two commonly known liquid biofuel are bioethanol and biodiesel (BD). Bioethanol is produced by fermentation of starch from sugar- and starch-laden crops such sugarcane and corn (Pousa et al., 2007). In developing countries, roots and tubers, and molasses are the main feedstock for bioethanol production (OECD/FAO 2010). Bioethanol production and consumption is dominated by the US, Brazil, and some countries of the EU (OECD/FAO 2010). BD is distinguished from bioethanol as the former is transesterified. The term BD is used to describe mono alkyl esters conventionally produced by the transesterification of oils and fats mainly from vegetables and animals sources in the presence of an alcohol and a catalyst that can be used as a fuel in compression ignition engines (Knothe, 2005b). The resulting alkyl esters are also collectively called fatty acid alkyl esters (FAAE), and fatty acid methyl esters (FAME) or fatty acid ethyl esters (FAEE) if methanol or ethanol is the alcohol used. BD can be used pure or blended with petrodiesel at any level and is designated Bxx, where xx represent the volume percentage of BD in the fuel. Thus B5, B10, B25, or B100 represent a fuel which contains 5, 10, 25, or 100% by volume BD, and 95, 90, 75, or 0% petrodiesel, respectively (Knothe, 2005b).

Biofuels are also generationally classified; first, second, or third. First generation biofuels are derived from terrestrial crops such as; sugar, starch, and oil bearing crops (soybean, maize, and rapeseed) or animal fats, which in most cases can also be used as food and feed or from food residues (OECD/FAO 2010). Their impact on global food markets and food security is often a controversial subject (Tilman et al., 2009; OECD/FAO 2010). Second generation biofuels are derived from lignocellulosic agricultural products (e.g. corn stover and corn cobs), forest residues, biomass, and non-food crops such as *Jatropha curcas*. Feedstocks in the third generation category include,

but not limited to, algae.

2.1.1. Prospects, Impact and Opportunities of Biofuels

Apart from the oil sands from Canada, biofuels are the other contributor of the 70% increase in total unconventional liquid fuels supply between 2007 and 2035 (IEO 2010). In fact, total global biofuel production reached 100 billion litres (bnl) in 2009; made up of 82 bnl of bioethanol and 18 bnl of BD, and contributed about 1.5% of the total global transportation fuel consumed (OECD/FAO 2010). This is expected to double (200 bnl) in 2019 with respectively, 159 and 41 bnl from bioethanol and BD. This increase has been linked to sustained higher prices of traditional liquid fuel such as petrodiesel, and the increasing support for alternative fuel due to its known advantages over petrodiesel (IEO 2010; OECD/FAO 2010). Another catalyst is the rising production and use of unconventional cars (e.g. hybrid and flex-fuel cars) which are capable of running on neat or blends of biofuels. On Sunday February 24th, 2008, Virgin Atlantic's Boeing 747 jet became the world's first commercial airline to fly on BD without redesigning its jet and engines (AP 2008). It flew on partially fuelled coconut and babassu oil BD from London's Heathrow to Amsterdam's Schiphol airport in one of its four main fuel tanks.

Many countries are in search of alternative energy, energy security, and sustainable and cleaner energy by taking advantage of their vast existing renewable natural resources (SDTC 2006; Pousa et al., 2007; Garofalo, 2007; Jobe, 2007; Hammond et al., 2008; OECD/FAO 2010). While Europe seems to be more inclined to reducing GHG, North America is more geared towards energy security (Garofalo, 2007; Jobe, 2007). The BD market is also very well developed in Europe. BD has been in commercial use as an alternative fuel since the 1980s in many European countries such as France, Austria, Germany, Sweden, Italy, Belgium, Hungary, and Czech Republic (Pousa et al., 2007; Amigun et al., 2008; Hammond et al., 2008). The EU is projected to be the largest global producer (50%) and consumer (60%) of BD in 2019 (OECD/FAO 2010). There has also been significant growth in the pursuit of alternative energy, and BD production these past few years in North America and Asia (Jobe, 2007; OECD/FAO 2010). Parts of Africa are seeing the shift too, and momentum is gathering in most countries, even those

with no specific national set targets (Amigun et al., 2008; OECD/FAO 2010; SNV 2010).

An influx of government subsidies and tax breaks as incentives to make BD competitive to petrodiesel, and tighter environmental standards are also shifting production forward (Henke et al., 2005; Bernard and Prieur, 2007; Hammond et al., 2008; OECD/FAO 2010). As indicated by Haas and Foglia (2005) and other researchers (Garofalo, 2007; Jie, 2007; Jobe, 2007), government policies will ultimately be a strong incentive for the development of biofuels. As such, the nature of the policy can also help develop one biofuel over the other and cause the demise of other types of biofuels. For instance, the pressure to grow certain crops like corn for bioethanol production over soybean for BD, or the higher tax incentive on vegetables oils over animal-based feedstocks for BD production (Henke et al., 2005; Adreani, 2007; Jie, 2007). Additionally, the introduction of government legislation in Canada to reduce sulfur levels in petrodiesel from 500 ppm to the current allowable level of 15 ppm might lead to the increase demand and incorporation of BD (SDTC 2006).

The EU for instance advocated for 5.75% biofuel as part of the overall transportation fuel mix by 2010, and the USA is calling for reduction in gasoline consumption by 20% by 2017 (Garofalo, 2007; Jobe, 2007). The transportation sector in Canada as a whole and Québec alone has been reported to contribute about 26% and 38% of the total GHG emissions, respectively (BIOBUS 2003). It is therefore of no coincidence that currently in Canada, transportation fuels are the only sector with specific target to reduce GHG emissions with the government's announcement of 5% (3.3 bnl/year) of biofuels by 2010 (BIOBUS 2003; SDTC 2006). This was expected to come from 2.8 bnl/year of bioethanol, and 0.5 bnl/year of BD (SDTC 2006).

In accordance with the federal government's mandate on renewable fuels, a few provinces most notably, Québec, adopted the use of BD (B5 and B20) made from 24% vegetable oil, 28% animal fat and 48% recycled cooking oil as a source of fuel supply for public transit in a project called BIOBUS (BIOBUS 2003). The project was to assess the viability of BD as part of routine operation of bus fleets, particularly in cold weather, and to reduce GHG emissions. The project, a first in North America involved 550 thousand litres of BD supplied to a fleet of 155 buses serving Montréal's downtown over a period of 1 year (March 2002-March 2003). At the end of the project, CO_2 emissions were

reduced by roughly 1300 tons. Despite the cloud point (CP) of the BD, and very cold weather where overnight temperatures sometimes dropped to between -20°C and -30°C, no specific complications were reported from using the BD blend.

2.1.2. Properties of Biodiesel (BD)

Biodiesel can be used unmodified and is distinguished from other biologically derived fuels such as straight vegetable oils or waste vegetable oils which are used in modified diesel engines (Knothe, 2005b). BD has received enormous attention because it is a readily available local derived resource, renewable, technically competitive with petrodiesel, and has environmental benefits (Knothe, 2005b; Akoh et al., 2007).

The properties of BD have been shown to approximate petrodiesel in terms of power, wear, and efficiency (Allen et al., 1999; Knothe, 2005b; Knothe and Steidley, 2005). Apart from its comparable fuel efficiency, BD has other numerous advantages and the notable ones include sulfur and CO_2 neutral, i.e. it burns cleaner with no net contribution of sulfur and CO_2 to the atmosphere (Wyatt et al., 2005; Bernard and Prieur, 2007; Hammond et al., 2008). Vehicles running on BD also emit less gaseous pollutants such as carbon monoxide (CO), aromatics, polycyclic aromatic hydrocarbons (PAHs), and partially burned or unburned hydrocarbon than petrodiesel. The generally lower CO emissions of BD compared to BD blends and petrodiesel has been attributed to the higher oxygen content of BD. CO emissions level thus decreases with increasing BD percentage in the blend (Behçet, 2011) i.e., B100 < B40 < B20. BD has a higher flash point making it safer to handle and store (Knothe, 2005b). Another advantage of BD is its excellent lubricity (Knothe, 2005b). New government legislation requires petrodiesel to have reduced sulfur content (<15 ppm) (SDTC 2006). Though sulfur *per se* is not a lubricant, the sulfur reduction process also reduces the fuel's naturally occurring lubricating agents and properties (Schumacher, 2005). BD has been proposed as an alternative additive to improve the lubricity of ultra-low sulfur diesel (ULSD) due to its superior lubricity; at 1-2% BD blend at least, the lubricity of petrodiesel is significantly improved (Schumacher, 2005). BD has thus also been registered as a fuel additive. Additionally, unlike bioethanol, BD can be transported in existing petrodiesel delivery lines and distribution facilities thus there is no need for new/additional infrastructure (Knothe and Steidley,

2005).

Despite the above mentioned attributes, BD has some undesirable characteristics and properties associated with low-temperature operation properties, oxidative stability, and exhaust emissions (Knothe, 2005a; Van Gerpen, 2005; Wyatt et al., 2005). The low CP of BD results in poor performance in cold weather with increased gaseous and particulate emission. Higher levels of nitrogen oxides (NOx), another identified GHG are also emitted during the combustion of BD. NOx emissions generally occur under opposite emissions analogy, i.e. increasing BD concentration increases NOx emissions. Even though increasing NOx emissions is tied to increasing BD concentration, combustion is improved in engines running on BD (Behçet, 2011). The ability of some microbes to survive in closed tanks, form colonies, and eventually feed on the BD especially in warmer temperatures has also been speculated (Meher et al., 2006). The probable presence of residual catalyst, alcohol, and bound and free glycerol are also undesirable features of BD (Knothe and Steidley, 2005; Fernando et al., 2007). BD is a solvent and thus can corrode the metal parts of a car and through which leaks may pass and eventually end up in water bodies (Meher et al., 2006). Various remedies have been suggested to ease these shortfalls of BD such as the use of antioxidants and additives to minimize oxidation and degradation, improve fuel stability and cold flow and low-temperature performance properties, and exhaust emissions (Behçet, 2011).

2.2. Biodiesel Feedstock: Current and Future Potentials

When Rudolf Christian Karl Diesel invented the diesel engine in the 1890s; he also coined the idea of using alternative/cheaper fuels to power the engine. He is quoted as saying; “the use of vegetable oils for engine fuels may seem insignificant today, but such oils may become, in the course of time, as important as petroleum and the coal tar products of the present time” (Knothe, 2005c). The use of oils from peanut and oil palm which grew in many then European colonies in Africa were suggested; to provide some degree of energy self-sufficiency to these colonies. Vegetable oils were also used as emergency fuel during WW II, and refined soybean oil as bunker fuel in Japan’s battle ship; Yamato (Knothe, 2005c). The use of these oils as fuel source is thus not a new idea.

Biodiesel is commonly produced using feedstocks from refined rapeseed (canola),

soybean, and oil palm (Demirbas, 2008; Brennan and Owende, 2010). The choice of BD feedstock differs around the world. It is primarily dependent on the geographical location of the producer, abundance, availability, and economies of production among others (Weber, 2009; OECD/FAO 2010). For instance, rapeseed oil is the preferred feedstock in Europe and Canada, while soybean and palm oil is preferred in the USA and the tropics, respectively (Lang et al., 2001; Demirbas, 2008).

It has been stated that the use of food crop for fuel will result in fuel and food industries competing for the same resources but at the detriment of food supply and prices (Brennan and Owende, 2010; OECD/FAO 2010). Even though only about 1% (14 million hectares) of the world's available arable land is used for biofuel production, providing about 1% of global transportation fuels (Brennan and Owende, 2010), it has also been predicted that food, water, and scarce arable land resources could be sources of conflict as governments scramble to face the realities of food supply, soaring population, and struggles with the number of the ever-increasing mouths to feed (Stein, 2007; Tilman et al., 2009). With BD demand predicted to stay strong, the concern and debate on food versus fuel competition are apparently both moral- and sustainability-based (Brennan and Owende, 2010; OECD/FAO 2010). Apart from the debate on the use of food crops for fuel, these feedstocks are expensive and have been estimated to account for a huge chunk of the total BD production cost (70-85%) (Haas and Foglia, 2005). The price gap between BD and petrodiesel can also be as large as 4-fold when petrodiesel prices are low (Demirbas, 2003; Haas and Foglia, 2005). Coupled with the cost of some downstream processes, the price of BD can be less competitive to petrodiesel (Veljković et al., 2006; Berchmans and Hirata, 2008; Oliveira et al., 2008).

An alternative approach is to expand the feedstock base to include biomass such as animal processing by-products which are currently available at low or no cost (Salameh, 2003; Steubing et al., 2011). Biomass; a term use to describe materials such as forestry and agricultural residues; is the oldest source of energy and an immense source of renewable energy. It has been estimated to contribute about 10-14% of the world's primary energy supply and as much as 30% of energy demand in developing countries (Salameh, 2003; OECD/FAO 2010; OECD/IEA 2010). Demands for alternative BD feedstock to include not only food crops but by-products are becoming increasingly

intense and crucial. The search also involves sustainable and environmental friendly potential sources of feedstocks. Feedstocks such tobacco oil, coffee oil, used frying oil, tallow, lard, and grease have been explored and used in BD production (Alcantara et al., 2000; Veljković et al., 2006; Berchmans and Hirata, 2008; Oliveira et al., 2008). The proposed increase in biofuel production in the US is expected to be met in part by BD with greater contribution from animal fats, grease, and other non-edible oil-bearing crops like camelina, *jatropha cacus*, recovered oil from distillers dry grains with solubles (DDGS)-a co-product from corn ethanol production, and algae (Duffy et al., 2009; Weber, 2009; Brennan and Owende, 2010). Canada's target of 0.5 bnl/y of BD by 2010 was expected to be generated from vegetable oils (33%) and rendered animal fat (66%) (SDTC 2006). In 2011, Rothsay® Biodiesel, Canada, produced 45 million litres of BD from rendered animal fat and recycled restaurant oil (Rothsay 2011).

The potential to add new and unconventional feedstocks is promising (Knothe, 2005b; Tilman et al., 2009). An example is microalgae; which have rapid generation rates and accumulate large amounts of fatty acids (FA) and hydrocarbons with no need or use of arable land (Cantrell et al., 2008; Duffy et al., 2009). Sludge; the residual organic matter from wastewater or sewage treatment, is also another feedstock being explored (Campbell et al., 2011; Siddiquee and Rohani, 2011). There are also new biotechnological advancements to increase crop yield and oil content without increasing acreage (Weber, 2009). In fact, more than 12 million, 430 million, and 1 billion USD is respectively spent by the Australian, Canadian, and USA governments to boost biofuel research and development (OECD/IEA 2010).

2.2.1. Fishery and Animal Waste as Potential Biodiesel Feedstock

2.2.1.1. Availability and Uses of Fishery and Animal Waste

Livestock, poultry and fisheries resources are important food industries (Thassitou and Arvanitoyannis, 2001; FAO 2010). The total global livestock and poultry production reached 21 billion in 2009; made up of 1.3 billion heads of cattle, 941 million heads of pigs, and 18 billion poultry birds (FAO 2010). Canada is a leading producer and exporter of these commodities, an important contributor to export trade and employment (FAO 2010). However, the production and processing of these resources generates significant

amount of waste and by-products that may have profound impact on human health and the environment (Meeker and Hamilton, 2006; Rustad, 2007).

The distinction or classification of processing by-product, waste or discards is often ambiguous (Guerard, 2007; Rustad, 2007; Gwyther et al., 2011). In animal processing, by-product refers to the remaining tissue after much of the muscle is removed or the secondary products obtained during the manufacturing of a principal commodity (Meeker and Hamilton, 2006). Processing discards are deem waste or by-product if the former cannot find use and often incinerated or dumped. However, if the discard can be transformed from its previous "waste" status into useful products, it can then be said to be a by-product.

According to Thassitou and Arvanitoyannis (2001), the meat, poultry, and fish industries produce the largest amount of discards within the food industry. Approximately 25 billion kg (25 MMT) and 2 billion kg (2 MMT) of by-products and on-farm mortalities are generated annually by the animal and meat production industries in the USA and Canada, respectively (Meeker and Hamilton, 2006). Only 50 to 72% of the live weight of livestock and poultry are used for human food (Thassitou and Arvanitoyannis 2001; Hamilton et al., 2006; Kalbasi-Ashtari et al., 2008). The remaining component (28 to 50%) may be converted by rendering, and incorporated in many applications and products such as animal feed, soaps, paints, cosmetics, lubricants, and pharmaceuticals (Hamilton et al., 2006; Kalbasi-Ashtari et al., 2008). However, a large amount of these by-products often exceed these traditional market demands or maybe deem unsuitable for food or feed. Where these recourses cannot be utilized, the most widely used routes are incineration, composting, burial, illegal dumping or just abandoned (Hamilton et al., 2006; Kalbasi-Ashtari et al., 2008; Gwyther et al., 2011). For instance, carcasses from perished diseased animals during outbreaks such as bovine spongiform encephalopathy (BSE) or through natural causes cannot be rendered for human consumption. The perceived risk of disease-causing pathogens entering the feed/food chain from their incomplete destruction is enforced by stringent public health and environmental safety regulations (Hamilton et al., 2006; Kalbasi-Ashtari et al., 2008; Gwyther et al., 2011). Regulatory agencies such as the Food and Agriculture Agency (FDA) of the USA have banned the feeding of ruminant materials back to ruminants

following the outbreak of BSE in 1997 (Meeker and Hamilton, 2006; Kalbasi-Ashtari et al., 2008). In fact, bovine composting may render the site unsuitable for grazing according to FDA laws.

Like animal production, fisheries and aquaculture resources have been an important constituent of human diet for generations. Approximately 19% of the 142 MMT of wild stock captured and aquaculture production in 2008 was not used for food (FAO 2010). Large amounts of by-catch, underutilized, unsalable, undesirable, unconventional or unexploited species are also often discarded and lost to landfills and incinerators. Additional source of discards are by-products; such as skin, head, viscera, liver, trimmings, frames and other solid waste generated during filleting, gutting, and other fish processing operations (Ekanayake et al., 2005; Nomura et al., 2005). With aquaculture production at an average annual growth rate of 6.2% from 38.9 MMT in 2003 to 52.5 MMT in 2008 (FAO 2010), concomitant increase in waste is inevitable. Rustad (2007) reported that 57% of the 0.85 MMT of fisheries resources in the UK were classified as waste. While the total volume, and composition of wastes are not always precisely known, it is estimated to range between 13-65% of the total live weight (Falch et al., 2006a) (Table 2.1) and varies with the type of fish and processing. The *ca* 44% by-products generated in lobster processing constitute the entire body after the tail and claw meat is removed. Approximately 33-50% and 60-65% of the total weight of salmon and cod, respectively, were described as discards (Gildberg et al., 2002; Falch et al., 2006a).

Species	**Production (ton)**	**Discards (ton)**	**By-product fraction (amount of total weight) (%)**
Salmon	1,025,000	>512,500	Cut off (15), viscera (13), head (10)
Cod	1,300,000	>858,000	Cut off (18-30), viscera (12-15), head (15-20)
Pollock	2,938,230	>1,000,000	Cut off (17), viscera (12-17), head (12-17)
Lobster	73,000	>31,974	Body (43.8)

Table 2.1: Selected fishery/aquaculture by-products by quantity and composition.

While the high susceptibility of fishery raw materials to lipid oxidation, enzymatic hydrolysis and changes in quality, sparsely situated fish processing plants, and the heterogeneity of waste generated may be reasons for discarding or low retail value;

the fisheries and aquaculture industries have traditionally been wasteful considering the large amount of untapped and unutilized resources such as proteins and lipids that are discarded (Jobling, 2002; Falch et al., 2006a; Rustad, 2007; FAO 2010). Current use of these resources and the dwindling stock of fishery reserves are unsustainable (Njinkoue et al., 2002; Falch et al., 2006a). A better understanding of these wastes, will allow the design of practical strategies to enhance their value and marketability as by-products. Some of these resources have been used in product development while others have been identified as potential biomaterials (Sathivel et al., 2004; Falch et al., 2006a) (Table 2.2).

Ingredient	**Tissue**	**Potential/Application**	**References**
Antioxidants	Shell skin	Food and pharmaceutical	Ekanayake et al., 2005
Chitosan and chitin	Frame, shells	Food, cosmetics, water purification, nutraceuticals-glucosamine, antimicrobial, fertilizers	Simpson et al., 1997; Kim and Mendis, 2006
Collagen	Skin, bones, scales, cartilage	Pharmaceutical, medicinal, food, cosmetics	Ogawa et al., 2004; Nomura et al., 2005
Enzymes	Viscera, waste water from fish and shellfish	Cheese making, deskinning, herring fermentation, medical, food, roe processing, meat tenderization	Tschersich and Choudhury, 1998; Kuraishi et al., 2001
Minerals	Bones, shells, frame	Nutraceuticals, fish processing (degradation of fish bone)	Jung et al., 2005a
Oil	Skin, muscle, waste stream	Nutraceutical, cosmetics, food, dietary supplement, biofuel	Garcia-Sanda et al., 2003; Njinkoue et al., 2002.
Peptide	Gonad, head, muscle	Flavor, fish soup, fish paste, protein hydrolysate	Sathivel et al., 2004
Pigments	exoskeleton of crustacean, skins	Nutraceuticals, food (colorants, feed and supplement), cosmetics	Benjakul et al., 2005
Protein/nucleic acid/	Roe, backbone, skin, head, milt, viscera	Protein concentrate, food (fish sauce, surimi-based products, texture-enhancer), silage, immune-enhancers, collagen, gelatin	Bledsoe et al., 2003; Gildberg et al., 2002

Table 2.2: Alternative and potential uses of fishery waste.

For instance, interest in fish oil stems from its unusual characteristics which differ significantly from other oils, and its suitability in many applications such as cosmetics, food, and pharmaceuticals (Njinkoue et al., 2002; Garcia-Sanda et al., 2003) (Table 2.2). Fish oils are known to generally contain high amount of polyunsaturated fatty acids (PUFA) especially the ω-3 FAs and its long chain metabolites particularly eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3). There is a large scientific literature supporting their role in human health, nutrition, and disease prevention (Njinkoue et al., 2002; Zuta et al., 2003).

2.2.1.2. Current Uses of Fish Oils and Animals Fats

Like fish oils, the global statistics on rendered beef, pork, and poultry fats produced or used are scanty. An estimated 4.4 MMT of fat and grease is produced annually by USA rendering industries from animal by-products, on-farm mortalities (Fig. 2.1), and used cooking oil (Meeker and Hamilton, 2006).

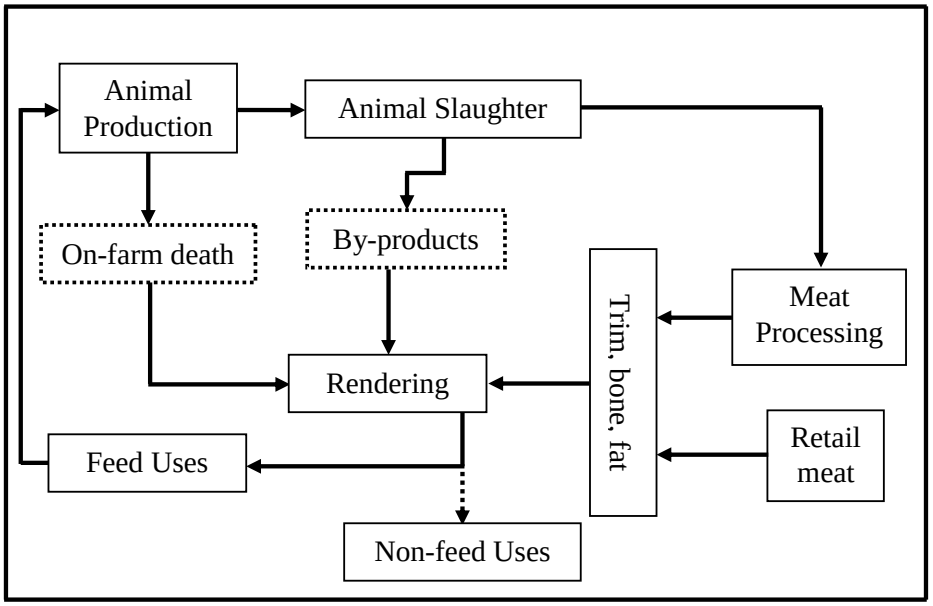


Fig. 2.1: Interrelationships of rendering with animal production and processing. (Hamilton et al., 2006).

Inedible tallow contributes the largest proportion of 1.8 MMT, followed by grease, edible tallow, poultry fat, and lard at 1.2, 0.8, 0.5, and 0.1 MMT, respectively. Approximately 39% of rendered animal fat and protein produced enter international

trade, generating about $1 million each from proteinaceous feed ingredients, and rendered fats and grease (Hamilton et al., 2006). Animal fats are a relatively inexpensive lipid source, with tallow widely used in non-food applications (Wyatt et al., 2005).

Over the years, fish from target catch as well as processing waste have been recovered and converted to fishmeal and oil for use primarily in aquaculture, poultry, and livestock feed formulation, and for human consumption (Swisher et al., 2006; Kerry and Murphy, 2007; Shahidi, 2007) (Fig. 2.2). Approximately 27.2 MMT or 19% of global fishery production was used to produce fish oil and meal (FAO 2010). Projected growth in fish oil is based on the growing international market to meet the increasing demand from aquaculture and the emergence of new markets such as pet food (Swisher et al., 2006). For instance, the use of fish oil in aquaculture feeds grew nearly threefold (0.23 to 0.78 MMT) between 1992 and 2006 (FAO 2008). The market value also determines if some fish species are profitable if processed for human consumption or as fishmeal and oil. For instance, it is economically wiser to convert pelagic species such as capelin and blue whiting to fish oil and meal than for direct human consumption.

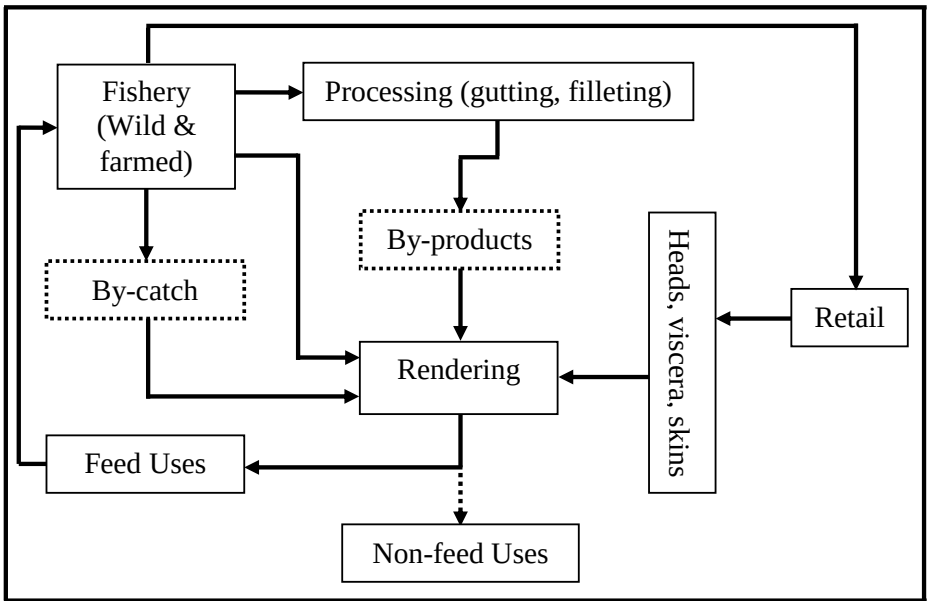


Fig. 2.2: Interrelationships of rendering with fisheries production and processing.

2.2.1.3. Fish Oils and Animals Fats for Biodiesel

For fishery and animal production and processing industries looking at exploring

new technologies and markets to propel their resources beyond feed ingredients and other traditional products; the use or increase use of recovered oil from these resources for BD production has been suggested (Cantrell et al., 2008; Geller et al., 2008; Weber, 2009; Steubing et al., 2011). Cantrell et al. (2008) reviewed various biological and thermochemical approaches to convert livestock waste to bioenergy for heat, power, and as transportation fuel. This approach will also contribute to the additional feedstock needed to meet set targets, broadened feedstock base, relieve the perceived burden on food crops for biofuel production, while contributing to the development of sustainable feedstocks (Swisher et al., 2006; Weber, 2009; Steubing et al., 2011). Additionally, this will also alleviate the pressure of producing BD feedstock on arable land, support local development, and contribute to other policy agendas such as cutting the need for more landfills. The use of these resources is not only a great potential to ensure their full utilization, but also expand the usefulness of processing by-products in new and unconventional bioprocesses.

The contribution of vegetable oils in global BD production is projected to decrease from almost 90% to about 75% by 2019 (OECD/FAO 2010). This is mainly due to the development and availability of alternative non-edible oil bearing crops such as *jatropha curcas*, animal fat, and biomass (second generation) feedstocks. Various workers are analyzing the availability of domestic feedstock that could be used to meet increasing demands of BD without affecting their traditional/existing uses. Records show the availability of fats and oils sources to meet demand without forestalling traditional demands. There is a potential of producing *ca* 964 million gallons (4.4 bnl) of BD from the animal fat and grease rendered in the USA (Weber, 2009) and this accounted for the increase in BD production in 2008.

While availability, demand, and use of fish oils as alternative BD feedstock may not match or be as popular as vegetable oils or animal fats, there are reports even if limited of both laboratory and commercial scale BD production from fish oils (ONC 2004; Reyes and Sepúlveda, 2006; WHH 2007; USDA 2009; Fan et al., 2010; Behçet, 2011). In Canada, residual fish oil from ω-3 supplement production is converted by Ocean Nutrition and Wilson's fuels to FAME and FAEE, respectively and used internally in a boiler or sold as home heating oil, and fuel for cars (ONC 2004; WHH 2007).

Opened in 2006, Aquafinca, a tilapia farm in Honduras converts oil recovered from tilapia waste to BD, producing about 1.4 million litres of BD annually (USDA 2009). Andersen and Weinbach (2010) also reported the estimated potential of BD production from residual animal and fish oil in Norway. Of the 8.2, 24, and 100 kilo tonnes (kt) of oil recovered from trap grease, slaughterhouse and poultry, and fish by-products, there is a potential to produce 6.4, 22.1, and 92 kt of BD, respectively, based on 95% yield estimate. Results of ongoing investigations on fish oil or rendered fat-derived BD approximating properties and performance of petrodiesel also looks promising (Fan et al., 2010).

Even though the role of fish oil in food, industrial, and pharmaceutical applications may become limited due to their increasing demand in aquaculture feeds, (FAO 2008), and the sparseness and remoteness of processing plants make large and continuous collection difficult, and central processing, often impractical or costly (Swisher, 2006). The undesirable, unexploited or unutilized resources from both the fishery and animal industries still represent an abundant resource for the production of biomaterial and now bioenergy (Swisher et al., 2006; Andersen and Weinbach, 2010). It is therefore of great interest for the animal, and fishing industry to utilize all for high-value products.

2.2.2.1. Overview on Fish and Animal Waste Rendering

Waste and animal mortalities are an inevitable consequence of fish and animal production and processing (Kim and Mendis, 2006; Kalbasi-Ashtari et al., 2008; Gwyther et al., 2011). While waste from production and processing cannot be entirely eliminated, the next best option involves the conversion of these perishable materials to valuable ingredients through rendering (Figs. 2.1 and 2.2). The main foci of rendering therefore are value-adding and conforming to regulatory provisions. According to Hamilton et al. (2006), rendering is the optimal and best available control technology for addressing the handling of animal waste, mortalities, and used cooking oils.

Fishery and animal waste, by-catches, and mortalities may be, for a number of stated reasons; be composted, buried, incinerated, illegally dumped or just abandoned (Hamilton et al., 2006; Gwyther et al., 2011). These approaches may however produce

odor, attract flies, birds, and rodents. Incineration can be expensive considering the cost of fossil fuel used. Like disposal in landfills, composting also takes up land space. Rendering on the other hand can efficiently be used to manage animal mortalities and processing waste to produce high-value resources, and lower the amount and cost of waste disposal both to human health and the environment (Hamilton et al., 2006; Kalbasi-Ashtari et al., 2008; Gwyther et al., 2011).

Rendering these resources to provide ingredients for the feed and oleochemical industries also minimizes the need to use marginal farm lands to provide these inputs (Meeker and Hamilton, 2006). As already stated, majority of the raw materials for rendering originate from slaughterhouses, entire condemned animal carcasses; by-catches, fatty tissue, offal, and trimmings from the butchery, as well as viscera, head, skin, and muscle of fish. The raw materials for production of fish oil are usually small, oily and bony, which may be edible, inedible or not required for human consumption. Examples include; menhaden, anchovy, herring, and mackerel (in lesser quantities), (Meeker and Hamilton, 2006).

The primary products of rendering include fats and oils, meat and bone meal, meat meal, hydrolyzed feather meal, blood meal, and fish meal. These are incorporated into animal feed as proteins and energy sources, and also used by the oleochemical industries (Hamilton et al., 2006; Swisher et al., 2006). Furthermore, studies indicate that rendered fat from BSE-infected animals present no risk to human when used as feedstock in BD production (Haas and Foglia, 2005), because the infectious proteins (prions) of BSE partition to the meat and bones and not to the lipid fraction during rendering.

2.2.2.1.1. Lipid Extraction Methods

Oils have been extracted using several methods such as thermal, non-thermal, physical or mechanical, chemical, or a combination of these (Chantachum et al., 2000; Guderjan et al., 2007; Perretti et al., 2007). The choice between the methods is dependent on several factors such as; type and fat content of the sample, cost of supplies or the safety of the technique to humans and the environment. The quantity of oil generated by rendering is directly related to the species processed and the degree of further processing that is associated with marketing the product (Falch et al., 2006b). Moreover, for fish and

fishery samples, oil yield and composition are also dependent on feeding habits, spawning cycle, season, and water temperature of habitat, and the portion (skin, head or muscle), or type of tissue of the sample used (Jørgensen et al., 1997; Jobling et al., 2002; Falch et al., 2006b). An estimated 3:1 portion of fishmeal and oil, respectively, are obtained during production, representing 1/6 to 1/8 of the total weight of the original material (Ockerman and Hansen, 2000). Nevertheless, the influence of extraction time, and temperature, solvent type and sample pretreatment are important process parameters (Kalbasi-Ashtari et al., 2008; Sayyar et al., 2009).

During rendering, there is quantitative separation of a substance or group of substances from a mixture of materials. The raw materials are crushed into pieces of uniform size, heated with or without added steam and then separated into fat, proteinaceous material, and water (Meeker and Hamilton, 2006; Kalbasi-Ashtari et al., 2008). The traditional method of fish oil production is the continuous wet reduction process which involves; cooking, pressing, and centrifugation (Chantachum et al., 2000; Iverson et al., 2001). The rendering process may be batch or continuous using wet or dry methods. The choice of wet or dry method is dependent on the type of raw material, production temperature, and the intended use of the rendered lipid (Meeker and Hamilton, 2006; Kalbasi-Ashtari et al., 2008).

In wet rendering, steam is added to the material to cause fat to rise to the top, while the raw material is dehydrated to release fat in the dry method. After a few hours, the fat is decanted, centrifuged, and separated from the liquid (water), and solids. The cracklings "solids"/cake maybe further pressed, dried, and incorporated in food or feed. Tallow and lard is produced from the fatty tissue of cattle and pigs, respectively, while chicken fat is from the skin and trimmings of chicken. These fats are further categorized as edible or inedible. Inedible rendered fat is produced from viscera, non-edible parts, and other by-products of animal processing. Extracted oil from fishery resource are collectively called fish oil or prefixed with the name of the fish species and/tissue, for instance cod liver oil. Rendered used-frying oil from restaurants is called yellow grease.

Many solvents have also been used in lipid extraction (Folch et al., 1957; Bligh and Dyer 1959; Sayyar et al., 2009). These include alcohols, chlorinated hydrocarbons, and ethers. The solubility characteristics of these various solvents have been shown to

significantly affect yield (Sayyar et al., 2009). Some solvents have been found to be ineffective, while others are disallowed because of health and environmental concerns (Reuber, 1979; Radin, 1981). The classical solvent extraction methods of Folch et al. (1957), and Bligh and Dyer (1959), with polar and non-polar solvents have been extensively employed in lipid extraction. The Bligh and Dyer method for instance employs relatively polar solvents system and may account for the low solubility and extraction of triacylglycerols (TAGs) (Iverson, 2001). TAGs are soluble in non-polar solvents and their solubility decreases with increasing polar solvent content (Smedes and Thomasen, 1996). The chloroform and methanol extraction mixture used in these classical methods offers additional undesirable features of dissolving appreciable amounts of non-lipids and even proteins (Radin, 1981). Furthermore, there is growing health and environmental concerns over the use of chlorinated solvents, a suspected carcinogenic agent (Reuber, 1979; Radin, 1981). Radin (1981) described a method that uses hexane and iso-propanol as the extraction solvents. The Soxhlet technique is a commonly used solvent extraction system, while Edward Randall's Soxtec is a modification of the Soxhlet technique. The main difference between the two techniques (Soxtec and Soxhlet) is that the solubilization of extractable components is performed by a cold solvent dropping from a reflux condenser into the thimble (containing the sample) in the latter, while a faster solubilization is achieved by the direct immersion of the thimble (containing the sample) in the hot solvent in the former resulting in a marked reduction in extraction time.

Other novel extraction methods such as supercritical fluid extraction (SFE), pulsed electric fields (PEF), and enzyme assisted extraction (EAE) (Guerard, 2007; Perretti et al., 2007) have been used to improve oil recovery without compromising quality, and to increase their industrial applications. SFE employs the dissolving power of fluids such as CO_2 and water at temperatures and pressures above their critical values (Perretti et al., 2007). Though SFE is expensive, the conventional extraction methods are usually fraught with solvent and thermal degradation of labile essential components. In addition to the use of flammable and toxic solvents and its associated health and environmental implications, extraction durations are longer with limited recovery efficiencies, and the extraction of undesirable components (Perretti et al., 2007).

The PEF and EAE techniques are non-thermal methods which share similar advantages with SFE. PEF was previously known as electroporation and was originally used to extract biomolecules (Guderjan et al., 2007; Min et al., 2007). It involves the application of an external electric field to induce critical electrical potential across the cell membrane and the formation or enlargement of existing, transient or permanent pores on the cell membrane (Fincan and Dejmek, 2002). Electroporation increases the permeability of the membrane to either intrusion of surrounding media or diffusion of cell content. PEF is widely used in microbial inactivation, pasteurization of milk, and sterilization of fruit juices (Praporscic et al., 2007), and has been used in extraction or as a pretreatment step of materials to increase extraction efficiency (Guderjan et al., 2007). The main limitation to its use is energy cost. Like PEF, EAE is environmentally friendly because it avoids the use of organic solvents and high temperatures and thus protects labile components (Gbogouri et al., 2006; Guerard, 2007). EAE either uses a single protease or a combination of proteolytic enzymes to hydrolyze the subcellular structure of oil bearing matrices (Linder et al., 2005; Abdulkarim et al., 2006; Guerard, 2007). EAE has also been used to increase yields in fish oil extraction as well as concentrate ω-3 FAs (Linder et al., 2005; Guerard, 2007). The main drawback is the cost of the enzyme, long extraction time, and lower oil yields in aqueous media (Zhang et al., 2007).

2.2.2.1.2. Lipid Feedstock Quality Characterization

Oil characterization provides indispensable information about the oil, and essential quality traits using a variety of indices. The indices most relevant to BD feedstocks and the transesterification reaction include free fatty acid (FFA), and moisture contents, and FA composition. These indices have been found to influence fuel quality, performance, emission, and engine durability (Berchmans and Hirata, 2008; Demirbas, 2009; Fjerbaek et al., 2009; Behçet, 2011). Knowledge of the composition, chemical and physical characteristics of the oil affords the application of appropriate conversion processes. Additionally, monitoring feedstock stability during storage provides useful information on changes in quality which might require modifications to the BD production process design (Berchmans and Hirata, 2008; Fan et al., 2010).

Fats and oils are broadly termed lipids and subdivided into polar and neutral or

non-polar lipid classes (Riegel, 2005). Polar lipids include FAs which are generally soluble in water and other polar solvents, while non-polar lipids such as TAGs are insoluble in water but soluble in organic solvents. The main constituent of animal fats and vegetable oils is TAG (Knothe, 2005b; Holčapek et al., 2005). In fish lipids, TAGs occur as storage fat in hydrophobic aggregates (Aursand et al., 1994; Jobling, 2002). There is however substantial variation in lipid composition and quality, and this is apparent among and within species depending on feed, sex, age, environment, and season (Jobling, 2002; Njinkoue et al., 2002), and account for the limited uniformity, and heterogeneity of crude fish oils compared to crude vegetable oils. Different fish species also utilize different energy sources during different stages of their life cycle. For instance, during starvation, energy depots in the form of lipids and protein are utilized, thus depending on the period and season the amounts of these components are affected or maybe depleted. TAGs are glycerol esterified with one, two, or three FAs, and designated monoacylglycerol (MAG), diacylglycerol (DAG), and TAG, respectively. Decreasing number of fatty esters on the glycerol backbone increases polarity, thus; TAG < DAG < MAG. In nature, different FAs with various acyl chains length, orientation, degree of saturation, position or number of double bonds may be attached to the glycerol backbone, and these impart different physical and chemical characteristics on the lipid. Based on the degree of saturation, FAs are categorized as; saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) or polyunsaturated fatty acids (PUFA) if only single bonds, one double bond, and >1 double bond are respectively present.

The FFA content is an important quality indicator as well as a useful parameter in the method of refining, and choice of catalyst in the transesterification reaction (Berchmans and Hirata, 2008). Non-food or unrefined lipids used in BD transesterification reactions usually contain high amounts of FFAs, phospholipids, sterols, moisture, and some other impurities (Veljković et al., 2006; Di Serio et al., 2008), and as a result of the contribution of other components such as amino acids and oxidized lipids to acidity (Zhou and Ackman, 1996). In base-catalyzed BD production, the presence of <0.5% FFA in the feedstock leads to the consumption and reduction in the effectiveness of the catalyst, and increases the formation of soaps and emulsions (Freedman et al., 1984; Canakci and Van Gerpen, 2001) (Eq. 2.1).

$$\underset{\text{(FFA)}}{\text{R-COOH}} + \text{NaOH} \rightarrow \underset{\text{(Soap)}}{\text{R-COONa}} + \text{H}_2\text{O}$$

Eq. 2.1: Soap formation in high free fatty acid feedstock.

Soaps are difficult, time consuming, and costly to separate from the product, and result in low conversion and BD yield (Berchmans and Hirata, 2008). An additional pretreatment step with an acid catalyst to convert the FFA to esters, and reduce the FFA content (Eq. 2.2) is usually undertaken in such oils (Canakci and Van Gerpen, 2001).

$$\underset{\text{(FFA)}}{\text{R-COOH}} + \underset{\text{(Methanol)}}{\text{CH}_3\text{OH}} \rightarrow \underset{\text{(FAME)}}{\text{R-COOCH}_3} + \text{H}_2\text{O}$$

Eq. 2.2: Acid-catalyzed pretreatment of high free fatty acid feedstock.

FFA content is quantitatively determined by titration according to the official method of the American Oil Chemists' Society (AOCS [Ca 5a-40]) and other newer techniques such as Fourier transform infrared (FTIR) spectroscopy (Al-Alawi et al., 2004; Aryee et al., 2009).

Another important quality index in BD transesterification reactions is the moisture content (Freedman et al., 1984; Fjerbaek et al., 2009) and it is believed to pose a greater negative effect than FFA in conventional BD production. In addition to increasing the FFA content of the feedstock which forms soaps subsequently, hydrolysis of the formed alkyl esters (FAME) to FFA can occur (Eq. 2.3). The presence of hydrous reactants will thus require an additional acid catalyst to neutralize the FFA produced (Eq. 2.2). It is thus imperative to ensure that the reaction system remains anhydrous for efficient catalysis and smoother downstream processing.

$$\underset{\text{(FAME)}}{\text{R-COOCH}_3} + \text{H}_2\text{O} \rightarrow \underset{\text{(FFA)}}{\text{R-COOH}} + \underset{\text{(Methanol)}}{\text{CH}_3\text{OH}}$$

Eq. 2.3: Hydrolysis of formed alkyl esters to free fatty acid.

The absence of sufficient moisture or the presence of high moisture content in the feedstock have also been linked to low activity and product yield in lipase-catalyzed BD transesterification reactions (Kaieda et al., 2001; Noureddini et al., 2005). In lipase-

catalyzed transesterification reactions, a small amount of water is needed to maintain conformation and activate the enzyme (Noureddini et al., 2005; Li et al., 2006), while excess water on the other hand promotes the competing hydrolysis reaction resulting in increased FFA content.

Another lipid characterization index of importance in BD production is the FA composition. The characteristics of the resulting BD can be deduced from the FA profile of the parent oil (Dunn, 2005; Fernando et al., 2007; Behçet, 2011). Various workers have shown the correlation between unsaturated FA and fuel stability, low-temperature operations, as well as engine performance (Wyatt et al., 2005). Compared to vegetable oils, animal fats generally contain higher proportion of SFA. For instance, the composition of beef tallow and pork lard is typically *ca* 40% SFA, 30% in chicken fat, and 14% and 6% in soybean and canola oils, respectively. While animal fat-derived BD generally exhibit poorer cold flow properties compared to vegetable oil-derived BD, they have comparable or better lubricity and oxidative stability due to their high SFA content (Ramos et al., 2008). BD derived from feedstocks with high SFA were reported to reduce NOx emissions when used either as pure BD or as blends at 20% (B20) (Wyatt et al., 2005; Behçet, 2011). The high proportion of PUFA in fish oils on the other hand raises its propensity to oxidation and affects other fuel-related properties (Fan et al., 2010).

2.3. Biodiesel Production and Process Variables

The main objective for the modification of vegetable oils or animal fats to BD is to reduce viscosity, impart better fuel-related properties to meet engine requirements, fuel test standards and manufacturer's warranty (Knothe and Steidley, 2005; Wyatt et al., 2005; Meher et al., 2006). The viscosities of vegetable oils and animal fats are several orders of magnitude (10-20 times) greater than that of petrodiesel (Srivastava and Prasad, 2000; Knothe and Steidley, 2005). Direct use of these oils as fuel may compromise injector function and cause other engine-related problems. These include; poor fuel atomization upon injection, poor oil-air mixing leading to incomplete combustion and carbon deposits (ring carbonization) (Dunn, 2005; Meher et al., 2006). Others include; choking of the fuel injectors, accumulation of fuel in the lubricating oil, and oxidation and polymerization of the oil during storage. Several strategies proposed to reduce

viscosity and increase volatility such as engine modification, and preheating of the oil have been argued to be impractical (Fukuda et al., 2001; Dunn, 2005).

Four practical approaches namely; dilution with petrodiesel, pyrolysis, microemulsification, and transesterification have been suggested (Freedman et al., 1984; Srivastava and Prasad, 2000; Cantrell et al., 2008). Dilution of oil with petrodiesel is not encouraged for long term use as the propensity for injector choking, carbon build-up, and subsequent engine failure is high (Dunn, 2005). Pyrolysis; a thermochemical decomposition process which involves the use of high temperatures of about 500^{o}C in the absence of oxygen, have been shown to produce modified oils which contain similar amount of sulfur as petrodiesel (Ma and Hanna, 1999), trampling one of the objectives of reducing these compounds linked to GHG. The third approach involves the addition of aliphatic compounds to disperse, dilute, and increase the solubility of the oil-alcohol mixture (Dunn, 2005). Microemulsions can remain at a single phase without stirring, and do not require a catalyst during production. Although microemulsions are cheaper owing to the simplicity of production; mixing oils with alcohol, like oil diluted with petrodiesel, is not recommended for long term use due to limited durability (Dunn, 2005). Of the approaches listed, transesterification is the most widely suggested and used, and has been the subject of numerous studies (Freedman et al., 1984; Meher et al., 2006).

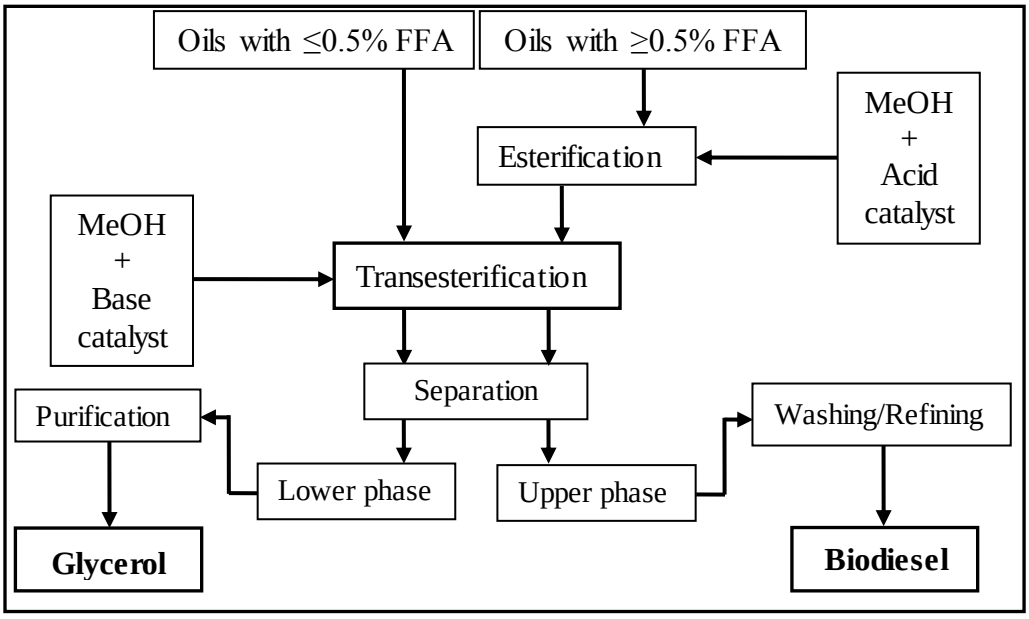
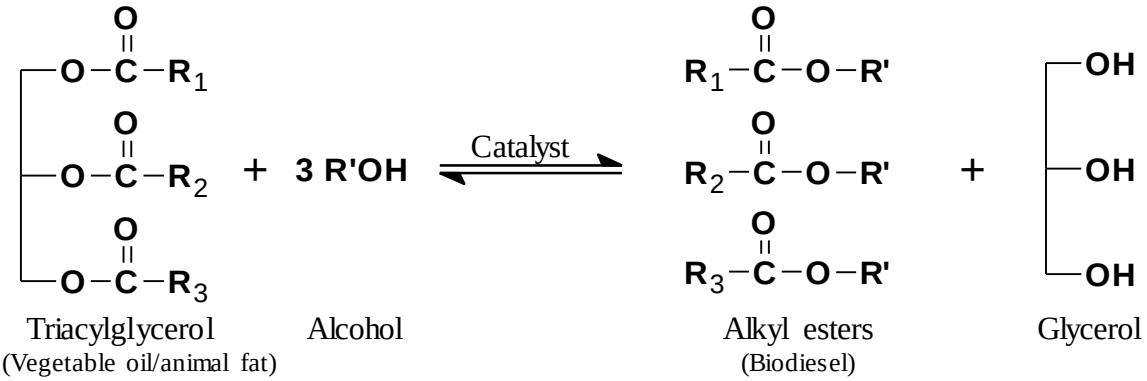


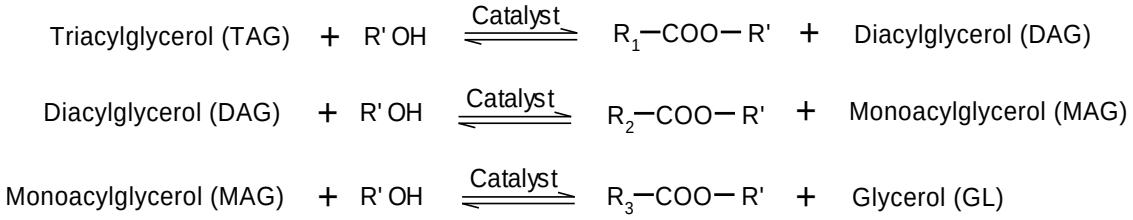
Fig. 2.3: Conventional biodiesel production scheme.

Depending on the quality of the oil, conventional BD production can follow one of the routes in Fig. 2.3 (Freedman et al., 1984). The resulting modified oil; BD, is less viscous with improved fuel-related properties such as higher cetane number, lower pour, cloud and flash points, and good long-term performance in diesel engines (Srivastava and Prasad, 2000; Lang et al., 2001). The transesterification reaction involve the displacement of an alkyl moiety between an alkyl glycerol and an alcohol in the presence of a catalyst (acid, base or lipase) to produce the corresponding FAAE found in the parent oil, and glycerol (Eq. 2.4); this reaction can also be termed alcoholysis. A complete conversion of one mole of TAG will yield three moles of FAAE and a mole of free glycerol.



Eq. 2.4: Transesterification reaction scheme.

The transesterification reaction has been explained to occur sequentially in three-steps with the production of FAAE (R-COOR) in each step, DAG and MAG as intermediates, and free glycerol in the last step (Eq. 2.5).



Eq. 2.5: Transesterification reaction scheme showing the three sequential steps.

The transesterification reaction forms a biphasic system of alcohol and oil, and glycerol and FAAE, not miscible at the beginning and end of the reaction, respectively. The immiscibility of the reactants account for the initial lag, however the solubility of

methanol increases in the course of the reaction with the formation of BD (Freedman et al., 1984). Although in principle the reaction is reversible, the backward reaction, i.e., conversion of BD and glycerol to reactants does not occur or is negligible if it occurs, due to the immiscibility of the products.

At the end of the reaction the upper phase consisting of FAAE is separated from the lower phase by gravity or centrifugation (Fig. 2.3). The lower phase consist of glycerol, unreacted alcohol, remaining catalyst together with the soaps formed during the reaction, some retained BD, and partial glycerides (MAG and DAG). FAAE is first neutralized with an acid to minimize the amount of water needed for washing, and formation of emulsions. The acid also splits the soaps into salts and FFA (Eq. 2.6).

$$\underset{\text{(Soap)}}{\text{R-COONa}} + \underset{\text{(Acid)}}{\text{HAc}} \rightarrow \underset{\text{(FFA)}}{\text{R-COOH}} + \underset{\text{(Salt)}}{\text{NaAc}}$$

Eq. 2.6: Reaction of soaps with acid to form free fatty acid and soluble salts.

Washing removes the remaining catalyst and alcohol together with the salts, soaps, and free glycerol in the FAAE. However this procedure may also leave residual acid in the fuel that may exceed the allowable amount in the fuel. The bulk of the alcohol is removed from the FAAE before washing. The FAAE is then dried and ready for use.

The free glycerol co-product account for 10% of the total BD reaction yield (Berchmans and Hirata, 2008; Adhikari et al., 2008). As stated above, crude glycerol also contains the remaining alcohol, catalyst, and formed soaps. Like the fossil fuel refinery, substantial amount of profit can be generated from by-products for many industrial applications after purification. Glycerol is treated with acid to split the soaps, the resulting FFA separates on top, the salts may precipitate out, and the remaining alcohol is removed. The purified glycerol can be used to produce hydrogen to be sold as fuel (Adhikari et al., 2008), and the CO_2 can be sold to fizzy drink producing companies (Eq. 2.7), among other potentials. Alternatively, the CO_2 and H_2 can be used to produce methanol or ethanol to sustain BD production.

$$C_3H_8O_3 + 3H_2O \rightarrow 3CO_2 + 7H_2$$

Eq. 2.7: Conversion of glycerol to carbon dioxide and hydrogen.

The production, yield, and recovery of BD and glycerol have been shown by several studies to be influenced by various reaction parameters such as alcohol type, oil:alcohol molar ratio, catalyst type and concentration, reaction temperature and time, mixing intensity, moisture and FFA content as well as their interactions (Freedman et al., 1984; Srivastava and Prasad, 2000; Meher et al., 2006; Marchetti et al., 2007).

2.3.1. Alcohol

TAG stoichiometrically reacts with alcohol at a ratio of 1:3 in BD transesterification reactions. However in practice, alcohol in excess of 1:6 is needed to drive the equilibrium to the right for higher BD yield (Freedman et al., 1984). Excess alcohol molar ratio can also complicate BD purification (Freedman et al., 1984; Tamalampudi et al., 2008). Apart from imparting characteristic properties on the resulting fuel, the fuel takes on the name of the alcohol as well, to distinguish it from BD produced with other alcohols, such as FAEE or FAME when ethanol or methanol is respectively used. Methanol is the conventional alcohol in commercial BD production in most countries due to its relatively lower price (Shimada et al., 2002; Samukawa et al., 2000; Noureddini et al., 2005; Li et al., 2006; Tamalampudi et al., 2008). Existing regulations also require BD to be made from methanol (Srivastava and Prasad, 2000; Tamalampudi et al., 2008). Ethanol has a higher dissolving power than methanol thus transesterification reactions with ethanol can proceed in solvent-free systems. Ethyl esters have also shown interesting advantages over methyl esters. The main advantage of FAEE over FAME is the extra carbon atom in the ethyl esters. The extra carbon in the fuel increases the heating value, FP, and CN, but lowers the PP and CP (Encinar et al., 2002). FAEE is however less reactive and volatile compared to FAME. Long chain alcohols such as butanol, iso-propanol, and 2-methyl-1-propanol have also been used (Iso et al., 2001; Xu et al., 2004), producing BD with increased freezing point (Knothe and Steidley, 2005). However, their high prices make them unattractive for commercial use (Alcantara et al., 2000; Haas and Foglia, 2005). Apart from the price of these alcohols, considerably higher alcohol:oil molar ratios and temperatures are needed for the preparation of their esters.

In lipase-catalyzed BD production, the choice and amount of alcohol may also alter the catalytic efficiency and stability of lipase, and properties of the BD produced

(Noureddini et al., 2005; Yasmin et al., 2006). Methanol is insoluble in oil and exists as droplets, upon contact with lipase, the propensity to deactivate the enzyme increases and increases with its increasing concentration in the reaction media. Excess amount of methanol have also been described to suppress catalytic efficiency and transesterification rates (Samukawa et al., 2000). Negligible amounts of BD were recorded when methanol was used in some lipase-catalyzed transesterification reactions (Iso et al., 2001; Li et al., 2006). The effect of alcohol type on lipase and FAEE yield was evident in several studies (Abigor et al., 2000; Du et al., 2004; Modi et al., 2007). For instance, Abigor et al. (2000) reported that the yield of alkyl esters from the transesterification of palm kernel oil with PS30 lipase resulted in; 72, 62, 42, 42, 24, and 15% FAAE with ethanol, t-butanol, 1-butanol, n-propanol, iso-propanol, and methanol, respectively. Sixty seven and 65 mol% of FAME and FAEE were respectively produced from soybean oil when 7.5 and 15.25 molar ratios of methanol and ethanol were used (Noureddini et al., 2005).

2.3.2. Catalysts

The transesterification reaction can be catalyzed by an acid, base, lipase or catalyst-free with supercritical fluids (SCF) (Freedman et al., 1984; Chongkhong et al., 2007; Marchetti et al., 2007; Demirbas, 2008). Acids and bases are usually homogenous after solubilizing in the alcohol and before the addition of the oil, while lipases are often immobilized on supports such as resins, thus forming a heterogeneous mixture with the reactants.

Variable	Base	Acid	Lipase	SCF
Reaction temperature	60-70°C	55-80°C	30-40°C	239-385°C
FFA in raw material	Saponified product	Esters	FAAE	FAAE
Moisture in raw material	Interference with reaction	Interference with reaction	No influence	-
Yield of FAAE	High	High	Low	Good
Recovery of glycerol	Difficult	Difficult	Easy	-
Purification of FAAE	Repeated washing	Repeated washing	Minimal	-
Cost of catalyst	Cheap	Cheap	Expensive	Medium

Table 2.3: Comparison of catalysts for biodiesel production (Marchetti et al., 2007).

The FFA and moisture contents of the oil or fat are the main reaction variables which directly affect the choice of catalyst (Canakci and Van Gerpen, 2001; Marchetti et al., 2007). As listed in Table 2.3, each catalyst type has its own merits and drawbacks on the BD production process. Chemical catalysis is also faster compared to enzyme-catalyzed reactions (Marchetti et al., 2007). The difficulty with downstream purification is a major drawback of chemical catalysis.

2.3.2.1. Base Catalysts

Base catalysts are employed in industrial applications because of their higher reaction rates and shorter reaction time (Freedman et al., 1984; Schwab et al., 1987; Alcantara et al., 2000; Oliveira et al., 2008). Alkali metal alkoxides as well as their carbonates have been used in BD production (Schwab et al., 1987; Vicente et al., 2004; Marchetti et al., 2007). Base catalyst such as the hydroxides of sodium and potassium (NaOH and KOH), especially NaOH has been widely used (Karmee and Chadha, 2005; Arzamendi et al., 2006) due to its lower cost and high solubility in methanol. The hydroxide ions react with methanol to form methoxide anions, which are considered the active species, which then react with TAG to form FAME. Base catalysts are normally used in the concentration range of 0.5-1% (w/w), oil:alcohol molar ratio of 1:6, and near or at the boiling point of the alcohol to obtain >98% ester yield (Freedman et al., 1984; Ma and Hanna, 1999; Fukuda et al., 2001; Marchetti et al., 2007).

Lang et al. (2001) did not report any significant differences between methyl esters yields from the same oil catalyzed by KOH or sodium methoxide (CH_3ONa); however taking cost into consideration, KOH is preferred over CH_3ONa. Schwab et al. (1987) and Vicente et al. (2004) also respectively reported 98 and near 100% conversion yield at 60 and 65^oC using CH_3ONa under anhydrous condition. CH_3ONa is moisture-sensitive and will thus require completely anhydrous oil and alcohol for maximum conversation yield (Akoh et al., 2007; Vicente et al., 2004; Demirbas, 2008). This largely excludes most of the typical BD feedstocks. KOH has been reported to perform better than other base catalysts (Arzamendi et al., 2006). To prevent the formation of soaps and water, KOH content should not exceed 2% weight of the oil (Santori et al., 2009). KOH is quite popular in Europe and it offers additional advantage as a fertilizer source that can be

recovered in the polar layer after the transesterification reaction (Haas and Foglia, 2005).

Antczak et al. (2009) and other authors (Demirbas, 2003; Meher et al., 2006) listed the conditions required for base catalysis in BD production and the difficulty with moisture and FFA in the feedstock. Unlike acids, base catalysts do not convert FFA to ester thus soaps are rather form in high FFA oils (Eq. 2.1). Base catalysts are intolerant to moisture and FFA level above 0.1-0.3% and 0.5%, respectively. The presence of even moderate amount of FFA (0.5%) will result in soap and pigment formation, consumption of catalyst, reduce BD yield and complicate the separation of BD and glycerol. For oils containing ≤0.5% FFA, base-catalyzed transesterification is suitable (Fig. 2.3). Even though water is used during washing as part of the purification process to separate or neutralize the base catalyst, remove glycerol, and soaps (Karmee and Chadha, 2005; Meher et al., 2006), there is increased risk of incorporating dispersed water in the BD, becoming problematic during usage. This may result in poor combustion, smoking, and plugging (Fernando et al., 2007). Washing also generates large volume of wastewater that needs to be treated before disposal (Arzamendi et al., 2006).

2.3.2.2. Acid Catalysts

Acid catalysts are rarely used in industrial BD production, but when used hydrochloric and sulfuric acids are preferred (Freedman et al., 1984; Zhang et al., 2003; Chongkhong et al., 2007). Acid-catalyzed transesterification of oils proceeds slower than base-catalyzed reactions, and also requires higher reaction temperatures (Srivastava and Prasad, 2000; Fukuda et al., 2001; Arzamendi et al., 2006), thus BD yield is lower for the same reaction time and temperature. Acid catalysts are also corrosive and will require specialized reaction vessels. An excess molar ratio of the acids favors satisfactory amount of product formation (Meher et al., 2006). For instance, a 1:30 molar ratio of soybean oil:acid yielded 95% FAAE after 69 h, 22 h, and 3 h with methanol, ethanol, and butanol, respectively. However the use of high amount of acids complicates downstream processes and increases overall production cost.

Acid catalysts are however insensitive to FFA in the feedstock and forms no soaps, thus can accommodate a wide variety of lipids and have accordingly been used to esterify FFA prior to the incorporation of other catalysts in two-step acid-base catalysis

(Canakci and Van Gerpen, 2001; Veljković et al., 2006; Berchmans and Hirata, 2008). Acid-catalyzed esterification can reduce the FFA content of the oil to <1% in the first step, after which the mixture is allowed to settle for phase separation. The methanol-water mixture which separates at the top layer is removed, while the denser esterified oil remains in the bottom. In the second step, the esterified oil is catalyzed by a base to its corresponding alkyl ester and glycerol. Acid-catalyzed esterification of tobacco seed oil at a molar ratio of 1:18 reduced the FFA level from *ca* 35% to <2% in 25 min, and the base-catalyzed alcoholysis of the esterified oil yielded 91% of methyl esters in 30 min (Veljković et al., 2006).

2.3.2.3. Lipase Catalysts

The cost associated with energy, and disposal of large volumes of base/acid waste water generated from washing, and post-synthesis purification cast some reservations on the cost-effectiveness of chemical catalysts (Tamalampudi et al., 2008). In addition to the requirement of an almost anhydrous and a minimal to no FFA feedstock (≤0.5%), direct use of most of the available BD feedstocks is excluded. In commercial BD production, the ability to process a wide range of feedstock grades is favorable economics since feedstocks account for a large chunk of production cost (Haas and Foglia, 2005). Cheap but low quality feedstocks are underutilized in BD production due to their high moisture and FFA contents and the related difficulty associated in using such oils under conventional BD production processes. Although the oil can be refined, it requires high proportions of solvents which will add cost, and several steps to the production process.

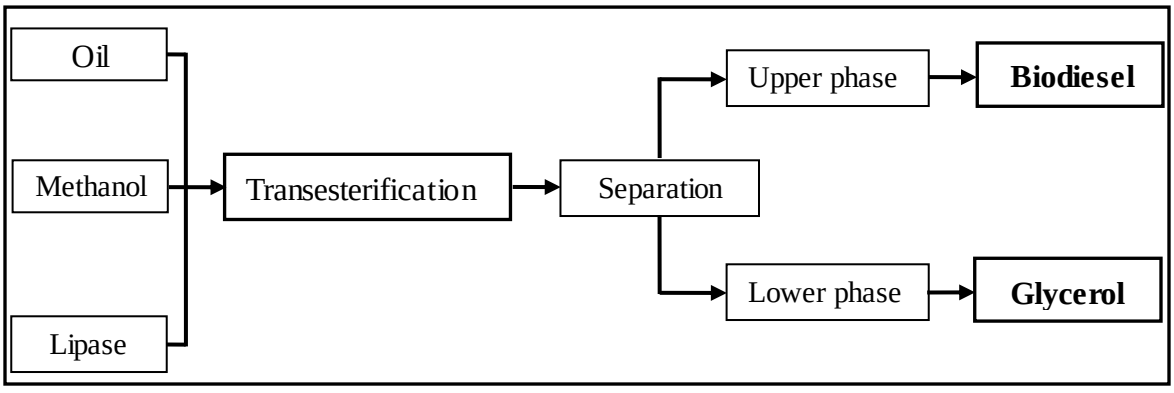


Fig. 2.4: Lipase-catalyzed biodiesel production scheme.

Lipases have shown some greater advantages over chemical catalysts in BD production (Iso et al., 2001; Li et al., 2006; Tamalampudi et al., 2008). Lipases are considered alternative catalyst devoid of the major drawbacks associated with chemical catalysts. Lipases have been used to catalyze reactions involving cheap and low quality feedstocks with high FFA content without additional refining steps, eliminated waste acid and alcohol streams, and enhanced the quality of BD and glycerol co-product formed, unmatched by chemical catalysts. Enzymes are choice catalysts because of their higher activity at milder reaction conditions and thus reactions are less energy intensive, with no requirement for specialized reaction vessels thereby significantly reducing production cost. While enzyme-catalyzed reactions are longer, time is somewhat compensated by the lower need for downstream processing (Tufvesson et al., 2011). Enzyme-catalyzed reactions offer a simpler technical route (Fig. 2.4) as opposed to multi-step chemical-catalyzed reactions (Fig. 2.3) (Marchetti et al., 2007). Separation of catalyst is simplified by its insolubility in the reaction mixture, and much simpler if immobilized on a support, with the possibility of repetitive use (Iso et al., 2001; Mateo et al., 2007; Tufvesson et al., 2011).

Lipases are ubiquitous enzymes well distributed in animals, plants, and microorganisms for lipid metabolism and wide range of biological functions and applications (Delorme et al., 2011). Although lipases perform similar functions, due to their different properties and size, they consequently differ in their capacity to act as catalyst and in various media. Lipases are naturally glycerol ester hydrolase whose natural substrates are lipids (Derewenda et al., 1992; Verger, 1997; Noureddini et al., 2005). Lipases can be intra or extracellular or classified based on their source (microbial, animal or plant). Commercial lipases such as Novozym® 435 and Lipozyme® RM from *Candida antarctica* B and *Mucor meihei*, respectively are examples of microbial lipases and the former is the most widely used lipase in BD production, while pancreatic lipase is an animal sourced lipase (Du et al., 2004; Modi et al., 2007; Royon et al., 2007). Lipases can also be classified based on the reaction they catalyze. For instance, lipase promote ester formation in low water, solvent or solvent-free media through the reaction of esters with acids (acidolysis), alcohols (alcoholysis), acids and alcohols (esterification) or other esters (interesterification). These reactions are collectively termed transesterification.

Lipase hydrolytic reaction yields however do not necessarily equate to transesterification yields (Pencreac'h and Baratti, 2001). Lipases may also be classified based on their specificity as; substrate-, regio-(discriminate between similar parts of the molecule), and stereo-(discriminate between optical isomers) specific lipases.

Lipase-catalyzed transesterification reactions for BD production have been extensively studied (Akoh et al., 2007; Fukuda et al., 2007; Li et al., 2007; Tamalampudi et al., 2008). As described by Noureddini et al. (2005); the initial reaction system using heterogeneous catalysts such as immobilized lipases and at low oil:alcohol molar ratio resembles a three phase system made up of oil, alcohol, and the lipase. As the reaction progresses, the formation of the BD results in a two-phase system made up of solids and liquids. As reaction moves towards completion, the reaction system reverts to a separated three-phase system again now made of lipase, glycerol, and BD phases. Lipases can also catalyze the esterification of the FFA in the feedstock, thus instead of two catalysts; acid for esterification and base for transesterification, lipase can perform both functions. The high initial reaction rates of lipase-catalyzed BD production were attributed to the high FFA content in waste oil compared to the refined oil (Li et al., 2006). The first lipase catalyzed alcoholysis with the aim of producing BD was carried out by Nelson et al. (1996) with tallow, soybean, rapeseed, and recycled restaurant oils, and hexane as solvent. The reactions catalyzed by *Rhizomucor miehei* and *C. antarctica* lipases with short chain primary alcohols and secondary alcohols resulted in 95% and 80% conversion yield, respectively. Sim et al. (2010) recorded an optimal BD yield of 85% when crude palm oil was transesterified at 30^{o}C, using oil:alcohol molar ratio of 1:6.5, enzyme load of 6.67%, and an agitation speed of 130 rpm.

Lipase selection also affects FAAE yield (Lee et al., 2006; Salis et al., 2008). A known 1, 3-positional specific lipase; Lipozyme®-catalyzed reactions theoretically results in *ca* 67% ester yield, with acyl transfer accounting for higher yields (Xu et al., 2004; Du et al., 2004; Lee et al., 2006; Li et al., 2006). Lee et al. (2006) reported that BD production from soybean oil reached a maximum of 70% in 18 h using 1, 3-specific *Rhizopus oryzae* lipase, and less than 20% with non-specific *C. rugosa* lipase after 30 h. Iso et al. (2001) reported various triolein and safflower oil BD yields with various lipases and organic solvents. For instance, immobilized *Pseudomonas fluorescens* lipase showed

the highest activity, while *P. cepacia* lipase resulted in 32% propyl oleate, and 20% butyl oleate yield after 25 h, whereas *Mucor javanicus*, *C. rugosa*, and *R. niveus* were completely inactive. Soumanou and Bornscheuer (2003) also investigated lipase catalysis in both solvent and solvent-free media. The highest conversion in *n*-hexane and petroleum ether was 80%. In the solvent-free system, *P. fluorescens* gave the best yield of >90% at the highest oil:methanol molar ratio (1:4.5), while the rate of alcoholysis was decreased in both lipases from *R. miehei* and *Thermomyces lanuginosa* using >3.0 molar equivalents of methanol. They attributed the high conversion obtained by *P. fluorescens* lipase to its tolerance to methanol.

2.3.2.3.1. Lipase in Organic Solvent Synthesis

The use of lipase is extensive and is a well-known strategy employed in either solvent (aqueous and organic) or solvent-free milieu (Gagnon and Vasudevan, 2011; Tufvesson et al., 2011). The latter is an issue of interesting debate on the function of water in catalyzed reactions with sometimes diverging views presented (Zak and Kilbanov, 1985; Berchmans and Hirata, 2008). This stems from the need for a small amount of water to activate the enzyme and preserve its conformational structure to retain catalytic activity in organic solvents (Osório et al., 2001). Water also maintains the hydration and stability of the enzyme through the formation of hydrogen bonds and van der Waals interaction (Zak and Kilbanov, 1985; Osório et al., 2001). Increasing water activity also increases the amount of water droplets available to form the oil-water interface and subsequently increase interfacial activation (Noureddini et al., 2005; Delorme et al., 2011). However, excess amount of water may act as a competitive inhibitor in transesterification reactions, and promote hydrolysis instead. Novozym® 435 from *C. antarctica* B lipase prefers a nearly anhydrous reaction medium to be effective and appears to contain sufficient water to preserve its conformation (Salis et al., 2005; Tamalampudi et al., 2007). Depending on the lipase, a compromise between maintaining enzyme conformation, enhancing interfacial activation, and suppressing hydrolysis will have to be struck to determine the optimum water for transesterification reactions (Noureddini et al., 2005; Li et al., 2006; Tamalampudi et al., 2008).

The log *P* values have been used to predict the behavior of lipases in organic

media (Zaks and Klibanov, 1985; Laane et al., 1987; Pogorevc et al., 2004). The hydrophobicity or log *P* value relates to the interaction between the water molecules and enzyme, and it is defined as the partition coefficient of an organic solvent between water and 1-octanol (Laane et al., 1987). As a rule, the lower the hydrophobicity, the lower the log *P* value, the greater the affinity of the solvent to water, and the higher the propensity to strip off the essential water molecules that surrounds the enzyme. Thus for instance, organic solvents with log $P < 2$ will enhance loss of lipase activity; solvents with log *P* values between 2 and 4 are moderate to highly effective in catalysis; whereas lipases are completely deactivated in more hydrophobic solvents with log $P > 4$.

2.3.2.3.1.1. Lipase Stability in Biodiesel Production, Effect on Yield, and Remedies

The operational stability of lipases is affected by the low solubility of methanol and glycerol in the oil and BD respectively (Li et al., 2006; Du et al., 2007; Mido et al., 2007). While the stoichiometry of the transesterification reaction requires the addition of 3 molar equivalent of alcohol to 1 mole of oil (Eq. 2.4), lipases are inhibited and may be deactivated beyond half the molar equivalent (1:1.5 oil:alcohol molar ratio) in alcohols such as methanol. At >1:1.5 oil:alcohol molar ratio, methanol remains immiscible with the oil and is observed as dispersed droplets in the oil (Nelson et al., 1996; Shimada et al., 2002; Chen and Wu, 2003; Tamalampudi et al., 2008). The droplets and non-solubilized methanol have been suggested to inactivate lipase upon contact (Watanabe et al., 2000; Shimada et al., 2002). Another possible explanation of such alcohols as lipase denaturant relates to their smaller molecular weight in relation to the substrate (Chen and Wu, 2003). The alcohol thus has easier access to the active site of the enzyme than the substrate. This phenomenon may lead to competition between the substrate and the alcohol for the active site. Lipases are also more labile to deactivation in lower and linear alcohols than long chain and branched alcohols, with the degree of deactivation found to be inversely proportional to the number of carbon atoms in linear short chain alcohols (Chen and Wu, 2003; Mido et al., 2007). The loss of lipase activity has also been attributed to the formation of a hydrophilic hindrance around the enzyme due to the adsorption of polar components such as water and glycerol onto the enzyme limiting the diffusion of the hydrophobic substrate to the enzyme (Stevenson et al., 1994; Marty et al., 1997).

Various strategies and remedies have been devised to reduce the inhibitory effects of these polar alcohols, relieve inactivation, maintain lipase stability, improve yield, promote reusability, and invariably improve the cost-efficiency of the lipase. Polar alcohol inactivation was relieved by the stepwise addition of the alcohol (Shimada et al., 2002) which improved yield, stability (up to 100 d) and reusability (up to 50 times), or the addition of co-solvents to the reaction (Karmee and Chadha, 2005; Li et al., 2006), or the regeneration of the enzyme by washing with C3-C5 alcohols, and continuous removal of glycerol (Du et al., 2007), and the use of alternative acyl acceptors (Lang et al., 2001). Other approaches involve the pre-treatment of lipase by pre-incubation in methyl oleate for 30 min before use (Samukawa et al., 2000), and maintaining very low oil:methanol molar ratios (1:0.33), among others.

The stepwise addition of methanol is a widely suggested remedy to minimize the deactivating effect of these short-chained alcohols on lipase (Shimada et al., 2002; Soumanou and Bornscheuer, 2003; Xu et al., 2004). A two and three-step methanolysis of waste oil was investigated by Shimada et al. (2002). In the first of the three-step reaction also known as pretreatment; 1/3 molar equivalent of methanol was added to the waste oil, while a second and third 1/3 molar equivalent of methanol were added at 10 h and 24 h resulting in a total BD yield of 90%. In the two-step reaction, 1/3 and 2/3 molar equivalent of methanol were added initially and after 10 h, respectively. The increase in solubility and dispersion of methanol in the second step which contained formed esters from the first step minimized the deactivating effect of methanol on lipase.

Another source of lipase deactivation in alcoholysis is the glycerol co-product. Glycerol has the tendency to accumulate in the reaction mixture, block the active site of lipase, limit substrate and product diffusion to and from the enzyme respectively, and shorten the life span of the lipase. Glycerol is also insoluble in oil or organic solvents, and leads to hydrophilic hindrance around the enzyme (Soumanou and Bornscheuer, 2003; Xu et al., 2004; Du et al., 2007). The remedies devised to improve glycerol solubility and minimizing its adsorption onto the surface of the immobilized lipase, include; recovery and intermittent washing of the enzyme during use with polar solvents such as iso-propanol or tertiary alcohols. The inclusion of a suitable absorbent such as molecular sieves or silica gel to the reaction mixture preferentially desorbs the glycerol

produced away from the enzyme (Stevenson et al., 1994; Dossat et al., 1999; Xu et al., 2004; Li et al., 2006; Du et al., 2007). In a similar development, Chen and Wen (2003) devised a method that reactivated 56% and 75% of the original activity of completely deactivated immobilized *C. antarctica* lipase by incubating and washing the lipase in 2-butanol and t-butanol, respectively.

Another strategy involves the use of hydrophobic solvents such as *n*-hexane, t-butanol, or iso-propanol as co-solvents, solvents modifiers, or diluent (Nelson et al. 1996; Dossat et al., 1999; Chen and Wu, 2003; Karmee and Chadha, 2005; Iso et al., 2006; Li et al., 2006; Du et al., 2007; Modi et al., 2007). The co-solvent mitigates the two-phases created by the polar alcohol with the oil by simultaneously solubilizing the alcohol, creating an oil-dominant one-phase system, and limits the concentration of alcohol surrounding the enzyme (Gagnon and Vasudevan, 2011). Royon et al. (2007) reported a 97% BD yield within 24 h in a Novozym® 435-catalyzed reaction with 13.5% methanol and 32.5% t-butanol as co-solvent, while using similar reaction conditions but in a solvent-free media, Watanabe et al. (2002) reported 93.8% BD yield in 48 h. In comparison, the lipase was more efficient in the solvent media than the solvent-free media in terms of reaction time, yield, and amount of lipase added. The yields of 1, 3-regiospecific lipases such as Lipozyme® have been reported to be improved by a series of processes which accelerate acyl migration, for instance the addition of hexane or silica gel (Du et al., 2007; Rodrigues et al., 2010). 1, 4 dioxane was the most effective solvent and improved homogeneity when ethanol and methanol were used but was not needed when 1-propanol or 1-butanol was used as acyl acceptors (Iso et al., 2006). The choice of the co-solvent is also important, for instance, tetrahydrofuran (THF) is miscible with both methanol and water and has a boiling point close to that of methanol thus can be co-distilled with methanol upon completion of the reaction. The main drawback to the use of co-solvents or modifiers is that, organic solvents can be flammable and toxic and may require additional investments to meet safety requirements (Royon et al., 2007), and the inconveniences involved in their recovery and disposal, sometimes in large quantities.

Methyl and ethyl acetate are two other novel acyl acceptors with no apparent inhibitory effect on lipases in BD production (Du et al., 2004; Xu et al., 2005; Modi et al., 2007). These authors reported improved BD yield and prolonged reusability of the

enzyme. Unlike methanol, these acyl acceptors have been used beyond 11 molar equivalents. A 12:1 molar ratio of methyl acetate:soybean oil (both crude and refined) at 40^{o}C yielded 92% of methyl ester with no negative effect on Novozym® 435 even after 100 cycles (Du et al., 2004). A similar trend was reported by Mido et al. (2007), in that study, the reusability of Novozym® 435 was maintained over 12 cycles with ethyl acetate as acyl acceptor while it dropped to zero after only 7 cycles with ethanol. Other advantages of these novel acyl acceptors include the production of triacetylglycerol instead of glycerol as co-product. Triacetylglycerol has no inherent capabilities of blocking the active sites of the enzymes and have higher value as co-product than glycerol (Du et al., 2004; Xu et al., 2005; Modi et al., 2007).

Combined use of two lipases in the same reaction is another strategy proposed to minimize the negative effects of methanol on lipases and enhance BD yield (Lee et al., 2006; Li et al., 2006; Tongboriboon et al., 2010). Using methanol and t-butanol at 35^{o}C, BD yield was 85% in 12 h when 20% Lipozyme TL IM and 2% Novozym® 435 was used separately and 95% when 3% Lipozyme TL IM and 1% Novozym® 435 lipases were used together under the same conditions as when used separately (Li et al., 2006). Used palm oil in a solvent-free transesterification reaction gave higher BD yield when combinations of 5% each of lipase AK from *P. fluorescens* and AY from *C. rugosa* were used than AK alone (Tongboriboon et al., 2010).

Other authors have used supercritical fluids (SCF) *via* catalytic and non-catalytic transesterification pathways to circumvent the drawbacks of lipase catalysis in organic solvents (Kusdiana and Saka, 2004; Demirbas, 2008). Methanol has a lower dielectric constant and is less polar at supercritical state due to the change in properties and structure, thus overcoming the problems associated with the two-phases created by the methanol-oil mixtures. Other advantages of SCF include simpler separation especially in non-catalytic-SCF reactions (Demirbas, 2002; Kusdiana and Saka, 2004; Rathore and Madras, 2007). Where SCF is combined with enzymes, separation is simply by reducing the pressure of the system (Rathore and Madras, 2007). The main disadvantages of supercritical alcohols is the high temperature (>350°C) and pressure (45-65 MPa), and high oil:alcohol molar ratio (1:42) used. Recent technologies use co-solvents such as CO_2 to lower these reaction temperatures (Demirbas, 2008; Yin et al., 2008).

2.4. Lipase Immobilization

As previously stated, enzyme offer an environmentally benign way to perform reactions under mild conditions and with high degree of selectivity (Tischer and Wedekind, 1999; Tamalampudi et al., 2008; Garcia-Galan et al., 2011). However the cost of enzymes reduces their competiveness to chemical catalysts (Fernández-Lorente et al., 2011; Tufvesson et al., 2011). The production of cheaper and robust lipases with enhanced stability and catalytic efficiency tailored for BD production is warranted. While commercial lipases are usually sourced from microorganisms in high yield from cultures and more easily genetically engineered than from animals, the prospects of alternate sources of lipase nevertheless from aquatic environment such as the waste generated from fishery processing have received less attention (Kurtovic et al., 2011). Although fish enzymes exhibit similar biochemical characteristics with similar microbial and mammalian enzymes, fish enzymes exhibit distinctive characteristics due to their relatively unique habitats such as higher catalytic activities at low reaction temperature than their mammalian or microbial counterparts (Simpson and Haard, 1984; Gjellesvik et al. 1992).

Lipase from both extracellular and intracellular sources have been employed in catalysis either as whole cells, free powder or immobilized on a suitable support (Fukuda et al., 2007; Li et al., 2007; Tamalampudi et al., 2008). The use of either the soluble or lyophilized form of the enzyme is not convenient or practical in large-scale or continuous production processes and present several major drawbacks such as; thermal instability, low activity, susceptibility to proteolytic attack, and difficulty in separation and reuse (Sheldon, 2007; Garcia-Galan et al., 2011). The lyophilized form may also aggregate, sequestering the active site away from the reactants or may be denatured during lyophilization. Immobilization has been suggested as an alternative approach to improve the thermal, operational and storage stabilities, and reusability with minimal changes in efficiency over time to compensate for the cost of the enzyme (Tischer and Wedekind, 1999; Tamalampudi et al., 2008; Garcia-Galan et al., 2011). Immobilization involves the attachment, dispersion, localization or confinement of the free form of the enzyme in a defined region of space.

2.4.1. Immobilization Methods

Immobilization methods belong to four general categories namely; adsorption on a carrier material, covalent binding to a solid matrix, entrapment or microencapsulation in a solid support, and carrier-free cross-linked immobilization (Tischer and Wedekind, 1999; Garcia-Galan et al., 2011) (Fig. 2.5).

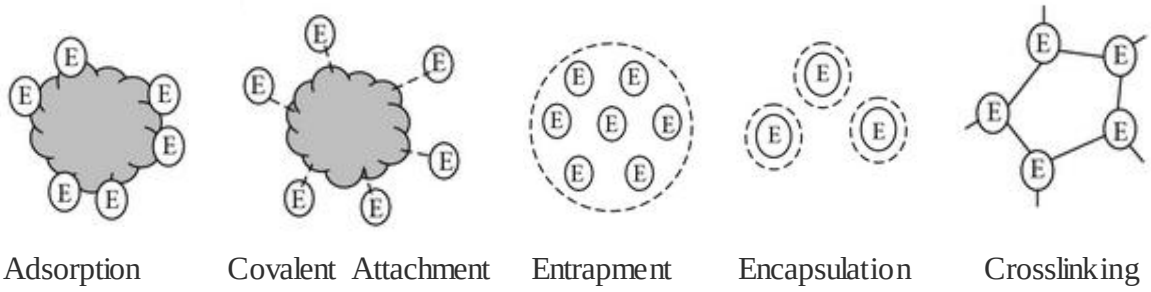


Fig. 2.5: Immobilization methods.

2.4.1.1. Adsorption

Adsorption involves mixing enzyme solutions and support resin or precipitating of the enzyme on a resin with organic solvents. Upon completion, the buffer or solvent is reduced by drying. Enzymes have been adsorbed on several resins such as; sepharose, sephadex, celite, collagen, silica, and alumina with charged or neutral surfaces, and may also be used as a technique for separating proteins (Bastida et al., 1998; Tischer and Wedekind, 1999; Garcia-Galan et al., 2011). Adsorption is known to have the least effect on the enzyme compared to other immobilization methods. Adsorption is also a relatively simple and low cost technique and is widely used (Brígida et al., 2008; Fernández-Lorente et al., 2011). However, due to the relatively weak enzyme-support complex formed, there is greater possibility of leaching and desorption (Iso et al., 2001; Brígida et al., 2008; Liu and Chang, 2008) with the possibility of reusing the support. Depending on the nature of the support, the binding of the enzyme can vary, and as result of either ionic interaction, physical adsorption, hydrophobic, van der Waals forces or a combination of these interactions (Sheldon, 2007; Brígida et al., 2008). Consequently, in the presence of the substrate, variable temperature, pH or ionic strength, the enzyme can be adsorbed or desorbed from the support (Brígida et al., 2008). The interfacial activation mechanism of lipase has also been used to adsorb lipase onto hydrophobic resins (Mateo et al., 2007; Fernández-Lorente et al., 2011) (Fig. 2.6).

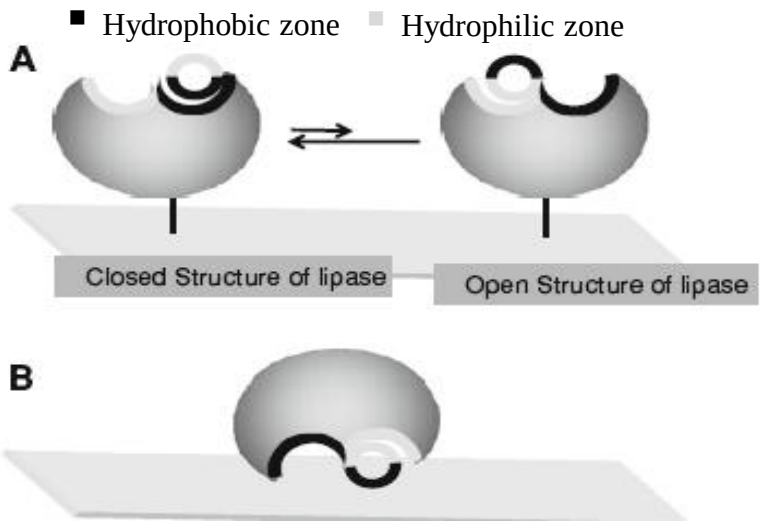


Fig. 2.6: Lipase immobilized on two supports with different hydrophobicity. **A**. Lipase is immobilized on a less hydrophobic resin with equilibrium shifted to the closed inactive conformation to protect the hydrophobic active site. **B**. Lipase is adsorbed on a more hydrophobic resin with the open active form stabilized on the resin to receive the substrate (Fernández-Lorente et al., 2011).

This adsorption mechanism is thought to involve the large and hydrophobic active center of the lipase and hydrophobic supports which mimics the interaction of lipases undergoing interfacial activation in the presence of its preferred substrate and surface active agents (Mateo et al., 2007; Fernández-Lorente et al., 2011). Increased lipase activity upon adsorption on hydrophobic supports is due to improved enzyme-support affinity, stabilized open conformation, and greatly enhanced substrate-active site interaction (Bastida et al., 1998; Mateo et al., 2007; Sörensen et al., 2010).

2.4.1.2. Covalent Attachment

Covalent binding offers a stronger multipoint enzyme-support complex which is irreversible with minimal enzyme loss by desorption or leaching from the support, however the attachment may involve several costly steps (Iso et al., 2001; Liu and Chang, 2008; Garcia-Galan et al., 2011). Covalent attachment is achieved through the covalent binding of amino/carboxyl, sulfhydryl or hydroxyl groups of the enzyme to the support (Yagiz et al., 2007; Garcia-Galan et al., 2011). However since these functional groups are

essential for catalysis, other non-essential groups present such as the carbohydrate moiety of glycoprotein lipases can be used, to avoid inactivating the enzyme or blocking the active site. The presence of functional groups on the support material is thus crucial, but since most support polymers lack active functional groups, surface modification coupling and activation agents such as carbodiimide (EDC) and glutaraldehyde act as important interface. The treatment of silica with γ-aminopropyltriethoxysilane introduces amino groups to the otherwise chemically inert support (Weetall, 1976). Like other immobilization methods the support may positively or negatively affect the properties of the enzyme. The immobilized enzymes may exhibit higher resistance to temperature, denaturants, and organic solvents. The drawbacks of this type of immobilization is the possibility of loss of enzyme activity or partial reduction in the external pore opening of the support as a result of the modifications and several sequences of steps involved. The reagents used during modification also add to production cost.

2.4.1.3. Entrapment and Encapsulation

Enzymes are entrapped inside gel matrices such as alginate, agarose, and gelatin, or gels are allowed to form in an aqueous solution containing the enzyme. The relatively high activity displayed by entrapped enzymes has been attributed to the extensive dispersion of the enzyme on the matrix (Reetz et al., 1995). The binding forces are however weak and enzyme leaching is high (Sheldon, 2007). The matrix may also act as a diffusion barrier as a result of smaller pore size in relation to the enzyme. Microencapsulation is a modified entrapment procedure where the enzyme is immobilized within microcapsules prepared from organic or synthetic polymers such as carrageenan and hydrogels. Although the catalyst is effectively retained within the capsule, the effectiveness of this method largely depends on the stability of enzyme and its accessibility to large substrate molecules.

2.4.1.4. Carrier-free Immobilization

Enzymes aggregates may be cross-linked to a multifunctional reagent without any solid support using reagents like glutaraldehyde (Garcia-Galan et al., 2011). Enzymes can be immobilized as cross-linked enzyme crystals (CLEC) or aggregates (CLEA). CLECs

however require the use of pure enzyme and the immobilization protocol which involves crystallization can be complex, while CLEA is a simpler aggregation technique. Due the high viscosity of some of the precipitants used in making CLEA, the recovery of immobilized enzyme as well as final cross-linking can be complicated. Overall, enzyme mobility may be reduced.

2.4.2. Immobilization Supports

The support materials for immobilization are broadly grouped as inorganic, synthetic polymers, and natural macromolecules. Inorganic support resins include porous glass, silica, and diatomaceous earth. Styrene-divinylbenzene is an example of hydrophobic polymer, while agarose, gelatin, chitosan, and cellulose are examples of natural macromolecules (Tischer and Wedekind, 1999; Sheldon, 2007). Other commercially available resins include Accurel EP00 particles and polystyrene latex which are porous and non-porous lipophilic polymers, respectively. As important component of the immobilization procedure and subsequently the properties of enzyme, support materials play an important role in the usefulness of the immobilized enzyme (Arica et al., 2000; Chen and Wu, 2003; Tamalampudi et al., 2008). The selection of a support for immobilization depends on the type and stability of lipase, intended reaction system, and ease of regeneration (Garcia-Galan et al., 2011). The chemical properties of the support (composition, hydrophobicity/hydrophilicity, functional groups) as well as mechanical properties (flow resistance in fixed bed reactors or abrasion resistance in stir tank reactors) both influence the immobilized enzyme (Tischer and Wedekind, 1999; Yagiz et al., 2007). Different support has different surface area, average pore diameter which can affect the activity and selectivity of the enzyme once immobilized on it, thus the same enzymes may present very different catalytic properties when immobilized on different supports (Samukawa et al., 2000; Sörensen et al., 2010; Kurtovic et al., 2011). Support materials should be low-cost, have large surface area with optimal/well defined/tunable pore size, and minimal diffusion limitation in the transportation of reactants and products.

The efficiency of the immobilization procedure is evaluated through the characterization of the immobilized enzyme.

2.5. Biodiesel Quantification Methodologies and Instrumentation

The components of the BD transesterification reaction has been quantified using a variety of instruments and methodologies such as; GC (Iso et al., 2001; Noureddini et al., 2005; Lee et al., 2006; Li et al., 2006; Sim et al., 2010), HPLC (Wyatt et al., 2005; Arzamendi et al. 2006), FTIR (Dubé et al., 2004; Mahamuni and Adewuyi, 2009) and ^{1}H NMR (Karmee and Chadha, 2005). Quantification determines the amount of remaining reactants (TAG, alcohol and/solvents), reaction intermediates (DAG and MAG) and products (BD and glycerol) as well as other minor components such as FFA, to evaluate the various degree of transesterification conversion, and as a quality control measure (Knothe, 2001). The latter is very essential due to the negative effects of remaining and residual contaminants on fuel quality and engine operation. A quick and easy analytical method that is also reproducible and allows the simultaneous determination of all the components with no ambiguity is preferred. The quantification of compound classes (TAG, DAG, MAG) and not individual compounds is sufficient for official BD testing so long as the limit of each class is met (Knothe, 2001; ASTM D6751; CEN EN 14214).

GC is the most widely used method for BD analysis and often coupled with a flame ionization detector (FID) (Knothe, 2001; Li et al., 2006; Sim et al., 2010), and forms the basis for the official protocols for determining total glycerol in BD. The GC analysis however requires sample derivatization to reduce the polarity of the analytes and improve thermal stability. This can be time and reagent consuming and the analyses may also be affected by baseline drift, and an output of several peaks in one chromatogram.

The HPLC method on the other hand allows the direct analyses of all components without derivatization. Analytes subjected to HPLC are usually non-volatile (Holčapek et al., 2003). The HPLC method may employ size exclusion chromatography (SEC) which separates different components according to their molecular size. Separation may be based on lipid class with overlapping physical/chemical and chromatographic properties. The main advantage of SEC is the short analytical time due to minimal interaction between the analyte and the column; as such, separation of individual compounds is based solely on their hydrodynamic volume (Holčapek et al., 1999; Darnoko et al., 2000; Arzamendi et al., 2006) or effective molecular size in solution. Retention is accordingly dependent on the size and shape of the solute molecule solvated in the mobile phase

relative to the size of the pores in the column packing. Small molecules will permeate the smaller pores, intermediate-sized molecules will permeate only part of the pores, and very large molecules will be completely excluded thus eluting first. The different glycerides produced during the transesterification reaction differ in molecular weight by *ca* 250 units, while their steric hindrances are different; it still results in only one peak for each lipid class (Knothe, 2001; Kittirattanapiboon and Krisnangkura, 2008). Thus species of close molecular weight which are eluted from the column at very close retention times, are detected together as a single peak (Arzamendi et al., 2006), while in the GC method a difference of 2 carbon atoms results in another completely different peak (Darnoko et al., 2000). The HPLC system has been used in combination with several detectors such as ultraviolet-visible (UV-Vis), refractive index (RI), mass spectrometer (MS), evaporative light scattering (ELS), and fluorescence (F) detectors (D) (Darnoko et al., 2000; Knothe, 2001; Arzamendi et al., 2006; Kittirattanapiboon and Krisnangkura, 2008).

Using THF as mobile phase and one of two columns (Waters 300 mm x 7.8 mm Styragel® HR0.5 and HR2 columns, with 100 and 500 Å single-pore size, respectively), Arzamendi et al. (2006) did not obtain complete separation of the transesterified sunflower oil. When only HR0.5 was used, TAG and DAG were co-eluted, when HR2 was connected in series with HR0.5, TAG and DAG were only partially separated; only when two HR2 and one HR0.5 columns were connected in series did complete separation occur.

Darnoko et al. (2000) did not observe different elution times for different classes of TAG; tripalmitin and triolein, using gel permeation chromatography (GPC) with THF as mobile phase, two Phenogel® columns coupled in series and a RI detector. However, different methyl esters gave slightly different elution times when injected separately but when present together in the same sample, for instance; methyl palmitate and methyl oleate, only one asymmetric peak was produced.

Dubé et al. (2004) compared two analytical methods; GPC with RI, and attenuated total reflectance (ATR)-FTIR spectroscopy to monitor the products of acid-catalyzed transesterified waste frying oil. The reproducibility of each method was found to be within ±1-5%. The differences between the results of the two methods were less than ±2%. The GPC method showed good separation of MAG and glycerol from TAG

and FAME, but DAG was not completely separated from TAG. GPC gave good quantitative results for MAG and FAME, but TAG and DAG analyses required correction, depending on the mole ratio of TAG/DAG. In contrast, the ATR-FTIR method could only give quantitative data for the sum of TAG, DAG, and MAG.

Mahamuni and Adewuyi (2009) developed a FTIR method to monitor the transesterification reaction, determine BD and oil content in BD-petrodiesel, and adulterated BD-petrodiesel blends, respectively. The method could measure up to 98.11% accuracy the amount of BD in BD-oil mixtures, 99.99% and 95.32% accuracy the BD, and oil and BD contents in BD-petrodiesel mixture (blend) and BD-petrodiesel-oil mixture (blend adulteration), respectively. The method however could not quantify DAG and MAG individually due to their similar structures to TAG.

The glycerol content is often extremely difficult to directly and simultaneously analyze and quantify (Darnoko et al., 2000; Li et al., 2006; Kittirattanapiboon and Krisnangkura, 2008). Even with two columns connected in series, glycerol could not be analyzed (Darnoko et al., 2000; Dubé et al., 2004, Arzamendi et al., 2006). It is possible that the washing step prior to sample injection removes all the glycerol or only small quantities were present in the alkyl ester-rich phase. In comparison with TAG, DAG, MAG, and BD, the glycerol molecule is extremely polar and hydrophilic; however, by using a polar column the other components might not be separated. Very few authors have simultaneously detected glycerol with the other analytes (Li et al., 2006).

2.6. Fuel Properties and Testing

The concept of BD production as previously stated is a simple transesterification reaction, but the technical components and issues associated with feedstock and BD quality are copious (Knothe and Steidley, 2005; Fernando et al., 2007; Behçet, 2011). BD inherits most of its properties from the parent oil, and it is compared to petrodiesel No. 2 (Knothe, 2005a). The American society for testing and materials (ASTM), and the European committee for standardization (CEN) set BD standards in the United States and Europe, respectively. ASTM D6751 and EN 14214 detail the official chemical and physical tests, standards and specifications of BD fuels, and are independent of the oil or fat used to produce the BD, but solely performance-based. The specifications provide the

limits of acceptability and safe levels of BD properties in diesel engines. ASTM D6751 among other things indirectly measures the completeness of the reaction by the absence of glycerol, residual catalysts, and unreacted alcohol in the fuel (Fernando et al., 2007). Nevertheless, ASTM D6751 does not include a test that directly measures total FAAE content. The CEN method (EN 14103) however measures the FAME and linolenic acid methyl ester content of the fuel. BD is most commonly used as blends and BD-petrodiesel blends are allowed in the range of 5 to 7 vol. % (B5 to B7) for any application as if it were pure petrodiesel, and 6 to 20 vol. % (B6 to B20) in other applications that use petrodiesel, and are respectively covered by their own set of standards; ASTM D975, and ASTM D7467. A compilation of the ASTM D6751 test methods is given in Table 2.4 and the intent of some of these quality assessment requirements is briefly described.

Fuel Property	**Test method**	**Limits**	**Units**
Acid number	D664	0.80 max	mg KOH/g
Carbon residue (100% sample)	D4530	0.050 max	% mass
Cetane number	D613	47 min	-
Cloud Point	D2500	Report	^{o}C
Copper strip corrosion	D130	No 3 max	-
Distillation temperature	D1160	360 max	^{o}C
Flash point (closed cup)	D93	130.0 min	^{o}C
Glycerol (Free)	D6584	0.020 max	% mass
Glycerol (Total)	D6584	0.240 max	% mass
Heat of combustion/caloric value (gross)	D240	Report	kJ/kg
Heat of combustion/caloric value (net)	D240	Report	kJ/kg
Kinematic viscosity (40^{o}C)	D445	1.9-6.0	mm^2/s
Phosphorus content	D4951	0.001 max	% mass
Pour Point	D97	Report	^{o}C
Sulfated ash	D874	0.020 max	% mass
Sulfur (or 0.05 max for S 500/500 ppm)	D5453	0.0015 max	% mass
Water and sediment	D2709	0.050 max	% volume

Table 2.4: Specification and standard for biodiesel (B100) (D6751).

The cetane number (CN) measures the tendency of a fuel to auto-ignite when injected at the engine cylinder's temperature and pressure (Knothe, 2005a). The CN is measured by comparing the auto-ignition tendency of BD with a blend of two reference fuels, cetane (hexadecane) and heptamethylnonane. The higher the CN the shorter the ignition delay; i.e. the shorter the time period between fuel injection and auto-ignition. The CN increases with increasing SFA content in the oil but decreases with branching (Van Gerpen, 2005; Behçet, 2011).

The gross and net heat of combustion of BD is determined by the ASTM D240 specification. The heat content of vegetable oils is *ca* 90% that of petrodiesel No. 2 and the heat of combustion of BD do not vary greatly from the parent oil (Van Gerpen, 2005). The low power output of engines running on BD and BD-petrodiesel blends have been attributed to their low heating values (Van Gerpen, 2005; Behçet, 2011).

The standardized test to measure the resistance to flow of the fuel from one part to the other is the kinematic viscosity (Knothe, 2005d). According to ASTM D445 specification, the viscosity for BD should be between 1.9 and 6.0 mm^2/s. Studies have shown that viscosity increases with increasing chain length in saturated FA esters but is dependent on the nature and position of the double bond in unsaturated FAs (Knothe and Steidley, 2005). For instance, the *cis* configuration gives lower viscosity than *trans*. The viscosity of the resulting fuel is also affected by the type of alcohol used in the transesterification reaction (Knothe, 2005d; Knothe and Steidley, 2005).

The poor low-temperature operability property is one of the two main inherent technical deficiencies of BD, the other being long-term fuel storage stability. A huge constraint with BD usage in low temperature conditions is the possibility of the fuel to crystallize (Dunn, 2005; Fernando et al., 2007; Behçet, 2011). The test used to measure the tendency with which fuel form crystals is the cloud point (CP). Further temperature depression causes these crystals to agglomerate, and beyond which the fuel may solidify. Increasing chain length and the presence of saturated esters increases the CP of BD. For instance, the CP of soybean and tallow methyl esters are -12 to 0^oC and 9 to15^oC, respectively (Dunn, 2005; Fernando et al., 2007). The pour point (PP) measures the extremes of the CP. It measures the lowest temperature at which fuel can be poured. Operation difficulties develop beyond the CP but before the onset of PP (Dunn, 2005).

The lower amount of waxes in petrodiesel No. 1 makes it a common PP depressants for low temperature operations (Van Gerpen, 2005). The small temperature differences between the CP and PP of BD further aggravate the problems of low temperature operation. While the difference between the CP and PP of petrodiesel No. 2 is 18^{o}C, it is only 2^{o}C in soybean methyl esters (Dunn, 2005). Like the CP and heat of combustion, the ASTM does not set limits, however it must be reported to the consumer.

The flash point (FP) test measures the temperature at which the fuel gives off enough vapor to form a flammable mixture in air. BD has a higher FP than petrodiesel, usually >150^{o}C, which gives it a safer handling advantage over petrodiesel with a FP of 52-66^{o}C (Van Gerpen, 2005).

The unique oxygen-containing, polar chemical structure of the carboxyl group (-COO^{-}) of BD gives it a much stronger tendency to absorb moisture. BD can absorb *ca* 30 times more dissolved water than petrodiesel (1500 ppm against 50 ppm) (Van Gerpen, 2005). The presence of free or dissolved water and sediments in fuel has been shown to initiate and propagate corrosion as well as affect the stability of the fuel (Van Gerpen, 2005). Water in the fuel can also serve as media for microbial growth, leading to filter blockage or corrosion of metal parts. When chicken fat BD was blended with petrodiesel between 20 to 80%, the viscosity and specific gravity of the blends had minimal changes over the 1 year storage period, however, sediments accumulated with increasing BD concentrations (Geller et al., 2008).

The acid number (AN) test measures the residual FFA content of the fuel. The test also serves as a quality control guide to monitor fuel degradation during storage. In BD, fuel aging and oxidation can lead to high AN, while in petrodiesel, increase in acidity suggest a build-up of petroleum gums and varnishes in the engine (BIOBUS 2003; Behçet, 2011).

The glycerol component of the unreacted TAG, and residual DAG and MAG makeup bound glycerol, the glycerol co-product the free glycerol, while the sum of the bound and free glycerol constitute the total glycerol content. This implies that incomplete conversion of the oils into BD can lead to high total glycerol content while incomplete removal of the glycerol co-product can lead to both high free glycerol and total glycerol contents. Excessive amounts of glycerol in the BD can cause problems in the engine, foul

injectors, and form deposits on valves and injector nozzles, and also affect BD quality during storage (Van Gerpen, 2005). A 99.76% reaction completion is necessary to meet the ASTM D5684 specification since the maximum allowable total glycerol content is 0.24%.

Fish oil from anchovy waste (Behçet, 2011), salmon (Reyes and Sepúlveda, 2006) were converted to BD in a two-step acid-base-catalyzed transesterification reaction with methanol as alcohol. Fish oil-derived BD was reported to have met some fuel property specifications such as; FP, free, and total glycerol, AN, water and sediment content. The oxidative stability and cold soak filtration-(the time in seconds it takes for cold soaked BD to pass through two 0.8 micron filters and the amount of particulate matter collected on the filter) tests were however out of specification and were attributed to the high PUFA content of fish oils (Fan et al., 2010). Long filtration times indicate a higher potential for fuel filter plugging. Fuel testing also revealed slight to high power loss when used unblended (B100), but lower particulate material emission when blended with petrodiesel (AIDEA 2002; Reyes and Sepúlveda, 2006; Behçet, 2011). Having a higher CN than petrodiesel, the BD improved the ignition performance of blends and reduced NOx emissions.

CHAPTER III

CONNECTING STATEMENT

In addition to their value as edible oils, feedstock for BD production cannot be met solely by vegetable oils, thus the exploration and use of non-traditional feedstocks is warranted. The first part of the thesis outlined established and emerging biomass to biofuel conversion approaches; from waste to a value-added product, and the availability of fishery and animal processing waste as feedstock for BD production. In the second part of the thesis the potential of recovered oils from fishery and animal processing waste as BD feedstock and the use of lipase in the conversion of these oils to BD were assessed.

The large amounts of waste generated by the fishery industry from processes such as gutting and filleting are often underutilized, disposed at sea or in landfills. This chapter details the recovery of oil from salmon skin generated during these processes. The influence and efficiency of various extraction parameters, solvent systems and techniques on oil yield and quality were investigated. The ideal extraction technique was evaluated in terms of economic (cost of solvents, ease, and extraction time) and environmental (amount of solvents used and disposed) benefits on the production of oil for BD production. The oil will fill specific needs such as reducing the bulk of processing waste currently going to low dollar value products and landfills, and as an alternative BD feedstock.

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CHAPTER III

COMPARATIVE STUDIES ON THE YIELD AND QUALITY OF SOLVENT-EXTRACTED OIL FROM SALMON SKIN

3.1. Abstract

Oil was extracted from the skin of Atlantic salmon by solvent extraction with various solvent systems and analyzed for efficiency in terms of oil yield and quality. The yield of salmon skin oil (SSO) was significantly lower ($p < 0.05$) with the hexane-isopropanol solvent system versus either of the chloroform-methanol systems, i.e., 32.21% on dry weight basis (dwb) against 35.15% dwb and 43.82% dwb, respectively. Second and third extractions were performed on the residue using the same solvent systems to verify any biases and to test the completeness of the first extraction. These successive extractions resulted in nominal increases in yield. The yield of SSO from Soxhlet-hexane compared favorably with Soxhlet-petroleum ether at all the extraction times investigated. Soxtec-hexane gave the highest oil yield of *ca* 62% dwb. Both hexane and petroleum ether were suitable solvents for the extraction of SSO, though the yield obtained with hexane was significantly higher ($p < 0.05$). The study further indicated that salmon skin was a rich source of oil (23.32-61.53% dwb), and for the various solvent systems, the free fatty acid (FFA) content was quite low (0.60-1.19%).

3.2. Introduction

Globally, *ca* 1/4 of the total fish catch is discarded, and these are either generated from fishery processing or as by-catch (Falch et al., 2006). Fish processing discards include head, skins, bones, and trimmings; and these components form the major residues from the processing of fish (Aidos et al., 2001; Rustad, 2007). Some of these discards have been collected and used as sources of various valuable bioingredients such as proteins, minerals, and lipids (Haard et al., 1994; Sathivel et al., 2004).

In marine species, fat is deposited in three main sites; the viscera, beneath the skin, and in the muscle, and these sites of fat deposition have been used in their partial classification. The fat content varies within the different body parts for various fish species. For example, the fat content of Atlantic salmon muscle (fillet) can range from 2

to 7% (Jørgensen et al., 1997), to >15% for farmed Atlantic salmon (Aursand et al., 1994; Hemre and Sandnes, 1999; Jobling et al., 2002). Aursand et al. (1994) also reported that *ca* 57% of the total body fat of farmed Atlantic salmon is deposited in the edible portion, while on wet weight basis the fat content of the skin is *ca* 18%.

Generally, lipids are classified into two main groups, namely; neutral or non-polar lipids such as triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), and sterols, and polar lipids such as free fatty acids (FFA), phospholipids (PL), and sphingolipids. Fish lipids are primarily TAGs and occur as storage fat in hydrophobic aggregates (Aursand et al., 1994; Jobling et al., 2002), and fatty acids of various chain lengths and degree of unsaturation (Jobling et al., 2002). TAGs are soluble in non-polar solvents and their solubility decreases with increasing amount of polar solvents (Smedes and Thomasen, 1996), while extraction of polar lipids is enhanced with the addition of polar solvents such as methanol and iso-propanol.

The classical solvent extraction methods of Folch et al. (1957), and Bligh and Dyer (1959) have been employed extensively in lipid extraction. However, there is growing health and environmental concerns with the use of chloroform, a suspected carcinogenic agent (Reuber, 1979; Radin, 1981). Petroleum ether (PE) and hexane have also been extensively used as solvents for oil extraction (Anonymous, 2002); the latter however has a lower vaporization temperature (boiling point: 68.7°C), higher stability, cheaper, less corrosive, and lower toxicity.

To increase the industrial application and utilization of these oils from marine origin, recovery procedures that result in high yields without compromising the quality of the extracted oil are required. The traditional method of fish oil production is the continuous wet reduction process and it involves cooking, pressing, and centrifugation (FAO, 1986; Chantachum et al., 2000). Other methods such as supercritical fluid extraction (Dunford et al., 1997; Esquível et al., 1997; Nurhan et al., 1998), fractionation (Hirata et al., 1993), and low temperature solvent extraction (Moffat et al., 1993) have been reported. Enzymes have also been used to increase oil yield during extraction as well as increase the concentration of ω-3 fatty acids (Shahidi and Wanasundara, 1998).

The main objectives of this study were to: (i) study the extraction of oil from salmon skin using various solvents, (ii) evaluate the effect of solvent type and extraction

time on oil yield, and to (iii) investigate the influence of extraction methods on the FFA content in the oil recovered. The choice of salmon skin as source material for this study was based on availability and potential for commercial exploitation.

3.3. Materials and Methods

3.3.1. Materials

The salmon skin samples (Picture 3.1) from farmed Atlantic salmon were obtained from a local fish market (Waldman Plus, Montreal, QC). The sample was kept between crushed ice in a plastic bag, and transported directly to the laboratory where they were stored frozen at -20°C. Chloroform, methanol, petroleum ether, hexane, iso-propanol, and methanol (all HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Potassium chloride, sodium hydroxide, and sodium sulphate were purchased from Sigma-Aldrich Canada (Oakville, ON), and Whatman filter paper No.1 was obtained from Whatman International Ltd. (Maidstone, Kent).

3.3.2. Methods

3.3.2.1. Sample Preparation

The frozen fish skin was placed in a Labconco flask and attached to the flange of a bench top ModulyoD-115 freeze dryer (Thermo Savant, Holbrook, NY) which had been pre-cooled to a condenser temperature of -50°C and freeze dried for 24 h. After freeze drying, the sample was grinded in a Waring blender and stored vacuum-sealed at -20°C until needed for oil extraction. Based on the intended purpose of the oil, the proximate composition of the fish skin was not determined except for moisture and oil content.

3.3.2.2. Solvent Extraction Methodologies

The solvent extraction methods investigated employed extraction at room temperature (ERT), Soxhlet or the Soxtec extraction systems using hexane or petroleum ether as solvents. The other extraction procedures used include the Folch method (FM) (Folch et al., 1957), the Bligh and Dyer method (BDM) (Bligh and Dyer, 1959), and the Radin method (RM) (Radin, 1981) using solvent mixtures of chloroform, methanol, and iso-propanol (Fig. 3.1). These last three methods were used as originally described and

outlined. With the exception of the Soxtec method, oil was collected in a preweighed flask and evaporated to dryness under reduced temperature in a rotary evaporator (Büchi-Rotavapor®, Flawil, St. Gallen) at 40°C. Final traces of solvent were removed by flushing with nitrogen. The weight of the oil was then gravimetrically determined.

3.3.2.2.1. Extraction at Room Temperature (ERT)

To one part of dried powdered fish skin was added 20 parts of hexane (ERT-Hex) or petroleum ether (ERT-PE) (1:20 w/v) in a 250 ml Erlenmeyer flask. The flask and content were shaken at 150 rpm for 2 h at 25°C. After the incubation period the mixture was homogenized in a Waring blender for 1 min and filtered under suction. The homogenate was re-suspended with 1/6 of the initial volume of solvent for *ca* 2 min and filtered again. This was repeated twice with the same volume of solvent.

3.3.2.2.2. Soxhlet Extraction

One part of the dried powdered fish skin was weighed into a 33 x 94 mm cellulose extraction thimble and covered with a silanized glass wool and extracted with 20 parts of petroleum ether (PE) or hexane (1:20 w/v) for 1, 2, 3, 4, 6, 8, 10, or 12 h at the boiling point of the solvent (Table 3.1) in a Soxhlet extractor. For each experimental condition, six replicates were run simultaneously.

3.3.2.2.3. Randall Extraction (Soxtec)

One part of the dried powdered fish skin was weighed into a 33 x 94 mm cellulose extraction thimble and covered with a silanized glass wool. To each part of sample were added 10, 12 or 14 parts of PE or hexane (w/v) in the extraction cup and then placed in a SER 148 Velp solvent extractor (Velp Scientifica, Usmate, Lombardy). The thimble with its contents were directly immersed in the boiling solvent for 60 min, and then suspended below the cooling condenser (from which the cold refluxed condensed solvent drops and wash the sample) to remove residual extractable materials for 30 min. Much of the solvent was recovered in the solvent reservoir during 15 min. The remaining solvent in the extraction cup with the oil was removed by flushing with nitrogen and the weight of the oil was determined gravimetrically.

The oil content from each of the methods described above was quantified on a dry weight basis (dwb) with reference to the total sample and subsets of the extracted oil subjected to further analysis. The precision of the techniques investigated was expressed by the relative standard deviation (RSD), and though generally there were significant differences ($p < 0.05$) in the yield of oil extracted within each method studied, RSD was <2.5%.

3.3.2.3. FFA Determination

FFA was determined according to the AOCS method (Ca 5a-40) (AOCS, 1998) and % FFA was calculated as oleic acid equivalents as follows:

$$\%\ \boldsymbol{FFA\ (oleic\ acid)} = \frac{\boldsymbol{(S-B)\ x\ N\ x\ 28.8}}{\boldsymbol{w}} \qquad (\mathbf{3.1})$$

where S and B are titre values of sample and blank respectively, N is the normality of NaOH, and w is the weight of the oil.

3.3.2.4. Data Analysis

Fish oil yields from the methods investigated were evaluated and compared using univariate (mean and standard deviation) statistical analysis. The data were subjected to *t-test* (confidence interval 95%) to compare differences between mean yields using the General Linear Model (GLM) procedure of SAS® (Statistical Analysis Systems, Version 9.1, SAS Institute Inc., Cary, NC). All extraction runs were carried out at least in triplicate and in randomized order.

3.4. Results and Discussion

Six solvent extraction techniques namely FM, BDM, RM, ERT, Soxhlet, and Soxtec were applied to the salmon skin samples and evaluated for SSO recovery; as well as the effect of mode and extraction time on the yield and quality of the oil recovered. The moisture content of salmon skin based on the lyophilization process ranged between 59 and 65%, similar to the 56.3% reported by Aursand et al. (1994) for Atlantic salmon skin.

The method of Folch et al. (1957) that was further extended by Bligh and Dyer (1959) has been adopted as the standard method for lipid extraction in marine organisms (Smedes and Thomasen, 1996; Smedes and Askland, 1999). An oil yield of *ca* 22.27% dwb was obtained with the BDM after the first extraction (Table 3.2). An additional 3-10% of SSO was obtained after the second and third extractions using the residue (Table 3.2). This is in agreement with the reports of Roose and Smedes (1996) and Smedes and Askland (1999), who attributed additional recovery after the first extraction to the absorption of the organic phase by the tissue. The FM gave higher SSO yield (38%) in comparison with the BDM during the first extraction, but as shown in Table 3.2 only *ca* 0-7% SSO was recovered in the second and third extractions. In total, the FM recovered about 25% more SSO than the BDM. The BDM has been reported to produce lower estimates of lipid content than the FM with increasing lipid content (Iverson et al., 2001) since the composition of the BDM mixture is relatively more polar than the FM solvent mixture. Increasing total lipids in animal tissue often corresponds to an increase in TAGs. In relatively polar solvent systems such as the one employed by the BDM, there is limited solubility of TAGs, and may have resulted in the poor oil yield. The use of mineral salts in the washing step of these methods has also been explained to have a distribution altering effect by shifting the lipids into the lower lipid phase, eliminating the lipid from the upper aqueous phase (Folch et al., 1957). Washing also removes non-lipid contaminants from the crude extract and negligible amounts of lipids.

The FM and BDM employ mild extraction conditions; neither heat nor high pressures are applied; which avoid potential alterations to the oil extracted. The main difference between the two systems is the volume of the solvent used. The BDM uses chloroform:methanol:water ($CHCl_3$:CH_3OH:H_2O) at 1:2:0.8 and 2:2:1.8 (v/v) before and after dilution, respectively, resulting in a monophasic system, the FM also uses $CHCl_3$:CH_3OH:H_2O but at 8:4:3 (v/v) resulting in a biphasic system. In general, the FM required a large amount of $CHCl_3$:CH_3OH; 1 part of sample to 20 parts of 2:1 $CHCl_3$:CH_3OH followed by several washings of the extract. In contrast, the BDM uses 1 part of the sample to 3 parts of 1:2 $CHCl_3$:CH_3OH followed by 1 part of $CHCl_3$. The FM is thus expensive owing to the high amount of solvents required. Both methods are too exhaustive, time consuming, and involve the use of chloroform, a suspected carcinogen.

Due to issues of chloroform toxicity and stricter solvent disposal programs, a relatively safer organic solvent was proposed by Radin (1981). The RM employs the hexane-isopropanol solvent system, and extracts both simple lipid classes and more complex polar lipids bound to cellular constituents like membrane proteins. In this study, the RM yielded *ca* 32% dwb of SSO versus 35% by BDM and 43% by FM. The low yield of the RM has been attributed to the low polarity of iso-propanol in comparison to methanol, and the limited efficiency of hexane in extracting polar lipids compared to chloroform (Gunnlaugsdottir and Ackman, 1993; Undeland et al., 1998). According to Christie (1992), extraction solvents or mixtures of solvents should be sufficiently polar to remove lipids from their association with other cell constituents, but not too polar that the solvents do not readily dissolve all the TAGs and other non-polar lipids.

The low yield achieved with the room temperature (25°C) extraction method; ERT can also be attributed to the relatively lower interaction between the solvent and the matrix at that reduced temperature (de Boer, 1988). The ERT-PE and ERT-Hex yielded *ca* 23% and 25% SSO, respectively. Extraction time and temperature have been shown to be important variables which affect oil recovery (de Boer, 1988). Similar to the classical methods (FM, BDM, and RM), insufficient contact time between the solute and the solvent may have accounted for the poor SSO yield (Gunnlaugsdottir and Ackman, 1993). This could also be due to decreasing oil solubility in the solvents at ambient temperature as a result of decreased ease of penetration of the solvent molecules into the matrix. Solute diffusivity and solute solubility have been reported to be affected by extraction temperature (Akaranta and Anusiem, 1996; Hu et al., 2007); however this is dependent on specific solute-solvent systems (Hu et al., 2007).

After establishing that none of the above methods (FM, BDM, and RM) were distinctly superior to each other in terms of SSO yield, the Soxhlet technique was employed to study the efficiency and effectiveness of petroleum ether (PE) and hexane in extracting SSO at their respective boiling points. There was no significant difference ($p > 0.05$) in oil yield between extraction times 1 or 2 h (Fig. 3.2) but an appreciable increase beyond 2 h and up to 3 h, indicating that increasing contact time between the solvent and salmon skin resulted in a much greater yield. Though statistically significant ($p < 0.05$), SSO recovery beyond 3 h showed no appreciable increase. The use of hexane, in general,

resulted in higher oil recoveries than PE at all the extraction times employed. For instance, hexane recovered 17, 9, 11, 6, 7, and 9% more SSO than PE at 3, 4, 6, 8, 10, and 12 h of extraction, respectively and this can be attributed to the higher solvation power of hexane. However, at extraction time 1 and 2 h, PE recovered 10 and 6% more SSO than hexane, respectively.

One of the numerous automated appliances and principles developed after Soxhlet was Soxtec. It considerably decreases the extraction time required to complete a batch of samples, reduces solvent required and facilitate solvent recovery at the end of a run. The permanent contact with hot solvent may be the reason for its success. To optimize oil extraction yield, extraction time in terms of immersion and washing, and solvent volume were varied. Higher yields were obtained when immersion and washing times were 30 min and 1 h, respectively, with 10 parts of solvents to 1 part of sample. The Soxtec technique extracted 0.2% and 0.7% less SSO at sample:solvent ratio of 1:12 and 1:14, respectively. Thus increasing solvent volume did not lead to an increase in SSO recovery. As shown in Fig. 3.3, the use of hexane provides an alternative to PE for lipid extractions as it gave higher oil recovery than PE ($p < 0.05$). Hexane recovered 5.2, 5.3, and 4.5% more SSO at 1:10, 1:12, and 1:14 sample:solvent ratio, respectively than PE. Unlike Soxhlet, Soxtec reduces exposure to solvents since >85% of the solvent was recovered by the instrument and only a diminutive fraction of solvent remained with the oil in the extraction cup, which was flushed with a stream of nitrogen at the end of the run.

The FFA content is an important quality index for oils, thus studies were conducted to determine the effect of extraction method on the FFA content of the oil. Due the relatively high unsaturated fatty acid content of fish oils, mild extraction methods are preferred to minimize oxidative damage, and formation of undesirable co-products. This in part is the reason why higher boiling point solvents like toluene were not investigated, as higher temperatures will be needed during extraction and also after extraction to rid the oil of solvent. The data for the FFA content in the extracted SSO are presented in Fig. 3.4. The allowable amount of FFA in edible crude fish oil is suggested as 2-5% (Young, 1985) with maximum acceptability at 4%. In general, no significant FFA degradation occurred in any of the extraction methods employed in this study as shown in Fig. 3.4. It can be seen that the temperatures employed were not extremely high to cause hydrolysis

of the ester bonds in TAG to produce high levels of FFA. As shown in Fig. 3.4, SSO obtained from the Soxtec, RM, and FM had appreciably less FFA than the other methods. Relatively higher amounts, though not excessive, FFA were recorded in both solvents and at all the extraction times in the Soxhlet technique. A relative lower FFA content was measured in the Soxtec-hexane-extracted-SSO and this can be attributed to relatively short extraction time, and this trend was similar to varying solvent volumes and extraction times. This, in addition to ease of the Soxtec method and higher SSO yield are the reasons why it is the method of choice for subsequent oil recovery from salmon skin.

3.5. Conclusion

The methods investigated extracted varying amounts of SSO depending on solvent type, extraction time and temperature. At ambient temperature, neither hexane nor petroleum ether was a good solvent for SSO extraction but at their boiling points their efficiency to extract SSO increased appreciably. Both FM and BDM gave lower oil yield. Three hours was adequate extraction time for the Soxhlet technique to obtain high yields of SSO, while longer extraction times led to no significant increase in SSO recovered. The results from the Soxtec extraction technique indicated significant reduction in extraction time and volume of solvent, with higher oil yields compared to the Soxhlet technique. Higher amount of oil was recovered when hexane was used as solvent coupled with safer handling and cheaper price in comparison with petroleum ether. This afforded the convenience of a fast and safe extraction procedure. Additionally, greater than 85% of the solvent was recovered at the end of each run with the Soxtec technique. Relatively low FFA content was recorded in most of the techniques investigated.

3.6. Acknowledgements

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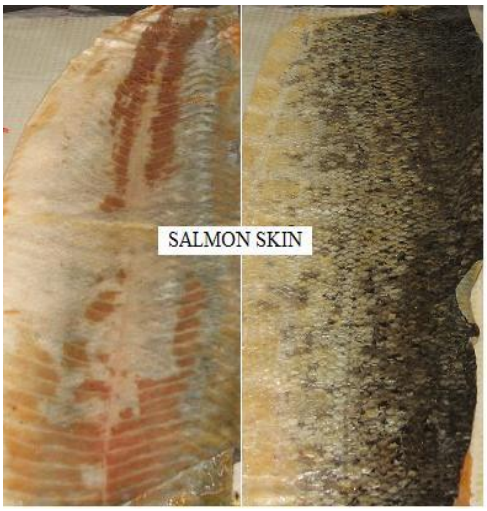

Picture 3.1: Dissected salmon skin.

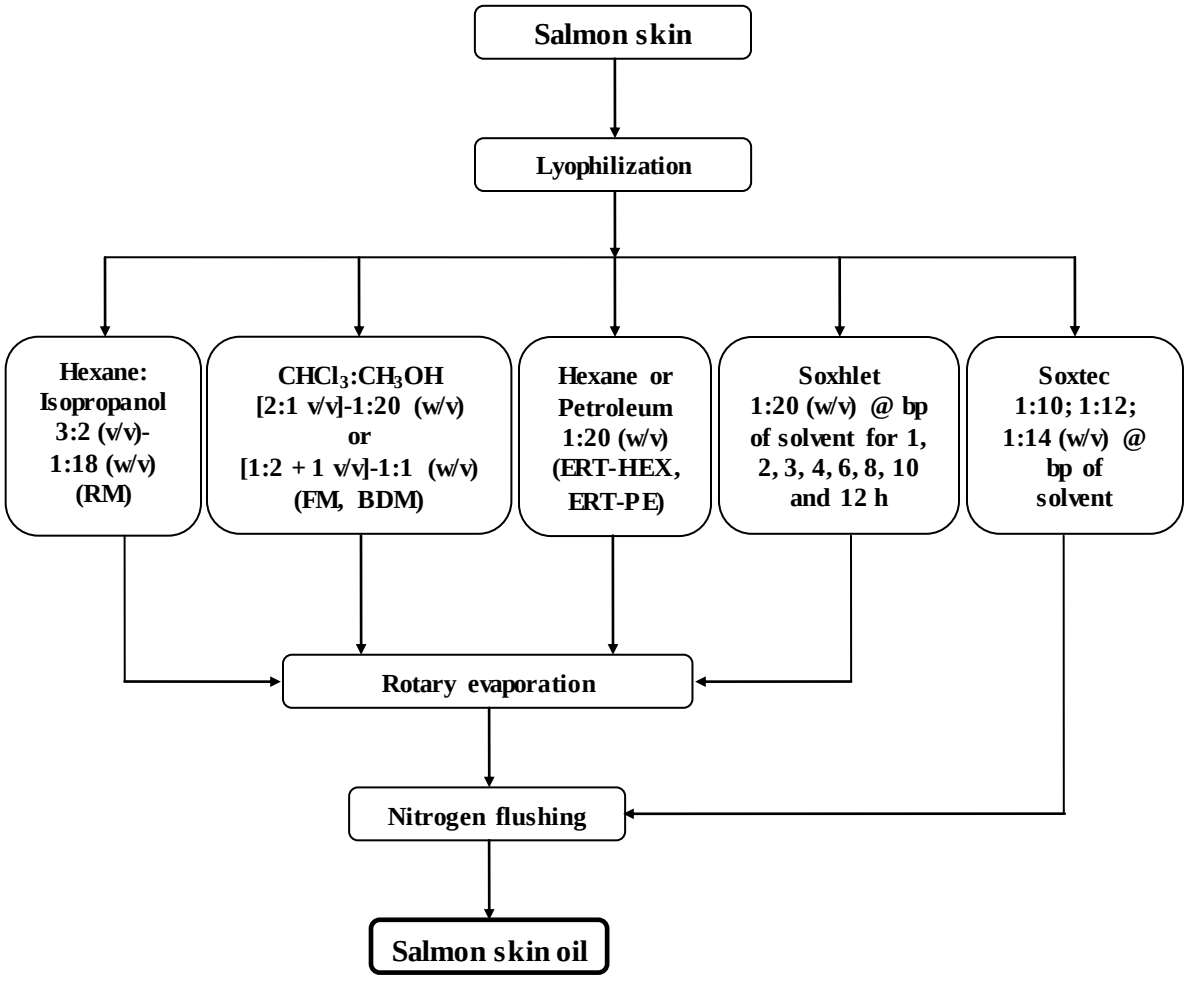

Fig. 3.1: Schematic diagram of the extraction protocol.

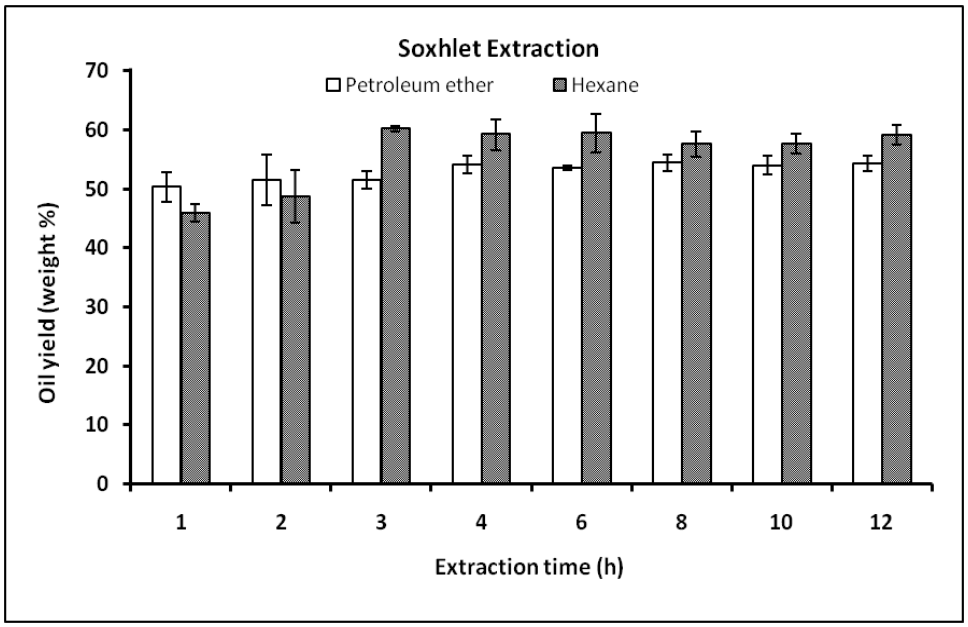

Fig. 3.2: Effect of solvent type and extraction time on the yield of salmon skin oil (SSO). Columns and bars represent average oil yield and their respective standard errors.

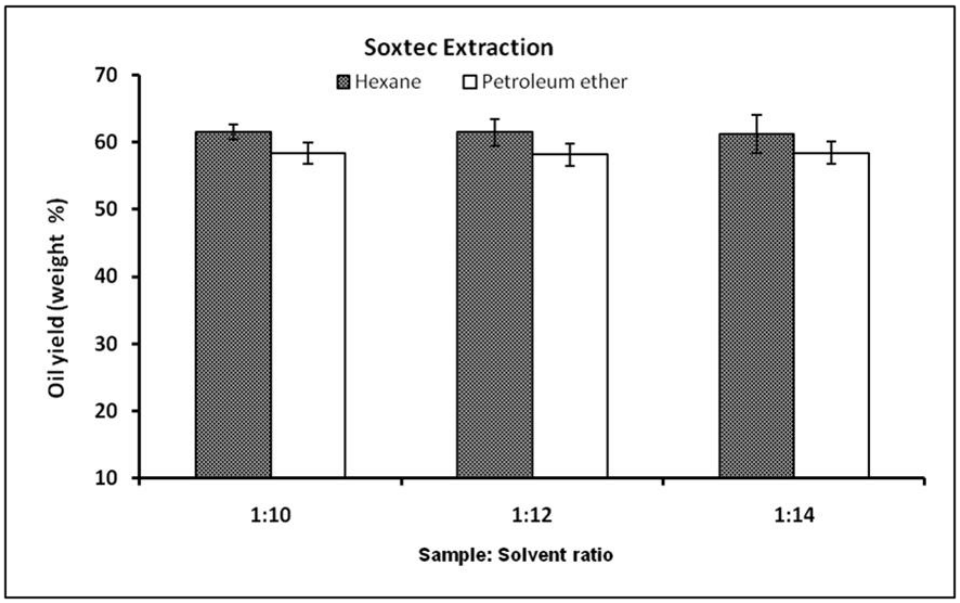

Fig. 3.3: Effect of solvent type and sample:solvent ratio on the yield of salmon skin oil (SSO). Columns and bars represent average oil yield and their respective standard errors.

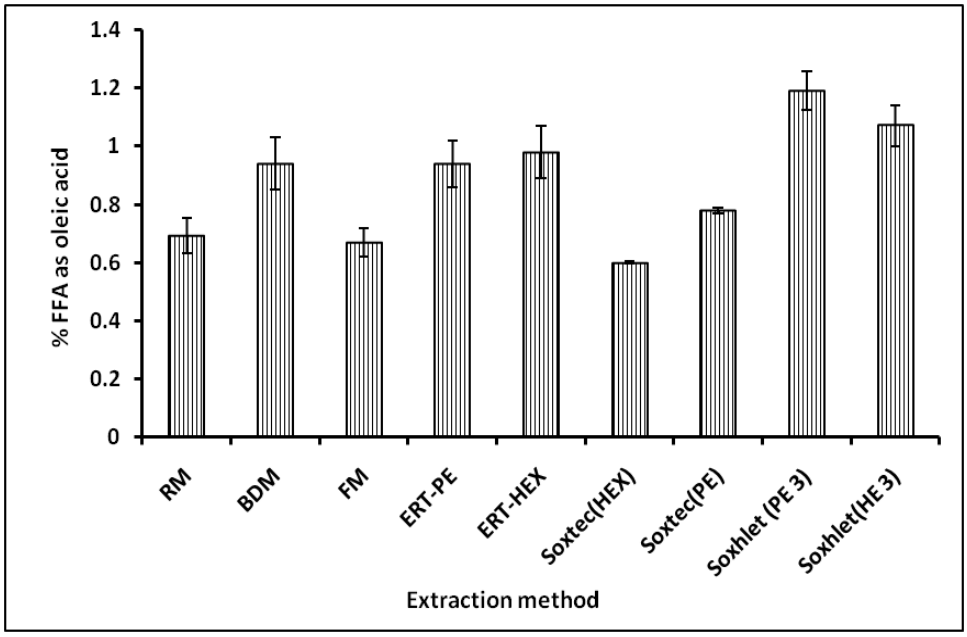

Fig. 3.4: Effect of extraction method on the free fatty acid (FFA) content of salmon skin oil (SSO). RM (Radin method), BDM (Bligh and Dyer method), FM (Folch method), ERT-PE, and ERT-HEX (extraction at room temperature using Petroleum Ether {PE} or Hexane {HEX}), Soxtec, and Soxhlet (using PE and Hex; 3 h of extraction). Columns and bars represent average oil yield and their respective standard errors.

Solvents	Structure	Boiling point (^oC)	Refractive index at 25^oC	Density (g/ml) at 25^oC	Cost of solvent ($/L)
Petroleum ether	Mixture of hydrocarbon	20-75	1.379	0.656	11.15
Chloroform	$CHCl_3$	61.2	1.446	1.490	13.69
Hexane	C_6H_{14}	68.7	1.378	0.648	6.56
Methanol	CH_3OH	64.7	1.328	0.791	4.16
Iso-propanol	$CH_3HCOHCH_3$	82.2	1.377	0.785	7.04

Table 3.1: Characteristics of the solvents used for oil extraction.

Trial	Mass of oil after 1st extraction, g (% oil)	Mass of oil after 2nd extraction, g (% oil)	Mass of oil after 3rd extraction, g (% oil)	Total oil, g (% oil)
		BDM		
1	2.24 (22.40)	0.81 (9.54)	0.23 (3.25)	3.28 (35.19)
2	2.39 (23.90)	0.90 (9.89)	0.31 (3.64)	3.60 (37.43)
3	2.05 (20.50)	0.88 (9.65)	0.23 (2.68)	3.16 (32.83)
AVE	2.23 (22.27)	0.86 (9.69)	0.26 (3.19)	3.35[b] (35.15)
SD	0.17	0.05	0.05	0.23
		FM		
1	3.90 (39.00)	0.39 (4.33)	0 (0)	4.29 (43.33)
2	3.66 (36.60)	0.57 (6.33)	0 (0)	4.23 (42.93)
3	3.83 (38.30)	0.62 (6.89)	0 (0)	4.45 (45.19)
AVE	3.80 (38.00)	0.53 (5.85)	0 (0)	4.32[a] (43.82)
SD	0.12	0.12	0	0.11
		RM		
1	2.99 (29.90)	0.10 (1.11)	0 (0)	3.09 (31.01)
2	3.19 (31.90)	0.06 (0.67)	0 (0)	3.34 (32.57)
3	3.26 (32.60)	0.04 (0.44)	0 (0)	3.30 (33.04)
AVE	3.15 (31.47)	0.07 (0.74)	0 (0)	3.21[b] (32.21)
SD	0.14	0.03	0	0.11
		ERT-PE		
1	2.29 (22.90)	0.04 (0.42)	0 (0)	2.33 (23.32)
2	2.21 (22.10)	0.02 (0.21)	0 (0)	2.23 (22.32)
3	2.21 (22.10)	0.20 (2.20)	0 (0)	2.43 (24.32)
AVE	2.24 (22.37)	0.09 (0.94)	0 (0)	2.33[c] (23.32)
SD	0.04	0.10	0	0.05
		ERT-HEX		
1	2.41 (24.10)	0.13 (1.30)	0 (0)	2.54 (25.40)
2	2.35 (23.50)	0.33 (3.32)	0 (0)	2.68 (26.82)
3	2.29 (22.09)	0.20 (2.0)	0 (0)	2.49 (24.09)
AVE	2.35 (23.23)	0.22 (2.20)	0 (0)	2.57[c] (25.24)
SD	0.06	0.10	0	0.08

Table 3.2: Mean gravimetric yield of salmon skin oil (SSO) and their respective standard deviation (SD). Means with same letter are not significantly different ($p < 0.05$).

CHAPTER IV

CONNECTING STATEMENT

In the previous manuscript (Chapter III) the effect of extraction parameters (technique, solvent type, extraction time, and temperature) on the yield and free fatty acid (FFA) content of salmon skin oil (SSO) were comparatively assessed. Oxidative stability is a major technical issue in oils, especially fish oils, and markedly in unrefined fish oils. This is due to the propensity of the unsaturated component of the oil to oxidize during storage. As alternative biodiesel (BD) feedstock, unrefined oils will require ample evaluation of their stability during storage to track quality changes to allow requisite modification to the transesterification protocol to accommodate any variations in the quality of the oil. The study in this chapter intends to gather information on the stability of the unmodified salmon skin oil monitored during storage at various temperature and duration. Some of the most relevant quality indices of BD feedstock during storage were evaluated. Generally an inverse relationship between storage duration and temperature on the stability of SSO was observed.

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CHAPTER IV

EFFECT OF TEMPERATURE AND TIME ON THE STABILITY OF SALMON SKIN OIL DURING STORAGE

4.1. Abstract

The effect of 45 days of storage at 25^{o}C, 4^{o}C, -18^{o}C, and -80^{o}C on the quality indices; free fatty acid (FFA) content, peroxide value (PV), thiobarbituric acid reactive substances (TBARS), and changes in the fatty acid (FA) profile of crude oil recovered from salmon skin were evaluated at 5 day intervals using spectrophotometric and titrimetric methods. Higher temperatures and longer storage time resulted in higher quantities of oxidative products in the salmon skin oil (SSO). By day 45, SSO stored at 25^{o}C and 4^{o}C had 8.50 and 8.29% FFA, 32.43 and 26.33 µg malondialdehyde (MDA) eq/ g oil, and 88.19 and 64.53 mequiv peroxide/kg oil, respectively. No significant ($p > 0.05$) changes in FA profile were observed at all the storage temperature and time studied.

4.2. Introduction

Fish oils have been known for their beneficial role in health and nutrition for decades. These benefits have been attributed to the high content of polyunsaturated fatty acids (PUFA) especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Rafflenbeul, 2001). Fish oils are generally extracted from targeted catches or recovered from processing by-products (Aidos et al., 2002; Huang and Sathivel, 2008).

Fishery processing by-products are an abundant and relatively underexploited source of oil which can be used as a non-food feedstock for biodiesel (BD) production. With increasing acceptance and demand of BD as an alternative fuel, there has been significant interest in the search for newer and cheaper renewable feedstocks (Berchmans and Hirata, 2008) of minimal or no food value.

Salmon is an important finfish of the Canadian fishery and aquaculture industries. In 2008 alone, salmon harvest in Canada was estimated at over 107 thousand metric tonnes and valued at more than $642 million (DFO 2008). The by-products generated during salmon processing include head, offal, scales, fins, and skin, of which the skin constitutes about 7% (Falch et al., 2006). The skin is often combined with other fish offal

for fish meal and oil production or discarded (Falch et al., 2006; Huang and Sathivel, 2008). Depending on the extraction parameters and techniques, *ca* 23-62% oil (on dry weight basis) can be obtained from salmon skin (Aryee and Simpson, 2009), making salmon skin, a processing by-product, a source of abundant oil.

The high PUFA content of fish oil however increases its susceptibility to oxidative spoilage (Aidos et al., 2002; Huang and Sathivel, 2008) leading to the formation of primary peroxides (hydroperoxides) in the presence of oxygen, the degradation of the peroxides, and formation of secondary products such as volatile and non-volatile aldehydes, ketones, alcohols, lactones, and tertiary oxidation products (Aidos et al., 2002; Bower et al., 2009). Additionally, high amounts of PUFA in fuels have been linked to engine failure and sediment formation (Schober et al., 2009). The composition of oxidized oils is an important quality index (Berchmans and Hirata, 2008); however it is embodied in a complex mechanism with intricate implications on the intended use. The quantification of these oxidation products is important in describing the quality status of the oil for food and non-food application.

Lipid oxidation studies have led to the development of rapid quantification methods for routine assessment of quality indices. Given that there is no single specific satisfactory index for lipid oxidation, several analytical techniques, some of which may often partially overlap have been used to describe and quantify the composition of oxidized oils. The primary and secondary products of oxidation for instance are quantified by the peroxide value (PV) and *p*-anisidine value (AnV) tests, respectively (Aidos et al., 2002; Bower et al., 2009). Some of the many techniques and instruments proposed to measure lipid stability and quality include traditional methods such as titration and iodometry, and instrumentation methods such as UV-Visible spectroscopy, liquid chromatography, Fourier transform infrared spectroscopy, and differential scanning calorimetry (Pokorný et al., 2005; Huang and Sathivel, 2008; Aryee et al., 2009).

Fish oils from various fish species and parts of the fish exhibit varying levels of oxidation and stability under storage (Aidos et al., 2002; Boran et al., 2006; Wu and Bechtel, 2009). These workers identified temperature and storage duration as important influences on lipid oxidation. Although studies on fish oils abound and a number of references are available on the physicochemical composition and properties, oil content,

and fatty acid (FA) profile (Aidos et al., 2002; Boran et al., 2006; Huang and Sathivel, 2008), detailed studies on solvent-extracted salmon skin oil are scanty. This study is part of research work aimed at converting and adding value to otherwise discarded fishery processing by-products. This present study describes the effect of storage temperature and time on the quality of SSO to be used for BD production.

4.3. Materials and Methods

4.3.1. Materials

Salmon skin samples were obtained from a commercial processor in Mont-Louis (Atkins et Frères Inc., Mont-Louis, QC) (n = 36, 75.5-89.5 g, and 43-47 cm L x 9-20 cm W), vacuum-packed, and shipped under frozen conditions on February 13th, 2008 to the laboratory where they were stored at -20^{o}C until needed. Chloroform, acetic acid, hexane, iso-octane, sodium hydroxide (NaOH), sodium thiosulphate ($Na_2S_2O_3$), and potassium iodide (KI) were purchased from Fisher Scientific (Whitby, ON). Trichloroacetic acid (TCA), 1,1,3,3-tetraethoxypropane (TEP), and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (Oakville, ON). Ethanol was purchased from Commercial Alcohols (Boucherville, QC). Standards of 20:5, 22:5, and 22:6 as well as two standard mixes of 10:0, 12:0, 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, and 18:3 were purchased from Supelco (St. Louis, MO).

4.3.2. Methods

4.3.2.1. Oil Extraction

The fish skins were freeze-dried for 24 h in a bench top ModulyoD-115 freeze dryer (Thermo Savant, Holbrook, NY) and stored at -20^{o}C until needed for extraction. Salmon skin oil (SSO) was extracted with hexane from the freeze-dried salmon skin according to a method previously described (Aryee and Simpson, 2009). It involved extraction of 1 part of the crushed fish skin with 10 parts of hexane (w/v) using a SER 148 Velp solvent extractor (Velp Scientifica, Usmate, Lombardy).

4.3.2.2. Effect of Storage Temperature and Time on Quality Parameters

Pooled samples of SSO were stored in amber glass vials at 25, 4, -18, and -80°C for 1, 5, 10, 15, 20, 25, 30, 35, 40, and 45 days. Aliquots from each treatment combination were analyzed in duplicates for FFA, PV, and TBARS at the respective storage day. The FA profile of SSO was determined at day 1, 25, and 45.

4.3.2.2.1. Free Fatty Acid (FFA) Content

FFA content was determined by titration according to the AOCS method (Ca 5a-40) with standardized 0.1 N NaOH, to a phenolphthalein endpoint and expressed as % oleic acid equivalent:

$$\%\ \boldsymbol{FFA\ (oleic\ acid)} = \frac{\boldsymbol{(S-B)\ x\ N\ x\ 28.8}}{\boldsymbol{w}} \qquad (\mathbf{4.1})$$

where S and B are titre values of sample and blank, respectively, N is the normality of NaOH, and w is weight of the oil.

4.3.2.2.2. Peroxide Value (PV)

PV was determined by titration according to the AOCS method (Ca 8-53) with standardized 0.01 N $Na_2S_2O_3$ and starch as indicator. PV was calculated as:

$$\boldsymbol{PV\ (meq\ peroxide\ kg^{-1}\ oil)} = \frac{\boldsymbol{(S-B)\ x\ N\ x\ 100}}{\boldsymbol{w}} \qquad (\mathbf{4.2})$$

where S and B are titre values of sample and blank respectively, N is the normality of $Na_2S_2O_3$, and w is weight of the oil. PV was expressed as mequiv peroxide/kg oil.

4.3.2.2.3. Thiobarbituric Acid Reactive Substances (TBARS)

TBARS value of SSO was measured according to the method of Salih et al. (1987) with 1,1,3,3-tetraethoxypropane [Malondialdehyde bis (diethyl acetal), (MDA)] as standard. Sample solutions were prepared by homogenizing approximately 0.1 g of SSO in 25 ml of 5% TCA for 5 min at 25°C with Polytron® (PT-MR 3000, Kinematica AG, Littau, Lucerne) at 4500 rpm. Two milliliter aliquots of the homogenized sample were

added to 3 ml of 0.02 M TBA in 5% TCA in screw cap tubes. The contents were heated in a boiling water bath for 45 min and rapidly cooled on ice. The absorbance of the cooled solution was measured at 532 nm in a DU® 800 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). TBARS values were expressed as total µg of MDA equivalents/g oil.

4.3.2.2.4. Fatty Acid (FA) Profile

FA profile of SSO from the various treatment combinations were determined using gas chromatography (GC) (Hewlett-Packard, Palo Alto, CA) without replication, according to the method of Metcalfe et al. (1966). In brief, 70-100 mg of SSO was accurately weighed into tubes. To this was added 5 ml boron trifluoride in methanol, dimethoxypropane, and decanoic acid (C10:0) as an internal standard, and fitted with a PTFE-lined screw cap. After careful mixing, the contents were heated at 70°C for 10 min. Hexane and water were added to the sample and thoroughly vortexed. The hexane (organic) layer was transferred into tubes and dried with anhydrous sodium sulfate for 20 min, after which the supernatant was carefully transferred to amber GC vials for injection or storage at -15°C.

The GC was equipped with an Omegawax 32 (Supelco, St. Louis, MO) 30 m x 320 µm x 0.3 µm capillary column, and a flame ionization detector (FID). The oven was set for a 5°C/min ramp, from 80°C to a final temperature of 240°C and a total run time of 42 min. Helium was used as the carrier gas at a flow rate of 21.3 ml/min. An autosampler injected 1 µl of the sample or standard with a 10:1 split, and a 5 min solvent delay. Peaks were identified by comparison with standards. The results were expressed as percentage of total fatty acids.

4.3.2.3. Data Analysis

The results were subjected to statistical analysis using the General Linear Model (GLM) of SAS® (Statistical Analysis Systems, Version 9.1, SAS Institute Inc., Cary, NC). Tukey's test for multiple comparisons was used to compare the data for significant differences ($p < 0.05$) among storage temperatures as well as respective days. Values used for the graphs were means ± standard deviation (SD). The Wilcoxon's matched

pairs test was used to evaluate significant differences in individual FAs between and within treatment combinations (Wu and Bechtel, 2009).

4.4. Results and Discussion

4.4.1. Salmon Skin Oil (SSO) Extraction

Salmon skin usually contains *ca* 60% moisture which reduces the efficiency of oil recovery by direct extraction (Aursand et al., 1994; Aryee and Simpson, 2009). To improve oil extraction, salmon skins were freeze-dried to reduce the moisture content (Aryee and Simpson, 2009). Extraction yielded approximately 58% (dry weight basis) SSO and this value is consistent with our previous report (Aryee and Simpson, 2009).

4.4.2. Free Fatty Acid (FFA) Content

Irrespective of storage temperature, there were fluctuations in FFA content until day 35 (Fig. 4.1). This suggests that consistent and significant hydrolysis of the oil did not occur during day 1 to 35. This may possibly be due to the low moisture content of SSO, and storage duration. The FFA content increased from 6.58, 7.32, 7.14, and 7.09% to a maximum at day 45, with 8.50, 8.29, 8.19, and 7.86% FFA content at 25, 4, -18, and -80^{o}C, respectively. There were marginal but significant increases ($p < 0.05$) in FFA content between samples stored at days 1 and 45 at all the temperatures investigated. SSO stored at -80^{o}C recorded the lowest FFA content during the storage period while generally higher amounts of FFA were recorded at 25^{o}C. There were no significant differences ($p > 0.05$) in the samples stored at either -80 or -18^{o}C during the storage period. Similar studies by Bower et al. (2009), on the oxidation of oils from raw and smoked pink salmon heads found samples stored at 4^{o}C to be more stable than samples stored at 35^{o}C. Wu and Bechtel (2009) and other workers (Boran et al., 2006) observed increasing FFA content with increasing temperature and time. The acidity of unrefined oils has been attributed to the presence of components such as amino acids, phospholipids and oxidized lipids (other than FFA) (Zhou and Ackman, 1996). The presence of moisture and long chain PUFA may also accelerate hydrolysis of the oil and increase FFA content (Boran et al., 2006). Boran et al. (2006) and other workers (Wu and Bechtel,

2009) attributed the higher FFA content in the oils to higher hydrolytic rate at higher temperatures.

The FFA content is an important quality indicator as well as a useful parameter for the selection of a catalyst in transesterification reactions and thus an important index in BD production (Berchmans and Hirata, 2008; Aryee et al., 2009). Non-conventional unrefined oils and fats do not always meet the highest quality standard as BD feedstocks. In base-catalyzed transesterification reactions, feedstocks with a FFA content of >0.5% require multistep processes, such as acid esterification pretreatment to lower the FFA content and avert soap formation, while oils with <0.5% FFA can be directly transesterified by a base catalyst in a one-step process (Berchmans and Hirata, 2008).

4.4.3. Peroxide Value (PV)

The PV test is a conventional method for quantifying total hydroperoxides content. The PV test measures the hydroperoxide concentration of oxidized oils. The titrimetric method is based on the reaction of hydroperoxides with potassium iodide in an acidic medium to produce iodine. PV generally increased in all the samples at the various storage temperatures during the study period (Fig. 4.2). Similar trends were observed by various workers who studied oxidative stability of various fish oils (Aidos et al., 2002; Boran et al., 2006; Zuta et al., 2007). There was an initial increase of *ca* 23, 5, 18 and 13% in PV at 25, 4, -18, and -80^{o}C from day 1 to 5, but these increases were not significant ($p > 0.05$). SSO stored at -18 and -80^{o}C showed small increases in PV over time, while samples stored at 4^{o}C recorded larger increases. There were appreciable increases and significant differences ($p < 0.05$) between the PV of samples stored at days 1 and 45 at 25, 4, -18, and -80^{o}C. After 45 days of storage, the PV of SSO at 25, 4, -18, and -80^{o}C were 88.19, 64.53, 59.38, and 34.47 mequiv peroxide/kg oil, respectively. The increases in PV with temperature and time suggest the development of primary oxidation products; hydroperoxides, from thermo-oxidative or lipid oxidation processes. These oxidation products may later break down to produce lower molecular weight compounds, such as FFAs, alcohols, and aldehydes (Aidos et al., 2002; Boran et al., 2006; Wu and Bechtel, 2009). Various workers observed from studies on several fish species such as herring (Aidos et al., 2002), horse mackerel, shad, garfish, and golden mullet (Boran et

al., 2006), and salmon (Wu and Bechtel, 2008), that storage temperature and storage time had significant effects on the quality and stability of the oils.

4.4.4. Thiobarbituric Acid Reactive Substances (TBARS)

The TBARS test involves the quantification of secondary oxidation products from the breakdown of hydroperoxides (Dahle et al., 1962). The assay is based on the reaction of 2-thiobarbituric acid (TBA) and malonaldehyde or malonaldehyde-type products which results in the formation of a colored compound that corresponds to a maximum absorbance at a wavelength of approximately 530 nm (Pokorný et al., 2005). Figure 4.3 shows the changes in TBARS of SSO as a function of time at the various storage temperatures. The results obtained at the various treatment combinations did not show significant differences ($p > 0.05$) in TBARS values until day 30. This is consistent with results from other workers (Boran et al., 2006; Zuta et al., 2007; Wu and Bechtel, 2009). There were 4.32, 4.13, 2.86, and 3.89 µg MDA eq/g oil on day 1 at 25, 4, -18, and -80^{o}C, respectively. By day 45, the levels had increased to 32.43, 26.33, 25.80, and 17.04 µg MDA eq/g oil. The higher TBARS values towards the end of the storage period (beyond day 25) may be ascribed to higher oxidation rate and the decomposition of hydroperoxides to secondary oxidation products (Boran et al., 2006; Wu and Bechtel, 2009).

4.4.5. Fatty Acid (FA) Profile

As shown in Table 4.1, the major FAs identified in SSO were C16:0 (palmitic), C18:1*n*-9 (oleic), C18:2*n*-6 (linoleic), C20:5*n*-3 (eicosapentaenoic), and C22:6*n*-3 (docosahexaenoic), constituting *ca* 76-79% of the total FAs. The highest concentration of saturated fatty acid (SFA) was palmitic acid (C16:0) with values ranging from 17.31 to 19.85%. High SFA content has been reported to improve the cetane number; a measure of the combustion quality of diesel fuels during compression ignition (Lin and Li, 2009; Fan et al., 2010). The most abundant monounsaturated fatty acids (MUFA) was oleic acid (19.42 to 21.84%), while the most abundant PUFA was EPA with values ranging from 12.69 to 14.97% of the total FA. A high PUFA content is known to increase susceptibility to oxidation (Aidos et al., 2002; Huang and Sathivel, 2008; Wu and Bechtel, 2009). Even

though there were small changes in the proportions of individual FA stored at 25, 4, -18, and -80^{o}C at days 1, 25, and 45, there were no significant ($p > 0.05$) changes in the FA profile. These results show that SSO could be kept at these respective temperature and time with no major changes in the FA composition. Although fish oils are cheaper feedstocks compared to vegetable oils, various workers have called for limited quantities in BD because of the established link between high PUFA content and the propensity for engine failure, and sediment and polymer formation (Schober et al., 2009; Fan et al., 2010). In fact a 1% PUFA maximum has been set by the EN 14214 (European Standard for Biodiesel) specifications (Schober et al., 2009). Fish oil blended with used cooking oil was shown to meet BD specification (Fan et al., 2010).

4.5. Conclusion

This study indicated that temperature and duration were important factors that affect oil oxidation during storage. PV, TBARS, and FFA content generally increased with storage temperature and time, while the FA composition was generally not significantly affected by temperature and time. Recovered SSO could be stored for up to 45 days at either -18^{o}C or -80^{o}C with minimal increases and variations in PV, TBARS, and FFA content. These tests which monitored the stability or deterioration of SSO during storage will serve to expand its use as a non-food feedstock for BD production by controlling oxidative damage, and devising the most suitable transesterification reaction process.

4.6. Acknowledgments

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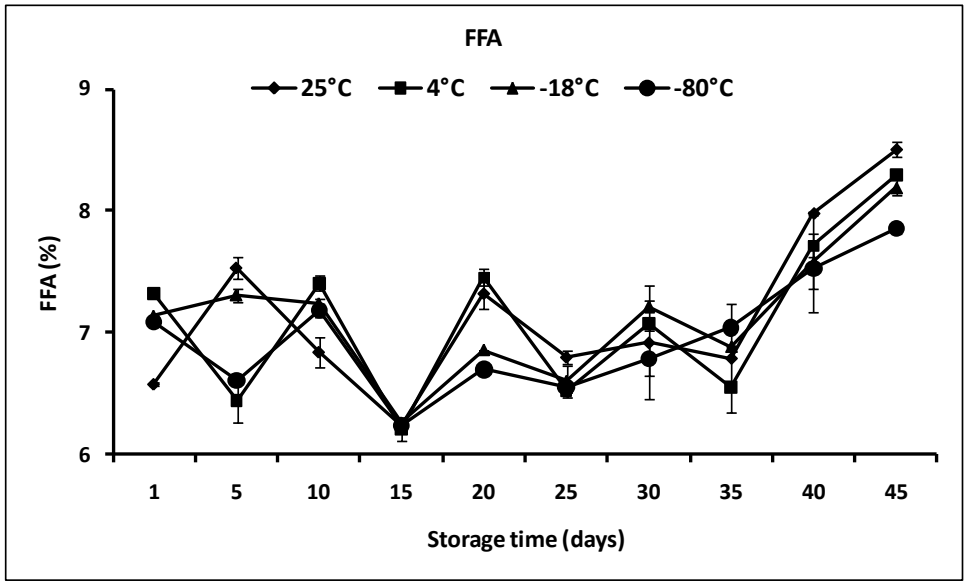

Fig. 4.1: The effect of storage temperature and time intervals on the free fatty acid (FFA) content in salmon skin oil (SSO).

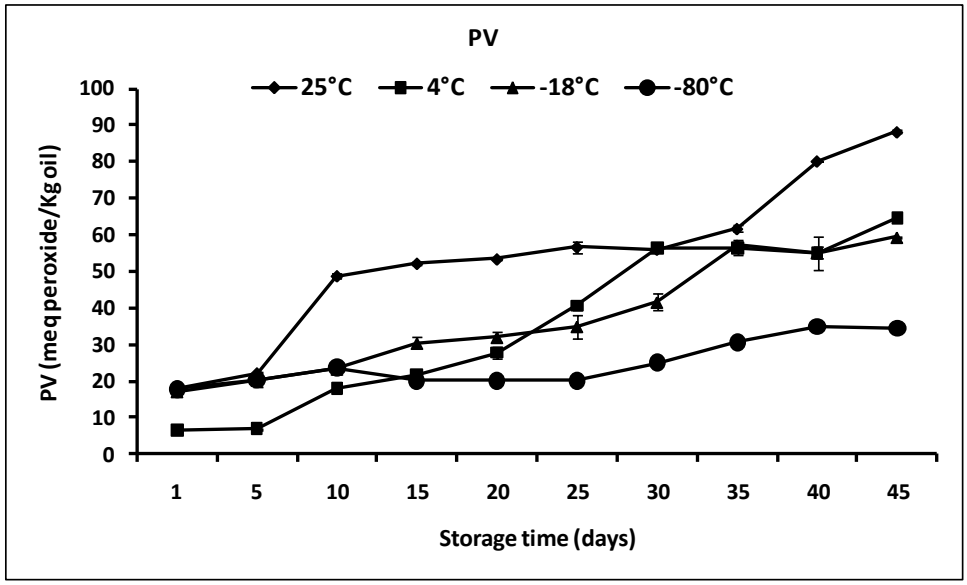

Fig. 4.2: The effect of storage temperature and time intervals on the peroxide value (PV) in salmon skin oil (SSO).

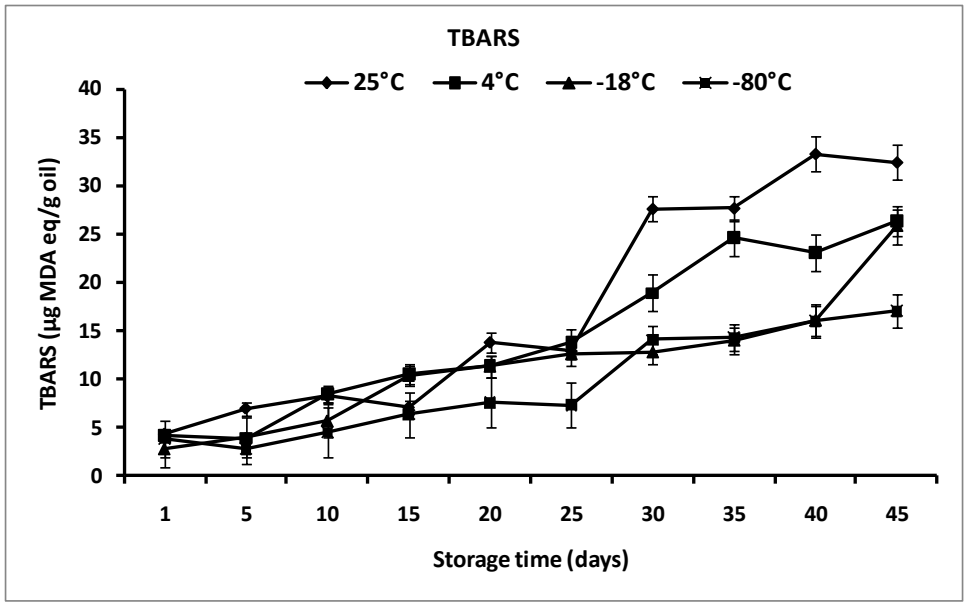

Fig. 4.3: The effect of storage temperature and time intervals on the thiobarbituric reactive substances (TBARS) in salmon skin oil (SSO).

Fatty Acids	% FA											
	25°C			4°C			-18°C			-80°C		
	Day 1	Day 25	Day 45	Day 1	Day 25	Day 45	Day 1	Day 25	Day 45	Day 1	Day 25	Day 45
10:0	3.28	3.76	3.35	4.75	3.96	3.02	2.41	3.36	2.18	3.79	2.04	2.79
12:0	0.29	0.39	0.29	0.36	0.38	0.20	0.31	0.31	0.15	0.53	0.21	0.11
14:0	6.78	6.73	6.16	6.06	6.75	6.07	5.97	6.73	6.15	6.10	6.84	6.14
16:0	17.54	19.49	18.72	18.86	19.62	18.12	17.31	19.85	18.05	17.77	18.72	18.78
16:1*n*-7	6.78	6.56	6.06	6.04	6.68	5.88	5.84	6.86	5.92	6.34	6.43	6.04
18:0	4.96	4.79	4.59	4.60	4.79	4.46	4.38	4.95	4.40	4.39	4.66	4.36
18:1*n*-9	20.77	21.15	20.85	20.52	21.84	21.11	20.05	21.30	20.47	19.42	21.30	20.65
18:2 *n*-6	17.89	14.60	17.68	18.71	14.92	17.94	16.93	14.74	16.36	18.12	13.99	16.28
18:3*n*-3	1.86	1.65	1.94	1.96	1.59	1.94	1.99	1.62	1.83	1.99	1.68	1.86
20:5*n*-3	13.16	13.11	13.23	12.79	12.69	13.71	14.97	12.78	14.89	14.43	13.97	14.56
22:6*n*-3	6.55	7.77	7.12	5.36	6.79	7.57	9.85	7.51	9.61	7.12	10.16	8.44
∑ SFA	32.85	35.16	33.11	34.63	35.50	31.87	30.38	35.20	30.93	32.58	32.47	32.18
∑ MUFA	27.55	27.71	26.91	26.56	28.52	26.99	25.89	28.16	26.39	25.76	27.73	26.69
∑ PUFA	39.46	37.13	39.97	38.82	35.99	41.16	43.74	36.65	42.69	41.66	39.80	41.14

Table 4.1: Fatty acid profile of salmon skin oil (SSO) stored at various temperature and selected time. Values are % of total fatty acids. Saturated fatty acid (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA). No significant difference ($p > 0.05$) were recorded in individual fatty acid stored at the four temperatures during the three storage days with the Wilcoxon's matched paired test.

CHAPTER V

CONNECTING STATEMENT

As indicated in Chapters III and IV the free fatty acid (FFA) content of oils is an important index and one of the numerous routine evaluations of biodiesel feedstock quality. Titrimetry is the widely used measurement, but to satisfy current demands for analytical techniques which afford quantitative, non-destructive, rapid, less solvent consumption with minimal impact on the environment, an alternative approach such as Fourier transform infrared (FTIR) spectroscopy which addresses the aforementioned was assessed. The rapid and efficient technique employed by FTIR in quantifying FFA in solvent extracted fish oil intended for biodiesel production are much desired in this automated and environmentally conscious world as a quality control and assurance tool.

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CHAPTER V

FTIR DETERMINATION OF FREE FATTY ACIDS IN FISH OILS INTENDED FOR BIODIESEL PRODUCTION

5.1. Abstract

Biodiesel is commonly derived from vegetable oils and animal (livestock) fats by alkali- or lipase-catalyzed transesterification reactions. Since free fatty acid (FFA) content is a critical parameter in the conversion of fish oils to methyl esters, the performance of a Fourier transform infrared (FTIR) spectroscopic method was assessed as an alternative to the conventional AOCS titrimetric method. The FTIR method involves the simultaneous extraction of FFAs and their stoichiometric conversion to their salts using a weak base; sodium hydrogen cyanamide (NaHNCN) dissolved in methanol, followed by measurement of the carboxylate band, $\nu(COO^-)$, at 1573 cm^{-1} relative to a baseline at 1820 cm^{-1} in the differential spectrum of the methanol extract. With minor modifications, this method was found to be capable of responding linearly to oleic acid (0-6.5%) addition, producing a FFA calibration equation having a S.D. of ±0.014% FFA. FTIR and titrimetric analytical results were compared for samples prepared by standard addition as well as for fish oils extracted from Atlantic salmon (*Salmo salar*) skin which had been stored up to 120 days at -20°C. Both methods responded in a comparable manner; however, the FTIR method was more reproducible and accurate as well as simpler to carry out and was deemed to be a better primary method than the titrimetric method. The FFA content of salmon skin lipids increased linearly from *ca* 0.6% to 4.5% within 120 days, likely as a result of autoxidation. It was concluded that the NaHNCN-based FTIR method is a flexible, viable instrumental alternative to the AOCS titrimetric procedure for the determination of FFA content of fish tissue lipids destined for biodiesel production.

5.2. Introduction

The fish processing industry generates large quantities of tissue waste and byproducts which tend to be either discarded or retailed at low value for fertilizer or animal feed. Fish processing activities may generate byproducts such as: heads,

trimmings (tail, fin), viscera, and skin. In some fish species such as salmon, the skin is peeled off longitudinally before the flesh is sold or sent for canning or smoking. The skins like the other byproducts listed above are mostly not put to profitable use and are discarded or dumped as waste in landfills or in the ocean (Atkins, 2008, personal communication; Atkins et Frères Inc., Mont-Louis, QC). In the processing of Atlantic salmon (*Salmo salar*), between 33% and 50% of the total body weight constitutes by-product, much of which is discarded and ends up in the waste stream (Ockerman and Hansen, 2000). In studies conducted in our laboratory, the lyophilizing of salmon skin for 24 h resulted in 58% moisture loss and 61.53 ±1.14% extractable lipids. Given the high volumes of fish skins generated by commercial fish processing plants such as Atkins et Frères Inc. (typical samples are shown in Fig. 3.1), fish skin and tissue discards could be a potential source of fats/oils for biodiesel production. However, due to the relatively high autolytic activity associated with fish tissue in combination with its high polyunsaturated fatty acid (PUFA) content, the lipids are very prone to both lipolysis and oxidation. Thus, the oils extracted from fish tissue will tend to have high levels of free fatty acids (FFAs), which are problematic during the conversion of oils to methyl esters for biodiesel. In general, the transesterification reaction is carried out in methanol with alkaline catalysis; however, if the lipid contains more than 0.5% FFA, soaps can form and the efficiency of the catalyst can be compromised (Freedman et al., 1984). As such, the FFAs first need to be removed by refining, and accurate determination of the FFA content of the refined oil is required to ensure that all the FFAs have been neutralized, to minimize any inhibition of subsequent catalytic transesterification reaction with methanol to produce biodiesel.

FFA determinations are traditionally carried out by titration, a reasonably sensitive, but cumbersome and subjective procedure if not carried out using an auto-titrator. FTIR spectroscopy has been advocated as an alternative means of determining a variety of oil quality parameters (van de Voort et al., 2001), including FFA content, by making use of the extensive functional group information available in the mid-infrared (MIR) portion of the spectrum. FTIR determination of FFAs may be based on measurement of their characteristic functional group absorption at 1711 cm^{-1} (ν(C=O) of dimerized carboxylic acid groups) (Ismail et al., 1993). However, the partial overlap of

this absorption band with the strong ester ν(C=O) band of triacylglycerols gives rise to several limitations. Although this overlap is accounted for during calibration of the FTIR method, the intensity of the ester ν(C=O) band (and hence its contribution to the measured "FFA absorbance") depends on the saponification number of the oil, making it necessary to develop an individual calibration equation for each type of oil (Verleyen et al., 2001). In addition, matrix effects arise because the position of the ester ν(C=O) band (and hence the extent to which it overlaps with the FFA ν(C=O) band) changes with changes in polarity of the oil, such as it occurs upon autoxidation (Dubois et al., 1996). These limitations may be circumvented by reacting the FFAs with base to convert them to carboxylate salts and measuring the absorption of the COO^- group which occurs in a region where spectral interferences are minimal.

The McGill IR Group has developed several methods based on the latter approach for the determination of FFA content in edible oils (Ismail et al., 1993; Al-Alawi et al., 2004; Al-Alawi et al., 2006) as well as acid number (AN) in lubricants (van de Voort et al., 2003). Originally, the base used was KOH (Ismail et al., 1993), but errors due to saponification of triacylglycerols could be encountered if the analysis was not carried out very quickly (Ismail et al., 1993; Cañada et al., 2001). Subsequently, saponification was avoided by using a weak base; potassium phthalimide added to the oil in *n*-propanol (Al-Alawi et al., 2004); however, this method required FTIR analysis of the sample both before and after treatment with the base in order to compensate for matrix effects. A more recently developed method (Al-Alawi et al., 2006), which uses a methanol solution of a weak base, sodium hydrogen cyanamide (NaHNCN), to extract the FFAs from the oil and convert them to their salts, avoids both saponification and the need to analyze two samples to obtain a single result. Furthermore, since the FFA salts are concentrated in the extraction solvent, analytical sensitivity is increased and automation of the FTIR analysis is facilitated because the samples are methanol solutions rather than viscous oils (Al-Alawi et al., 2006).

To date, none of the MIR FFA methods described above has been applied to FFA determination in fish oils whereas near-infrared (NIR) spectroscopy has been used to analyze FFA content in mackerel oil (Zhang and Lee, 1997) and in salmon fillets (Isaksson et al., 1995). Although MIR and NIR spectroscopy are related analytical

methodologies, NIR methods rely on advanced chemometrics, typically partial least squares (PLS) regression, to extract the pertinent data from overlapping overtone bands, and they characteristically require a large number of reference samples to obtain a representative calibration (Stallard, 1997). Thus, the NIR FFA methods (Isaksson et al., 1995; Zhang and Lee, 1997) are secondary methods reliant on a primary reference method for calibration and applicable only to the oils represented by the calibration standards. In contrast, the MIR NaHNCN-based FFA method is a straightforward primary method in its own right, based on a simple, defined, stoichiometric reaction and measurement of a fundamental, well-defined absorption.

The objective of this study is to evaluate the suitability of the NaHNCN-based FFA method (Al-Alawi et al., 2006) for FFA determination in fish oils destined for biodiesel production. This method was originally designed to measure the low levels of FFA (<0.1%) in refined edible oils, and its performance at the high FFA levels commonly encountered in oils extracted from fish tissue has not previously been examined. Thus, this study assesses the reproducibility and accuracy of the NaHNCN-based FFA method relative to the AOCS titrimetric method in relation to tracking the changes in FFA content of lipids extracted from dried fish skins stored over time and compares their relative performance in that regard.

5.3. Materials and Methods

5.3.1. Materials

Sodium hydrogen cyanamide (NaHNCN 99+%), oleic acid (90% technical grade), activated silica gel, sodium hydroxide, and potassium acid phthalate were all obtained from Sigma-Aldrich (Oakville, ON). Anhydrous methanol (MeOH) and ethanol (EtOH) were purchased from Fisher Scientific (Fair Lawn, NJ), and refined canola oil was purchased locally. Salmon skin samples from salmon with almost no adherent tissue (muscle/flesh) were obtained from a fish market in Montreal (Waldman Plus). The skin was cut into smaller pieces, lyophilized for 24 h and stored at -20°C. The lyophilized skin was extracted for 1 h with hexane using a SER 148 Velp solvent extractor (Velp Scientifica, Usmate, Lombardy) at a sample:solvent ratio of 1:10 (w/v). Following evaporation of the solvent, the salmon skin oil (SSO) was stored at -20°C until needed for

analysis. The NaHNCN-MeOH reagent was prepared by dissolving 4 g of NaHNCN in 1 l of anhydrous MeOH and was kept dry over 4 Å molecular sieves at all times. Prior to use, the solution was allowed to age (Al-Alawi et al., 2006) at room temperature (25°C) until the ν(C≡N) band at 2100 cm^{-1} had completely disappeared (*ca* 4 days).

5.3.2. Instrumentation

The FTIR spectrometer employed for this study was a Bomem WorkIR spectrometer (Bomem, Québec City, QC) equipped with a deuterated triglycine sulfate (DTGS) detector and purged with dry air from a Balston dryer (Balston, Lexington, MA). The spectrometer was controlled by an IBM compatible Pentium 150-MHz PC running under proprietary Windows-based UMPIRE® (Universal Method Platform for InfraRed Evaluation) software (Thermal-Lube, Pointe-Claire, QC). Sample analysis was carried out using a 100-µm CaF_2 transmission flow cell (International Crystal Laboratories, Garfield, NJ); the output line was connected to a trap and vacuum, and the input line was equipped with a valve to allow samples to be loaded by aspiration. All spectra were collected by co-adding 16 scans at a resolution of 8 cm^{-1} and a gain of 1.0 and were ratioed against an open-beam background spectrum.

5.3.3. Methods

5.3.3.1. Sample Preparation/Analytical Protocol

A 1:5 (w/v) ratio of oil:NaHNCN-MeOH reagent solution was used for all analyses. Five grams of oil were weighed into a 50 ml tarred centrifuge tube to which 25 ml of the NaHNCN-MeOH solution was added. The sample was vortexed for 30 s and then centrifuged at 6000 x *g* for 15 min to separate the oil and solvent layers, with the upper layer being the analyte. The analytical protocol is summarized in Fig. 5.1. Prior to analysis, the cell and transfer lines were rinsed with MeOH. The NaHNCN-MeOH solution and the upper solvent layer in the centrifuged sample were then sequentially aspirated into the transmission flow cell to collect their spectra. Prior to the spectral subtraction step shown in Fig. 5.1., an intermediate step was added to compensate for any dilution of the solvent layer of the sample by oil whereby a dilution factor was determined by dividing the height of the methanol overtone band at 2045 cm^{-1} (measured

relative to a baseline point at 2136 cm^{-1}) in the spectrum of the sample by its height in the spectrum of the NaHNCN-MeOH solution. Following multiplication of the spectrum of the sample by the inverse of this dilution factor, the spectrum of the NaHNCN-MeOH solution was subtracted from it to produce the differential spectrum of the analyte, the spectral features of the solvent being removed in the process. The absorbance of the carboxylate [$\nu(COO^-)$] band was then measured at 1573 cm^{-1} relative to a single baseline point at 1820 cm^{-1}.

5.3.3.2. IR Calibration and Titration

FFA calibration standards were prepared by gravimetrically adding 0-6.5% (w/w) oleic acid to refined canola oil, which had been passed through a column of activated silica to remove any residual FFAs. The standards were then treated with the NaHNCN-MeOH reagent as per the analytical protocol described above. A calibration equation was obtained by linear regression of %FFA (% oleic acid) against the peak height at 1573/1820 cm^{-1}. FFAs were determined by titration as per the AOCS method (Ca 5a-40), where alcohol-diluted oil is titrated with standardized 0.1 N NaOH to a phenolphthalein endpoint to obtain %FFA (expressed as % oleic acid).

5.4. Results and Discussion

As noted above, the NaHNCN-based FTIR FFA method was originally designed as a sensitive procedure to determine low levels of FFAs in refined oils. In an initial evaluation of its application for the determination of higher FFA levels (up to *ca* 7%), a variety of oil:NaHNCN-MeOH ratios were assessed. It was found that even at oil:reagent ratios of 1:4 and 1:5, the reagent was limiting beyond *ca* 4% FFA, as indicated by an asymptotic response of the carboxylate absorption at 1573 cm^{-1} to increasing %FFA. However, by increasing the concentration of NaHNCN in the reagent solution from 0.2% (as specified in the original method) to 0.4% and setting the oil:NaHNCN-MeOH ratio to 1:5 (w/v), a linear spectral response was observed. Fig. 5.2 presents the spectra obtained when the calibration standards were taken through this modified analytical protocol, and the corresponding calibration plot is shown in Fig. 5.3. This plot confirmed that a linear

spectral response is obtained over an FFA range of 0-6.5% and produced the following linear regression equation:

$$\%FFA_{(oleic)} = 0.2626 + 22.2362\ \ Abs_{(1573/1820\,cm^{-1})}\ \ R = 0.999,\ \ S.D. = 0.014 \qquad (5.1)$$

To assess the accuracy of the method, standard addition experiments were performed by spiking canola oil with oleic acid. The spiked oils were analyzed in triplicate by the FTIR method and the AOCS titrimetric method for FFA determination, and the results obtained were compared with the %FFA added. Both plots were linear, and their respective regression equations (Eqs. 5.2 and 5.3) as well as their relationship to each other (Eq. 5.4) are presented below:

$$\%FFA_{FTIR} = -0.054 + (1.027)\%FFA_{spiked}\ \ R = 0.999,\ \ S.D. = 0.031 \qquad (5.2)$$

$$\%FFA_{titration} = 0.015 + (1.054)\%FFA_{spiked}\ \ R = 0.999,\ \ S.D. = 0.053 \qquad (5.3)$$

$$\%FFA_{FTIR} = -0.067 + (0.975)\%FFA_{titration}\ \ R = 0.999,\ \ S.D. = 0.055 \qquad (5.4)$$

These results are in agreement with those previously obtained by Al-Alawi et al. (2006), with the FTIR method having better precision than titration, the S.D. values being ±0.031 and ±0.053, respectively. Fig. 5.4 presents the plot of the data represented by Eq. (5.4), showing the means of triplicate analyses of the standard addition samples by both FTIR spectroscopy and titration, the slope of 0.975 indicating their overall concurrence. Further analysis of the FTIR and titration data in terms of the mean difference (MD) and standard deviation of the differences (SDD) for reproducibility (r) and accuracy (a) indicates that the FTIR method performs significantly better than titration (Table 5.1).

The validity of employing calibration Eq. (5.1), derived using canola-based calibration standards, for the analysis of SSO samples was then examined by standard addition of oleic acid to freshly extracted SSO. The use of a canola-based calibration equation which is advantageous because it avoids the need to extract and purify SSO for the purpose of preparing calibration standards, is predicted on the assumption that the efficiencies of extraction of FFAs or any co-extracted species from SSO and canola oil by the NaHNCN-methanol reagent do not differ significantly. It may be noted that this

statement raises the issue of co-extracted species, which has not been considered thus far but merits some discussion at this point. In principle, species co-extracted with FFAs into the methanol phase could give rise to spectral interferences, matrix effects, or dilution errors. However, as mentioned at the outset, the possibility of spectral interferences can largely be discounted because the $\nu(COO^-)$ absorption of the FFA salts is observed in a region of the spectrum devoid of other absorptions, and examination of the neat-oil spectra of the calibration standards and the SSO samples confirmed the absence of any potentially interfering absorptions. Similarly, interactions between the charged carboxylate group, solvated by methanol, and any co-extracted species may be presumed to be negligible, thereby precluding matrix effects and leaving only dilution effects as potential sources of error. Referring back to the calibration spectra (Fig. 5.2), the split absorption band observed in the 1750-1720 cm^{-1} range is attributed to the ester $\nu(C=O)$ absorption of triacylglycerols and is indicative of the extraction of a small amount of oil into the methanol phase. To evaluate the resulting dilution of the methanol, dilution factors were calculated by dividing the absorbance of an overtone band of methanol at 2045 cm^{-1} in the spectrum of each calibration standard by its absorbance in the spectrum of the NaHNCN-methanol reagent solution. The mean dilution factor calculated in this manner was 0.9829 with an S.D. of 0046, indicating that the extent of dilution of the methanol phase by extracted oil was <2% and could be regarded as constant for the calibration set. As such, the slope of calibration Eq. (5.1) effectively has this dilution factor built in, and thus application of this equation to oils that have a higher (lower) miscibility in the extraction solvent would underestimate (overestimate) the FFA content of the oil. To eliminate this potential source of error, a "dilution-corrected" calibration equation was generated by multiplying each calibration spectrum by the inverse of its spectrally determined dilution factor, and the spectra of the extracts of all samples subsequently analyzed were corrected for dilution in the same manner, allowing their FFA contents to be predicted reliably from the "dilution-corrected" calibration equation.

This dilution correction procedure was applied to the spectra of the methanol extracts of the five spiked SSO samples prior to prediction of their FFA content from the dilution-corrected calibration equation. The mean dilution factor for the SSO samples was 0.9696 (S.D. = 0.069), as compared with 0.9829 for the canola-based calibration

standards, indicating that the miscibility of the SSO in methanol was very slightly higher than that of canola oil. Linear regression of the FTIR-predicted %FFA against the spiked amounts yielded a slope very close to unity, with an intercept well within the regression S.D., indicating full recovery of the fatty acid spiked into the SSO samples.

$$\%FFA_{FTIR} = -0.113 + (1.005)\%FFA_{spiked} \quad R = 0.996, \quad S.D. = 0.185 \qquad (5.5)$$

These results confirmed the efficacy of the dilution correction procedure and the validity of employing the canola-based calibration equation for the analysis of SSO samples.

5.4.1. FFA content of SSO

The ultimate objective of the present study was to determine whether FTIR FFA analysis can be used to track FFA formation in stored crude fish oil (SSO) destined for biodiesel production. This objective was addressed by periodically taking samples of SSO (stored at -20°C) over a period of 120 days and analyzing them in triplicate for FFA content by the FTIR method, using the dilution-corrected form of calibration Eq. (5.1), and by titration (Table 5.2). As shown by linear regression (Eq. 5.6), the results from the two methods correlated very well, with an S.D. lower than that of the corresponding relationship for the standard addition experiment (Eq. 5.5):

$$\%FFA_{FTIR} = -0.011 + (0.987)\%FFA_{titration} \quad R = 0.999, \quad S.D. = 0.069 \qquad (5.6)$$

To examine the reproducibility data in more detail, one replicate was paired with each of the other two, and the MD_r and SDD_r of the paired combinations were calculated and averaged to provide an alternative comparison of performance. As shown in Table 5.2, the MD_r of the FTIR procedure is closer to the ideal value of zero (±0.016 vs. ±0.049) and the SDD_r is lower (±0.002 vs. ±0.021), which is indicative of better overall reproducibility. Further comparison between the methods can be made in terms of the corresponding measures for accuracy (MD_a and SDD_a), with the titrimetric method being considered, by convention, the primary or reference method against which the FTIR

method is compared. On this basis, the values in Table 5.2 would indicate that the FTIR predictions have no bias, as the MD_a is within the SDD_a.

As a final note, this fish oil storage study assessed the stability of SSO, from the standpoint of changes in FFA content, under relatively ideal conditions (storage at -20°C). Over a period of 120 days, an effectively linear trend of increasing FFA content as a function of time was indicated by both the FTIR and the titrimetric data (Fig. 5.5). These changes in FFA content, which are quite significant, may be a result of autoxidation, which is an avenue by which substantial levels of FFAs may be produced.

5.5. Conclusion

This study has demonstrated that the NaHNCN-based FTIR FFA method originally developed for the measurement of the low FFA levels in refined edible oils can readily be modified to cover a wider range of FFA contents. This method should be a useful tool for determining FFA content in oils destined for biodiesel production, thus enabling the refining step to be carried out more accurately to enhance the catalytic efficiency of the transesterification process.

5.6. Acknowledgements

The authors gratefully acknowledge the financial support provided by the Natural Sciences and Engineering Research Council (NSERC-Strategic Program) of Canada and thank Thermal-Lube for providing the spectrometer and software for this study.

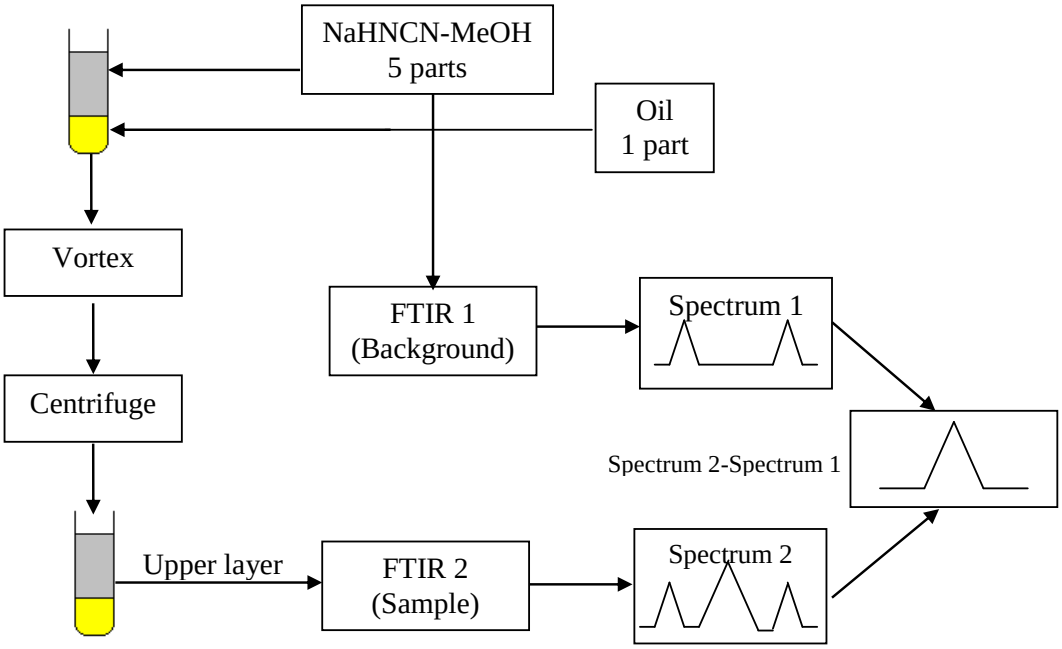

Fig. 5.1: Schematic illustration of the sample preparation procedure and analytical protocol (adapted from Al-Alawi et al. (2006).

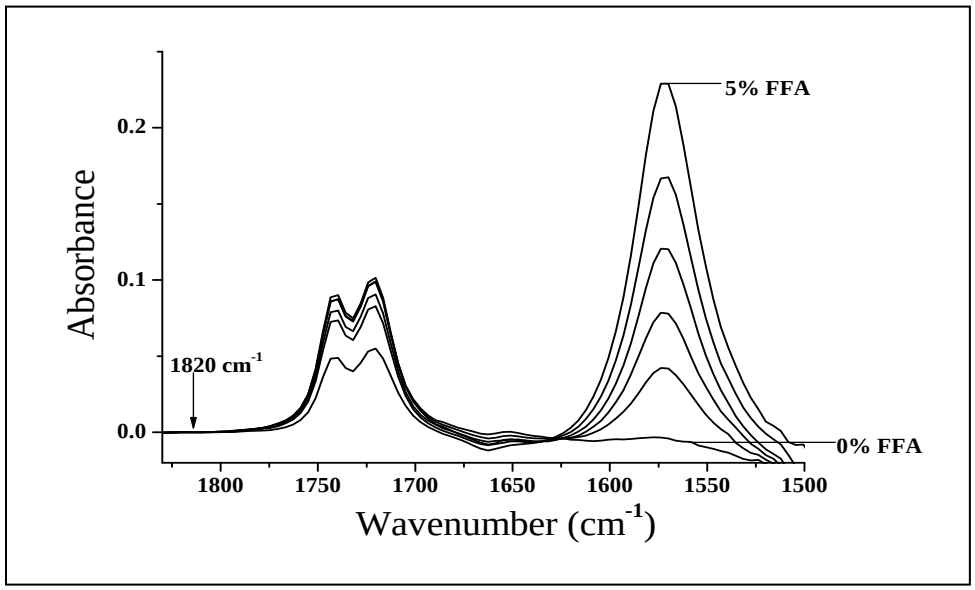

Fig. 5.2: Selected differential spectra (methanol layer) obtained following treatment of FFA-spiked oils with NaHNCN-MeOH (4 g/l), showing proportionate response of FFA salt [$\nu(COO^-)$] absorption with increasing FFA content.

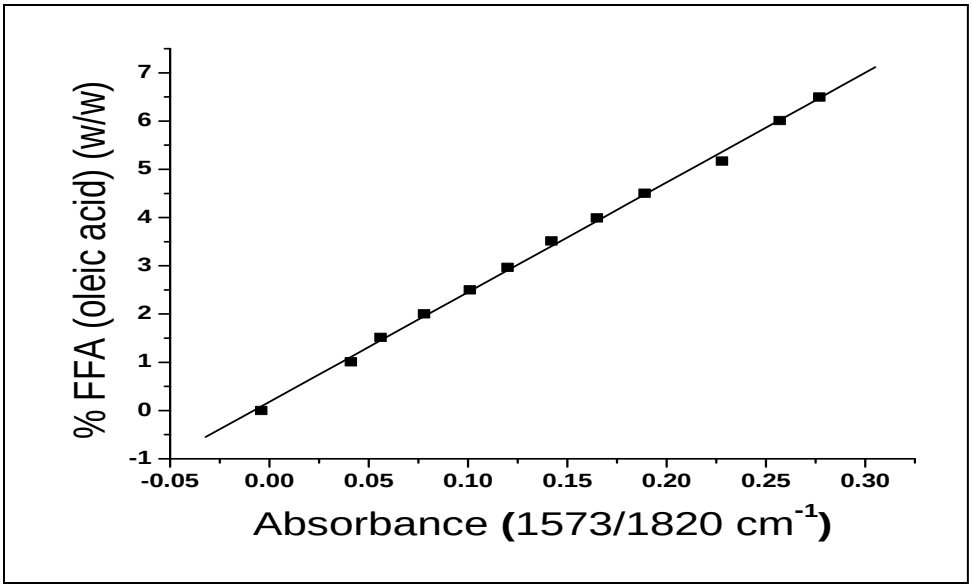

Fig. 5.3: Calibration plot for the determination of FFA obtained from the differential spectra in Fig. 5.2.

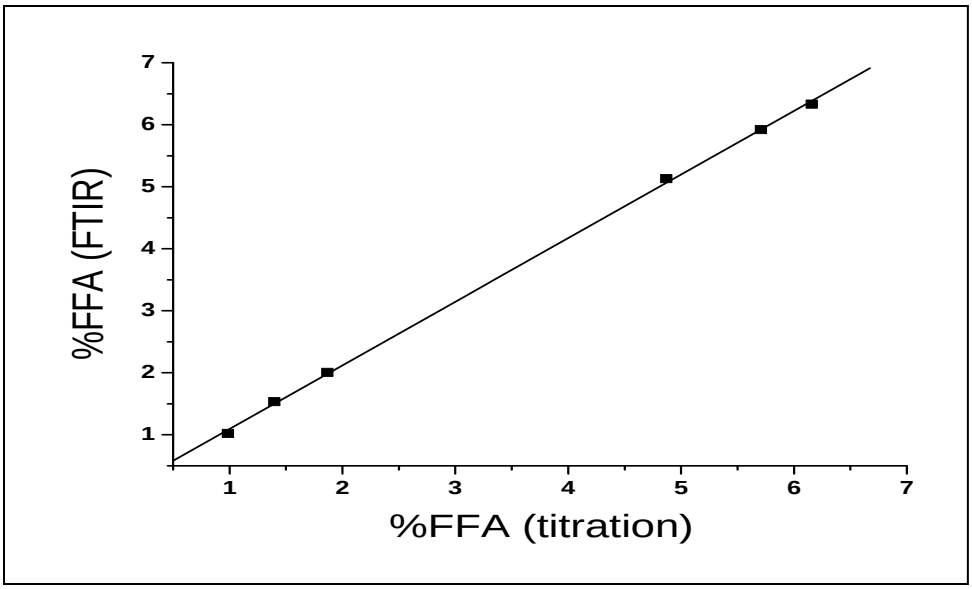

Fig. 5.4: Plot of the means of triplicate FFA results for standard addition samples obtained by FTIR and titration.

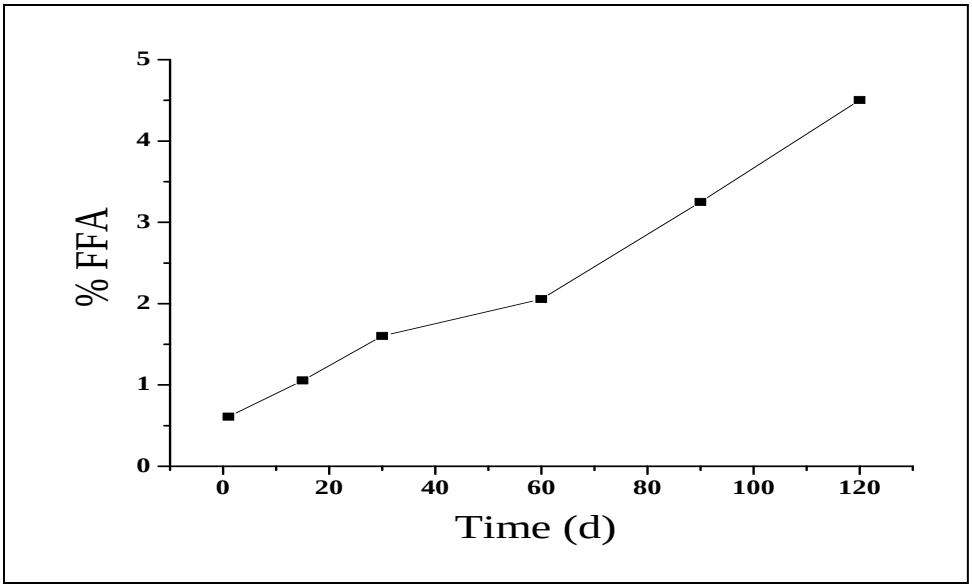

Fig. 5.5: FFA development in salmon skin oil (SSO) as a function of time.

Statistics	Titration	FTIR
MD_r	0.034	0.010
SDD_r	0.017	0.008
MD_a	0.202	0.054
SDD_a	0.128	0.062

Table 5.1: Mean difference (MD) and standard deviation of the differences (SDD) for accuracy (a) for the titrimetric and FTIR methods vs. gravimetric addition and reproducibility (r) for triplicate analyses carried out by the titrimetric and FTIR methods.

Time (d)	Titration		FTIR	
	FFA (%)	S.D.	FFA (%)	S.D.
1	0.609	0.032	0.618	0.014
15	1.056	0.026	1.042	0.014
30	1.061	0.021	1.570	0.012
60	2.057	0.064	1.992	0.011
90	3.251	0.052	3.317	0.013
120	4.505	0.031	4.474	0.010
MD_r	0.049		0.016	
SDD_r	0.021		0.002	
MD_a	-0.040			
SDD_a	0.065			

Table 5.2: FFA content in salmon oil as determined by titration and FTIR analysis (means of triplicate analysis) as a function of the time stored at -20°C as well as the mean difference (MD) and standard deviation of the differences (SDD) for reproducibility (r) of each method and the MD and SDD for accuracy (a) of the FTIR method in relation to the titrimetric method.

CHAPTER VI

CONNECTING STATEMENT

Chapter III, IV, and V provided insights into the extraction of salmon skin oil (SSO) and its quality assessment. This chapter details the initial assessment of the suitability of the recovered SSO, a commercial oil-fat blend of yellow grease and rendered animal fat, [Rothsay composite (RC)], and olive oil (OO) as feedstocks for biodiesel (BD) production, with immobilized lipase as catalyst, and the influence of the reaction parameters on the transesterification reaction. In this study, both issues of using alternative feedstocks and lipase from non-food competing sources to reduce the overall BD production cost were addressed. While methanol has been widely used, to our knowledge there are no previous reports of using ethanol as acyl acceptor with recovered fish oil or blend of animal fat and used restaurant oil with Lipozyme®-IM as the catalyst.

This chapter constitutes the text of a paper published as follows: Aryee, A.N.A., Simpson, B.K., Cue, R.I. and Phillip, L.E. (2011). **Enzymatic Transesterification of Fats and Oils from Animal Discards to Fatty Acid Ethyl Esters for Potential Fuel Use.** *Biomass Bioenerg. 35(10): 4149-4157*.

CHAPTER VI

ENZYMATIC TRANSESTERIFICATION OF FATS AND OILS FROM ANIMAL DISCARDS TO FATTY ACID ETHYL ESTERS FOR POTENTIAL FUEL USE

6.1. Abstract

Fatty acid ethyl ester (FAEE) for use as biodiesel (BD) was produced by enzymatic transesterification of salmon skin oil (SSO), a commercial oil-fat blend of yellow grease and rendered animal fat (referred to as Rothsay composite, RC), and olive oil (OO) with Lipozyme®-IM as the catalyst. The effects of temperature (25-65^{o}C), oil:alcohol molar ratio (1:1-1:6), alcohol type (ethanol or methanol), and reaction time (8-120 h) on the yield of FAEE were assessed. The reaction products as well as residuals were identified and quantified using an HPLC unit equipped with a size exclusion column and refractive index (RI) detector. The statistical procedures of General Linear Model (PROC GLM) and PROC MIXED of SAS were both used to analyze the data and to develop a prediction model that provides a good description of the effects of the reaction parameters on FAEE yield. The conversion rate of the oils to FAEE was generally moderate (*ca* 50%) at the temperatures, oil:alcohol molar ratios, and reaction times studied, with ethanol as co-substrate. At 45^{o}C and at an oil:alcohol molar ratio of 1:1, increasing reaction time from 12 to 24 h increased FAEE yield by *ca* 21%; while at oil:alcohol molar ratios 1:3 and 1:4, FAEE yield increased by 16% and 14% ($p < 0.05$), respectively. Triacylglycerol content generally decreased with increasing FAEE yield during the course of the reaction.

6.2. Introduction

The growing global demand for fossil fuels that have precipitated supply variability since the mid-70s, coupled with their non-renewable nature, and adverse environmental impacts have become the drivers to seek alternative sources of fuel (Fukuda et al., 2001; Canakci, 2007). Biodiesel (BD); the mono alkyl esters of fatty acids has been presented as a suitable, cleaner, biodegradable, and sustainable alternative fuel (Srivastava and Prasad, 2001; Demirbas, 2005; Meher et al., 2006). Majority of BD is currently produced from vegetable-based oils such as soybean and canola (Kaieda et al.,

2001; Demirbas, 2009; Silva et al., 2010). However, with increasing demand for BD and the high cost of traditional feedstocks, the use of alternative, cheap, and predominately non-food feedstocks is both sensible and preferable (Canakci and Gerpen, 2001; Veljković et al., 2006; Berchmans and Hirata, 2008).

The fishery and animal production and processing sectors generate large quantities of by-products that are either put to low value use or discarded as waste (Gair et al., 2006). It is estimated that about 25% of the global fishery catch end up as discards, from by-catch and processing (Stobutzki et al., 2001a; Falch et al., 2006; FAO 2008). Some fishery processing practices generate between 40 and 60% of the total body weight as waste, and this amount could increase from postharvest spoilage (Gildberg and Stenberg, 2001; Falch et al., 2006). Likewise, the animal production industry generates large quantities of discards which accumulates from abattoirs, processing plants, butcher, and grocery shops (Patkie et al., 2000; Mittal, 2006; Sharrock et al., 2009). According to Sharrock et al. (2009), the US National Renderers Association (NRA, USA), reported that 22-50% of turkey, chicken, pig, and cattle meat do not end up in the supermarket. Approximately 92% of the 24.6 Mt of animal by-products generated annually in USA and Canada is contributed by the USA (Meeker and Hamilton, 2006). The NRA also estimated that about $2 billion is used to dispose the 180 kt of used restaurant frying oil generated in Canada annually. Incineration of agricultural waste and/or dumping of the waste in landfills are expensive, pollute the environment, and are health threats (Catchpole et al., 2005; Pratt, 2008). It is thus expedient to engage in practices that enable the biotransformation of, and processing of discards into useful products, while protecting the environment (Gildberg and Stenberg, 2001; Catchpole et al., 2005). Biodiesel has been produced from a few non-edible feedstocks (Canakci and Gerpen, 2001; Veljković et al., 2006; Berchmans and Hirata, 2008). With no competing food uses, oil generated from food processing discards are more readily available, cheaper, and less controversial as BD feedstock.

Biodiesel may be produced by chemical catalysis either with acids or bases, or *via* enzymes (lipase) (Akoh et al., 2007; Oliveira et al., 2008; Fjerbaek et al., 2009). The suitability of a commercial immobilized lipase in carrying out the enzymatic transesterification of the recovered oil was evaluated in this study. The choice of lipase as

the catalyst is based on its unique specificity and ability to effectively catalyze the bioconversion reaction while minimizing undesirable side reactions compared to chemical catalysts (Fukuda et al., 2001; Akoh et al., 2007; Fjerbaek et al., 2009). Biodiesel produced by lipase catalysis requires relatively little or no further elaborate purification upon completion of the reaction, an important advantage over chemical catalysts which require extensive washing and purification at an additional operation cost, and generate large amount of wastewater and unused chemical catalyst with undesirable environmental implications (Akoh et al., 2007; Fjerbaek et al., 2009).

Lipase-catalyzed BD production has been reported for some oils and fats (Veljković et al., 2006; Akoh et al., 2007; Canakci, 2007) but not for SSO or RC from fishery processing and animal slaughterhouse discards, respectively. In this study, SSO obtained by solvent extraction (Aryee and Simpson, 2009) from salmon skin discards from salmon smoking operations, and a commercial sample obtained from Rothsay® Biodiesel Company (and designated as RC); a blend of rendered fat from animal processing (beef, pork, and chicken fat), and yellow grease from used restaurant frying oil were examined as potential feedstocks for BD production.

6.3. Materials and Methods

6.3.1. Materials

Salmon skin oil (SSO) was extracted as previously described (Aryee and Simpson, 2009). Rothsay composite (RC) was provided by Rothsay® Biodiesel Company (Ste. Catherine, QC); while olive oil (OO) and immobilized lipase from *Mucor miehei* (Lipozyme®-IM) (86.8 U/g) were obtained from Sigma-Aldrich (Oakville, ON). One unit of immobilized lipase activity is defined as the amount producing 1 mmol of stearic acid per min from the hydrolysis of tristearin at pH 8.0 and 70^{o}C. All chemicals and reagents were analytical grade or better. Ethanol (EtOH) was purchased from Commercial Alcohols (Boucherville, QC), and toluene, methanol, and acetic acid were from Fisher Scientific (Whitby, ON). Standards of fatty acid ethyl ester (FAEE), fatty acid methyl ester (FAME), triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), and free fatty acid (FFA) were purchased from Nu-Chek Prep Inc. (Elysian, MN).

6.3.2. Experimental Methods

A summary of the overall scheme is illustrated in Fig. 6.1.

6.3.2.1. Oil Characterization

The following properties of the starting raw materials (oils) were determined: (i) free fatty acid (FFA) content [by volumetric titration according to the AOCS method (Ca 5a-40)], (ii) fatty acid composition [using gas chromatography (GC)], and (iii) moisture content [according to the AOCS method (Ca 2c-25)], all in duplicate.

6.3.2.2. Preparation of Standard Solutions and Calibration Curves

Stock solutions (5 mg/ml) and 5 different concentrations of TAG, DAG, MAG, FFA, FAME, FAEE, and EtOH were prepared in toluene. The concentrations of the standard solutions ranged between 0.3125 mg/ml and 5 mg/ml of which 2 ml was transferred into screw-cap vials for injection into the HPLC system. Individual calibration curves were developed for each of the standards, and calibration equations were derived from the calibration curves of plots of peak area (μRIU) against the standard.

6.3.2.3. FAEE Transesterification Reaction Process Design

The production of FAEE from SSO, RC or OO with ethanol or methanol as co-substrate, and Lipozyme®-IM as catalyst was conducted in a 30 ml stoppered vial in a horizontal shaking water bath (model 25, Precision Scientific, Chicago, IL) at temperatures of [(Temp (25-65^{o}C)] at 60 shaker rate/min. The reaction mixture consisted of 5 g of the test oil, oil:alcohol molar ratios [(AlcoR) (1:1-1:6)], and a fixed Lipozyme®-IM amount [load (21.70 U)]. One hundred microliter (100 μl) aliquots of the reaction mixture were periodically withdrawn [(Time) (8-120 h)] and mixed with 5 ml toluene; 2 ml of this mixture were then transferred into screw-cap vials for injection and HPLC analysis.

The limits of the reaction parameters chosen for this study were based on available literature and the constraint of their effects on other reaction components such as the enzyme. For instance, the lower limit of the reaction temperature was set at 25^{o}C with consideration for the nature of the oils used, and the upper temperature limit was set

at 65°C, which is *ca* 13°C lower than the boiling point of ethanol and 5°C lower than the hydrolytic optimum temperature of Lipozyme®-IM (70°C).

6.3.2.4. Identification and Qualitative Analysis by HPLC

An HPLC system with a refractive index (RI) detector (Varian 365 LC RI detector; Varian, Palo Alto, CA) was used to monitor the reaction products, intermediates, and residuals (lipid classes-TAG, DAG, FAAE, and MAG), according to the method of Kittirattanapiboon and Krisnangkura (2008). The HPLC system was equipped with a 100 A° Phenogel™ column (300 mm x 7.8 mm ID), packed with styrene benzene copolymer beads with particle size of 5 mm (Phenomenex, Torrance, CA) and fitted with a pre-column of the same phase, and an autosampler. The column and RI detector temperatures were set at 25°C and 35°C, respectively, and the injection volume was preset at 25 µl. A mobile phase of 0.25 (v/v) acetic acid in toluene at a flow rate of 1 ml/min was used during the 25 min run time. A Galaxie™ chromatography software (Varian, Palo Alto, CA) was used for data acquisition and processing. Twenty five microliter (25 µl) of the calibration standard or sample was injected and recorded under the same conditions. Peak identification was made by comparing the retention time and spectrum of the sample with that of the standards.

6.3.2.5. Quantitative Analysis

Sample quantification was carried out using the external reference method with the calibration curves and linear regression equations. The yield of each component was expressed as the corrected area of the corresponding peak relative to the sum of the corrected area of all the peaks excluding the alcohol peak, as follows:

$$\%\ \boldsymbol{Yield} = \frac{\boldsymbol{Ax}}{\boldsymbol{At}} \qquad (\mathbf{6.1})$$

where Ax is the corrected area of the peak corresponding to component x, and At is the sum of the corrected areas of all the peaks, excluding the alcohol peak. For instance, the conversion to FAEE was expressed as the ratio of the amount of FAEE to the sum of the amount of products, reaction intermediates, and residuals, excluding the alcohol peak.

$$\%\ \boldsymbol{Yield}\ (\boldsymbol{FAEE}) = \frac{\boldsymbol{FAEE}}{\sum(\boldsymbol{TAG} + \boldsymbol{DAG} + \boldsymbol{FAEE} + \boldsymbol{FFA} + \boldsymbol{MAG})} \qquad (\mathbf{6.2})$$

6.3.2.6. Data Analysis

6.3.2.6.1. Statistical Analysis

The data were first fitted and analyzed separately at each discrete time and compared as classification or regression models using PROC GLM (Statistical Analysis Systems, Version 9.2, SAS Institute Inc., Cary, NC). The MIXED procedure (PROC MIXED) of SAS® (Version 9.2) was also used for data analysis with temperature and oil:alcohol molar ratio as fixed effects, vial as a random effect, and reaction time (8, 12, 24, 36, 48, 60, 72, 96, and 120 h) as repeated measure. The magnitude and nature of the correlation between measurements from each vial was modeled by a variety of variance-covariance structures either as compound symmetry (CS), autoregressive 1 (AR {1}), Ante-Dependence (ANTE {1}), Linear SP(LIN), Power SP(POW), or Gaussian SP(GAU). Among all competing variance-covariance structure, the AR(1) was selected as being the most appropriate because it was the best fit as indicated by the smallest/improved AIC (Akaike Information Criteria) or BIC (Bayesian Information Criteria) (Sawa, 1978; Akaike, 1987; Littell, et al., 2000). Scheffĕ's test for multiple comparisons was used to evaluate the fixed effect of temperature and oil:alcohol molar ratio on FAEE yield.

6.4. Results and Discussion

6.4.1. Oil Characterization

The FFA content of the oils (SSO, RC, and OO), were 6.26, 4.37, and 0.16%, respectively. The corresponding estimates of moisture content determined by the oven method were 0.02, 0.03, and 0.01%. The relatively higher FFA (>1%) in the SSO and RC would have required acid-catalyzed esterification pretreatment if alkali instead of lipase was the catalyst of choice, to make the feedstock suitable for the reaction (Akoh et al., 2007; Fjerbaek et al., 2009). The major (over 82%) FA in the SSO, RC, and OO were 18 carbons in length and included stearic, oleic, linoleic and linolenic acids (Table 6.1). As shown in Table 6.1, RC contained almost equal amounts of saturated and

monounsaturated FA, which is to be expected because it is a composite mixture of rendered fat (beef, pork, and chicken), and yellow grease, while OO is predominately a monounsaturated oil.

6.4.2. Fatty Acid Ethyl Ester (FAEE) Production

Due to its relative advantages over the free form of the enzyme and ultimately on the transesterification reaction (Dizge and Keskinlera, 2008; Pires-Cabral, 2010) an immobilized lipase was used for the transesterification of animal fats to FAEE in this study. In transesterification reactions, ester bonds of glycerides are cleaved to produce unesterified fatty acids (FA) and glycerol. The unesterified FA then reacts with alcohol to form FAAE, i.e., BD. The transesterification of TAGs to FAAE occurs in three reversible sequential steps, with the production of a monoester in each step. Although the enzymatic process has important advantages over the chemical process as alluded to above (e.g., more uniform products from batch to batch, as well as lower energy, and post-processing purification requirements), the latter approach achieves relatively higher conversion rates in a relatively shorter time (Akoh et al., 2007; Fjerbaek et al., 2009).

The various reaction products were separated according to their polarity, and simultaneously compared with external standards. The results obtained for the calibration curves showed high linear correlation (R^2 = 0.9943, 0.9971, 0.9984, 0.9960, 0.9984, and 0.9943) for TAG, DAG, FAEE, MAG, FFA, and EtOH, respectively. Fig. 6.2 depicts a typical chromatogram of an aliquot taken during the course of the reaction. The order of elution based on retention time was as follows; TAG < DAG < FAEE < MAG. A good baseline separation was achieved for all the components within 25 min and the shapes of the peaks were sharp and symmetrical as shown in Fig. 6.2. This suggests that the methodology reported can be used as a simple and rapid technique to separate, monitor, and quantify the reaction components and products of FAEE production. As the quantity of lipid classes is required in official BD testing (Knothe, 2001; ASTMD 6751; CEN EN 14214), and not individual FAs, this methodology will be suitable for commercial application.

6.4.2.1. The Effect of Reaction Parameters on FAEE Yield

In this study, particular emphasis is placed on the calculation of FAEE yield as indicated in equations (Eqs.) 6.1 and 6.2. Given that other workers have expressed FAEE yields differently, an unambiguous comparison of FAEE yield across published studies is often difficult. Refined olive oil was chosen as a representative vegetable oil. Overall, the yields obtained from SSO were higher than those from RC and OO although they were studied under identical conditions. This variation may be due to their different FA composition as shown in Table 6.1. Fig. 6.3 depicts the course of a typical transesterification reaction and the corresponding release of FAEE. Although some previous studies reported FAEE yield with one of the reaction parameters kept constant (Demirbas, 2005; Dizge and Keskinlera, 2008), in this study, there were statistical significant differences ($p < 0.05$) in FAEE yield as a function of temperature, oil:alcohol molar ratio, and time. These findings indicate that the yield of FAEE is dependent on these factors. Moderate yields of FAEE were reported in this study and are in agreement with previous studies using a similar alcohol and enzyme (Bernardes et al., 2007; Matassoli et al., 2009; Souza et al., 2009). Like other 1, 3-regiospecific lipase, Lipozyme®-IM has a theoretical alkyl ester yield of 66% (Rodrigues et al., 2009; Fernandez-Lafuente, 2010). There was progressive disappearance of TAG concurrently with the appearance of FAEE at most of the temperatures, oil:alcohol molar ratios, and reaction times, (treatment) studied. The estimated proportion of SSO TAG decreased from 85% to 20% of the total composition, while the proportion of FAEE increased from 8% to almost 50%. The enzyme consistently maintained a low initial activity and this could be attributed to the initial inactivation of the enzyme and/or the inadequate mixing of the two phases, i.e., oil and alcohol. A slow increase in FAEE yield was observed for most of the treatment combination during the first 48 h; beyond this time there were very little changes in FAEE yield (Figs. 6.4a-e).

6.4.2.1.1. Effect of Temperature on FAEE Yield as influenced by Time and Alcohol molar ratio

Fig. 6.4a shows a continuous increase in FAEE yield at 25^{o}C from 8 to 120 h at all the oil:alcohol molar ratios studied with yield leveling off after 72 h for 1:1 and 96 h

for 1:3, with moderately lower yield increases at 1:6. However at 35^{o}C (Fig. 6.4b) the gradual increase in FAEE was up to 48 h for 1:1 and then decreased at 60 h after which higher yield was recorded at 72 h followed by a decreasing trend afterwards. The inhibitory effect of ethanol on the enzyme was milder at 35^{o}C and 45^{o}C (Figs. 6.4b and c) with various increases in FAEE yield even at oil:alcohol molar ratios >1:4. At 55^{o}C (Fig. 6.4d), increasing alcohol concentration had pronounced effect on FAEE yield and even greater effect beyond 1:4 oil:alcohol molar ratio, while at a 65^{o}C (Fig. 6.4e), alcohol concentrations >1 molar were harsher and effectively inhibited lipase catalysis. As also shown in Table 6.2 for RC and OO, increasing alcohol molar ratios caused enzyme inactivation at all the temperatures studied. This inactivation was however relieved with time and also at all the temperatures studied for oil:alcohol molar ratios 1:1 and 1:3, but not at higher alcohol molar ratios. When oil:alcohol molar ratio was 1:1, FAEE yield increased with temperature at 25, 35, and 45^{o}C and declined beyond these temperatures. When oil:alcohol molar ratio was 1:3, FAEE yield increased with temperature only up to 35^{o}C and declined beyond that, i.e. at higher alcohol ratios (4, 5, and 6). The effect of temperature on FAEE yield did not show consistent pattern but generally declined with temperature at higher alcohol molar ratio. Higher temperatures (>65^{o}C) were not used in this study because of the possibility of irreversible deactivation of the enzyme. The effect of temperature on the reaction can be explained by the Arrhenius law, among others. It is known that higher temperatures increases the solubility of the solvent, decreases the viscosity of the oil and accelerates product formation. However, some enzyme catalysts may be deactivated at higher temperatures (Akoh et al., 2007; Dizge and Keskinlera, 2008; Hernández-Mártin and Otero, 2008).

6.4.2.1.2. Effect of Alcohol molar ratio on FAEE Yield as influenced by Time and Temperature

The stoichiometry of the transesterification of TAG requires 1:3 molar ratio of oil:alcohol, thus at equilibrium it is expected from Le Chatelier's principle that an increase in the amount of alcohol will progressively push the reaction to the right, i.e., towards more product formation. This general rule is however not obeyed when short chain alcohols such as ethanol are used with lipase as catalyst (Dizge and Keskinlera,

2008; Naranjo, 2010). An inherent characteristic of low molecular weight polar alcohols is their ability to distort or strip the ordered layer of water that surrounds the enzyme required for conformational integrity, stability and thus catalysis (Laane et al., 1987; Kaieda et al., 2001; Páez et al., 2003; van Rantwijk et al., 2003; Dizge and Keskinlera, 2008; Naranjo, 2010; Pires-Cabral et al., 2010). These alcohols, as well as the immobilization support, may also restrict the diffusion of the oil into the pore spaces of the enzyme (Chen and Wu, 2003), and limit product formation. Such an effect may have occurred in this study (Figs. 6.4a-e and Table 6.2). Even though the effect of oil:alcohol molar ratio on yield was not consistent, oil:ethanol molar ratio generally had greater influence on FAEE yield than temperature and time. As characteristic of competitive inhibition and also common with several enzymes (Souza et al., 2009) high concentrations of polar alcohols inactivates lipases. At lower alcohol concentration in relation to the substrate there is less probability of the alcohol attaching to the enzyme since all the enzyme is attached to the substrate. One of the objectives of this study was also to find the maximum alcohol molar ratio that this enzyme could tolerate under these reaction conditions. High amounts of TAG and DAG remained in the reaction mixture when >1:4 oil:alcohol molar ratios were used, further highlighting the inhibitory effect of short chain alcohols on the lipase-catalyzed FAEE production. Thus, despite the presence of marginal molar excess of ethanol, the equilibrium was not shifted to more FAEE production. Higher conversion rates were however achieved below and up to the stoichiometric ratio. When methanol was used as an acyl acceptor, only traces of FAEE were found at 1:1 oil:alcohol molar ratio and the enzyme was completely deactivated beyond this molar ratio (data not shown).

6.4.2.1.3. Effect of Time on FAEE Yield as influenced by Alcohol molar ratio and Temperature

It was generally observed in this study that increasing reaction time contributed to a slightly higher FAEE yield than increasing alcohol molar ratio (e.g. at 25^{o}C), and these values were statistically significant ($p < 0.05$). The low reaction rate during the first few hours could be due to mixing and dispersion of the alcohol. Increasing reaction time from 12 to 24 h at oil:alcohol molar ratio of 1:1 resulted in about 21% increase in FAEE yield,

and only 16 and 14% during the same period at 45^{o}C and 1:3 and 1:4 oil:alcohol molar ratios, respectively (Fig. 6.4c). Increasing reaction time from 12 to 36 h resulted in higher FAEE yield, for instance 3, 16, and 41% increases in FAEE yield were recorded at 45^{o}C. In contrast to 25-45^{o}C, at 55 and 65^{o}C, though statistically significant ($p < 0.05$), very little difference was observed in FAEE yield within the first 36 h and no appreciable increase in FAEE yield beyond 48 h.

The small amount of glycerol produced could not be simply detected by the method used, Kittirattanapiboon and Krisnangkura (2008), reported a similar limitation. The absence of glycerol in the reaction mixture drawn could also be attributed to the low degree of transesterification or the probable formation of an emulsion with FAEE. This HPLC method was able to monitor the varying content of residual alcohol, FFA, as well as the intermediates (DAG and MAG) produced during the course of the reaction (Fig. 6.2).

6.4.2.1.4. Statistical Management of Data

The PROC GLM and PROC MIXED of SAS® were both used in data analysis to develop a prediction model that provided a good description of the effect of the reaction parameters (independent variables) on FAEE yield (dependent). The data, first fitted to PROC GLM to compare as a classification or regression model at discrete time, revealed that the classification model was a significant ($p < 0.05$) improvement over the linear, quadratic, cubic, and quartic terms of the regression model. In this experiment, repeated FAEE yield measurements were taken over time (8, 12, 24, 36, 48, 60, 72, 96, and 120 h) from the same vial. Therefore, there is the likelihood that the observations from the same vial were correlated. Thus the PROC MIXED model with repeated measures was chosen and utilized for data analysis and presentation. The PROC MIXED model provides a flexible approach to model repeated measures data since it can accommodate both fixed and random effects and allows for their efficient estimation. Figs. 6.4a-e and Table 6.2 show the least square (LS) Means of FAEE yield obtained as a function of temperature, oil:alcohol molar ratio, and time, during the transesterification of SSO, RC, and OO. The interactions of the factors were significant ($p < 0.05$) at all the Type 3 tests of fixed effects which implied that these factors were not acting independently on FAEE yield and

thus their contributions were no longer additive. As the reaction progressed, the yields of FAEE varied and this variation was more pronounced among the different temperature and oil:alcohol molar ratio combinations. There appeared to be an interaction between time, temperature, and oil:alcohol molar ratio. However a comparison of these factors (temperature and oil:alcohol molar ratio) is dependent on reaction time (Figs. 6.4a-e, and Table 6.2). The treatment comparisons were thus analyzed separately by time. To compare the temperature, oil:alcohol molar ratio effects separately for each time, the interactions were sliced by time. This version of SAS® allows all possible slicing in a single step, thus overcomes the likelihood of running many individual analysis, and provides a meaningful joint interpretation of the results, for instance the interactions that are significant are detected and sliced in a single step (SAS 2010). In the PROC GLM model and at 5% significance, FAEE yield was significant at all the time tested. Based on a total of 225 error degrees of freedom and an error mean square estimate of 6.71, the F-statistic for example, for testing temperature and oil:alcohol molar ratio effects at 8 h was 17.08. This F-value and p-value were identical to that from the PROC MIXED analysis. Scheffé's test for multiple comparisons was applied to the estimates of all the possible contrasts among the factors means of the fixed effects, i.e., beyond the pairwise differences considered by Tukey's method; to adequately represent the treatment differences at the various levels of each factor.

6.5. Conclusion

This study evaluated the potential of processing discards from fishery (salmon skin oil) and animal origin (Rothsay composite) to produce FAAE for use as BD using Lipozyme®-IM and ethanol. Approximately 50% FAEE yield was recorded at 25^{o}C, 1:4 oil:alcohol molar ratio, and 96 h. PROC MIXED with repeated measures was compared to the traditional discrete time approach of assuming independence among the responses measured from the same vial over time. Although the two approaches adequately predicted the yield of FAEE over the range of the factor levels tested, the repeated measures approach with a randomized component (vial) as well as variance-covariance structure, proved to be more appropriate for the data. In this three-factor mixed analysis, significant ($p < 0.05$) main effects of the experimental trial and all interactions were

revealed. This study revealed that FAEE yield significantly differs throughout the course of the reaction and is dependent on temperature, oil:alcohol molar ratio, and reaction time.

6.6. Acknowledgments

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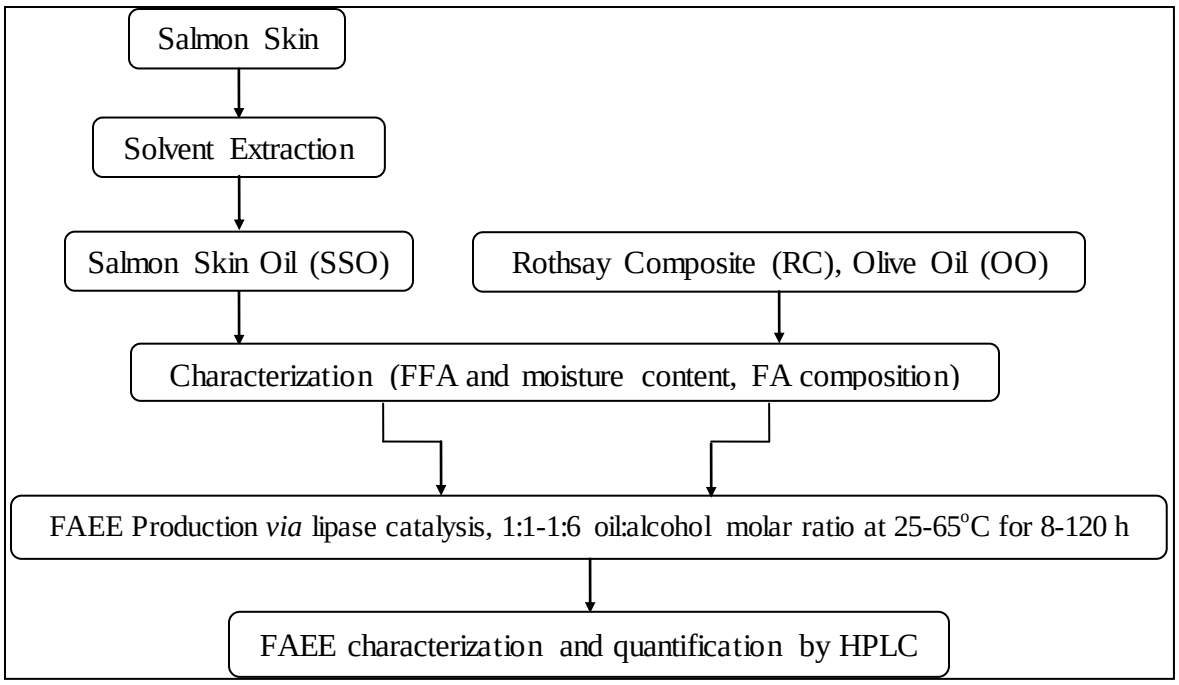

Fig. 6.1: Fatty acid ethyl ester (FAEE) production scheme.

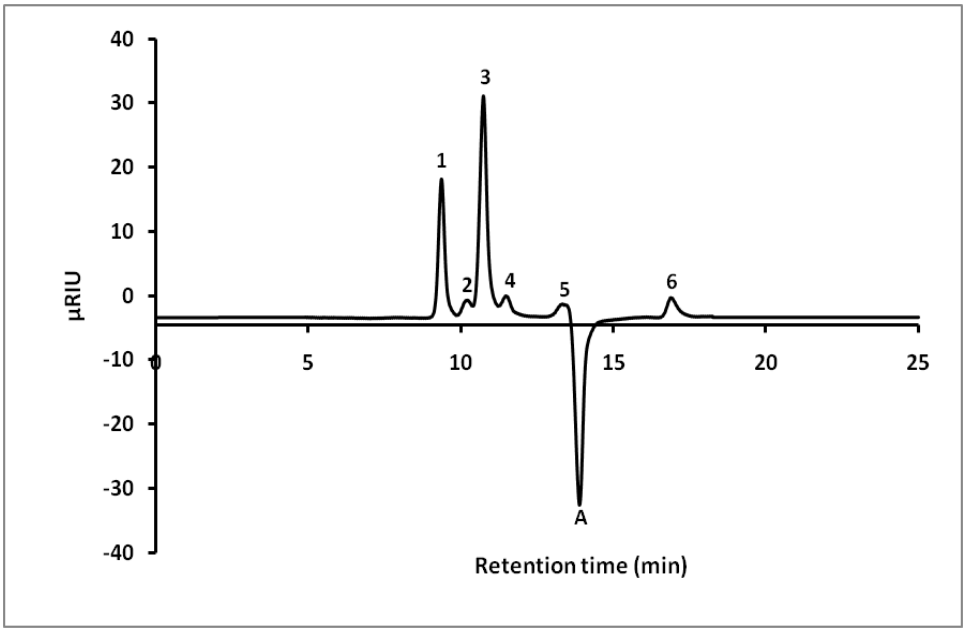

Fig. 6.2: HPLC chromatogram of transesterification components monitored with RI (isocratic elution mode 0.25 (v/v) acetic acid in toluene for 25 min). Peaks correspond to 1:- triacylglycerol (TAG), 2:- diacylglycerol (DAG), 3:- fatty acid ethyl ester (FAEE), 4:- free fatty acid (FFA), 5:- monoacylglycerol (MAG), 6:- ethanol (EtOH), and A: - internal flow marker.

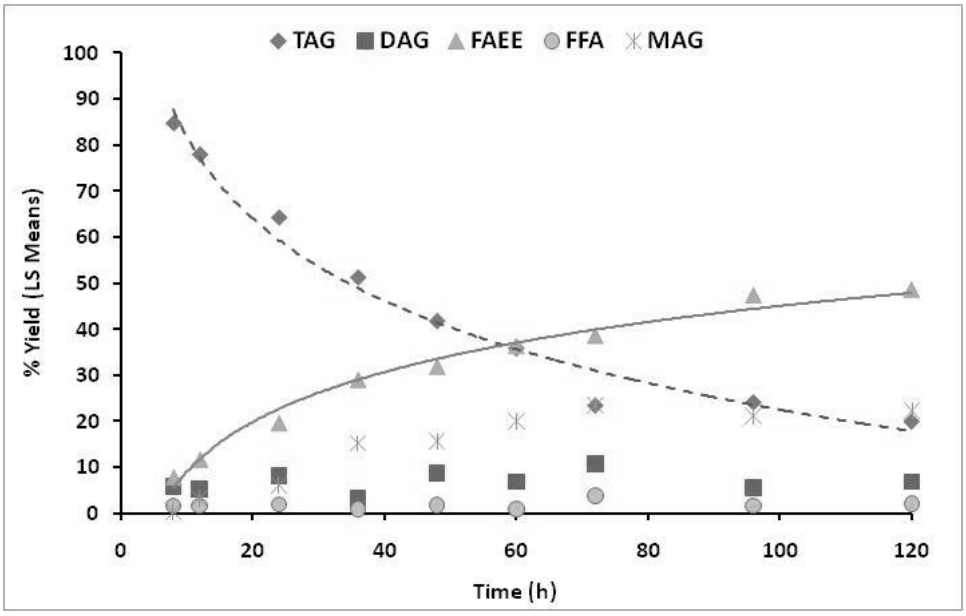

Fig 6.3: Evolution of fatty acid ethyl ester (FAEE) and other reaction components during the transesterification of SSO.

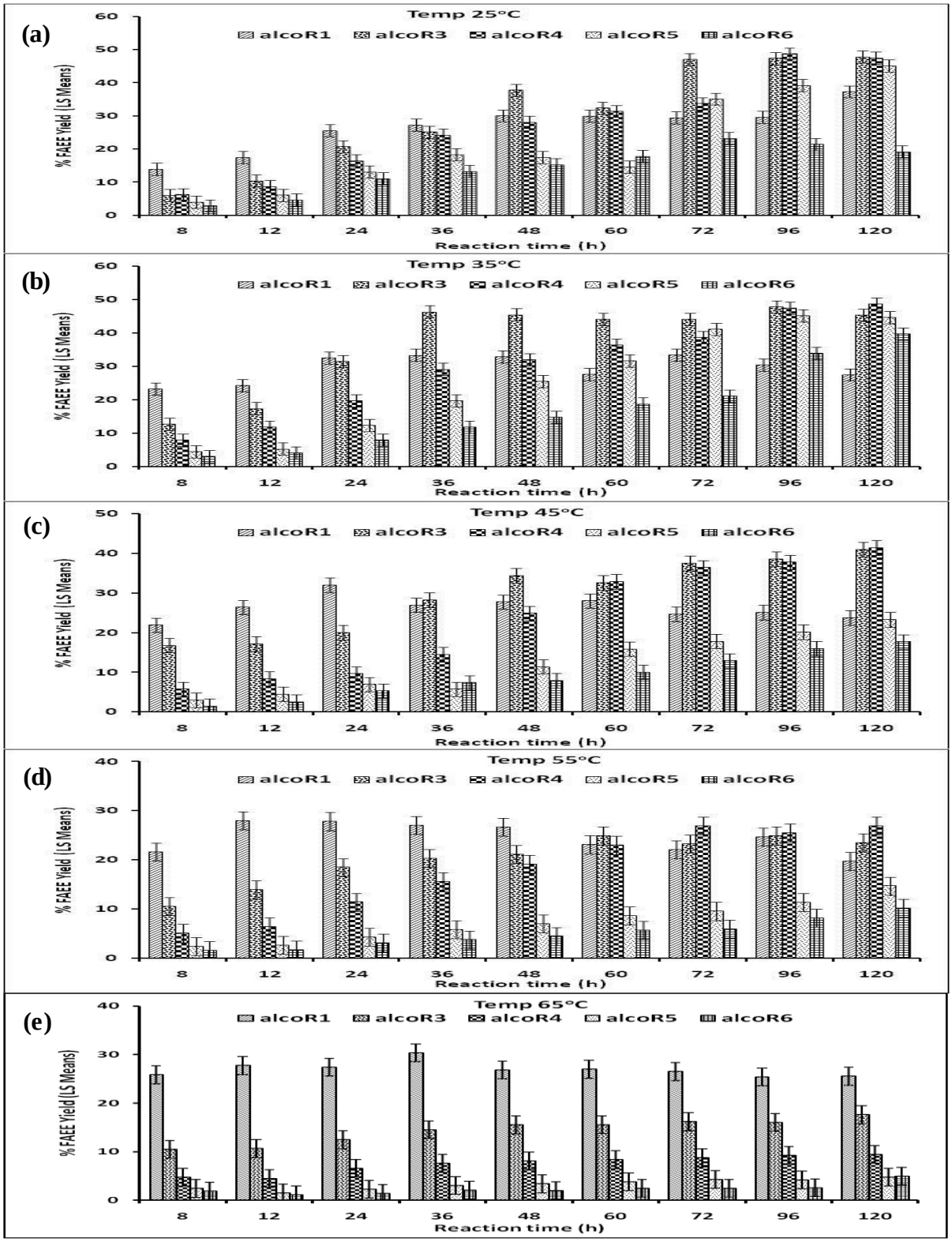

Fig.6.4: Least Square means (LS Means) ± standard error of treatment x time for FAEE yield of transesterified SSO at 25, 35, 45, 55, and 65°C.

Fatty Acid (FA)	Salmon Skin oil (SSO)	Rothsay Composite (RC)	Olive Oil (OO)
Lauric	0.14	0.00	0.00
Myristic	5.77	1.47	0.00
Palmitic	16.94	27.11	14.38
Palmitioleic	5.42	2.29	0.13
Stearic	4.31	12.79	3.75
Oleic	19.20	39.59	67.75
Linoleic	16.05	15.82	10.78
Linolenic	2.82	0.81	0.68
Eicosapentaenoic	15.55	0.00	0.00
Docosapentaenoic	2.45	0.13	0.00
Docosahexaenoic	11.36	0.00	2.58

Table 6.1: Fatty composition of SSO, RC, and OO (percent of total fatty acids).

Treatments		FAEE Yield (%)								
Temp (^{o}C)	Oil:alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
						RC				
	1:1	5.10	7.60	9.76	13.60	15.13	16.92	17.22	17.99	15.87
	1:3	3.81	7.19	10.28	12.32	13.75	13.38	17.39	16.96	17.48
25	**1:4**	2.17	5.11	8.19	10.71	14.28	19.02	22.95	22.40	26.57
	1:5	2.03	3.89	6.52	7.93	12.15	14.03	19.10	23.08	26.93
	1:6	2.50	3.25	5.37	8.28	8.46	13.00	14.73	19.36	23.91
	1:1	6.95	14.50	16.16	16.62	16.74	15.92	15.82	15.86	16.61
	1:3	6.36	7.89	9.91	11.07	10.92	11.84	12.23	12.94	13.39
35	**1:4**	4.90	8.08	14.73	16.32	19.46	20.70	23.21	23.13	27.00
	1:5	2.06	3.36	6.51	9.18	12.71	14.97	17.38	21.49	24.18
	1:6	1.64	2.36	4.11	6.54	8.82	11.22	13.65	17.01	19.68
	1:1	12.56	15.09	16.55	16.50	15.90	15.32	16.61	16.11	16.19
	1:3	10.18	11.42	10.92	11.62	12.42	12.97	13.20	13.77	14.33
45	**1:4**	3.77	5.89	10.73	9.75	10.28	10.72	10.67	11.40	11.33
	1:5	1.48	2.82	3.86	4.84	5.98	6.90	6.75	7.25	7.66
	1:6	1.64	1.45	2.59	3.52	5.08	5.82	6.52	7.09	8.59
	1:1	12.44	15.32	15.57	15.67	15.59	15.77	15.44	16.13	15.46
	1:3	7.05	7.67	8.55	8.83	9.26	9.53	9.70	10.08	10.69
55	**1:4**	1.91	2.35	3.83	4.51	5.03	6.02	6.96	7.91	7.99
	1:5	0.74	0.98	1.56	2.15	2.65	3.01	3.54	3.77	4.70
	1:6	0.58	0.66	1.33	1.69	1.70	2.03	3.44	3.02	3.83
	1:1	13.12	14.77	14.68	15.24	14.37	14.80	15.08	14.67	14.37
	1:3	4.32	4.94	5.57	6.29	6.40	6.60	7.07	7.16	7.65
65	**1:4**	0.83	0.89	1.34	2.07	2.74	4.53	4.85	6.34	6.53
	1:5	0.58	0.54	0.58	1.07	1.09	1.26	1.51	1.44	1.74
	1:6	0.35	0.52	0.60	0.73	0.86	1.03	1.18	1.44	1.32
						OO				
	1:1	6.63	9.00	12.92	16.59	16.79	18.78	16.36	17.12	18.17
	1:3	3.27	5.62	10.84	14.98	17.77	20.15	22.87	24.47	24.76
25	**1:4**	1.68	1.64	3.57	5.40	6.89	8.75	12.23	14.11	17.86
	1:5	1.01	1.11	1.84	2.83	3.58	4.41	3.87	6.05	6.40
	1:6	0.77	1.03	1.99	2.26	3.20	4.15	4.14	5.68	7.23
	1:1	9.39	13.18	16.40	16.98	18.28	18.37	17.47	16.80	17.07
	1:3	6.30	9.36	12.85	14.37	15.65	15.96	16.59	16.37	17.31
35	**1:4**	1.61	2.44	3.76	4.69	5.97	7.02	8.30	10.45	10.73
	1:5	0.35	1.10	1.85	2.00	3.07	2.95	3.49	4.79	4.96
	1:6	1.75	1.07	1.67	2.07	2.64	2.07	2.65	3.43	4.09
	1:1	14.17	15.32	17.09	17.67	17.26	17.37	17.49	16.50	16.89
	1:3	5.78	6.86	6.87	10.35	10.70	11.32	11.54	9.78	11.69
45	**1:4**	1.29	2.60	3.13	4.17	5.65	5.95	6.52	7.47	7.94
	1:5	0.66	0.58	1.26	1.83	2.87	2.38	3.06	4.00	4.45
	1:6	1.29	0.26	1.92	1.10	1.37	1.67	2.19	2.49	2.82
	1:1	10.50	12.99	15.97	15.96	16.22	16.89	17.19	16.66	16.19
	1:3	4.53	5.27	5.99	6.52	6.29	6.88	7.34	8.94	8.64
55	**1:4**	0.72	1.05	2.06	3.16	3.45	3.87	4.74	5.98	6.58
	1:5	0.68	0.55	0.68	1.17	1.37	1.74	2.98	4.33	4.31
	1:6	0.25	0.43	0.42	0.59	0.87	0.77	1.32	2.31	2.99
	1:1	11.17	13.42	15.76	15.86	16.09	15.05	15.53	15.94	15.55
	1:3	2.69	2.80	3.50	3.53	3.79	4.17	4.51	4.73	4.52
65	**1:4**	1.85	2.22	2.62	2.65	2.94	3.35	3.22	4.79	3.83
	1:5	0.97	0.96	0.83	0.94	0.83	0.97	0.88	0.95	0.70
	1:6	0.88	0.46	0.71	0.19	0.61	0.49	0.64	0.43	0.42

Table 6.2: Values are the LS Means obtained as a function of temperature, oil:alcohol ratio, time, and during the transesterification of RC and OO to FAEE (Standard error = 0.1675 for RC; and 0.6033 for OO).

CHAPTER VII

CONNECTING STATEMENT

In the presence of other acidic entities such as monoacylglycerol (MAG) and diacylglycerol (DAG) other than free fatty acid (FFA), a more sensitive method is required to accurately quantify these remaining products. Chapters VII complements Chapter VI and both collectively demonstrate the suitability of the HPLC-SEC-RI methodology in the identification and quantitation of reaction products, intermediates, and residuals for its intended application; as a detailed qualitative and quantitative assessment of biodiesel and other components of the transesterification reaction, and confirms that the method produces results equivalent to the accepted methods currently in use.

This chapter constitutes the text of a paper published as follows: Aryee, A.N.A., Phillip, L.E., Cue, R.I. and Simpson, B.K (2011). **Identification and Quantitation of Reaction Intermediates and Residuals in Lipase-catalyzed Transesterified Oils by HPLC**. *Appl. Biochem. Biotechnol. 165(1): 155-177*.

CHAPTER VII:

IDENTIFICATION AND QUANTITATION OF REACTION INTERMEDIATES AND RESIDUALS IN LIPASE-CATALYZED TRANSESTERIFIED OILS BY HPLC

7.1. Abstract

A high performance liquid chromatography (HPLC) unit equipped with size exclusion column and a refractive index (RI) detector was used for the simultaneous monitoring, identification, and quantitation of the reaction components from lipase-catalyzed transesterification of three oils. The procedure simultaneously separated and detected unreacted triacylglycerols (TAG), diacyl- and monoacyl- glycerol (DAG and MAG) co-products, residual alcohol as well as FFA based on retention time. The chromatograms showed well separated and resolved peaks. The elution of the components from the transesterification reaction in increasing order was: TAG < DAG < FFA < MAG. Generally, higher alcohol ratios decreased the conversion of TAG in all the oils studied with between 14 and 94% of TAG remaining in all the treatment combinations. Higher amount of salmon skin oil (SSO) TAG was generally converted to DAG than Rothsay composite (RC), and olive oil (OO) TAG. Relatively higher amount of OO DAG was converted to MAG than SSO and RC with only 5-14% DAG remaining in OO. RC and OO generally accumulated less MAG and this was reflected as lower MAG levels in RC (<6%) and OO (<14%) compared with SSO (<27%). For the various treatment combinations and the three oils used in this study, the least amount of FFA was recorded in transesterified OO with a maximum of approximately 4%. This HPLC method can be used as a simple and fast technique to analyze the reaction components and products of transesterification reactions without the need for additional derivatization steps.

7.2. Introduction

There has been considerable progress in the development of biodiesel (BD) production technologies over the past two decades (Demirbas, 2009; Fjerbaek et al., 2009). While the characterization of the feedstock is essential to accord the best

pretreatment regimes for the transesterification reaction, it is equally important to have in place accurate and rapid identification and quantitation techniques for the reaction products and co-products to assure better and continuous quality control (Dubé et al., 2004; Knothe, 2006; Canakci et al., 2009).

When the process of transesterification and/or purification post-synthesis is incomplete, significant amounts of residual mono-, di-, and triacylglycerols, free glycerol, catalyst and other minor components would remain in the fuel thereby contaminating it. These contaminants can affect fuel quality leading to engine problems and hazardous emissions (Monteiro et al., 2008; Canakci et al., 2009; Ranz et al., 2010). Standards for BD quality have been established, and these include test for free and total glycerol content, flash point, and acid number in Europe, North America, and elsewhere (ASTM D6751; EN 14214; Knothe, 2006; Canakci et al., 2009; Ranz et al., 2010) to monitor the completeness of the transesterification reaction and/or post-synthesis purification.

Although the transesterification reaction process is relatively straightforward, feedstock and product quality testing can be time consuming. Several separation and analytical techniques such as gas chromatography (GC), thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), and infra-red (IR) techniques (Dubé et al, 2004; Knothe, 2006; Meher et al., 2006) have been simplified, automated and used to identify and measure fuel quality indices. According to Plattner (1981) and other workers (Fillières et al., 1995; Lechner et al., 1997), the commonly used GC method for BD quantitation is not a convenient or direct analytical method for the detection and quantitation of the contaminants (TAG, DAG, MAG, FFA, glycerol, and unreacted alcohol) in the products. They further indicated that the GC method requires carefully controlled sample derivatization (trimethylsilylation or acetylation) prior to the GC analysis (Lechner et al., 1997; Knothe, 2001; Dubé et al., 2004). In addition, an inert, short capillary column with stable and high column temperature ($\geq 350^{o}C$) as well as elaborate pre-treatment steps to separate the glycerol from the BD is usually required (Plattner, 1981; Darnoko et al., 2000).

An alternative to GC and the other techniques mentioned above is the HPLC technique which is fairly sensitive, reproducible, and rapid (Plattner, 1981; Darnoko et al., 2000; Salis et al., 2005). Additional advantages of the HPLC method include direct BD

analyses, identification, and quantitation of the products and co-products of the reaction (Knothe, 2001; Meher et al., 2006; Kittirattanapiboon and Krisnangkura, 2008); thus minimizing the exposure to reagents, and the extra cost associated with reagents and time. The HPLC system has been used with various detectors with different selectivities and sensitivities (Türkan and Kalay, 2006; Meher et al., 2006; Santori et al., 2009). The HPLC-UV system is commonly used because of its high sensitivity and adaptability to the frequently used gradient elution technique (Plattner, 1981; Türkan and Kalay, 2006; Santori et al., 2009). However, it presents some constraints with respect to the monitoring of fuel contaminants such as TAG, DAG or MAG due to the unavailability of chromophores above 200 nm. Another limitation of the HPLC-UV system is its incompatibility with some commonly used solvents in lipid analysis such as chloroform and tetrahydrofuran; these solvents absorb strongly in the region of 190-220 nm; where most lipids absorb (Plattner, 1981; Holčapek et al., 1999; Arzamendi et al., 2006). In contrast, the refractive index (RI) detector is a robust instrument for liquid chromatographic studies (Fillières et al., 1995; Warabi et al., 2004; Kittirattanapiboon and Krisnangkura, 2008) that can be used with most solvent systems compared to the UV detector (Plattner, 1981). In spite of this, the HPLC-RI system has not been widely used in quantifying the products and co-products of transesterification reactions (Darnoko et al., 2000; Warabi et al., 2004; Arzamendi et al., 2006).

In this study, the co-products (TAG, DAG, MAG, and FFA) formed from the production of BD from salmon oil, olive oil, and a commercial fat-oil sample (referred to as Rothsay composite) were simultaneously verified and quantitated using an HPLC system equipped with a RI detector. While previous papers have mostly described quantitation of these components in methanol-based BD (methyl esters) (Darnoko et al., 2000; Arzamendi et al., 2006; Kittirattanapiboon and Krisnangkura, 2008), the present study was on ethanol-based BD (ethyl esters).

7.3. Materials and Methods

7.3.1. Materials

A commercial mixture of rendered animal fat and used frying oil (Rothsay composite, RC) was provided by Rothsay® Biodiesel Company (Ste. Catherine, QC);

salmon skin oil (SSO) was obtained from smoked salmon skin provided by Atkins et Frères Inc. (Mont-Louis, QC); olive oil and immobilized lipase (Lipozyme®-IM) (86.8 U/g) were purchased from Sigma-Aldrich (Oakville, ON). One unit of immobilized lipase activity is defined as the amount producing 1 µmol of stearic acid per min from the hydrolysis of tristearin at pH 8.0 and 70°C; anhydrous ethanol (EtOH) was purchased from Commercial Alcohols (Boucherville, QC); toluene and acetic acid were obtained from Fisher Scientific (Whitby, ON); while FAEE, TAG, DAG, MAG, and FFA standards were purchased from Nu-Chek Prep Inc. (Elysian, MN).

7.3.2. Methods

7.3.2.1. Oil Characterization

Salmon skin oil (SSO) was obtained from salmon skin by solvent extraction according to the method previously described by Aryee and Simpson (2009). The FFA content, moisture content, and the fatty acid composition of the starting oils were determined in duplicates according to the AOCS method (Ca 5a-40), AOCS method (Ca 2c-25), and with gas chromatography (GC), respectively.

7.3.2.2. Time-Course Transesterification Reaction

Transesterification of the test samples (5 g of SSO, RC, or OO) was carried out in 30 ml stoppered vials using a fixed load of Lipozyme®-IM [(Enz (21.70 U)], oil:alcohol molar ratio [(AlcoR), 1:1 to 1:6], at five different temperatures (25, 35, 45, 55, and 65^{o}C), and with uniform shaking in a water bath (model 25, Precision Scientific, Chicago, IL) at 60 shaker rate/min. Aliquots were withdrawn from the vial after reaction times ranging from 8 to 120 h and dissolved in toluene prior to injection into the HPLC-RI unit.

7.3.2.3. HPLC Identification and Quantitation Analysis

Calibration curves were developed for MAG, DAG, TAG, FAEE, FFA, and EtOH for the quantitation of the components produced during the transesterification reaction. Standard solutions of the each of the standard at 5 different concentration levels (0.3125-5 mg/ml) were prepared in toluene and injected and analyzed by the HPLC-RI system.

The data obtained were fitted by linear regression and the corresponding equations generated for quantitation.

The standards and reaction mixtures from the transesterification were analyzed using an HPLC system equipped with a 5 µm Phenogel™ 300 x 7.8 mm ID size exclusion column (Phenomenex, Torrance, CA) with a 50 x 7.8 mm guard column, and a RI detector. Elution was carried out in isocratic mode using 0.25% (v/v) acetic acid in toluene at a flow rate of 1.0 ml/min. An autosampler and injector were used to inject 25 µl of the standard or test sample into the HPLC system. The peak areas and response factors of the samples were recorded under the same conditions as the standards. A Varian Galaxie™ (Varian, Palo Alto, CA) software was used for data acquisition.

The criteria for identification of the compounds in the samples were established based on comparisons with the retention times and chromatograms of the standards. The peaks in the chromatograms were automatically integrated to generate the data for quantitation. The % yield of TAG, DAG, MAG, and FFA was determined using the integrated data of the corresponding peak and interpolated from the calibration curve constructed.

7.3.2.4. Statistical Analysis

The data obtained from the studies were analyzed using PROC MIXED (Statistical Analysis Systems, Version 9.2, SAS Institute Inc., Cary, NC) with temperature and oil:alcohol molar ratio as fixed effects, vial as a random effect, and reaction time (8, 12, 24, 36, 48, 60, 72, 96, and 120 h) as repeated measures. The autoregressive 1 [AR(1)] was selected among all competing variance-covariance structures to evaluate the correlation between measurements (Sawa, 1978; Littell et al., 2000).

7.4. Results

7.4.1. Oil Characterization

An important index used to monitor feedstock quality is acidity as measured by FFA. The FFA content of SSO and RC were relatively higher (4-6%) than that of OO (*ca* 0.2% FFA). The corresponding moisture contents of these oils (SSO, RC and OO) were

estimated as 0.02, 0.03, and 0.01%, respectively. As shown in Table 7.1, the predominant fatty acids (FAs) were C_{18}-fatty acids (42-82%); furthermore, there were *ca* equivalent amounts of saturated and monounsaturated FAs in the RC test sample (Table 7.1).

7.4.2. Characterization of Samples by HPLC

The transesterification reaction occurred in three sequential steps. TAG reacts with ethanol to produce DAG, MAG, and glycerol, sequentially, and each step produces a molecule of FAEE. Under optimum reaction conditions, to attain maximum FAEE yield, TAG, DAG, and MAG components would be expected to be nil or negligible. Characterization of BD produced by the transesterification of lipids for quality assurance is useful to avert engine and fuel injection problems associated with using poor quality fuels. For accurate and quantitative analysis, complete separation of all the reaction components is required to ensure unequivocal assignment of the peaks in the chromatographic spectra. The components produced from the various oils investigated in this study were verified using an HPLC method based on size exclusion with RI detection in a single 25 min run time. The RI detector responses were linear over the concentration range of 0.3125-5 mg/ml (Fig. 7.1), as indicated by the high correlation coefficients (R^2 > 0.99). TAG and DAG eluted ahead of MAG and FFA (Fig. 7.2).

7.4.3. Quantitation of the Reaction Components

The transesterification reactions were performed at 25-65^{o}C using various oil:alcohol molar ratio (AlcoR) for SSO, RC, and OO to evaluate the effects of the reaction parameters on the yield of co-products from the transesterification reaction.

The data for TAG, DAG, MAG, and FFA contents obtained from the transesterification of SSO, RC, and OO at 35^{o}C, AlcoR of 1:4 and at the different reaction times are shown in Fig. 7.3. The TAG content steadily decreased from *ca* 85% (8 h) to 20% (120 h) while MAG yield increased. The highest level of DAG (11%), MAG (24%) and FFA (4%) were attained at 72 h (Fig. 7.3a). In the RC sample, TAG content decreased from *ca* 81% (8 h) to 54% (120 h) with variable amounts of MAG. The highest amount of DAG (19%), MAG (5%), and FFA (6%) were recorded at 36, 24, and 72 h, respectively (Fig. 7.3b). The TAG content in transesterified OO decreased from *ca* 88%

(8 h) to 78% (120 h) with variable MAG content. The highest amount of DAG (10%), MAG (6%), and FFA (0.6%) were respectively recorded at 8, 96, and 60 h (Fig. 7.3c). The interactions of all the factors at Type 3 tests of fixed effects were significant ($p < 0.05$).

For the purpose of illustration and space limitation, the yields of TAG, DAG, MAG, and FFA for SSO for the various treatment combinations are presented graphically in Figs. 7.4-7.7 while those for RC and OO are tabulated in Tables 7.2-7.5. The data presented in Figs. 7.4-7.7 and Tables 7.2-7.5 show that ≥14% TAG content was recorded in all the three oils for all the treatment combinations (temperature and oil:alcohol molar ratio) investigated. In general, higher AlcoR decreased the conversion of TAG to BD with various amount of the other co-products (Figs. 7.4-7.7, Tables 7.2-7.5).

7.4.3.1. SSO Transesterification Residuals and Co-products

At 25^{o}C, there was substantial decrease in TAG content in the SSO sample at all the AlcoRs except at AlcoR 1:6 (Fig. 7.4a). While all 4 AlcoRs showed *ca* >64% decrease in TAG content between 8 and 120 h, AlcoR 1:6 showed only 35% decrease in TAG content and these were all significant ($p < 0.05$). The DAG content was fairly moderate (≤11%) at 25^{o}C (Fig. 7.5a) at all the AlcoRs studied up until 48 h, beyond which DAG content increased to between 1-20% at all the AlcoRs for the remainder of the experimental period. There was slow conversion of DAG to MAG and low accumulation of it during the initial period of the experiment at all the treatment combinations (Figs. 7.6a-e). Higher amounts ($p < 0.05$) of MAG were accumulated at AlcoRs 1:3, 1:4, and 1:5 beyond 72 h at 25^{o}C (Fig. 7.6a). With the exception of AlcoR 1:1 <7% of FFA was recorded at all the other treatment combination at 25^{o}C in transesterified SSO (Fig. 7.7a).

Similar pattern in TAG content was observed at 35^{o}C in transesterified SSO (Fig. 7.4b). DAG content increased at all the reaction times studied at 35^{o}C and AlcoR 1:1 (Fig. 7.5b), it however maintained a fairly constant though significant ($p < 0.05$) DAG content throughout the experimental period at the other AlcoRs. MAG content at 35^{o}C followed similar trend as 25^{o}C as evident by its appreciable increase with time at AlcoRs 1:3, 1:4, and 1:5 (Fig. 7.6b). FFA content was generally low at all the treatment combinations at

35°C with ≤6% FFA at all the AlcoRs except AlcoR 1:1 (Fig. 7.7b). At 45°C there was consistent and substantial decrease in TAG content at AlcoRs 1:1, 1:3 and 1:4 at all the reaction times studied but comparably high amount of TAG remained at AlcoRs 1:5 and 1:6 during the same period in transesterified SSO (Fig. 7.4c). A similar DAG pattern as 35°C was observed at 45°C but with occasional increases at some of the AlcoRs studied (Fig. 7.5c). The trend in MAG content at 45°C (Fig. 7.6c) was similar to 25 and 35°C. FFA content was also low at 45°C at all the treatment combinations but >15% at AlcoRs 1:5 and 1:6 at 120 h. Except for AlcoR 1:1, all the other AlcoRs at 55°C (Fig. 7.4d) and 65°C (Fig. 7.4e) showed only marginal decreases in TAG content in the course of the reaction in transesterified SSO.

The DAG content at 55°C (Fig. 7.5d) and 65°C (Fig. 7.5e) also followed similar trends as the DAG content at 35 and 45°C. At 55°C, AlcoRs 1:1, 1:3 and 1:4 showed clear increase in MAG content with time (Fig. 7.6d) up to 60 h and all these were significant ($p < 0.05$), while AlcoR 1:1 maintained the increase to the end of the experimental period. Similar to 55°C, the reaction at 65°C showed clear increases in MAG contents with time (Fig. 7.6e) at AlcoR 1:1 and all these were significant ($p < 0.05$). Reaction at 55 and 65°C revealed a generally constant FFA content over time at all the AlcoRs studied and an equally higher FFA content at AlcoR 1:1 in transesterified SSO (Figs. 7.7d and 7.7e).

7.4.3.2. RC Transesterification Residuals and Co-products

The remaining TAG content in the RC sample showed a decreasing trend at AlcoR 1:1 at 25°C throughout the course of the reaction from *ca* 84% at 8 h to 48% at 120 h (Table 7.2) with concomitant increase in DAG, MAG, and FFA content (Tables 7.3, 7.4 and 7.5). Similar TAG, DAG, MAG, and FFA content patterns were observed with AlcoRs of 1:3 and 1:4 (Tables 7.2, 7.3, 7.4, and 7.5). However the decreases in TAG content at AlcoRs 1:5 and 1:6 were comparably lower during the first 24 h (Table 7.2) while accumulation of DAG, MAG, and remaining FFA (Tables 7.3, 7.4, and 7.5) followed similar trends as described for AlcoR 1:1. At 35°C and AlcoR 1:1, there was *ca* 41-80% TAG remaining between 8 to 120 h (Table 7.2). Similar amounts of TAG remained at 35°C at higher AlcoRs (1:5 and 1:6) as 25°C. A general increase in DAG and

MAG content with time was observed at all the AlcoRs studied at 35°C ($p < 0.05$) (Tables 7.3 and 7.4). Similar to transesterified SSO and RC at 25°C, <7% FFA was recorded over time at all the AlcoRs at 35°C in transesterified RC (Table 7.5).

Almost similar patterns in TAG content were observed at all the AlcoRs studied at 45, 55 and 65°C (Table 7.2). For instance, TAG content decreased significantly ($p < 0.05$) from 71 to 34%, 68 to 36%, and 65 to 38% between 8 and 120 h at 45°C, 55°C, and 65°C, respectively. At 55 and 65°C, AlcoR 1:1 showed higher MAG content than the other AlcoRs studied ($p < 0.05$) but followed similar patterns as 25 and 35°C at all the other temperatures and reaction times studied in transesterified RC (Tables 7.3 and 7.4). Beyond 35°C, lower levels of FFA were generally observed at >1:1 AlcoR in all the other treatment combinations ($p < 0.05$) (Table 7.5).

7.4.3.3. OO Transesterification Residuals and Co-products

An AlcoR of 1:3 was found to decrease TAG content at 25°C more than the other AlcoRs studied with transesterified OO (Table 7.2). At 35°C, AlcoRs 1:1 and 1:3 showed <79% TAG content at the end of the reaction cycle (120 h), while >74% TAG remained at AlcoRs 1:4, 1:5, and 1:6 (Table 7.2). The results at 45, 55, and 65°C followed similar trends with substantial decrease in TAG at AlcoR of 1:1 and only minor decreases in TAG contents for the other AlcoRs studied.

The conversion of DAG to MAG was greater than the production of DAG from TAG in transesterified OO. The DAG contents were fairly constant throughout the course of the reaction and were comparably low, ranging between 5 and 14% at all the treatment combinations and these were significant ($p < 0.05$) (Table 7.3). It was between 1.4-39 and 0.5-35% for RC and SSO, respectively.

Like RC, higher amounts of MAG were accumulated at 55 and 65°C and at AlcoR 1:1 ($p < 0.05$) (Table 7.4). At 25, 35, and 45°C, more MAG accumulated over time at AlcoR 1:3 ($p < 0.05$). The transesterification of MAG was slow in the presence of high amounts of TAG. Overall, RC and OO accumulated less MAG (6 and 14%, respectively) over time at all the AlcoR and temperatures studied compared to SSO (27%). The least amount of FFA was recorded in transesterified OO with a maximum of ≤4% FFA at all the treatment combinations ($p < 0.05$) (Table 7.5). FFA content remained fairly constant

with AlcoR 1:1 giving higher FFA values compared to the other AlcoRs ($p < 0.05$) studied (Table 7.5). This study revealed that the rate of transesterification is dependent on temperature, reaction time, oil:alcohol molar ratio, thermal stability of the lipase and that there were significant ($p < 0.05$) differences in residuals yields between temperature, oil:alcohol molar ratio and reaction time as assessed during the transesterification reaction.

7.5. Discussion

7.5.1. The Effects of Reaction Parameters on the Residual Yield as Elucidated by Stoichiometry, Polarity, and Thermal Stability

Increasing oil:ethanol molar ratio seemed to have an initial inhibitory effect on the transesterification reaction in all the three oils studied. The low conversion of TAG and relative high amount of unreacted TAG quantified in the transesterified oils during the initial segment of the reaction may be due to a myriad of factors. These include the initial delay of the transesterification reaction due to minimal mixing and dispersion of the alcohol in the oil, poor solvation or low miscibility of TAG in the alcohol creating a two-phase/heterogeneous system, the deactivation effect of the polar ethanol on the enzyme, interfacial action of lipase and the nature of lipase catalysis in non-aqueous media (Laane et al., 1987; Kaieda et al., 2001; Páez et al., 2003; Dizge and Keskinlera, 2008; Antczak et al., 2009; Naranjo et al., 2010).

Polar alcohols such as ethanol possess the ability of distorting the ordered layer of water molecules that surround the enzyme needed for conformational integrity and stability (Salis et al., 2005; Fukuda et al., 2008). Beyond the stoichiometric (AlcoR 1:3) amount, this effect appeared to be more pronounced (Figs. 7.4-7.7 and Tables 7.2-7.5) and consistent with previous reports (Dizge and Keskinlera, 2008; Hernández-Mártin and Otero, 2008; Naranjo et al., 2010). In the presence of polar alcohols, the oil exists in a two-phase system with very minimal dispersion (Iso et al., 2001; Karmee and Chadha, 2005; Hernández-Mártin and Otero, 2008), creating diffusion limitations and a low concentration of oil in the alcohol phase. Lipases are easily deactivated when in contact with the insoluble alcohol (Shimada et al., 2002). In additional to this is another limitation from the immobilized lipase creating a three-phase system (Hernández-Mártin

and Otero, 2008; Freitas et al., 2009). For efficient catalysis, both the external diffusion limitation i.e. substrate from the bulk solvent through the boundary layer to the surface of enzyme, and the internal diffusion limitation of substrate to the active site of the enzyme must be overcome (e. g. through effective mixing).

Lipases exhibit maximum activity at the interface of oil and water by interfacial activation (Derewenda et al., 1992; Maruyama et al., 2000). The active site of the lipase which is covered by a lid, unhinges during interfacial activation for the substrate to reach the active site (Jaeger et al., 1994). In non-aqueous reaction systems, the absence of the oil-water interface keeps the lid in closed position and prevents contact and interaction between the substrate and enzyme. However an excess amount of water may lead to some unwanted side-reactions such as hydrolysis (Shimada et al., 2002; Dizge and Keskinlera, 2008; Lu et al., 2009). The amount of water required to either activate the enzyme for transesterification or the unwanted hydrolysis varies and it's based on several factors such as the lipase itself, the type of oil and alcohol used in the transesterification reaction (Shimada et al., 2002; Lu et al., 2009).

The observations in this study suggest that increasing the temperature or alcohol content did not appreciably decrease TAG content (though statistically significant, $p < 0.05$). Increasing reaction temperature is known to alter the limitations of mass transfers by reducing the viscosity of the reaction mixture (Hernández-Mártin and Otero, 2008). This increases solubility and facilitates the movement of the reactants to the catalytic site and the products formed away from the catalytic site of the enzyme and support (if immobilized). The low conversion of TAG with increasing temperature ($>45^{o}$C) maybe ascribed to thermal instability of the enzyme. The optimum temperature is dependent on other reaction parameters such as the oil:alcohol molar ratio (Dizge and Keskinlera, 2008; Antczak et al., 2009) as was observed in this study.

7.5.2. DAG, MAG, and FFA in the Transesterification Reaction

Transesterification reaction results in the formation of the intermediates; DAG and MAG. The high accumulation of DAG is consistent with the *sn*-1, 3 specificity of Lipozyme® (Türkan and Kalay, 2001; Rodriguez et al., 2010), and the synthesis of TAG (Shimizu et al., 2008), while the overall low MAG yield compared to DAG at all the

treatment combinations for all three oils may be due to faster conversion of the more soluble MAG (Arzamendi et al., 2006).

The source of FFA in the transesterified stock can be from; the starting oil, hydrolysis product of the moisture in the feedstock with lipase, and incomplete transesterification reaction (Shimada et al., 2002; Kumari et al., 2007; Shah and Gupta, 2007; Shimizu et al., 2008). Although volumetric titration is used to measure the FFA content in both ASTM D 664/974 [USA]; EN 14104 [EU], the HPLC method measures the FFA content exclusively, while the former measures the total acid value which will include other acidic components such as the MAG and DAG present. Some of the inherent problems associated with ASTM D 664 include variability in the electrodes used in the potentiometric titration (Knothe, 2006), and this is overcome by the HPLC method.

7.5.3. Suitability of the HPLC Analysis

The HPLC method was a straightforward approach with no requirement for prior derivatization or chemical modifications of the components, as is the case of the GC method. Each class (TAG, DAG, MAG, and FFA) eluted as a single peak each (Fig. 7.2) instead of several peaks as often seen with GC analysis. This simplifies the quantitation and avoids overlapping information. Previous reports of authors using size exclusion chromatography did not simultaneously detect FFA with the other reaction components (Darnoko et al., 2000; Dubé et al., 2004; Arzamendi et al., 2006).

Overall the results showed general agreement with previous size exclusion chromatographic (SEC) methods despite the differences in sample preparation and instruments (Darnoko et al., 2000; Dubé et al., 2004; Arzamendi et al., 2006; Kittirattanapiboon and Krisnangkura, 2008). Glycerol could not be detected similar to reports by Kittirattanapiboon and Krisnangkura (2008) and can be attributed to its small quantity in the ethyl ester-rich phase. Authors who detected glycerol had ≥2 columns coupled in series to the detector (Darnoko et al., 2000; Dubé et al., 2004; Arzamendi et al., 2006). However these authors often reported poor separation and resolution of the other lipids classes (TAG, DAG, and MAG) when only one column was used. Like Arzamendi et al. (2006), this method was able to monitor the residual ethanol in the transesterification reaction (Fig. 7.2). Additionally, since the analysis was performed at

room temperature (23^{o}C) with isocratic elution, new analysis could be started immediately after the 25 min since there was no need to change the column temperature or mobile phase.

7.6. Conclusion

The need for a reliable and fast technique for both qualitative and quantitative information about the alkyl esters (BD) and reaction components was achieved in a single HPLC-RI run. This paper summarized a simple, rapid, and practical HPLC technique for the unambiguous identification and quantitation of reaction residuals produced during lipase-catalyzed transesterification reaction of salmon skin oil, Rothsay composite, and olive oil samples to BD. The results revealed various degree of conversion during the reaction period and the interactive effects of reaction parameters on yield. Other lipids with similar transesterification scheme can be analyzed using this approach without any elaborate modifications. The accurate quantitation of components such as partial glycerides, unreacted TAG, FFA, and residual alcohol with tendencies to contaminate the fuel is very important in transesterified oils intended for use as fuel.

7.7. Acknowledgments

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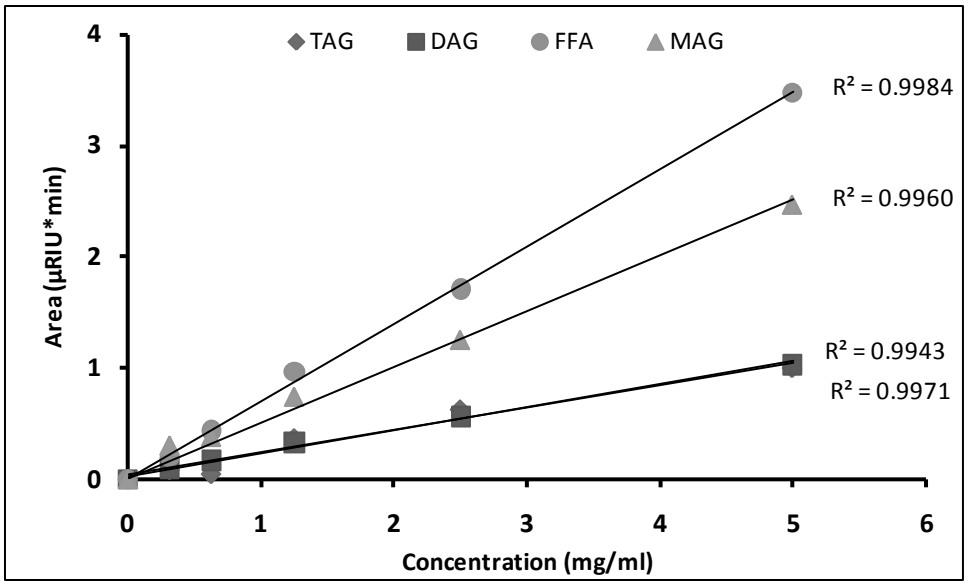

Fig. 7.1: Calibration curve for triacylglycerol (TAG), diacylglycerol (DAG) and monoacylglycerol (MAG) and free fatty acid (FFA).

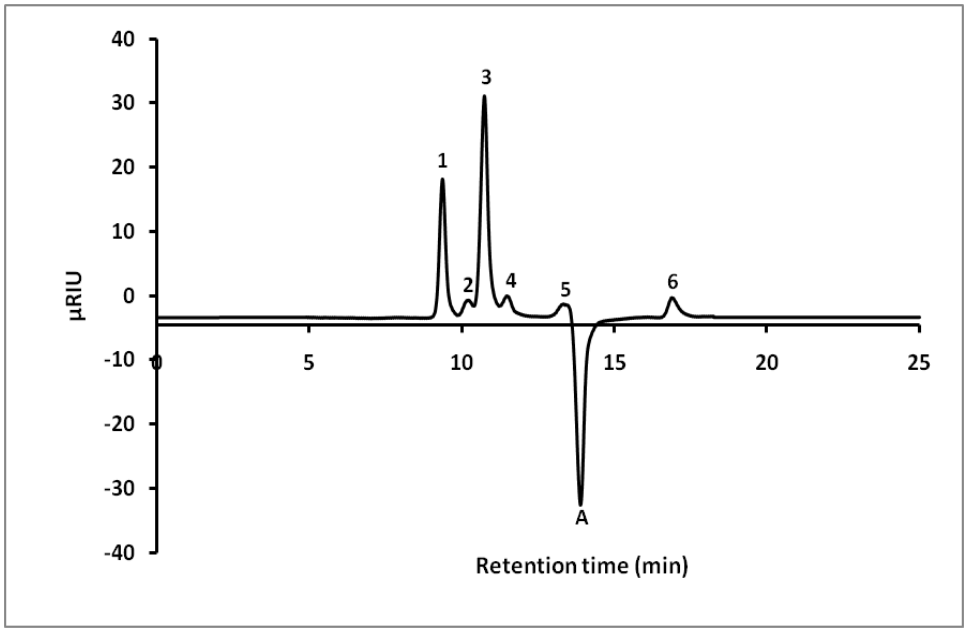

Fig. 7.2: HPLC chromatogram of transesterification components monitored with RI (isocratic elution mode; 0.25% (v/v) acetic acid in toluene for 25 min). Peaks correspond to 1:- triacylglycerol (TAG), 2:- diacylglycerol (DAG), 3:- fatty acid ethyl ester (FAEE), 4:- free fatty acid (FFA), 5:- monoacylglycerol (MAG), 6:- ethanol (EtOH), and A: - internal flow marker.

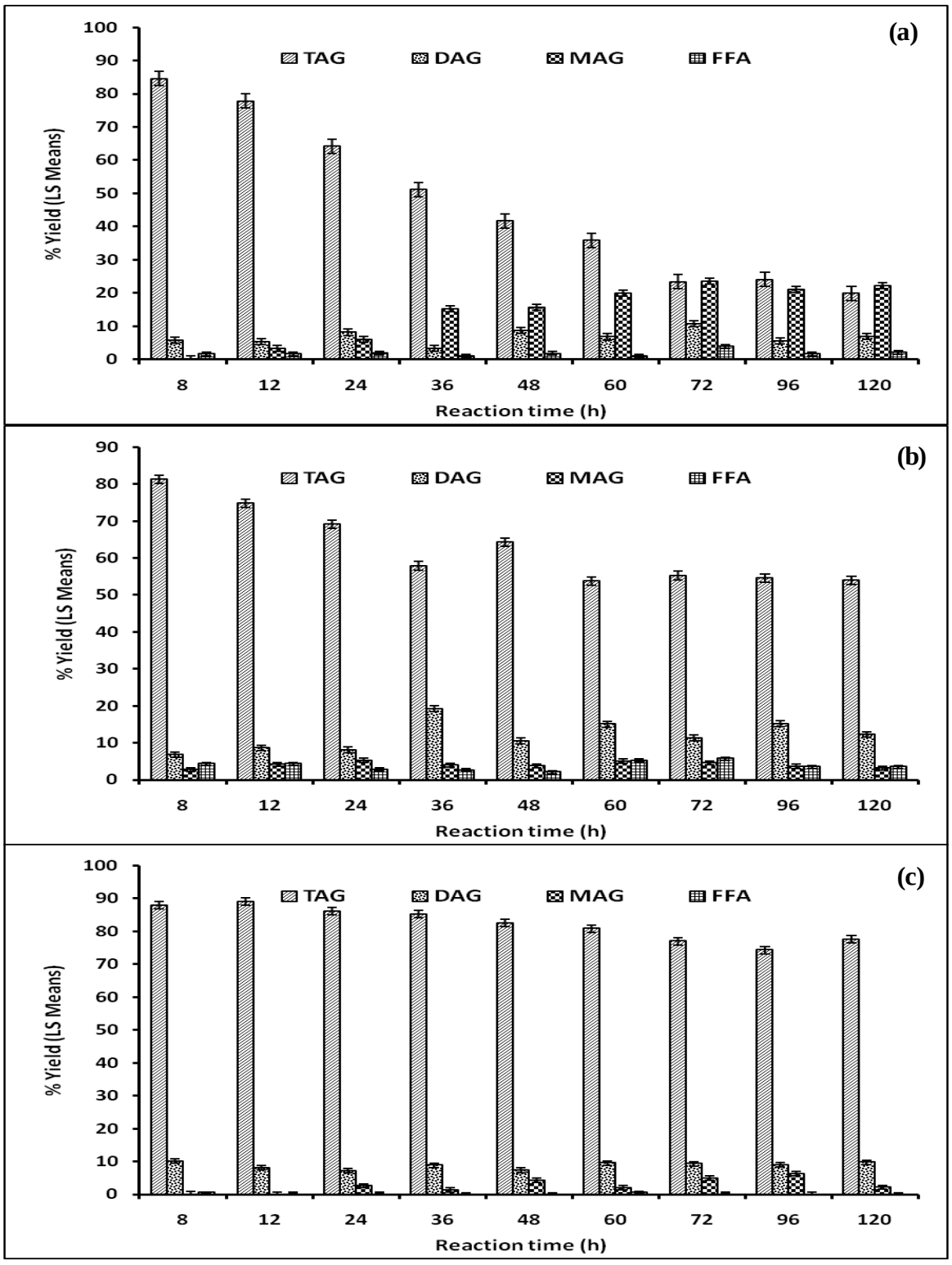

Fig. 7.3: Remaining and residuals components during the transesterification of (a): SSO, (b): RC and (c): OO (Reaction conditions:-oil:alcohol ratio 1:4, temperature 35^{o}C).

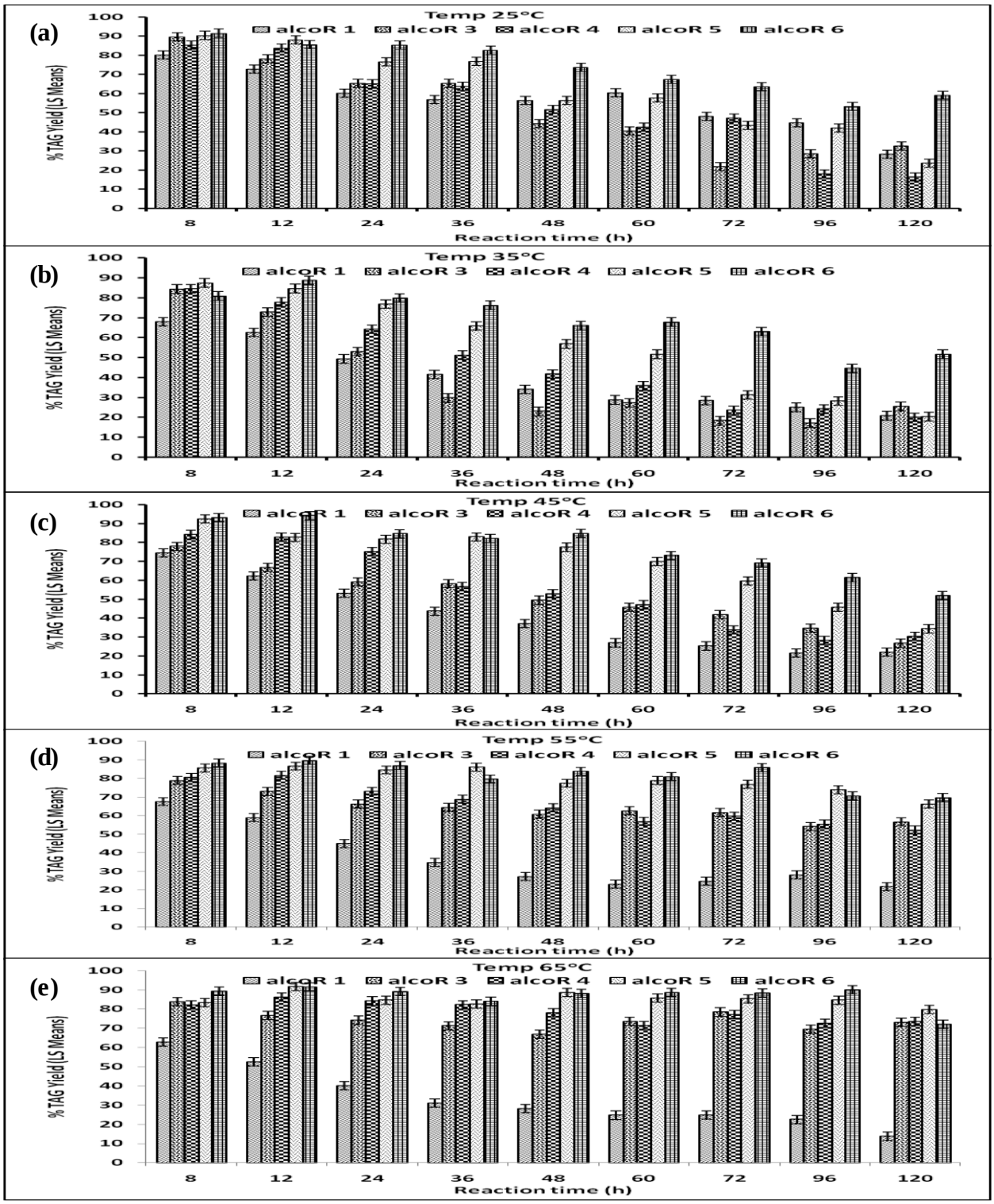

Fig. 7.4: Least Square means (LS Means) ± standard error of treatment x time of TAG (% TAG yield) of transesterified SSO at (a): 25°C, (b): 35°C, (c): 45°C, (d): 55°C, and (e): 65°C.

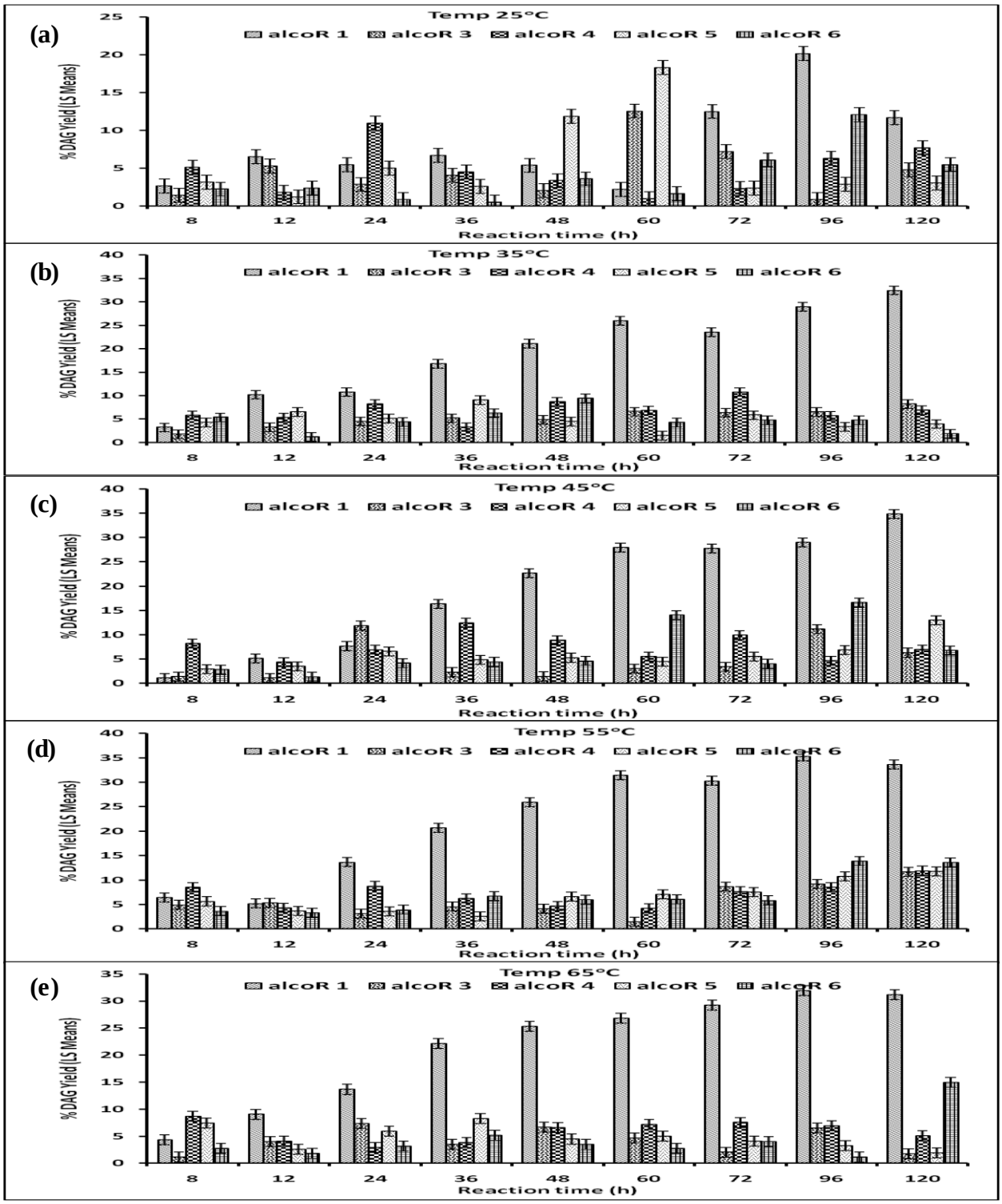

Fig. 7.5: Least Square means (LS Means) ± standard error of treatment x time of DAG (% DAG yield) of transesterified SSO at (a): 25°C, (b): 35°C, (c): 45°C, (d): 55°C, and (e): 65°C.

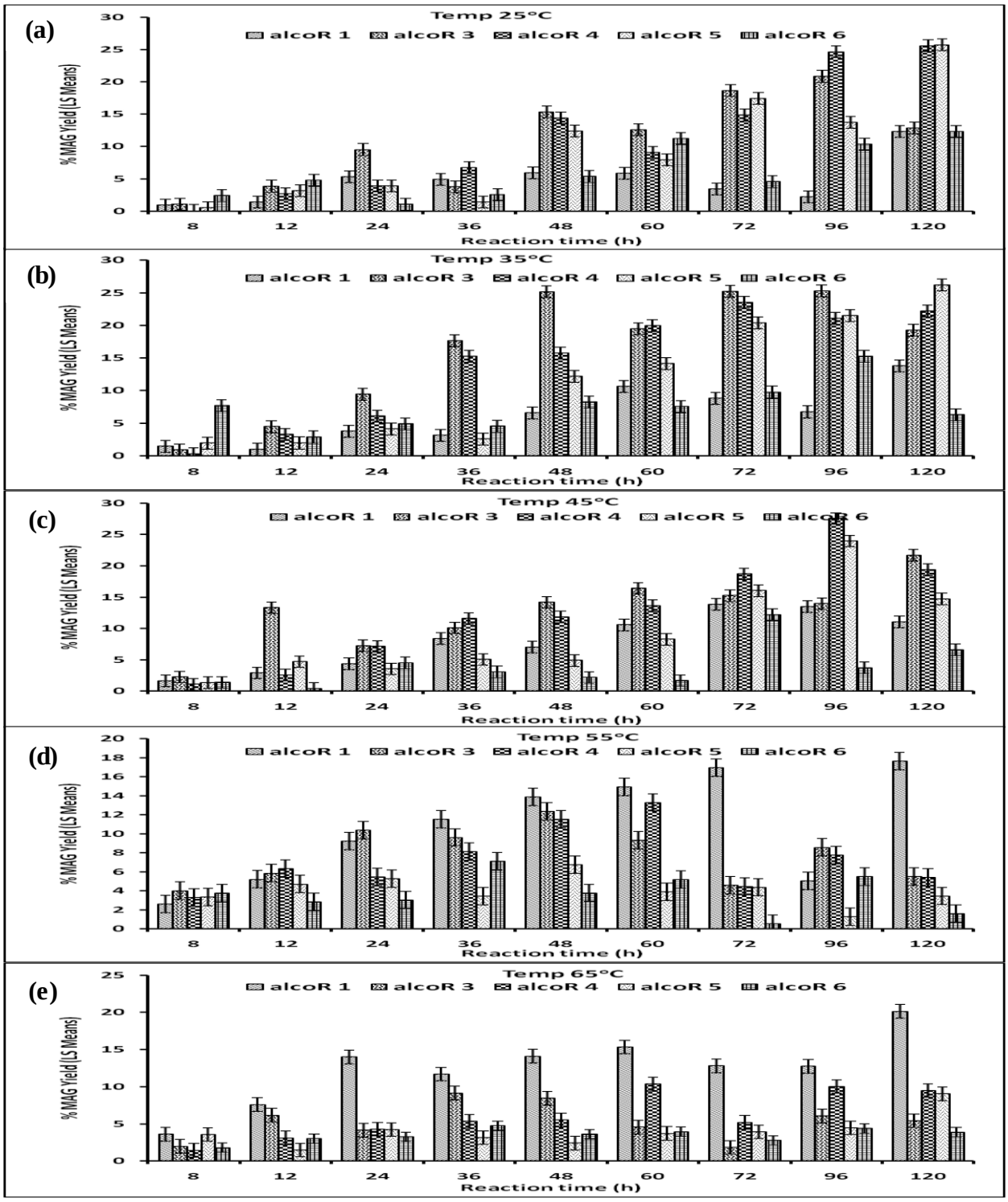

Fig. 7.6: Least Square means (LS Means) ± standard error of treatment x time of MAG (% MAG yield) of transesterified SSO at (a): 25°C, (b): 35°C, (c): 45°C, (d): 55°C, and (e): 65°C.

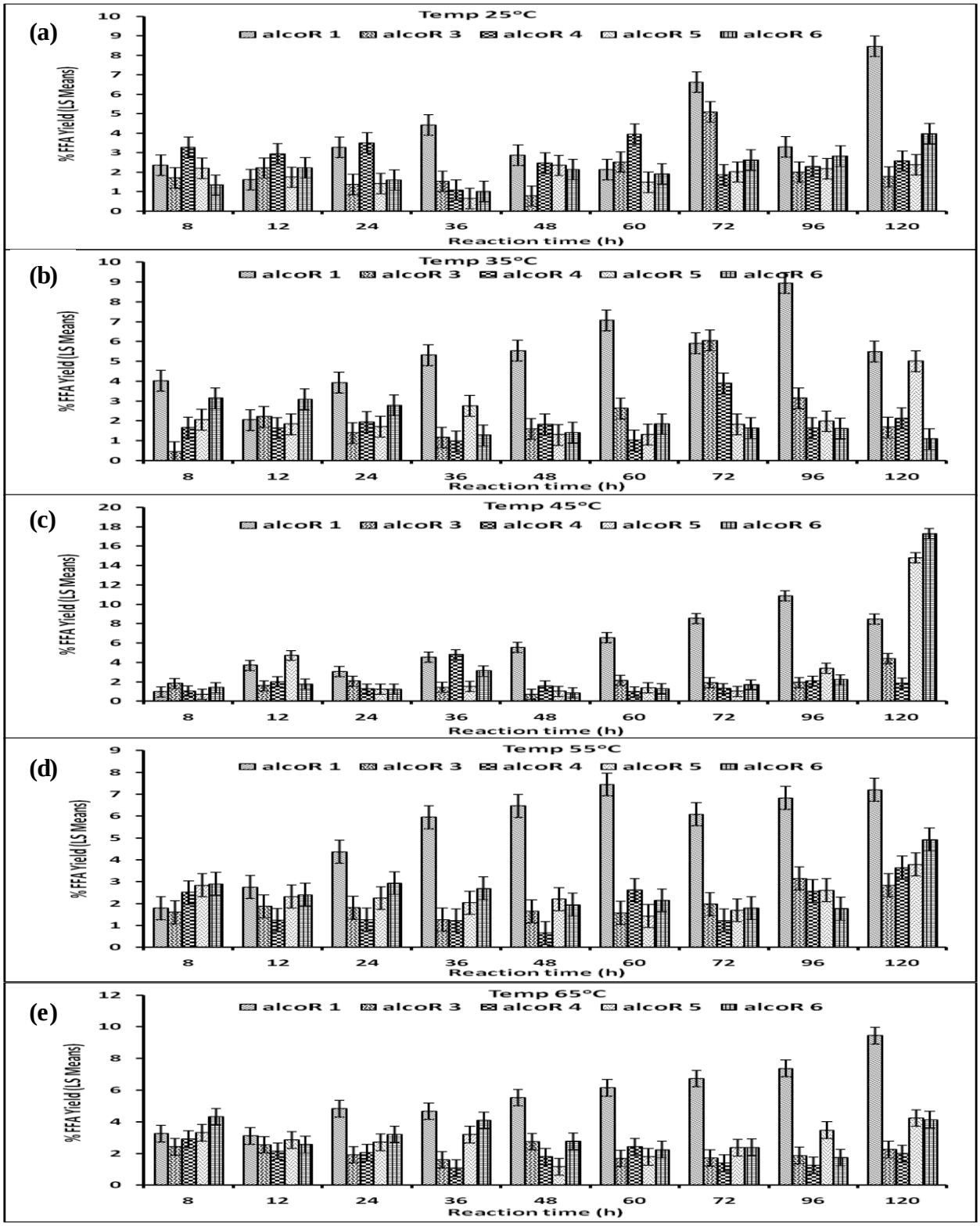

Fig. 7.7: Least Square means (LS Means) ± standard error of treatment x time of FFA (% FFA yield) of transesterified SSO at (a): 25°C, (b): 35°C, (c): 45°C, (d): 55°C, and (e): 65°C.

Fatty acid (FA)	Salmon Skin oil (SSO)	Rothsay Composite (RC)	Olive Oil (OO)
Lauric (12:0)	0.14	0.00	0.00
Myristic (14:0)	5.77	1.47	0.00
Palmitic (16:0)	16.94	27.11	14.38
Palmitioleic (16:1*n*-7)	5.42	2.29	0.13
Stearic (18:0)	4.31	12.79	3.75
Oleic (18:1*n*-9)	19.20	39.59	67.75
Linoleic (18:2*n*-6)	16.05	15.82	10.78
Linolenic (18:3*n*-3)	2.82	0.81	0.68
Eicosapentaenoic (20:5*n*-3)	15.55	0.00	0.00
Docosapentaenoic (22:5*n*-3)	2.45	0.13	0.00
Docosahexaenoic (22:6*n*-3)	11.36	0.00	2.58
∑ Saturated fatty acids (SFA)	27.16	41.37	18.13
∑ Monounsaturated fatty acids (MUFA)	24.62	41.88	67.88
∑ Polyunsaturated fatty acids (PUFA)	48.23	16.76	14.04

Table 7.1: Fatty acid composition of salmon skin oil (SSO), Rothsay composite (RC), and olive oil (OO). Values are % of the % total fatty acid.

Treatments		% TAG Yield (RC and OO)*								
Temperature (°C)	Oil:alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
						RC				
	1:1	83.63	78.19	73.29	71.37	62.25	59.71	52.94	48.87	48.30
	1:3	85.81	80.49	74.29	74.04	71.20	69.64	71.32	71.19	68.35
25	1:4	92.37	82.70	78.35	72.34	69.22	66.11	59.98	61.37	55.52
	1:5	89.02	85.63	86.43	78.95	74.17	71.63	67.28	63.42	58.23
	1:6	88.75	85.75	82.97	79.87	77.40	74.14	66.14	68.23	59.86
	1:1	79.53	62.28	51.54	45.58	45.73	41.87	43.11	40.66	40.78
	1:3	79.48	80.04	76.94	77.33	65.02	60.45	62.88	64.08	57.32
35	1:4	81.21	74.80	69.12	57.89	64.25	53.80	55.22	54.52	53.92
	1:5	86.06	83.96	76.57	75.67	71.06	68.01	62.38	62.01	58.73
	1:6	87.88	84.11	84.54	78.56	78.45	75.05	71.15	67.30	64.73
	1:1	70.55	62.37	56.41	45.87	44.76	39.23	34.63	35.21	33.64
	1:3	73.47	73.51	71.62	71.99	69.30	68.13	68.61	71.38	69.91
45	1:4	84.70	81.35	75.56	74.98	74.87	75.50	76.38	66.50	76.33
	1:5	88.31	85.31	84.53	82.91	80.96	82.45	79.92	81.36	79.54
	1:6	87.29	88.02	86.04	84.75	84.48	81.53	80.91	80.96	80.30
	1:1	68.01	58.99	45.55	40.74	37.70	37.26	36.80	35.91	36.03
	1:3	77.87	77.05	76.79	76.60	76.28	76.79	74.10	73.62	71.47
55	1:4	86.63	85.85	83.55	82.79	83.65	81.52	78.01	77.78	74.07
	1:5	87.97	88.21	87.26	86.59	85.27	85.53	83.66	84.91	80.88
	1:6	88.14	89.20	88.21	88.12	86.94	86.00	84.19	85.52	79.97
	1:1	64.94	54.63	45.61	39.72	40.42	39.49	38.38	37.97	38.35
	1:3	82.17	81.56	81.23	79.61	79.75	79.03	80.05	78.25	77.70
65	1:4	86.66	88.39	87.84	85.53	84.10	81.86	82.64	81.00	81.74
	1:5	89.76	89.10	87.49	87.18	86.83	88.08	85.89	87.34	86.22
	1:6	87.59	87.49	87.97	89.32	87.41	86.34	85.49	84.12	86.21
						OO				
	1:1	85.26	78.06	72.75	71.66	68.14	66.30	68.82	60.96	61.39
	1:3	84.36	80.98	69.12	62.84	62.34	55.66	55.03	51.15	52.87
25	1:4	89.74	90.33	85.33	82.61	82.33	76.20	69.99	63.90	59.96
	1:5	92.07	90.09	89.10	87.94	87.68	86.66	87.38	81.59	80.42
	1:6	91.36	89.36	91.80	88.42	85.37	83.73	87.14	88.01	84.57
	1:1	78.42	70.17	66.02	67.38	61.34	59.59	61.03	63.92	57.59
	1:3	78.91	73.53	67.83	67.68	65.35	64.18	65.24	64.88	63.19
35	1:4	87.87	88.97	86.06	85.19	82.47	80.84	76.98	74.28	77.56
	1:5	90.77	90.55	88.88	90.25	90.25	87.22	88.25	85.14	76.92
	1:6	87.40	87.73	89.72	89.58	89.08	89.27	87.95	87.04	83.80
	1:1	71.41	66.99	66.78	61.26	62.86	58.08	57.96	54.42	56.21
	1:3	81.28	80.92	77.59	73.39	74.07	71.89	72.94	76.12	71.52
45	1:4	91.84	89.39	85.82	85.28	82.89	82.42	80.15	80.35	78.40
	1:5	91.09	90.76	87.70	89.36	86.91	87.71	85.75	84.32	84.08
	1:6	92.34	90.73	86.29	91.02	88.95	88.66	86.02	87.26	85.92
	1:1	72.51	68.77	64.29	58.79	52.64	56.79	56.77	57.91	56.03
	1:3	82.55	82.26	81.50	81.11	82.79	81.67	78.55	71.93	73.32
55	1:4	89.83	89.21	87.29	86.55	85.28	87.28	82.25	76.31	76.45
	1:5	89.20	90.33	90.18	88.22	90.31	88.90	84.68	81.38	78.28
	1:6	90.96	89.23	90.36	90.34	89.27	89.30	87.83	85.71	79.83
	1:1	69.19	68.39	62.75	65.22	59.77	61.04	59.34	59.58	60.83
	1:3	85.45	85.38	84.98	82.86	83.83	83.65	83.06	85.03	84.57
65	1:4	86.74	86.78	86.40	84.73	85.83	86.20	85.84	84.46	85.67
	1:5	86.94	86.86	88.32	89.70	89.60	89.56	89.92	90.45	90.18
	1:6	86.35	87.68	88.38	91.42	89.36	89.95	90.84	90.46	89.22

Table 7.2: *Least Square means of triacylglycerol (TAG, % yield) at the various temperature, oil:alcohol ratio and reaction time during the transesterification of Rothsay composite (RC) and olive oil (OO). Standard error = 1.1463 for RC; and 1.1241 for OO.

Treatments		% DAG Yield (RC and OO)*								
Temperature (°C)	Oil:alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
						RC				
	1:1	6.13	8.62	10.17	10.92	13.80	14.44	22.39	22.54	24.60
	1:3	6.20	5.72	7.45	7.18	6.15	10.00	5.47	5.36	6.14
25	1:4	1.42	6.53	6.84	8.67	9.03	7.53	8.86	8.41	11.01
	1:5	4.67	5.15	2.76	6.05	6.35	5.36	5.50	6.17	8.56
	1:6	4.98	5.76	7.61	6.68	8.71	7.19	10.36	8.02	10.87
	1:1	7.38	15.69	22.80	28.73	28.76	32.85	32.01	32.01	31.17
	1:3	7.44	4.73	6.47	6.31	14.13	20.75	16.06	12.55	23.28
35	1:4	6.77	8.61	8.11	19.19	10.52	15.02	11.34	15.19	12.24
	1:5	6.61	6.64	9.38	8.06	11.15	11.57	12.17	9.97	10.55
	1:6	5.43	7.35	6.12	7.63	6.57	7.54	8.14	10.26	8.50
	1:1	10.41	13.82	19.15	29.15	30.83	34.28	35.63	38.22	39.59
	1:3	9.35	9.16	12.14	9.81	10.68	10.44	10.34	8.90	10.51
45	1:4	7.33	7.08	9.84	10.82	10.82	9.03	9.30	13.43	9.81
	1:5	5.47	7.61	7.38	7.96	8.59	6.82	8.92	7.71	8.60
	1:6	6.15	5.53	6.64	7.25	6.37	8.08	8.64	7.94	8.17
	1:1	11.84	17.05	29.00	33.72	36.40	37.27	36.69	38.12	37.71
	1:3	8.01	8.08	8.80	9.21	8.64	8.56	11.14	11.97	12.20
55	1:4	6.65	7.39	7.97	8.86	7.39	8.78	11.17	10.35	12.53
	1:5	6.04	5.44	6.48	6.58	7.77	6.95	8.55	7.88	9.89
	1:6	5.79	5.35	5.83	5.88	6.12	7.36	8.09	7.64	11.55
	1:1	13.50	19.82	29.30	34.32	34.11	36.07	36.32	36.26	36.09
	1:3	8.34	8.80	8.53	9.32	8.77	8.76	8.85	9.87	9.76
65	1:4	7.46	6.21	6.41	7.55	8.45	8.76	8.42	7.99	7.42
	1:5	4.85	5.55	6.32	6.81	6.84	6.34	7.34	6.69	7.72
	1:6	6.91	6.94	6.34	5.41	6.14	7.18	8.03	8.85	7.67
						OO				
	1:1	6.49	9.83	9.17	10.77	12.49	11.11	12.18	13.30	13.48
	1:3	8.06	10.88	10.96	9.45	9.31	11.07	8.00	8.77	10.46
25	1:4	7.36	7.43	9.53	9.30	7.36	10.12	7.86	11.58	11.98
	1:5	6.82	7.80	7.30	6.96	6.66	7.80	5.69	7.98	8.26
	1:6	6.62	8.21	5.70	8.11	8.93	9.51	6.58	5.67	5.79
	1:1	9.54	10.65	13.29	10.45	11.65	13.64	13.66	13.48	14.06
	1:3	6.74	10.97	11.36	10.67	9.12	11.63	9.09	10.53	10.99
35	1:4	10.08	8.10	7.11	8.81	7.32	9.52	9.28	8.93	9.75
	1:5	8.46	7.71	7.72	7.06	5.60	7.84	6.05	9.24	5.20
	1:6	8.25	9.32	7.78	8.25	6.50	6.98	7.77	6.55	9.25
	1:1	11.67	12.21	11.45	11.61	12.46	12.94	14.09	14.10	14.34
	1:3	10.29	9.67	10.48	11.76	10.14	10.06	10.44	9.64	10.35
45	1:4	6.17	7.08	8.71	8.32	9.11	8.50	9.47	9.30	10.59
	1:5	7.40	8.15	9.50	7.17	8.39	8.85	7.59	9.99	9.49
	1:6	5.02	8.01	11.54	6.71	7.14	8.31	8.79	7.90	9.20
	1:1	12.71	13.71	12.18	11.36	14.34	13.80	14.24	14.34	13.95
	1:3	9.74	9.85	9.79	9.49	8.94	9.05	10.28	14.31	13.32
55	1:4	7.31	8.44	8.90	9.44	9.64	8.48	10.58	13.34	13.38
	1:5	9.50	8.22	7.95	9.37	7.82	8.32	10.50	11.78	13.14
	1:6	8.08	7.97	8.63	8.27	8.10	9.17	9.17	10.33	13.74
	1:1	13.95	11.61	11.57	12.21	13.94	14.26	14.46	13.57	13.45
	1:3	11.04	10.64	10.20	11.02	11.15	10.05	10.34	8.78	8.66
65	1:4	10.11	9.94	9.47	9.42	9.76	8.94	8.57	9.38	8.54
	1:5	10.66	9.75	9.79	8.33	8.52	8.47	8.33	8.00	7.26
	1:6	11.30	10.33	9.76	8.15	9.53	8.80	8.31	8.67	6.81

Table 7.3: *Least Square means of diacylglycerol (DAG, % yield) at the various temperature, oil:alcohol ratio and reaction time during the transesterification of Rothsay composite (RC) and olive oil (OO). Standard error = 0.7725 for RC; and 0.6887 for OO.

Treatments		% MAG Yield (RC and OO)*								
Temperature (°C)	Oil:alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
						RC				
	1:1	1.86	1.79	1.50	1.16	3.32	1.66	1.82	4.36	4.98
	1:3	1.16	3.52	3.73	4.54	5.87	3.35	2.89	4.34	4.46
25	1:4	1.41	2.54	3.51	4.48	5.27	4.95	4.61	5.02	4.48
	1:5	1.26	2.26	2.13	5.27	5.46	5.44	4.22	5.41	5.07
	1:6	1.49	2.31	1.61	2.99	4.25	4.33	4.04	4.05	4.40
	1:1	1.79	1.94	3.91	3.76	3.68	3.16	3.72	4.55	4.89
	1:3	3.00	2.63	3.31	1.94	4.09	4.68	4.40	4.33	3.24
35	1:4	2.78	4.18	5.23	3.96	3.71	5.09	4.52	3.64	3.09
	1:5	1.53	2.02	3.94	4.38	3.10	3.76	4.68	4.86	3.44
	1:6	1.37	1.62	2.29	4.43	3.27	4.47	4.38	3.47	4.82
	1:1	1.59	2.22	3.23	3.35	3.70	5.23	5.37	4.40	5.10
	1:3	4.53	3.56	2.95	4.41	4.81	5.53	5.12	3.58	3.14
45	1:4	1.97	2.74	3.06	2.60	2.33	3.10	1.70	5.37	1.38
	1:5	1.29	1.55	1.39	1.87	1.92	1.95	2.82	2.07	2.90
	1:6	1.29	1.39	1.37	1.90	1.71	2.05	1.36	2.24	1.77
	1:1	3.14	4.29	4.24	3.96	4.01	3.98	5.50	4.91	5.57
	1:3	3.96	4.34	3.01	2.24	3.22	2.34	2.74	1.72	1.78
55	1:4	1.37	1.18	1.77	1.55	1.34	1.49	1.73	1.30	2.47
	1:5	1.40	1.45	1.63	1.52	1.39	1.18	1.58	1.70	1.79
	1:6	1.27	1.26	1.33	1.23	1.47	1.50	1.44	1.54	1.55
	1:1	2.38	5.22	4.56	5.02	5.47	4.26	4.76	5.58	5.79
	1:3	1.78	1.13	1.56	1.85	1.99	2.68	1.47	1.85	1.70
65	1:4	1.37	1.22	1.31	1.63	1.29	1.64	1.36	1.91	2.04
	1:5	1.26	1.22	1.82	1.34	1.72	1.24	1.38	1.72	1.54
	1:6	1.36	1.45	1.44	1.28	1.77	1.57	1.35	1.23	1.22
						OO				
	1:1	1.12	2.50	3.96	0.63	1.51	1.56	1.76	6.12	3.50
	1:3	3.81	2.25	8.33	11.93	10.18	12.54	12.85	14.71	9.84
25	1:4	1.02	0.23	0.72	2.68	3.52	3.99	9.38	9.91	9.70
	1:5	0.07	1.03	1.26	2.57	2.22	0.99	2.96	3.88	4.60
	1:6	1.18	0.84	0.52	1.22	2.14	1.96	1.89	0.64	0.90
	1:1	2.22	4.96	2.90	3.03	6.29	4.72	4.06	3.68	8.02
	1:3	3.77	5.85	7.95	6.08	8.77	7.58	8.82	6.89	7.92
35	1:4	0.26	0.13	2.56	1.31	4.24	2.02	4.94	6.25	2.11
	1:5	0.32	0.66	1.55	0.68	1.07	1.81	2.11	0.88	2.67
	1:6	2.60	0.87	0.89	0.34	1.78	0.94	1.40	2.97	1.37
	1:1	2.09	4.43	3.08	7.01	5.70	8.74	8.12	10.46	9.65
	1:3	2.15	2.54	5.06	4.50	4.55	6.01	4.54	3.96	5.62
45	1:4	0.44	0.68	1.85	2.23	2.29	2.63	3.86	1.88	2.79
	1:5	0.64	0.67	1.53	1.36	1.84	1.06	3.10	1.56	0.78
	1:6	0.64	0.25	0.43	0.71	1.95	0.86	2.80	2.30	1.28
	1:1	3.24	3.74	6.25	10.87	12.88	9.26	9.00	7.04	10.53
	1:3	3.03	2.39	2.72	2.74	1.81	1.97	3.33	2.71	2.93
55	1:4	1.14	1.31	1.74	0.86	1.63	0.36	2.04	2.34	2.13
	1:5	0.62	0.41	1.00	1.24	0.50	1.04	1.30	2.52	2.47
	1:6	0.71	1.37	0.59	0.80	1.26	0.77	1.40	1.24	1.42
	1:1	4.28	4.51	8.19	4.92	7.86	8.19	8.66	8.70	10.11
	1:3	0.29	0.61	0.93	1.91	1.05	2.06	1.45	1.26	2.21
65	1:4	0.53	0.59	1.22	2.94	1.18	1.14	2.06	0.87	1.72
	1:5	0.73	1.63	0.68	0.82	0.77	1.01	0.37	0.60	1.27
	1:6	0.93	0.83	0.74	0.24	0.41	0.75	0.21	0.44	2.05

Table 7.4: *Least Square means of monoacylglycerol (MAG, % yield) at the various temperature, oil:alcohol ratio and reaction time during the transesterification of Rothsay composite (RC) and olive oil (OO). Standard error = 0.5493 for RC; and 0.6194 for OO.

Treatments		% FFA Yield (RC and OO)*								
Temperature (^{o}C)	Oil:alcohol ratio	8 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	120 h
						RC				
	1:1	3.64	3.45	5.29	2.94	5.70	7.17	5.38	6.49	6.50
	1:3	3.07	3.34	4.26	2.03	3.03	3.69	2.93	2.15	3.63
25	1:4	2.54	3.41	2.92	3.55	1.96	2.40	3.35	2.60	2.41
	1:5	3.27	3.17	2.16	1.81	1.87	3.84	3.69	1.92	1.56
	1:6	1.91	2.93	2.39	2.53	1.61	1.37	4.83	0.75	1.00
	1:1	4.35	5.59	5.59	5.32	5.09	6.20	5.34	6.92	6.55
	1:3	3.72	4.46	3.37	3.35	5.85	2.27	4.44	6.25	2.77
35	1:4	4.33	4.33	2.81	2.65	2.07	5.25	5.71	3.52	3.53
	1:5	3.74	4.02	3.59	2.72	1.99	1.69	3.39	1.68	2.98
	1:6	3.68	4.57	2.95	2.84	2.88	1.72	2.69	1.95	2.27
	1:1	5.00	6.50	4.65	5.13	4.82	5.94	5.76	6.06	5.48
	1:3	2.47	2.35	2.36	2.17	2.79	2.92	2.73	2.13	2.11
45	1:4	2.47	2.95	0.85	1.86	1.70	1.65	1.96	3.24	1.31
	1:5	3.73	2.92	2.84	2.42	2.54	1.74	1.61	1.61	1.30
	1:6	3.93	3.39	3.36	2.59	2.36	2.53	2.57	1.76	1.18
	1:1	4.57	4.35	5.64	5.91	6.30	5.72	5.57	4.92	5.23
	1:3	3.11	2.86	2.86	3.11	2.61	2.77	2.32	2.62	3.86
55	1:4	3.43	3.23	2.88	2.29	2.59	2.23	2.13	2.66	2.94
	1:5	3.84	3.92	3.08	3.25	2.91	3.38	2.67	1.75	2.75
	1:6	4.23	3.52	3.30	3.07	3.51	3.10	2.84	2.43	3.10
	1:1	6.07	5.55	5.84	5.70	5.63	5.38	5.45	5.51	5.40
	1:3	3.39	3.56	3.11	2.94	3.09	2.94	2.56	2.86	3.18
65	1:4	3.69	3.28	3.10	3.22	3.42	3.17	2.73	2.75	2.27
	1:5	3.31	3.65	3.55	3.60	3.52	3.18	3.88	2.80	2.77
	1:6	3.85	3.61	3.81	3.31	3.82	3.88	3.95	4.37	3.65
						OO				
	1:1	0.69	0.75	1.33	0.60	1.37	2.50	1.23	2.49	3.47
	1:3	0.49	0.45	0.44	1.19	0.45	0.93	1.35	0.99	2.42
25	1:4	0.57	0.32	0.47	0.26	0.26	1.24	0.83	0.67	0.32
	1:5	0.15	0.09	0.32	0.17	0.26	0.23	0.49	0.22	0.37
	1:6	0.27	0.77	0.39	0.22	0.34	0.61	0.23	0.39	1.11
	1:1	0.50	0.82	1.39	2.16	2.44	3.68	3.79	2.32	3.26
	1:3	0.65	0.33	0.34	1.19	1.12	0.61	0.68	1.25	0.95
35	1:4	0.54	0.49	0.36	0.27	0.26	0.61	0.44	0.32	0.27
	1:5	0.33	0.30	0.36	0.18	0.30	0.18	0.53	0.23	0.35
	1:6	0.33	0.96	0.32	0.18	0.35	0.62	0.30	0.33	1.48
	1:1	0.84	0.90	1.60	2.44	1.72	2.89	2.45	4.37	2.90
	1:3	0.43	0.12	0.29	0.36	0.48	0.50	0.54	0.61	0.82
45	1:4	0.53	0.27	0.44	0.22	0.24	0.50	0.39	0.98	0.35
	1:5	0.43	0.29	0.32	0.17	0.24	0.24	0.50	0.32	1.07
	1:6	0.34	0.88	0.26	0.21	0.59	0.60	0.36	0.44	0.86
	1:1	1.05	0.80	1.31	3.03	3.91	3.01	2.81	4.05	3.30
	1:3	0.44	0.24	0.34	0.14	0.30	0.42	0.51	2.12	1.79
55	1:4	0.72	0.42	0.32	0.27	0.22	0.47	0.39	2.02	1.47
	1:5	0.42	0.34	0.58	0.19	0.26	0.16	0.54	0.27	1.80
	1:6	0.30	0.72	0.20	0.21	0.41	0.35	0.29	0.41	2.02
	1:1	1.40	2.08	1.74	1.79	2.48	1.66	2.21	2.20	0.31
	1:3	0.53	0.57	0.40	0.58	0.18	0.14	0.38	0.19	0.04
65	1:4	0.77	0.48	0.30	0.26	0.30	0.36	0.31	0.51	0.23
	1:5	0.70	0.80	0.38	0.21	0.28	0.22	0.57	0.31	0.65
	1:6	0.54	0.69	0.41	0.18	0.27	0.30	0.32	0.37	1.40

Table 7.5: *Least Square means of free fatty acid (FFA, % yield) at the various temperature, oil:alcohol ratio and reaction time during the transesterification of Rothsay composite (RC) and olive oil (OO). Standard error = 0.3913 for RC; and 0.2453 for OO.

CHAPTER VIII

CONNECTING STATEMENT

Statistical approach to interpolate enzyme-catalyzed reaction research outcome and generate models to predict patterns is becoming increasingly important. The screening experiment in Chapter VI revealed the process variables and interactions which influence biodiesel (BD) yield as well as the reaction intermediates and residuals in Chapter VII. This chapter extends studies on the transformation of animal fats to alkyl esters with in-depth application of experimental design to optimized BD yield. The application of central composite rotatable design (CCRD) and response surface methodology (RSM) substitute the original mixed factorial design to reduce the number of experimental treatments within a reasonable amount of time without remarkable loss of useful information. The design provides information on the direct additive, interaction, and bilinear variable effects on the response.

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CHAPTER VIII

OPTIMIZED TRANSFORMATION OF ANIMAL FATS TO ALKYL ESTERS

8.1. Abstract

The optimum conditions for the preparation of fatty acid ethyl ester (FAEE) were investigated in three experiments to assess the linear, quadratic, and bilinear effects of temperature, enzyme load, and oil:alcohol molar ratio on FAEE yield. In each experiment, second-order polynomial models fitted to the FAEE yield data provided response surfaces at various reaction times (8 h-48 h). These models were generally significant ($p < 0.05$) and produced reliable and stable predictions, especially at 24 h and 36 h. At these reaction times, optimum conditions were found to be close to the centre point values of the reaction variables (50^{o}C, using an enzyme load of 39.06 U, and an oil:alcohol ratio of 1:2). Fuel testing of the transesterified oils revealed various proportions of total and bound glycerol, acid number (AN), triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), and moisture.

8.2. Introduction

Biodiesel (BD) transesterification reactions have been reported to be affected by several reaction variables (Berchmans and Hirata, 2008; Rashid et al., 2009). These variables include alcohol type and its molar ratio to the oil, catalyst type and amount, reaction time and temperature, free fatty acid (FFA) and moisture content, among others. These variables tend to be examined one-at-a-time while keeping the other variables constant. This approach not only results in a large number of experiments which are time consuming and costly, but also ignores the interactions between the independent variables on the overall reaction and may result in loss of valuable information (Cochran and Cox, 1992; Bezerra et al., 2008). Statistical and mathematical techniques such as CCRD and RSM have proven to be powerful tools for designing experiments, building models, and analyzing the effects of the independent variables and their interactions on the dependent variable (Cochran and Cox, 1992; Bezerra et al., 2008). One main advantage of RSM is the reduction in the number of experimental trials needed to evaluate the effects of the factor variables and their interactions on the response. The suitability of the model to

produce appropriate polynomial equations to explain these relationships, and identify factor variables and their values which optimize the response are other valuable advantages (Cochran and Cox, 1992; Bezerra et al., 2008).

Biodiesel is conventionally produced by chemical catalyzed transesterification of fats and oils, with short-chain alcohols to yield the corresponding fatty acid alkyl esters (FAAE). Biodiesel is increasingly becoming an important alternative fuel due to its well documented and proven distinct properties and advantages over petrodiesel (Knothe et al., 2006). However, increasing BD demand comes with the need for cheaper but sustainable feedstocks, and less complicated production and downstream processes (Haas et al., 2005; Silva et al., 2010). Newer and underutilized resources with no food value or use are being considered to make BD production more cost competitive (Berchmans and Hirata, 2008; Rashid et al., 2009). Examples of such resources include fats and oils that can be harnessed from fishery by-catches, and by-products from fishery and livestock harvesting and processing (Falch et al., 2006; Meeker and Hamilton, 2006; Aryee et al., 2011). These resources are considered unsuitable for the human food chain or animal feed, and transformed into low value-added products, dumped into the environment causing pollution, or incinerated. In the quest to also simplify downstream processes and minimize purification cost, lipase is considered a preferred catalyst over bases and acids (Dzige et al., 2009; Fjerbaek et al., 2009). Lipase can tolerate feedstocks with high FFA and moisture contents, and catalyze reactions at lower temperatures and pressures, saving on energy and capital equipment costs.

As part of on-going research to enhance the value of recovered oils from processing discards, the objective of this study was to apply CCRD in order to model and assess the effects of temperature, enzyme load, and oil:alcohol molar ratio (considered solely, through linear and quadratic effects, or in combinations, through bilinear effects) on FAEE yield, and to generate response surface graphs and deduce the optimal reaction conditions.

8.3. Materials and Methods

8.3.1. Materials

A commercial mix of rendered animal fat and used frying oil (Rothsay composite, RC) was received from Rothsay® Biodiesel (Ste. Catherine, QC). Olive oil (OO), and immobilized lipase from *Mucor miehei* (Lipozyme®-IM) (86.8 U/g) were obtained from Sigma-Aldrich (Oakville, ON). One unit of immobilized lipase activity is defined as the amount producing 1 µmol of stearic acid per minute from the hydrolysis of tristearin at pH 8.0 and 70°C. Ethanol (EtOH) was purchased from Commercial Alcohols (Boucherville, QC). Toluene and acetic acid were purchased from Fisher Scientific (Whitby, ON). Triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), fatty acid ethyl ester (FAEE) and free fatty acid (FFA) standards were purchased from Nu-Chek prep (Elysian, MN).

8.3.2. Methods

8.3.2.1. Oil Extraction and Characterization

Salmon skin oil (SSO) was extracted as previously described (Aryee and Simpson, 2009). In brief, 1 part of crushed freeze-dried salmon skin was extracted with 10 parts of hexane (w/v) using a SER 148 Velp solvent extractor (Velp Scientifica, Usmate, Lombardy). The fatty acid (FA) composition, free fatty acid (FFA), and moisture contents of SSO, RC, and OO were determined using the methods of Metcalfe et al. (1966), Ca 5a-40, and Ca 2c-25, respectively.

8.3.2.2. Experimental Design

Three experiments (E1, E2, and E3) were run according to a three-variable, five-level CCRD, each with 20 combinations of values of the independent variables in total (Cochran and Cox, 1992). The selection of independent variables and their ranges were based on exploratory and preliminary experiments (Aryee et al., 2011), and reports in the literature on the probable effects of these parameters on the response, Y (FAEE yield). For instance, the thermal stability of lipase was an important influence on the temperature levels selected. The independent variables (factors) chosen were; temperature (Temp, ^{o}C), enzyme load [Enz], %/U), and oil:ethanol molar ratio (AlcoR, molar ratio). The levels

were coded -1.682, -1, 0, +1, and +1.682, as shown in Table 8.1, and the complete CCRD experimental matrix is presented in Table 8.2. The first eight combinations of values of the independent variables correspond to a standard 2^K factorial (where k is the number of variables) and coded +1 and -1. The next set of six combinations constitutes $2k$ points, known as the axial (star) points. They were fixed at a distance of 1.682 ($\alpha = 2^{k/4}$) from centre, to ensure rotatability (Mead, 1988). Axial points are also used to allow the inclusion of quadratic terms in the response surface model. The last 6 rows in the matrix form the centre points and were coded 0 (the mean values used here). Replication at the centre assures a greater uniformity in the precision of response estimation over the experimental domain.

8.3.2.3. Transesterification Reactions

All transesterification reactions were carried in a temperature-controlled horizontal shaker water bath (model 25, Precision Scientific, Chicago, IL) at 60 shaker rate per minute as previously described (Aryee et al., 2011). In a typical experiment, 5 g of SSO, RC or OO, ethanol (oil:alcohol molar ratio [AlcoR]) and Lipozyme®-IM (enzyme [Enz]) were placed in a 30 ml screw-capped vials and incubated (temperature [Temp]) in the water bath according to Table 8.2. Hundred microlitre aliquots of the transesterified oil were withdrawn (time: 8, 12, 24, 36, and 48 h) and diluted with 5 ml toluene for HPLC analysis.

8.3.2.4. High Performance Liquid Chromatography (HPLC) Analysis

Qualitative and quantitative analyses of the transesterified oils were performed in a HPLC unit equipped with a refractive index (RI) detector, an autosampler (Varian, Palo Alto, CA), and a Phenogel™ column (300 mm x 7.8 mm ID, Phenomenex, Torrance, CA) (Kittirattanapiboon and Krisnangkura, 2008; Aryee et al., 2011). Individual calibration curves were developed with standards of TAG, DAG, MAG, FAEE, and FFA, each in toluene at 5 different concentration levels (0.313-5 mg/ml). Twenty five microlitres (25 µl) of the standard or transesterified oil was injected for analysis. A mobile phase of 0.25% (v/v) acetic acid in toluene was used in isocratic mode at a flow rate of 1.0 ml/min during the 25 min run time. Varian Galaxie™ (Varian, Palo Alto, CA) was used for data

acquisition and processing. The reaction products and intermediates (lipid classes: TAG, DAG, FAEE, and MAG) as well as FFA were baseline separated. Peak identification and quantification of test samples were made by comparison with chromatograms and retention times of the standards. FAEE yield was calculated as a percent ratio of the integrated FAEE peak to the total integrated peaks (TAG, DAG, FFA, and MAG) excluding the EtOH peak as previously described (Aryee et al., 2011).

8.3.2.5. Fuel Testing

Transesterified SSO and RC were sent to the laboratories of Rothsay® Biodiesel in Guelph, ON, Canada for testing according to ASTM D6751-11. The following process-related tests were performed: total and bound glycerol, acid number (AN), TAG, DAG, MAG, and moisture content. Total and bound glycerol as well as TAG, DAG, MAG contents were determined by GC and expressed as mass percent. Karl Fischer titration was used to measure trace amounts of free, emulsified and dissolved water in the transesterified oils and expressed as parts per million (ppm). Acid number was as expressed in milligrams of potassium hydroxide required to neutralize 1 gram of the ester (mgKOH/g).

8.3.2.6. Statistical Analysis

The experimental data were analyzed with both the response surface regression procedure of SAS® (PROC RSREG; Statistical Analysis System, Version 9.2, SAS Institute Inc., Cary, NC) and similar tools from Design-Expert® (Version 8.0.6, STAT-EASE Inc., MN); to fit the response surface regression models using the second-order polynomial equation:

$$\boldsymbol{Y = \beta_o + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j} \qquad (\mathbf{8.1})$$

where Y is the response (FAEE yield, %) at 8, 12, 24, 36, and 48 h; x_i, x_j (i = 1, 2, 3; j = i + 1, 3) are the independent variables (uncoded or coded); x_1 = temperature (^{o}C); x_2 = enzyme load (%/U); x_3 = oil:alcohol molar ratio (molar ratio); β_0 is the intercept

(constant) term; and β_i, β_{ii} and β_{ij} are the linear, quadratic, and bilinear regression coefficients, respectively.

A preliminary power transformation was applied to the response data to avoid any lack of fit. The adequacy of models was tested using the analysis-of-variance (ANOVA) *F*-test and the significance of estimated coefficients was assessed with *t*-tests, all at 5% significance level (Cochran and Cox, 1992). Three-dimensional plots were produced from the second-order polynomial equations obtained from model fitting by holding one of the independent variables at a constant value and changing the other two variables with Design-Expert®. The canonical analysis option of SAS® PROC RSREG was used to determine the combination of values of the three independent variables corresponding to a maximum or a saddle point on the response surface.

8.4. Results and Discussion

8.4.1. Test Samples and HPLC Analysis

As previously reported (Aryee et al., 2011), the oils have varied FA profiles according to GC analysis. SSO is predominantly a polyunsaturate (48%) with nearly equal amounts of saturates (27%) and monounsaturates (25%). OO is principally a monounsaturate (68%) with low levels of saturates (18%) and polyunsaturates (14%). RC has a higher amount (42%) of monounsaturates than SSO but lower than OO, higher (41%) saturates content than both SSO and OO, and higher polyunsaturates content (17%) than OO but lower than SSO. The FFA and moisture contents of the oils ranged between 0.16-9.89% and 0.01-0.03%, respectively.

The Phenogel™ column used in the HPLC analysis afforded an excellent and stable baseline, and peak symmetry for determining the FAEE, TAG, DAG, MAG, FFA, and EtOH contents in the transesterified oils. The reaction components were thus easily and reliably quantified during the short analytical time (25 min) without compromising the resolution of the compounds characterized. The largest (TAG) and smallest (EtOH) molecules had the shortest (9.39 min) and longest (16.80 min) column residence times, respectively, as a result of interparticle permeation and molecular sieving. These observations were in agreement with previous studies (Kittirattanapiboon and Krisnangkura, 2008; Aryee et al., 2011). Conversion rates of SSO to FAEE were

relatively fast with >56% reached within 8 h at 50^{o}C, using 11.36%/49.32 U of enzyme and 1:2 oil:alcohol molar ratio. Changes in the amounts of glycerides (TAG, DAG, and MAG) and FFA contents with reaction time were also observed for all the oils. For instance, between 24 and 36 h, TAG and MAG contents decreased by *ca* 35% and 12% at the centre point (50^{o}C, using an enzyme load of 8%/34.72 U, and an oil:alcohol ratio of 1:2) in transesterified SSO, while both DAG and FFA contents respectively increased by *ca* 19% and 20% in the same reaction.

8.4.2. Response Surface Methodology-Central Composite Rotatable Design-Model fitting

A previous three-factor mixed exploratory analysis revealed that temperature, enzyme load and oil:alcohol molar ratio influence FAEE yield, depending on reaction time (Aryee et al., 2011). While a body of data and substantial amount of information on variables and levels were gained which provided pointers and offered information on which variables to select and initial ranges to explore in our study, it required at least 225 combinations of values without replication. A more concise design, CCRD, was thus chosen for our current work.

The current CCRD and the corresponding experimental domain (Table 8.1) are the outcome of initial runs (results not presented here), which did not result in the location of an optimum on the response surface within the domain. The design was thus augmented to a different location where the possibility of an optimum was more likely (Table 8.1). The entire experiment was run three times (E1, E2, E3) to demonstrate or assess the stability of the results. The response data, FAEE yield at 8 ($Y_{8\ h}$), 12 ($Y_{12\ h}$), 24 ($Y_{24\ h}$), 36 ($Y_{36\ h}$) and 48 ($Y_{48\ h}$) h, were quantified for all three oils. The response data were transformed by a power of 12.5 to improve the correlation between variables and improve model fitting.

The linear, quadratic, and bilinear coefficients of the fitted response surfaces at the reaction times studied are a mixture of positive and negative, significant and non-significant effects on FAEE yield (Tables 8.3, 8.5, and 8.7). Beyond this mixture, a response surface presents a maximum if the three estimated quadratic coefficients are negative, and a saddle point otherwise. At 24, 36, and 48 h, the models were significant

($p < 0.05$; 0.022 and below) and the lack of fit was non-significant ($p > 0.05$; up to 0.863), indicating that the models represent the experimental data and the variations accounted for by random error in a way sufficiently adequate for predicting the response. Another important use of the estimated coefficients reported in Tables 8.3, 8.5, and 8.7 is to produce response surfaces (Figs. 8.1 and 8.2), by substitution with the β_i, β_{ii} and β_{ij} coefficients in Eq. (8.1).

Combinations of values of the three independent variables, found by canonical analysis to correspond to a stationary point (maximum or saddle point), are reported in Tables 8.4, 8.6, and 8.8. The eigenvalues obtained in the analyses for reaction times of 24, 36, and 48 h were negative, indicating that the stationary point for the response surface was a maximum. At the reaction times studied, SSO FAEE yields are saddled or maximized when the independent variables are close to the mid-value (centre point) of their range (50^{o}C, 39.06 U, and 1:2 oil:alcohol molar ratio) (Tables 8.4, 8.6, and 8.8). For all three experiments (E1-E3), the neighbourhood of the estimated stationary point for SSO at 8 h and 12 h is shaped like a saddle, but shows a maximum at 24, 36, and 48 h. Through the fitted models, the relationships between the response and the independent variables are depicted in the form of surface and contour plots in Figs. 8.1a-c and 8.2a-c.

8.4.3. Effect of Variables on FAEE Yield

The influence of reaction variables on the amount of FAEE produced varied with feedstock, and ranged from 8.12% to 68.66%. The highest yields of RC FAEE and OO FAEE were 40.87% and 40.75%, respectively. Longer experimental time (>48 h) was avoided because it would not necessarily increase FAEE yield as reported in a previous study (Aryee et al., 2011) and would be uneconomical from an industrial point of view.

At 24 h, the linear effect of enzyme load is positive, while the linear effect of temperature as well as the quadratic effects of temperature and enzyme load and their bilinear effect are all negative (Tables 8.3, 8.5, and 8.7). Actually, FAEE yield initially increased when enzyme load increased. The negative bilinear effect of temperature and enzyme load indicates that higher enzyme load could not compensate for lower reaction rates at higher temperatures. Milder temperature and moderately higher enzyme load resulted in higher yield. A similar trend was observed at 36 h (Fig. 8.2a). Increasing

enzyme load increases the number of active sites available for substrate binding and subsequently increases yield. However, there were only small changes in yield with increasing enzyme load. The active sites of the enzymes may have been sequestered from the substrates with increasing enzyme load. Thus, even though enzyme concentration increased, it provided no appreciable contribution to the reaction (Halim et al., 2009). The minimal change in FAEE yield with time can also be ascribed to the probable agglomeration of the enzyme, and progressive absorption and accumulation of the hydrophilic and insoluble glycerol on the surface of the immobilized enzyme. Glycerol forms hydrophilic hindrance around the enzyme, which limits the diffusion of the hydrophobic substrate from the organic phase to the enzyme (Halim et al., 2009). The probable accumulation of water produced in the reaction may also have favoured hydrolysis instead of transesterification. Nevertheless, some amount of water is needed by lipase to retain its three-dimensional conformational structural integrity and active site polarity. Various enzymes in diverse reaction systems require different amounts of water to maintain activity in the organic media. The low conversion of TAG to FAEE at lower enzyme load can be attributed to relatively low enzyme activity, whereas the effect of temperature can be ascribed to conformational changes indicating greater unfolding of lipase at milder temperatures than at the lowest and highest temperatures used. Notwithstanding the increase in substrate solubility and mass transfer of reactants with temperature, proteins and lipases tend to denature at high temperature, resulting in loss of activity.

At 24 h, the linear effect of the reaction temperature as well as the quadratic effects of temperature and oil:alcohol molar ratio and their bilinear effect were found to be negative; only the linear effect of oil:alcohol molar ratio was positive. As a result of the negative interaction of these two factors, FAEE yield was found to decrease at higher temperature and oil:alcohol molar ratio (Fig. 8.1b). The low yield associated with higher alcohol concentration indicates that an excessive amount of alcohol may inhibit lipase activity by reducing the operational stability of the enzyme *via* stripping and distortion of the ordered water layer around the enzyme needed to maintain stability and conformation. The limiting effect of ethanol based on the reaction stoichiometry is also plausible. Although alcohol in excess of the stoichiometric molar ratio (>1:3) is required to shift the

reaction equilibrium to higher reaction rates, due to its limited solubility in TAG and its ability to enter the pore spaces of the immobilized enzyme, at higher alcohol concentration its inhibitory effect on enzymatic catalysis is pronounced. Enzyme catalyzed transesterification reactions seem to require lower proportions of alcohol than base and acid catalyzed reactions. A sufficient amount of alcohol is essential to break the glycerol-fatty acid linkages and act as acyl acceptor. Higher temperatures also increase the solubility of the alcohol and its effect on the enzyme. Due to the heterogeneous nature of the reaction mixture consisting of immiscible phases, mass transfer could be a critical rate limiting factor. Lower temperatures and oil:alcohol molar ratio also produced low yields, maybe because of lower catalytic rate and limited amount of ethanol respectively. The activation energy of the enzyme may also be rate limiting at lower temperatures. Since the reaction only happens at the interface of ethanol and the oil, the greater the surface area, the faster the reaction, but only up to the mid-values. A similar trend was observed at 36 h (Fig. 8.2b). Thus, milder reaction temperature and low oil:alcohol molar ratio gave better yield without exerting negative effects on enzyme activity and stability.

The linear and bilinear effects of enzyme load and oil:alcohol molar ratio on FAEE yield are positive at 24 h, with enzyme having a greater effect than alcohol (Tables 8.3, 8.5, and 8.7). Their quadratic effects were both negative, with enzyme load having a smaller effect than oil:alcohol molar ratio. Even though enzyme load was not significant ($p > 0.05$), it exhibited a synergistic effect with ethanol through a positive and strong bilinear effect. From the surface and contour plots produced for them (Fig. 8.1c), it can be seen that alcohol concentration initially increases yield but decreases yield with further increase in alcohol content. This result further suggests that high amounts of ethanol produces undesirable effects on lipase activity and/or stability. This may be a simultaneous effect of the inactivation caused by alcohol and the diffusion limitations caused by the accumulation of the glycerol by-product on the surface of the immobilized lipase. As seen in Fig. 8.1c, lower enzyme load and alcohol content result in low yield, due to incomplete conversion. Similarly, at 36 h (Fig. 8.2c), yields are lower at the extremes of oil:alcohol molar ratio, but increase with increasing enzyme load.

The absence of a maximum at 8 h and 12 h might be explained as follows; the effects of temperature, enzyme, and oil:alcohol molar ratio were probably not fully

developed to cause sufficient changes in yield. The enzyme might also have been inactivated in the presence of the bulk alcohol, but reactivated with time.

8.4.4. Fuel Properties

Untested fuels may be laden with detrimental contaminants which can affect fuel quality and engine performance (Atadashi et al., 2011). Fuel testing which uses reliable BD reference standards specifies test methods and indicates the maximum allowable concentration of contaminants in the fuel and fuel quality indices. The process-related parameters of total glycerol, bound glycerol, TAG, DAG, MAG, AN, and moisture content of transesterified SSO and RC determined according to ASTM D6751-11 were 0.949%, 0.608%, 1.980%, 1.903%, 0.454%, 6.65 mg/g, 785.4 ppm, and 1.540%, 1.269%, 1.886%, 1.735%, 3.140%, 7.420 mg/g, 1117.9 ppm, respectively. The recorded residual compounds (TAG, DAG, and MAG) in the transesterified SSO and RC, as detected by the GC method, confirm the HPLC analysis. The amount of dissolved glycerol in the transesterified oils were appreciable, and water wash would remove most of the glycerol, due to its high solubility in water. The residual and poorly water-soluble impurities (TAG, DAG, and MAG) can be reduced by a more complete reaction and by distillation. The GC procedure followed in this analysis was initially designed for methyl esters. Thus, substantial differences in column retention times of these ethyl ester compounds may exist (Knothe, 2006) resulting in measurement error of some compounds. The AN test measures the residual FFA content and serves as a quality control guide to monitor fuel degradation during storage. The relatively high AN seen in SSO and RC FAEE may not easily be removed by distillation due to a similar boiling point as BD or be reduced by water washing either (unless the acidity is caused by other compounds other than FFAs). Bringing the reaction to completion will also help to improve the AN, as will washing with a mild caustic mixture which brings with it post-process complications such as generation of large amount of wastewater. The high moisture content in the transesterified oils, especially in RC FAEE, might be due to the water by-product formed during the esterification of FFA to esters, in addition to dissolved moisture in the parent oil (Eq. 8.2).

$$\mathbf{RCOOH} + \mathbf{R'OH} \rightleftharpoons \mathbf{RCOOR'} + \mathbf{H_2O} \qquad (8.2)$$

Although acids are typically used in esterification reactions driving additional processes and costs up, lipase can simultaneously esterify and transterify the oil without the extra steps and processes. The remaining water can be removed by distillation or stripping. It is also possible to further reduce the moisture content by continuously removing the water from the mixture during the reaction with the addition of absorbents such as silica and molecular sieve or by pre-drying the oil before the transesterification reaction. Berrios and Skelton (2008) and Atadashi et al. (2011) discussed other available BD purification and refining techniques, such as wet and dry washing, and membrane technologies.

8.5. Conclusion

The CCRD proved to be an effective experimental design to model the various effects of temperature, enzyme load, and oil:alcohol molar ratio on FAEE yield. The optima were relatively easily located and a large amount of the variation was explained by the fitted response surface models. The second-order polynomial function provided a good description of the relationships between the reaction variables and the response. The three independent variables studied had varying effects on FAEE yield, depending on reaction time. The methodology also allowed yield prediction by interpolation over the experimental domain. With a reduced number of combinations of values of the independent variables (20) in the CCRD, as much information as in a full factorial design was generated in a concise manner. Process-related fuel testing of the samples revealed additional steps to improve fuel properties.

8.6. Acknowledgments

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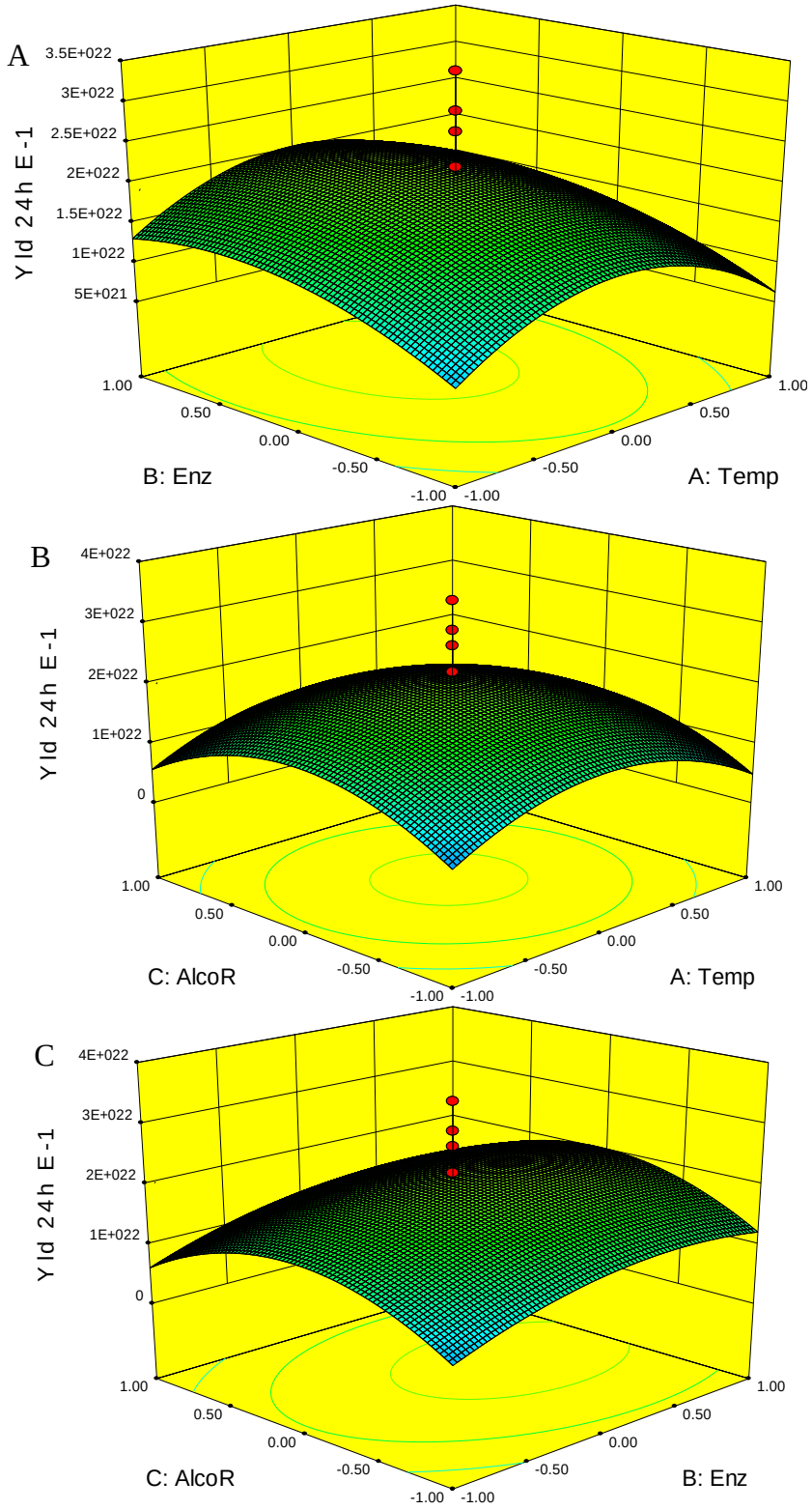

Fig 8.1: Response surface and contour plots for reaction time of 24 h for any two of the three parameters studied for their effects on FAEE production. The other factor is constant at coded value of 0 (Table 8.1).

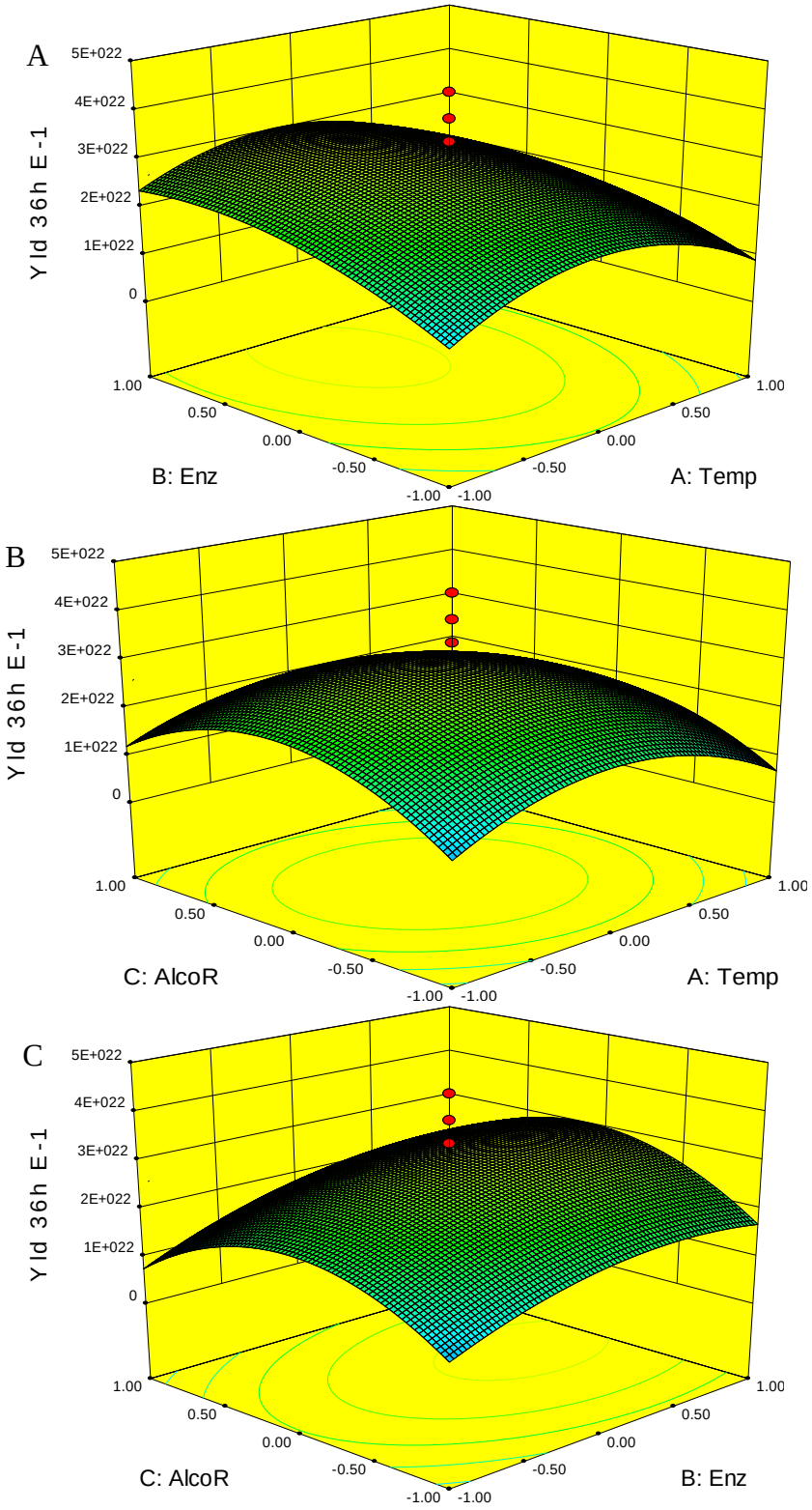

Fig 8.2: Response surface and contour plots for reaction time of 36 h for any two of the three parameters studied for their effects on FAEE production. The other factor is constant at coded value of 0 (Table 8.1).

Independent Variables	Experimental Domain				
	-1.682[a]	**-1**	**0**	**1**	**1.682[a]**
Temperature ([Temp], ^{o}C)	33.182	40	50	60	66.818
Enzyme load ([Enz], %/U)	4.636 /20.12	6 /26.04	8/34.72	10/43.40	11.364/49.32
Oil:alcohol molar ratio ([AlcoR], molar ratio)	0.318	1	2	3	3.682

Table 8.1: 3-factor-5-level experimental design.

Run	Temp	Enz	AlcoR
1	-1	-1	-1
2	-1	-1	1
3	-1	1	-1
4	-1	1	1
5	1	-1	-1
6	1	-1	1
7	1	1	-1
8	1	1	1
9	-1.682	0	0
10	1.682	0	0
11	0	-1.682	0
12	0	1.682	0
13	0	0	-1.682
14	0	0	1.682
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

Table 8.2: Complete CCRD matrix used in the transesterification reactions.

Coefficient Factor	8 h	12 h	24 h	36 h	48 h
	Estimates				
β_0	+2.629E+020	+2.822E+021	+2.115E+022	+2.984E+022	+5.200E+022
β_1	+1.795E+018[b]	-3.317E+019[b]	-3.172E+020[b]	-1.928E+021[b]	-1.445E+022[a]
β_2	+1.354E+021[a]	+1.584E+021[a]	+3.115E+021[b]	+5.408E+021[b]	+6.178E+021[b]
β_3	-8.921E+017[b]	+2.967E+019[b]	+6.214E+019[b]	+6.845E+020[b]	+4.629E+021[b]
β_{12}	+2.036E+018[b]	-4.745E+019[b]	-6.275E+019[b]	-1.107E+021[b]	-7.712E+021[b]
β_{13}	-1.510E+018[b]	-4.694E+019 [b]	-6.240E+019[b]	-1.114E+021[b]	-7.715E+021[b]
β_{23}	-1.521E+018[b]	+4.929E+019[b]	+6.574E+019[b]	+1.112E+021[b]	+7.724E+021[b]
β_{11}	-4.768E+020[b]	-1.391E+021[b]	-7.777E+021[a]	-9.824E+021[a]	-5.289E+021[b]
β_{22}	+1.465E+021[a]	+8.301E+020[b]	-3.746E+021[b]	-4.861E+021[b]	-1.710E+022[a]
β_{33}	-4.782E+020[b]	-1.401E+021[b]	-8.146E+021[a]	-1.169E+022[a]	-1.945E+022[a]

ANOVA

	df	Sum of Squares				
Model	9	6.630E+043[b]	1.026E+044[b]	1.875E+045[b]	3.672E+045[a]	1.395E+046[a]
Residual	10	4.719E+043	6.160E+043	8.520E+044	1.041E+045	3.519E+045
Lack of fit	5	4.717E+043[a]	5.865E+043[a]	2.208E+044[b]	4.932E+044[b]	1.702E+045[b]
Pure error	5	1.581E+040	2.951E+042	6.312E+044	5.476E+044	1.817E+045
Total	19	1.135E+044	1.642E+044	2.727E+045	4.713E+045	1.747E+046
R^2		0.584	0.625	0.688	0.779	0.799

Table 8.3: Estimated coefficients and ANOVA table for the response surface fitting for FAEE yield at the various reaction times for E-1.

[a]significant [b]not significant (based on *F*-test statistic).

	Optimum Values				
Factor	**8 h[a]**	**12 h[a]**	**24 h[b]**	**36 h[b]**	**48 h[b]**
Temp	50.003	49.937	49.847	50.196	48.316
Enz (%)	7.054	6.134	8.840	8.851	8.229
AlcoR	2.000	2.006	1.998	1.941	1.926

Table 8.4: Optimum values of temperature (Temp), enzyme load (Enz), and oil:alcohol molar ratio (AlcoR) for E-1.

[a]saddle point [b]maximum (based on canonical analysis of response surface data).

Coefficient Factor	8 h	12 h	24 h	36 h	48 h
	Estimates				
β_0	+2.447E+020	+3.470E+021	+1.566E+022	+3.470E+022	+6.869E+022
β_1	-6.082E+016[b]	-3.473E+019[b]	-3.432E+020[b]	-2.214E+021[b]	-1.363E+022[a]
β_2	+9.078E+020[a]	+1.977E+021[a]	+2.346E+021[b]	+5.511E+021[a]	+5.887E+021[b]
β_3	+3.559E+017[b]	+3.386E+019[b]	+1.473E+020[b]	+1.042E+021[b]	+4.267E+021[b]
β_{12}	-3.253E+017[b]	-5.456E+019[b]	-1.041E+020[b]	-1.588E+021[b]	-7.103E+021[b]
β_{13}	-5.004E+017[b]	-5.433E+019[b]	-1.037E+020[b]	-1.607E+021[b]	-7.126E+021[b]
β_{23}	+6.116E+017[b]	+5.455E+019[b]	+1.072E+020[b]	+1.596E+021[b]	+7.110E+021[b]
β_{11}	-3.435E+020[b]	-1.729E+021[b]	-5.682E+021[a]	-1.138E+022[a]	-1.168E+022[a]
β_{22}	+9.594E+020[a]	+1.059E+021[b]	-2.790E+021[a]	-6.624E+021[a]	-2.271E+022[a]
β_{33}	-3.442E+020[b]	-1.731E+021[b]	-5.968E+021[a]	-1.311E+022[a]	-2.516E+022[a]

ANOVA

	Df	Sum of Squares				
Model	9	2.967E+043[b]	1.596E+044[b]	1.012E+045[a]	4.799E+045[a]	2.034E+046[a]
Residual	10	2.122E+043	9.552E+043	1.601E+044	7.343E+044	3.122E+045
Lack of fit	5	2.121E+043[a]	9.196E+043[a]	1.226E+044[b]	4.071E+044[b]	1.488E+045 [b]
Pure error	5	9.303E+039	3.560E+042	3.750E+043	3.272E+044	1.634E+045
Total	19	5.089E+043	2.551E+044	1.172E+045	5.534E+045	2.346E+046
R^2		0.583	0.626	0.863	0.867	0.867

Table 8.5: Estimated coefficients and ANOVA table for the response surface fitting for FAEE yield at the various reaction times for E-2.

[a]significant [b]not significant (based on *F*-test statistic).

	Optimum Values				
Factor	**8 h[a]**	**12 h[a]**	**24 h[b]**	**36 h[b]**	**48 h[b]**
Temp	50.002	49.946	49.918	50.032	52.808
Enz (%)	7.069	6.382	8.481	8.900	8.836
AlcoR	2.000	2.005	2.000	1.958	1.688

Table 8.6: Optimum values of temperature (Temp), enzyme load (Enz), and oil:alcohol molar ratio (AlcoR) for E-2.

[a]saddle point [b]maximum (based on canonical analysis of response surface data).

Coefficient Factor	8 h	12 h	24 h	36 h	48 h
	Estimates				
β_0	+2.710E+020	+2.805E+021	+1.568E+022	+3.150E+022	+5.558E+022
β_1	-1.116E+017[b]	-3.606E+019[b]	-2.297E+020[b]	-1.708E+021[b]	-1.385E+022[a]
β_2	+1.227E+021[a]	+1.858E+021[a]	+1.693E+021[b]	+5.093E+021[a]	+8.068E+021[b]
β_3	+3.974E+017[b]	+3.599E+019[b]	+1.336E+020[b]	+7.224E+020[b]	+5.850E+021[b]
β_{12}	-4.200E+017[b]	-5.800E+019[b]	-9.500E+019[b]	-1.050E+021[b]	-9.751E+021[b]
β_{13}	-5.936E+017[b]	-5.803E+019[b]	-9.450E+019[b]	-1.061E+021[b]	-9.769E+021[b]
β_{23}	+8.672E+017[b]	+5.899E+019[b]	+9.958E+019[b]	+1.066E+021[b]	+9.758E+021[b]
β_{11}	-4.438E+020[b]	-1.467E+021[b]	-5.627E+021[a]	-1.058E+022[a]	-8.413E+021[a]
β_{22}	+1.317E+021[a]	+1.148E+021[b]	-5.627E+021[a]	-5.714E+021[a]	-1.657E+022[a]
β_{33}	-4.450E+020[b]	-1.468E+021[b]	-5.771E+021[a]	-1.205E+022[a]	-1.992E+022[b]

ANOVA

	df	Sum of Squares				
Model	9	5.440E+043[b]	1.330E+044[b]	9.829E+044[a]	3.999E+045[a]	1.546E+046[a]
Residual	10	3.882E+043	8.143E+043	8.942E+043	6.293E+044	3.118E+045
Lack of fit	5	3.881E+043[a]	8.098E+043[a]	5.839E+043[b]	4.196E+044[b]	1.451E+045[b]
Pure error	5	1.786E+040	4.543E+041	3.103E+043	2.097E+044	1.668E+045
Total	19	9.323E+043	2.145E+044	1.072E+045	4.628E+045	1.858E+046
R^2		0.584	0.620	0.917	0.864	0.832

Table 8.7: Estimated coefficients and ANOVA table for the response surface fitting for FAEE yield at the various reaction times for E-3.

[a]significant [b]not significant (based on *F*-test statistic).

	Optimum Values				
Factor	**8 h[a]**	**12 h[a]**	**24 h[b]**	**36 h[b]**	**48 h[b]**
Temp	50.009	49.912	49.862	50.035	46.061
Enz (%)	7.076	6.093	8.831	9.126	8.209
AlcoR	2.000	2.007	1.998	1.946	1.944

Table 8.8: Optimum values of temperature (Temp), enzyme load (Enz), and oil:alcohol molar ratio (AlcoR) for E-3.

[a]saddle point [b]maximum (based on canonical analysis of response surface data).

CHAPTER IX

CONNECTING STATEMENT

As stated in the previous chapters, the high cost of conventional feedstocks and value as food crops and edible oil are major bottlenecks for their use in BD production. With enzyme-catalyzed biodiesel (BD) production, the cost of the lipase is another limitation. On the other hand, heterogeneous immobilized enzymes for biotransformation are considered to be more advantageous than homogenous chemical catalysts as the former can be easily separated from reaction mixture, regenerated, and reuse multiple times. Adsorption on a hydrophobic resin was selected as the immobilization method for recovered fish lipase because it has been widely used with different enzymes and shown to be very versatile. In addition to converting processing waste to useful products, fish enzymes exhibit unique characteristics in comparison with their microbial counterparts.

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CHAPTER IX

IMMOBILIZATION OF LIPASE FROM GREY MULLET

9.1. Abstract

Grey mullet (*Mugil cephalus*) lipase was isolated using *para*-aminobenzamidine agarose (*p*-ABA) and immobilized on octyl Sepharose CL-4B (o-Sep). Immobilized grey mullet lipase (GML*i*) had a 10°C higher optimum temperature compared to the free enzyme (GML), and showed remarkable thermal stability. GML*i* was most active within the pH range of 8.0-9.5 with an optimum at 8.5. Immobilization also enhanced the storage stability and reusability of the enzyme with minimal changes to efficiency over a number of cycles. GML*i* showed variable stabilities in various organic solvents. A signal in the amide I absorption region of the FTIR spectrum of GML*i* was attributed to the protein layer on o-Sep. The surface morphology of o-Sep was visualized on a Zeiss stereomicroscope as globular shaped beads.

9.2. Introduction

The advantages of enzymes in catalysis include milder reaction conditions of temperature and pH. An example of such enzyme is lipase. Lipase is a ubiquitous enzyme occurring in organisms from bacteria to humans where it plays a key role in lipid metabolism (Delorme et al., 2011). Lipase often exhibit high catalytic efficiency in both hydrolytic and esterification reactions in low water milieu or in solvent and solvent-free reaction systems (Zaks and Klibanov, 1985; Pogorevc et al., 2002) as well as substrate-, regio- and stereo- specificities and selectivities (Bastida et al., 1998). These attributes make lipase highly useful in food, pharmaceutical and fine chemicals applications (Palomo et al., 2004; Mateo et al., 2007; Tufvesson et al., 2011).

Nevertheless, lipases are expensive which limit their use in industrial applications (Tufvesson et al., 2011). Widespread use of the free form of the enzyme in batch reactions is also restricted by limited stability, difficult separation, recovery, and reusability (Tischer and Wedekind, 1999; Palomo et al., 2004; Tufvesson et al., 2011). To enhance the convenience and cost effectiveness of enzymes in catalysis, immobilization among other strategies has been suggested (Palomo et al., 2004; Garcia-Galan et al.,

2011; Tufvesson et al., 2011). Immobilization involves the attachment of the soluble/free enzyme onto inert solid support materials such as resins. Immobilization may improve the stability of the enzyme, facilitate enzyme separation, recovery and reuse, and ease purification of reaction products. In organic solvents such as alcohols, immobilization may reduce the bulk solvent effect by protecting the enzyme from the relatively harsh polar solvent milieu (Iso et al., 2001; Fjerbaek et al., 2009). Some free enzymes may also show lower activities in some neat organic solvents as a result of aggregation and subsequent sequestering of the active site away from the reactants (Iso et al., 2001; Pencreac'h and Baratti, 2001). Immobilization on the other hand allows dispersion of the enzyme on the resin (Bastida et al., 1998; Palomo et al., 2004; Mateo et al., 2007).

Lipases have been immobilized by adsorption, covalent attachment, cross-linking, and entrapment/encapsulation, on biopolymers, organic, inorganic, and synthetic supports such as porous glass beads, ion exchange resins, silica, and chitosan (Tischer and Wedekind, 1999; Garcia-Galan et al., 2011). Immobilization of lipases *via* adsorption has been reported to occur through hydrophobic interactions between the surface of the resin and the hydrophobic pocket and face of the "lid" (Bastida et al., 1998; Garcia-Galan et al., 2011). Since the surface of hydrophobic resins resembles part of the interface that induces interfacial activation, immobilization of lipase on such supports is thought to result in a similar effect.

Recently the immobilization of lipase from Chinook salmon was reported by Kurtovic et al. (2011). Nevertheless, the prospects of immobilizing alternative and underutilized sources of lipase such as from fishery resources have received little attention. In our laboratory and elsewhere, fish digestive enzymes such as trypsin, pepsin, chymotrypsin, phospholipase A_2, and lipase have been characterized (Aryee et al., 2007; Yang et al., 2009; Sila et al., 2012). Due to their relatively unique habitats; these fish enzymes exhibit distinctive properties versus their counterparts from mammalian, plant, and microbial sources. In a previous study (Aryee et al., 2007), lipase from the viscera of grey mullet (*Mugil cephalus*) was shown to have exceptional activity and stability in organic solvents, making it a suitable candidate for potential applications in non-aqueous media, a fast growing industry. To prepare the enzyme for possible commercial

application, the advantages of increased operational stability as well as ease of enzyme recovery for repeated use *via* immobilization was explored in this study.

9.3. Materials and Methods

9.3.1. Materials

All solvents used in this study were purchased from Fisher Scientific (Whitby, ON) except ethanol which was obtained from Commercial Alcohols (Boucherville, QC); octyl Sepharose CL-4B (o-Sep), *para*-aminobenzamidine agarose (*p*-ABA), *para*-nitrophenyl palmitate (*p*-NPP), and *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BA-*p*-NA) were purchased from Sigma-Aldrich (Oakville, ON); while Grey mullet (*Mugil cephalus*) was purchased from a local fish market (Poissonnerie O-C-N, Montreal, QC).

9.3.2. Methods

Figure 9.1 provides a schematic illustration of the summary of the various steps involved in this study.

9.3.2.1. Sample Preparation: Isolation and Preparation of Delipidated Powder

Grey mullet viscera powder was defatted as previously described by Aryee et al. (2007) but with changes to the type and proportion of solvents used. Grey mullet was kept on crushed ice in a plastic bag and transported directly to the laboratory where the viscera were removed by hand. The viscera were first rinsed with distilled water followed by ice-cold 0.85% (w/v) sodium chloride. The viscera were then chopped into small pieces, rapidly frozen in liquid nitrogen, and comminuted to a fine powder in a Warring blender. The powder was delipidated with successive changes of cold ethyl ether and ethanol mixtures at ratios of 3:2, 1:3, 1:1, 3:1 (v/v) and then ethyl ether, all at -18^{o}C. The powder to solvent ratio was 1:5 (w/v). After each solvent treatment, the homogenate was filtered under vacuum through a medium coarse filter paper (pore size 10-15 μm) to separate the organic phase which contained the dissolved lipid. The delipidated material was air dried at 23^{o}C in a fume hood, and stored at -18^{o}C until needed.

9.3.2.2. Grey Mullet Lipase Extraction and Purification

9.3.2.2.1. Preparation of Extract

One gram of delipidated grey mullet visceral powder was homogenized with 20 ml of 20 mM Tris-HCl buffer (pH 7.2) at 4°C for 30 min. The homogenate was centrifuged at 10,000 g and 4°C for 15 min in an IEC multi RF™ centrifuge (Thermo Scientific, Asheville, NC). The recovered supernatant was designated crude extract.

9.3.2.2.2. Separation and Purification with *p*-ABA

The crude extract was batch loaded on a *p*-ABA column (30 cm x 1.5 cm ID) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.2) and eluted at a flow rate of 1.0 ml/min at 23°C. The column was washed with the same buffer and the combined elute and washing was denoted as grey mullet lipase (GML) fraction. Aliquots of the crude extract and GML fraction were scanned between 200-800 nm in a DU® 800 ultraviolet-visible (UV-Vis) spectrophotometer (Beckman Coulter Inc., Fullerton, CA) at 30°C against buffer (pH 7.2) as blank.

9.3.2.3. Immobilization of Grey Mullet Lipase (GML)

To 1 g of previously washed and equilibrated damp o-Sep were added 7 ml GML fraction and incubated on an Orbitron II™ 260250 platform rotator (Boekel Scientific, Philadelphia, PA) at 20 orbits/min for 4 h at 23°C. After the incubation period, the lipase-resin mixture was vacuum filtered on a Pyrex® Büchner funnel with a sintered (fritted) glass disc and the filtrate collected. The lipase immobilized was washed with 20 mM Tris-HCl buffer (pH 7.2) to remove unbound and loosely bound lipase, and filtrate was collected for protein and lipase assays. Subsequently, the resin was washed with the same buffer and filtrate was collected. The lipase immobilized was then incubated with 2.5 ml each of 10 mM sodium cholate and calcium chloride ($CaCl_2$) for 90 min after which it was filtered and vacuum dried in a desiccator over silica overnight. The dried immobilized lipase (GML*i*) was stored at -18°C.

9.3.2.4. Protein Content Determination

Protein content was measured according to Bradford's method (1976) with bovine serum albumin (BSA) as the standard.

9.3.2.5. Lipase Activity Assay

Adsorption of lipase onto the resin was monitored by measuring the residual activity of the crude extract, filtrate, washings as well as GML*i*. Free lipase activity was measured according to the method of Kordel et al. (1991) as previously described by Aryee et al. (2007) and consisted of a 16.5 mM stock solution of *p*-NPP and 50 mM Tris-HCl buffer, containing 0.4% (w/v) Tween 80®, and 0.1% (w/v) Arabic gum, and $CaCl_2$ and sodium cholate, both at 10 mM. The release of *p*-nitrophenol from the hydrolysis of *p*-NPP at 30°C was measured as the change in absorbance at 410 nm in a DU® 800 UV-Vis spectrophotometer (Beckman Coulter Inc., Fullerton, CA) against a blank without the enzyme. Enzyme activity was expressed as unit/ml (U/ml) and 1 unit of lipase activity (U) was defined as the amount of enzyme that released 1 µmol *p*-nitrophenol per min under the assay conditions described above.

For immobilized lipase (GML*i*) activity, Tween 80® was excluded from the substrate emulsion as it has been reported to desorb the immobilized lipase from the resin (Pencreac'h et al., 1997; Palomo et al., 2004). To 10 mg of GML*i* was added the substrate solution containing 10 mM each of $CaCl_2$ and sodium cholate. After 60 min of incubation on the Orbitron™ rotator at 23°C, the reaction mixture was separated in a Heraeus Biofuge 13 centrifuge (Thermo Scientific, Asheville, NC) at 3,000 g for 5 min at 23°C and the absorbance of the supernatant read at 410 nm in a DU® 800 UV-Vis spectrophotometer (Beckman Coulter Inc., Fullerton, CA) against a blank without the enzyme. Enzyme activity was expressed as unit/mg (U/mg) and 1 unit of lipase activity (U) was defined as the amount of enzyme that releases 1 µmol *p*-nitrophenol per min under the assay conditions described above.

9.3.2.6. Trypsin Activity Assay

The assessment of trypsin activity before and after *p*-ABA affinity chromatography was according to the method of Erlanger et al. (1961) with BA-*p*-NA as

substrate. Briefly, 0.2 ml enzyme aliquot was added to 1 mM BA-*p*-NA in 50 mM Tris-HCl buffer (pH 8.2, containing 10 mM $CaCl_2$ and 1% (v/v) DMSO) to make a final reaction volume of 3 ml. The enzyme was replaced with buffer for blank. The release of *p*-nitroaniline from BA-*p*-NA was measured at 410 nm in a DU® 800 UV-Vis spectrophotometer (Beckman Coulter Inc., Fullerton, CA). One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol *p*-nitroaniline per min under the assay conditions. To measure residual trypsin activity, 10 mg of GML*i* were added to 1 mM BA-*p*-NA in 50 mM Tris-HCl buffer (pH 8.2, containing 10 mM $CaCl_2$ and 1% (v/v) DMSO) and incubated for 60 min on the Orbitron™ rotator at 23°C. The reaction mixture was separated in a centrifuge at 3,000 g for 5 min and the absorbance of the supernatant read at 410 nm DU® 800 UV-Vis spectrophotometer (Beckman Coulter Inc., Fullerton, CA) against a blank.

9.3.2.7. Characterization of Free and Immobilized Grey Mullet Lipase

9.3.2.7.1. Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) Spectroscopy

Approximately 10 µl of GML and 1 mg GML*i* samples were separately deposited onto a diamond ATR accessory attached to a FTIR spectrometer (Excalibur, Agilent Technologies, Melbourne). The FTIR spectrometer was equipped with a deuterated triglycine sulfate (DTGS) detector and purged with dry air (Balston, MA). A total of 128 scans at 4 cm^{-1} were recorded. The spectral contributions of buffer, deuterium oxide (D_2O) or o-Sep to the IR spectra were digitally subtracted from the spectra of GML and GML*i*, respectively.

9.3.2.7.2. Effect of Immobilization on pH Optimum and Stability of GML

The effect of pH on the activity of GML and GML*i* was measured using a substrate emulsion with pH under study (from pH 6.5 to 9.5). Free and immobilized lipase activities were measured as described above. The extinction coefficient of *p*-nitrophenol for each pH buffered solution was determined. To evaluate pH stability, GML was incubated in a buffer with pH under study (2.0-9.5) for 30 min at 23°C and residual lipase activity measured as previously described. For the immobilized lipase, 10

mg of GML*i* was incubated at the various pH levels under study for 30 min at 23°C. At the end of the incubation period the sample was centrifuged at 3,000 g for 5 min and the supernatant (buffer) carefully removed. To the damp GML*i* was added the substrate emulsion and assayed as described above.

9.3.2.7.3. Effect of Immobilization on Temperature Optimum, Stability, and Thermodynamic Parameters of GML

The substrate emulsions were incubated at 25 to 65°C at 10°C increments for 15 min prior to lipase assay for temperature optimum studies. Free and immobilized lipase activities were measured as described above. The data were also used to determine the apparent Arrhenius energy of activation (*Ea*) from the slope of the plot of log of reaction rate (*k*) versus the reciprocal of absolute temperature (T) in kelvin according to the Eq. (9.1). Where *Z* is the Arrhenius frequency factor and *R* is the ideal gas constant in cal per kelvin per mole.

$$\boldsymbol{k = Ze^{-E_a/RT}} \qquad (\mathbf{9.1})$$

The enthalpy of activation (ΔH*), entropy of activation (ΔS*), and free energy of activation (ΔG*), all at an assay temperature of 30°C, were calculated using Eqs. (9.2), (9.3), and (9.4), respectively. The turnover number (*k*) was calculated from Eq. (9.5).

$$\boldsymbol{\Delta H^* = E_a - RT} \qquad (\mathbf{9.2})$$

$$\boldsymbol{\Delta S^* = 4.576\left(\log k - 10.735 - \log T + \frac{E_a}{4.576T}\right)} \qquad (\mathbf{9.3})$$

$$\boldsymbol{\Delta G^* = \Delta H^* - T\Delta S^*} \qquad (\mathbf{9.4})$$

The thermal stability of GML and GML*i* were assessed by incubating aliquots at 25 to 65°C for 30 min and residual activities assayed as described above with *p*-NPP. GML*i* was incubated at 25 to 65°C for 60, 90, and 120 min for additional studies.

9.3.2.7.4. Kinetic Parameters of GML and GML*i*

The apparent kinetic parameters (V_{max} and K_m) were determined by measuring the initial rates of the reaction of GML and GML*i* with varying concentration of *p*-NPP.

V_{max} and K_m were estimated by fitting the data to a Hanes-Woolf plot using Eq. (9.5). Reaction rate and substrate concentration are denoted by v and [S], respectively. A plot of [S]/v versus [S] yields $1/V_{max}$ as slope, $-K_m$ and K_m/V_{max} as x- and y-intercepts, respectively.

$$\frac{[S]}{v} = \frac{1}{V_{max}}[S] + \frac{K_m}{V_{max}} \qquad (9.5)$$

9.3.2.7.5. Stability of GML*i* in Organic Solvents

The stability of GML*i* in selected organic solvents was assessed. For easy comparison, the assay used the same hydrolytic reaction in both aqueous and organic media. Ten milligrams of GML*i* were incubated in anhydrous acetone, ethanol, methanol, butanol, iso-propanol, hexane, petroleum ether, and iso-octane at 23ºC for 1 h. After the incubation period the solvents were recovered and residual activities measured as described above. The $R_{O/A}$ (ratio of activity in organic solvents to activity in aqueous solvents) values were subsequently calculated.

9.3.2.7.6. Operational Stability of GML*i*

The operational stability of GML*i* was determined during 6 repeated batches (1 h/batch) of the hydrolysis of *p*-NPP. Each assay was carried out as previously described. Between two consecutive batches, GML*i* was separated and washed twice with 20 mM Tris-HCl buffer (pH 7.2). Residual activity was determined and compared with the activity of the first batch. Free lipase was excluded in this study since it was practically impossible to separate from the reaction medium for reuse, while GML*i* was easily recovered.

9.3.2.7.7. Storage Stability of GML and GML*i*

The stability of GML and GML*i* stored at -18°C were assessed on day 1, 30, 60, 90, and 180. GML was kept in small aliquots to minimize freezing and thawing damage, as well as contamination presented by pipetting from a single vial multiple times.

9.3.2.7.8. Statistical Analysis

All analyses were run in duplicate unless otherwise stated. Data were presented as mean ± standard deviation and subjected to one-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons ($p < 0.05$) using the General Linear Model (GLM) of SAS® (Version 9.1, SAS Institute Inc., Cary, NC).

9.4. Results and Discussion

9.4.1. Delipidation

Lipids were extracted and removed using various proportions of a mixture of ethanol and ethyl ether to accommodate the diverse lipids in fish viscera and minimize the denaturation of proteins. Polar solvents have also been shown to denature proteins when used alone and especially for this work, as the conformational integrity and activity of lipase has been shown to be compromised in the presence of high amount of polar alcohols (Zaks and Klibanov, 1985; Pogorevc et al., 2002; Soumanou and Bornscheuer, 2003). This was verified by enzyme activity assay which assesses if denaturation has occurred as proteins lose their catalytic activity on denaturation and since the assay involves interaction of the amino acid side chains present in the native conformation. Lipase assay of grey mullet visceral powder indicated that the procedure did not denature the proteins. The yield of the delipidated powder was *ca* 11% of the weight of the viscera. There was also limited exposure to solvents with the reduced number of solvents used; i.e., two instead of four (Aryee et al., 2007).

9.4.2. Separation and Purification on para-Aminobenzamidine Agarose (*p*-ABA)

In the extraction and preparation of enzymes, early removal of undesirable proteases minimizes the tendency of proteolytic degradation (De-Simone et al., 2005; Yang et al., 2009). The ability of *p*-ABA to selectively bind proteases and thereby partially purify the extract was observed in this study and confirmed by trypsin assay and SDS-PAGE. Due to the cost of purification, commercially available immobilized enzymes are also often partially purified (Pencreac'h and Baratti, 2001), and may contain non-protein additives as stabilizers. Greater than 95% decrease in trypsin activity was observed between the crude extract and GML fraction. Thus *p*-ABA was effective in

excluding trypsin-like proteases from the GML fraction, thereby, stabilizing the GML fraction.

The purification of lipase from grey mullet and other species is tedious involving a number of several chromatographic steps, and recovers the enzyme in low yield, relatively unstable and readily deactivated forms (Aryee et al., 2007; Garcia-Galan et al., 2011). This current procedure however offered a simple separation protocol which was less destructive to the enzyme. In addition to changes in the shape of the peaks of scanned aliquots of the crude extract and GML fraction shown in Fig. 9.2a is the reduction in relative absorbance intensity, indicative of the changes that occurred during separation on *p*-ABA.

9.4.3. Immobilization of Grey Mullet Lipase on octyl Sepharose CL-4B (o-Sep)

GML was absorbed onto o-Sep under mild conditions (23^{o}C, 4 h) and at a low protein concentration and ionic strength; i.e., 2.66 mg/ml and 20 mM Tris-HCl (pH 7.2), respectively. The resulting immobilized lipase, GML*i*, was active against *p*-NPP. Minimal activity differences were observed between batch preparations of GML*i*. Using a Zeiss Discovery.V20 stereomicroscope, dried o-Sep was observed as globular shaped beads (Fig. 9.2b). Since most enzymes lose their stability once isolated from their natural environment, immobilizing the free enzyme on resins has been suggested to mimic their natural mode of attachment to cellular membranes (Palomo et al., 2004**)**. The selection of a suitable support is thus an important factor besides the immobilization method due to the strong effect of the microenvironment of the resin on the stability and activity of the enzyme (Bastida et al., 1998; Sörensen et al., 2010; Garcia-Galan et al., 2011). In this instance the hydrophobic sites of lipase and its high affinity to the hydrophobic resin are plausible explanations for the enhanced activity and stability of GML*i* observed. The surface of the hydrophobic resin has been reported by various workers to resemble the natural substrates of lipases thereby interfacially activating it upon binding (Bastida et al., 1998; Sörensen et al., 2010). The washing solutions contained enzymes not captured or immobilized by o-Sep, evident of the reported high selectivity of these resins (Bastida et al., 1998; Palomo et al., 2004). Indeed, only the first washing showed *ca* 24% of total initial lipase activity indicating that the immobilization protocol was efficient.

In an initial immobilization assessment using 3-aminopropyl-triethoxysilane (APTES)-silanized, glutaraldehyde-activated silica (Weetall, 1976), >80% of total initial lipase activity was recovered in the washings, and the immobilized lipase concomitantly showed no or very limited activity. Comparative studies have shown that immobilizing the same enzyme on different resins (Sörensen et al., 2010; Kurtovic et al., 2011) or by different immobilization methods (Mateo et al., 2007; Garcia-Galan et al., 2011) result in immobilized enzymes with different characteristics. The observation in the initial assessment was attributed to the high hydrophilicity of silica which was unfavorable and limited lipase-resin interaction influencing the adsorption process. The low activity observed with the silica resin-immobilized lipase might also be due to limited diffusion of the emulsified substrate through the pores of the resin to the active sites of the enzyme for catalysis. On the other hand, o-Sep enhanced the occurrence and availability of lipase resulting in increased catalytic activity. The o-Sep resin did not require any complex pre-conditioning employing any additional steps prior to use. The relatively fast adsorption onto the resin strongly suggests a pronounced adsorption ability of the resin, and high affinity of the enzyme to the resin.

The improved activities of lipase adsorbed on hydrophobic resins have been related to the mode of lipase catalysis (Derewenda et al., 1992; Bastida et al., 1998; Mateo et al, 2007; Delorme et al., 2011). It has been suggested that the hydrophobic binding domain involved in interfacial activation of the enzyme are involved in the binding of lipase onto o-Sep resulting in the active center oriented towards the reaction medium with the open form stabilized on the resin (Bastida et al., 1998; Palomo et al., 2004; Sörensen et al., 2010). It was also shown that the activation promoted by the hydrophobic resin upon lipase adsorption was additional and much higher than by the emulsified substrate alone.

Other possible explanations to the improved activity of the adsorbed lipase are the presence of a spacer on the resin which might have minimized stearic hindrance during immobilization (Palomo et al., 2004), and the better dispersion of the lipase on the resin (Pencreac'h et al., 1997; Mateo et al, 2007). The enhanced activity of GML*i* may also be related to altered available water (Palomo et al., 2004). The dried immobilized lipase (GML*i*) presents just enough water needed for hydrolysis as oppose to the free enzyme

with high water activity, thus the probable hindering of access of the hydrophobic substrate to the active site or the stripping of the essential layer of water needed for conformation and catalysis were averted.

9.4.3.1. Effect of Detergents and Emulsifiers on Immobilization and Lipase Catalysis

Lipase conformational changes are primarily induced by detergents, emulsifiers, lipids, surfactants, and other surface active agents (Tiss et al., 2001; Delorme et al., 2011). Bile salts were included in both the coupling and assay solutions and may have induced/enhanced lid opening, and stabilized the exposed hydrophobic surface around the active site, and on the resin, and also helped to disperse the lipase on the resin (Fernandez-Lafuente et al., 2007; Mateo et al, 2007). While the enzyme showed activity without bile salts, activity was markedly increased (9-12 fold) with its inclusion in the assay mixture. The increased activity may also be due to a more optimal orientation of the lipase in the presence of bile salts for interaction with the substrate.

9.4.3.2. Effect of Enzyme Loading and Reaction Time on the Activity of GML*i*

The immobilization of enzymes has been shown to be affected by pH, temperature, enzyme loading, and coupling time (Palomo et al., 2004; Mateo et al, 2007). Of these parameters, optimal enzyme loading is an important process cost variable. GML*i* loaded with 9 parts of GML gave significantly ($p < 0.05$) higher hydrolytic rates than with 5 parts of GML (Fig 9.3a); however no significant difference ($p > 0.05$) were observed with GML*i* loaded with 7 parts of GML. At 1:9 o-Sep:GML (w/v), GML*i* showed *ca* 26 and 14% more activity than 1:5 and 1:7 o-Sep:GML (w/v) GML*i*, respectively. These results indicate that higher activities were obtained with higher loading compared to lower loading. This also suggests that enzyme loading did not cause aggregation or blocked the pores of the resin, or limited by internal diffusion. Even at the highest proportion (1:9), loading was still moderate and mass transfer limitations of the lipase to the resin were prevented (Pencreac'h et al., 1997; Garcia-Galan et al., 2011).

Figure 9.3b shows the time course of the hydrolysis of GML*i*. Hydrolytic rates were relatively fast with >67% reached within 30 min and very little changes after 45 min. In fact there were no significant difference ($p > 0.05$) between rates at 60, 75, and 120

min. For commercial-scale application, higher conversion yields in relatively short time and low enzyme loading are preferred.

9.4.4. Characterization of Free (GML) and Immobilized Grey Mullet Lipase (GML*i*)

9.4.4.1. ATR-FTIR Spectroscopy

ATR-FTIR spectroscopy was employed for the characterization of both GML and GML*i*. Figure 9.4a shows the amide I (1700-1600 cm^{-1}) and amide II (1600-1550 cm^{-1}) absorption regions of GML on ATR surfaces. The bands at *ca* 1625 cm^{-1} and 1550 cm^{-1}, were assigned to the C=O stretching vibration of the peptide groups on the protein backbone (amide I), and to the N-H bending and C-N stretching vibrations (amide II), respectively (Blout et al., 1960). A weak band in the amide II absorption region could be discerned at *ca* 1550 cm^{-1} in the spectrum of GML*i* (Fig. 9.4b**)**. The contribution of adsorbed water in the resin obscures the observation of the amide I band even though the sample was vacuum dried. This overlaps with protein at the amide I region making unambiguous band assignment difficult. GML*i* was thus suspended in D_2O to observe the hydrogen-deuterium (H-D) exchange and also as an additional measure to confirm the immobilization of GML on the resin. If the weak signal at 1550 cm^{-1} observed in the spectrum of the GML*i* is indeed due to amide II (N-H) absorption, then upon H-D exchange the band will be expected to shift by *ca* 100 cm^{-1} (Blout et al.,1960, Pelton and Mclean, 2000) to *ca* 1450 cm^{-1}. Indeed, the spectrum revealed a drop in absorption at 1550 cm^{-1} and a new band at *ca* 1450 cm^{-1} (Fig. not shown) that may be attributed to the N-D absorption of the amide II band.

9.4.4.2. Effect of Immobilization on pH Optimum and Stability of GML

The optimum pH of GML and GML*i* for the hydrolysis of *p*-NPP were 8.0 and 8.5, respectively (Fig. 9.5). Kurtovic et al. (2011) reported pH optima of 8.5 and 9.0 for free and immobilized Chinook salmon lipase (on a similar resin), respectively but using tributyrin as substrate. The disparity can be attributed in part to the different lipase source, substrate, and method of analysis. Figure 9.5a demonstrates that GML*i* was highly active between pH 8-9.5 with relative activities of more than 69%. Lower pH values led to lower reaction rates compared to hydrolysis performed close to the optimum pH of the

enzyme. As discussed by various authors (Tischer and Wedekind, 1999; Palomo et al., 2004; Mateo et al, 2007), when the pH of the reaction medium is a factor, the formation of reaction-generated proton gradients shifts the pH of the reaction medium producing a pH value different from the optimum of the enzyme, and this may have reduced the activity. Higher pH values (>9.5) were avoided due to the instability of the substrate. GML*i* was stable within a wider pH range (7.0-9.5) compared to GML (7.5-8.5) (Fig. 9.5b). The improvement in pH optimum and stability of GML*i* may be a result of alterations to the microenvironment (o-Sep residues) surrounding the enzyme upon adsorption.

9.4.4.3. Effect of Immobilization on Temperature Optimum and Stability of GML

Figure 9.6a shows the temperature profile of GML and GML*i*. GML and GML*i* showed optimum temperatures of 45 and 55^{o}C, respectively. These were both 10^{o}C higher than reported by Kurtovic et al. (2011) for free and immobilized Chinook salmon lipase using tributyrin as substrate. As reaction temperature increased above 45^{o}C, increase in the activity of GML*i* was generally higher than that of GML (Fig 9.6a). The enhanced activity of GML at higher temperature levels with immobilization opens broader range of possible industrial applications.

The Arrhenius plot for GML and GML*i* was linear within the temperature ranges of 25-45^{o}C and 25-55^{o}C, respectively. The E_a of GML and GML*i* from the slope of the regressed lines of the plot showed that immobilization of GML lowered the E_a; from 2.02 to 1.17 kcal/mol (1 cal = 4.187 J). The lowered E_a implies a decrease in energy barrier; as such GML*i* will not require an elevated temperature to reach its highest activity.

The ΔH^* and ΔG^* values of GML*i* were 1.10 kcal/mol and 16.93 kcal/mol, respectively. These values were respectively, >313 cal/mol and 1315 cal/mol lower than that of GML. These results suggest that immobilization made GML thermodynamically more efficient for catalysis with GML*i* requiring less energy to form the enzyme-substrate activated complex compared to GML. These lower values of GML*i* also confirm the thermal stabilization of GML *via* immobilization on o-Sep.

The temperature stability profile (Fig. 9.6b) indicates that immobilization stabilized the enzyme and improved its resistance to thermal denaturation with GML

showing greater inactivation at higher temperatures (>45^{o}C) than GML*i*. This might have resulted from the enhanced rigidity of the protein structure upon attachment on the resin. Additional experiments on the thermostability of GML*i* reveal that incubation for various times and temperatures resulted in varied lipase activities (Fig. 9.6c). For instance, GML*i* lost approximately half of its initial activity after 90 min of incubation at 65^{o}C while it showed minimal changes in stability during the various incubation times at 55^{o}C. According to Hong et al. (2008) and other workers (Sörensen et al., 2010), immobilized enzymes usually exhibit broader pH and temperature regions, are more resistant to pH and temperature inactivation compared to the free form of the enzyme. The increased thermostability of GML*i* can also be assumed to be due to improved conformational stabilization by a stronger degree of attachment of the enzyme on the resin through hydrophobic interaction.

9.4.4.4. Kinetic Parameters of GML and GML*i*

The activities of the free and immobilized lipase at varying substrate concentrations were used to calculate the values of the fitted parameters from the Hanes-Woolf plot (Fig. 9.7). GML exhibited a lower apparent V_{max} (1.44 µmol/mg protein) compared to GML*i* (3.45 µmol/mg protein). The ratio of the maximum reaction rates of the immobilized (GML*i*) to the free (GML) enzyme as denoted by the efficiency factor (η) was 2.40. The apparent K_m; Michaelis-Menten constant, on the other hand is used to measure the affinity between the enzyme and substrate and also defines the substrate concentration that gives $1/2V_{max}$. An alteration in the affinity of the enzyme to *p*-NPP was observed as measured by a different and lower K_m for GML*i* (0.18 mM) compared to GML (0.32 mM), indicating an increase in affinity of the immobilized enzyme to *p*-NPP. This may be a result of structural changes and altered orientation of the active site, as well as the retention of the lipase in its open form upon binding to o-Sep making it more readily available for catalysis. The corresponding catalytic efficiency (V_{max}/K_m) of GML and GML*i* were found to be 4.64 and 19.17, respectively indicating a >4-fold increase in catalytic efficiency upon immobilization.

9.4.4.5. Stability of GML*i* in Organic solvents

As shown in Table 9.1, the order of decreasing stability of GML*i* after 1 h of incubation was; methanol > iso-propanol > ethanol > butanol > acetone > petroleum ether > hexane. The stability of lipase in organic solvents is an important feature as it determines its suitability for catalysis in non-aqueous media (Zaks and Klibanov, 1985; Pogorevc et al., 2002; Gagnon and Vasudevan, 2011). For instance, a diverse variety of organic solvents have been tested and used in biodiesel production under solvent-free conditions with lipase from various sources (Iso et al., 2001; Aryee et al., 2011; Gagnon and Vasudevan, 2011). Each behaves and interacts with the catalyst differently in addition with other process parameters such as temperature, time, and oil:alcohol molar ratio (Pencreac'h and Baratti, 2001; Aryee et al., 2011). It is thus of particular interest to compare the stability of GML*i* in these solvents to determine which solvent is ideal for use in biodiesel transesterification reaction.

In a previous study (Aryee et al., 2007), the free enzyme showed greater stability in water-immiscible organic solvents (benzene, toluene, hexane, heptane, and iso-octane). While the free enzyme was completely deactivated in 50% (v/v) water-miscible solvents (dimethyl sulfoxide, dimethyl formamide, methanol, iso-propanol, and acetone), the immobilized form in this present study was stable up to 51% in anhydrous water-miscible solvents. Immobilization seems to have protected the enzyme from solvent denaturation. This indicates that solvent penetration and interaction with the enzyme were altered, and inactivation was relieved with immobilization (Iso et al., 2001). These results further confirm that immobilization of GML on o-Sep promoted stronger adsorption interaction with the hydrophobic surface thereby minimizing direct and full lipase-solvent interaction, negative partitioning of the solvent from the enzyme environment, and possible unfolding (Iso et al., 2001; Mateo et al., 2007; Fjerbaek et al., 2009). The extremely low stability of GML*i* in methanol and iso-propanol may be due to the distortion and stripping of the essential ordered layer of water molecules that surrounds the enzyme needed for conformational integrity and stability thereby fractionally inactivating the enzyme and leaving only a partially active enzyme (Zaks and Klibanov, 1985; Pogorevc et al., 2002; Gagnon and Vasudevan, 2011). GML*i* incubated in acetone, petroleum ether, and hexane respectively, retained *ca* 51, 61, and 69% of its activity compared to the control after 1 h

of incubation. Reported activities in organic media are usually lower than those observed in aqueous media (Iso et al., 2001), thus the <1 $R_{O/A}$ (ratio of activity in organic solvent to activity in aqueous solvent) values shown by GML*i* are to be expected. This may be ascribed to the additional reduction in the amount of available water, structural flexibility of the lipase upon immobilization, and the effects of the organic solvents on lipase (Iso et al., 2001; Lima et al., 2004).

While lipases from various sources have been shown to exhibit diverse sensitivity to solvents, the logarithm of the partition coefficient, log *P*, has generally been used to predict their behavior in solvents (Laane et al., 1987; Pogorevc et al., 2002; Gagnon and Vasudevan, 2011). Polar solvents (log $P < 2$) are assumed to be more detrimental to lipase than more hydrophobic solvents ($2 \geq \log P \leq 4$), while solvent with log $P > 4$ are more viscous and less favorable for catalysis. GML*i* incubated in iso-octane (log $P = 4.5$) showed no activity. According to Zaks and Klibanov (1985), polar solvents have greater affinity to water and hence more likely to strip essential water layer that surround the enzyme needed to maintain conformational integrity than non-polar hydrophobic solvents. However some exceptions have been reported further confirming that the effect of organic solvents on enzyme stability differs among lipases, and may be more dependent on the lipase itself and the nature of the solvent (Pencreac'h and Baratti, 2001; Gagnon and Vasudevan, 2011). For instance, *Aspergillus niger* lipase exhibited better activity in acetone than in hexane and heptane (Lima et al., 2004). Some suggested strategies to minimize enzyme inactivation in high polarity media include the stepwise addition of solvents (Soumanou and Bornscheuer 2003; Fjerbaek et al., 2009). The ability of GML*i* to remain active and stable in organic solvents will open opportunities for its use in synthetic reactions.

9.4.4.6. Reusability of GML*i*

As shown in Fig. 9.8a, GML*i* retained respectively, *ca* 88, 79, 62, 49, and 32% of its initial activity after the second, third, fourth, fifth, and sixth cycles. The marked activity retention displayed by GML*i* during the first three cycles implied that minimal desorption of and loss of enzyme from the resin occurred on repeated use. This can be attributed to the strong affinity between the resin and the enzyme produced by the

immobilization procedure. This also indicates that lipase remained adsorbed on the resin after incubation with the substrate during repeated use. While the free enzyme could not be reused after the first cycle because of difficulty in separation, immobilization was an effective way to produce a heterogeneous system which enabled easy separation and recovery from the reaction media for repetitive use. The capacity to reuse the enzyme will decrease the cost of this relatively expensive catalyst.

9.4.4.7. Storage Stability of GML and GML*i*

The effect of storage conditions on the stability and activity of immobilized enzymes is an important aspect of shelf life. In Fig. 9.8b the storage stabilities of GML and GML*i* are shown. GML*i* and GML maintained 92% and 18% of their respective initial activity after 30 d of storage, beyond which GML lost all its activity. The absence of cryoprotectants like glycerol in the extraction buffer may have caused the proteins to denature during freezing and thawing. Glycerol and other protectants such as chelating agents (e.g., ethylenediaminetetraacetic acid and benzamidine hydrochloride) regularly included in extraction buffers which act as stabilizing additives were excluded because these agents are known to interfere and compete with the hydrophobic interaction needed for adsorption (Ryczkowski, 2007). Additionally, proteins are known to be more susceptible to degradation when stored at low concentrations. These results presented in Fig. 9.8b suggest that immobilization improved the stability of the enzyme and helped maintain its activity during longer storage time.

9.5. Conclusion

Grey mullet lipase was not inactivated by the solvents used for delipidation. The hydrolytic activity and stability of immobilized grey mullet lipase (GML*i*) in both aqueous and organic solvents were determined for first time. The hydrophobic interaction between the enzyme and resin, and moderate protein loading may have minimized stearic hindrance during the immobilization of GML on o-Sep. o-Sep has shown to be a good adsorbent and provided a unique means for immobilizing and studying GML. The enhanced activity of GML*i* was attributed to the favorable microenvironment provided by o-Sep for lipase-resin interaction and interfacial activation. Immobilization enhanced the

tolerance of the enzyme to pH and temperature and produced a more thermodynamically efficient catalyst. The study demonstrated the simplicity of the separation and immobilization protocols together with the high thermal stability and activity of GML*i* during repeated use.

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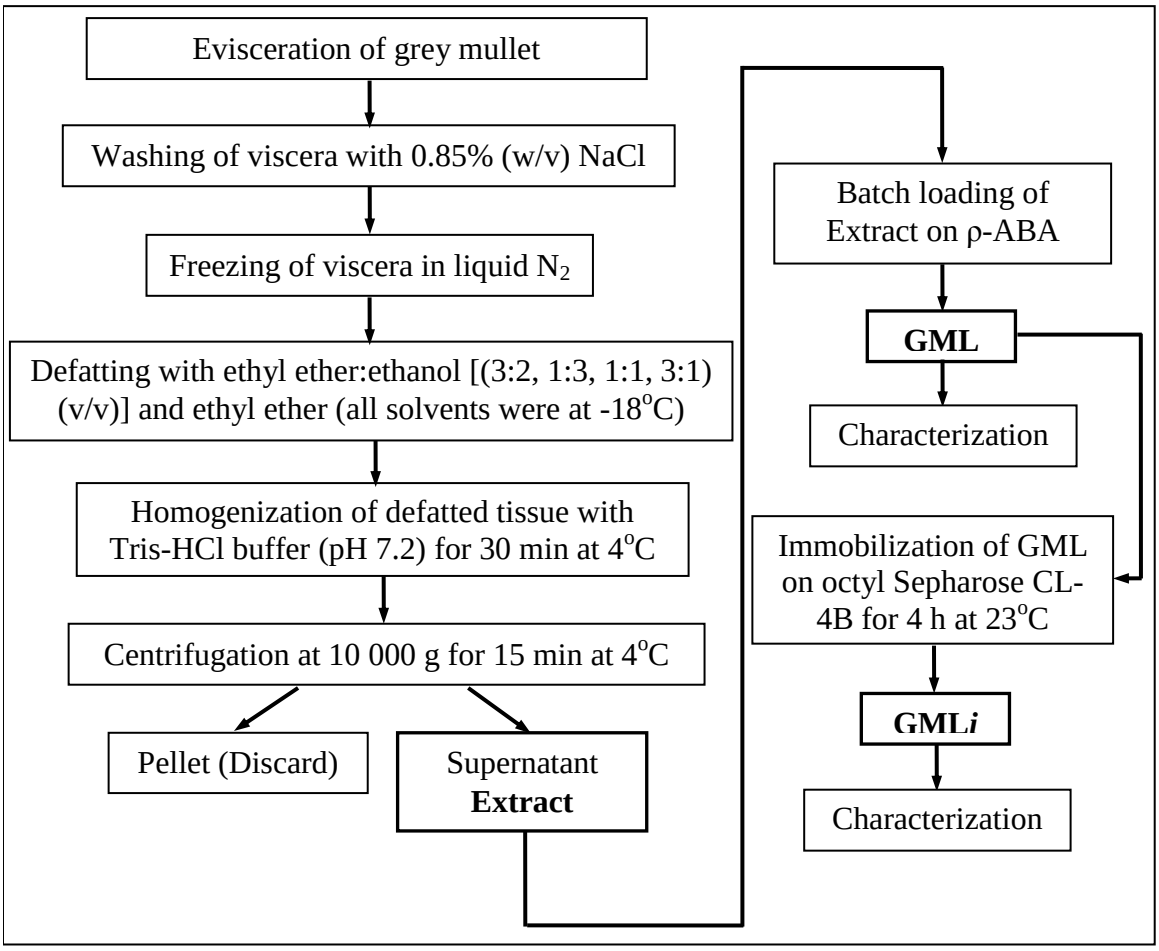

Fig. 9.1: Schematic overview of the extraction, separation, and immobilization procedures.

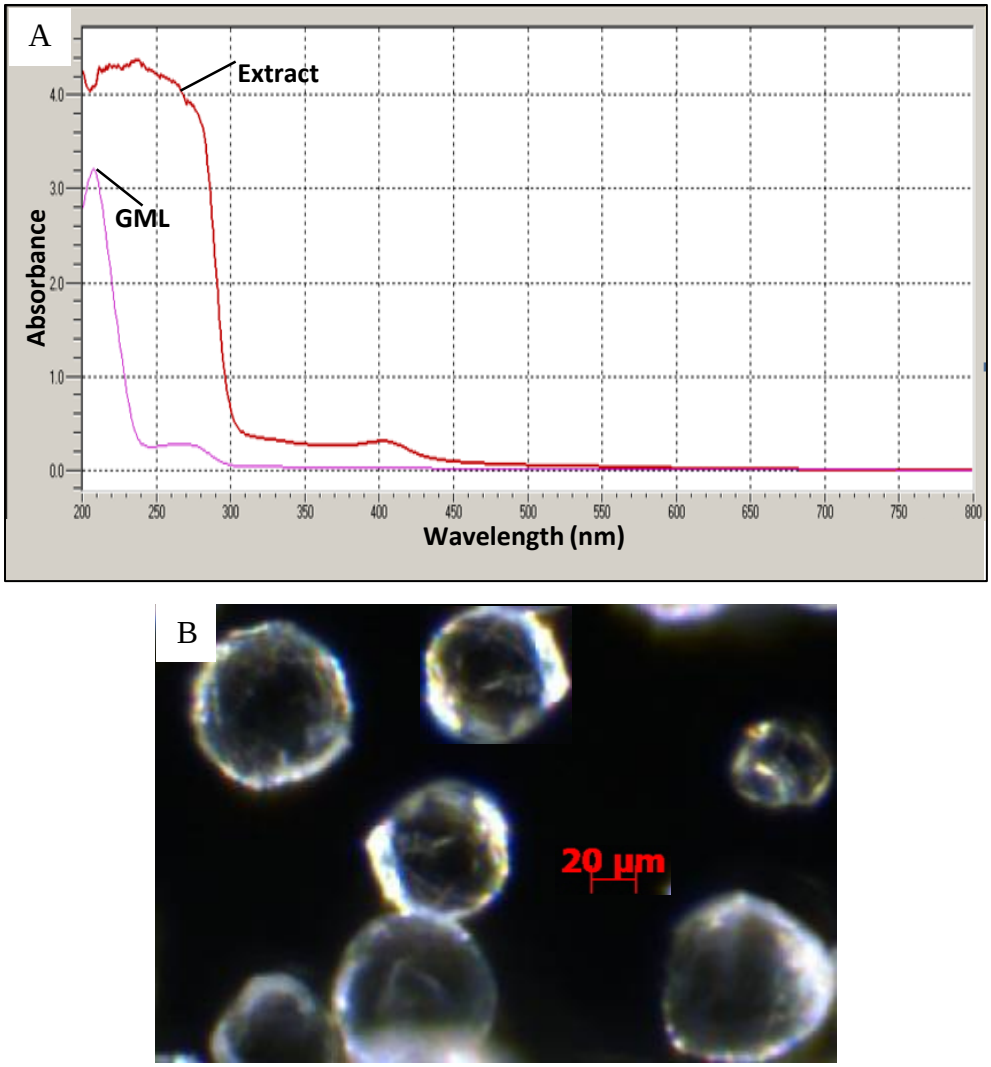

Fig. 9.2: **A)** UV-Vis absorbance spectrum of the crude extract and GML fraction and **B)** Captured image of o-Sep using a Zeiss Discovery.v20 stereomicroscope (magnification 125x).

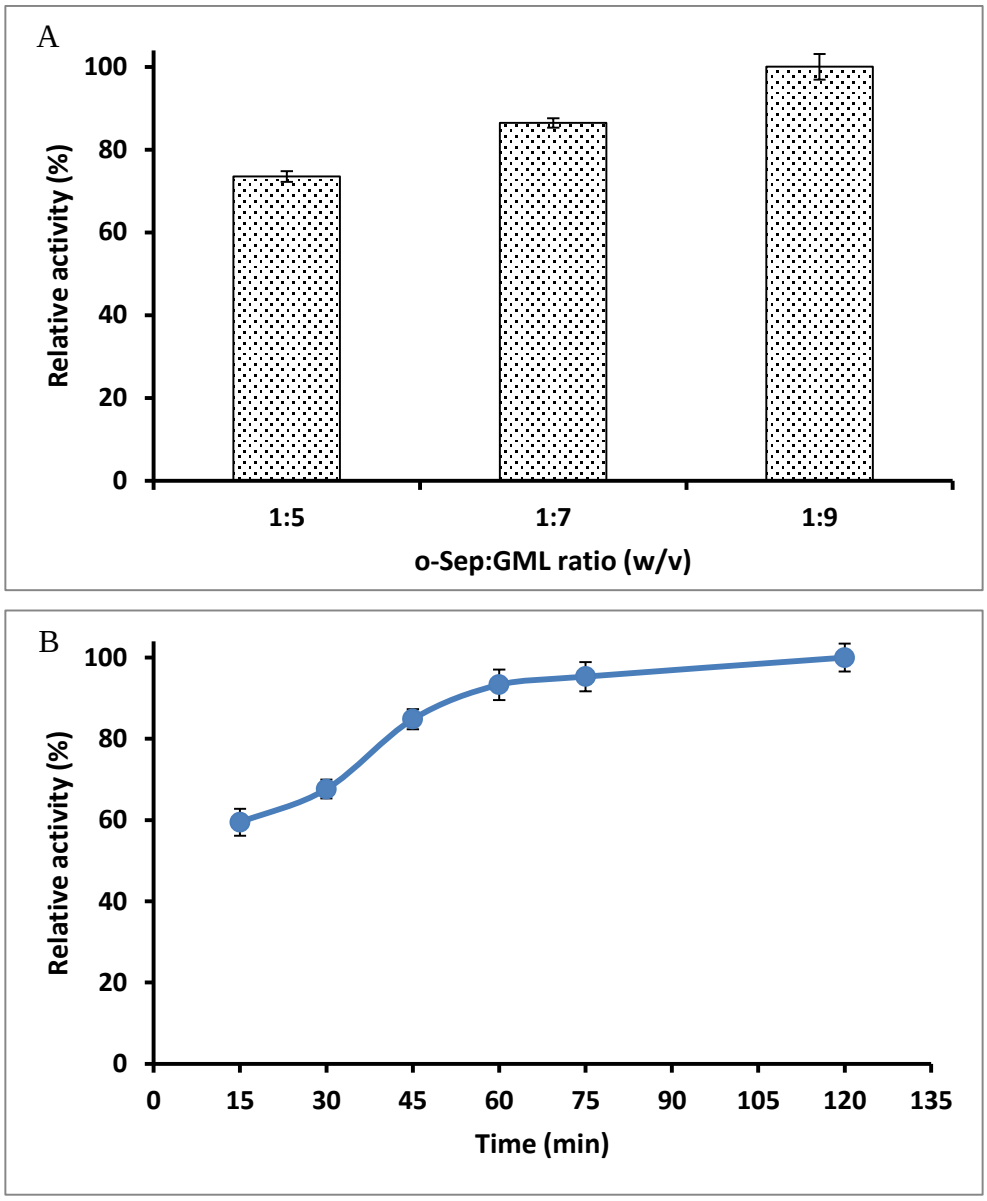

Fig. 9.3: Effect of **A)** enzyme loading and **B)** reaction time on the activity of GML*i*. Activities are relative to the respective measured parameter showing the highest activity.

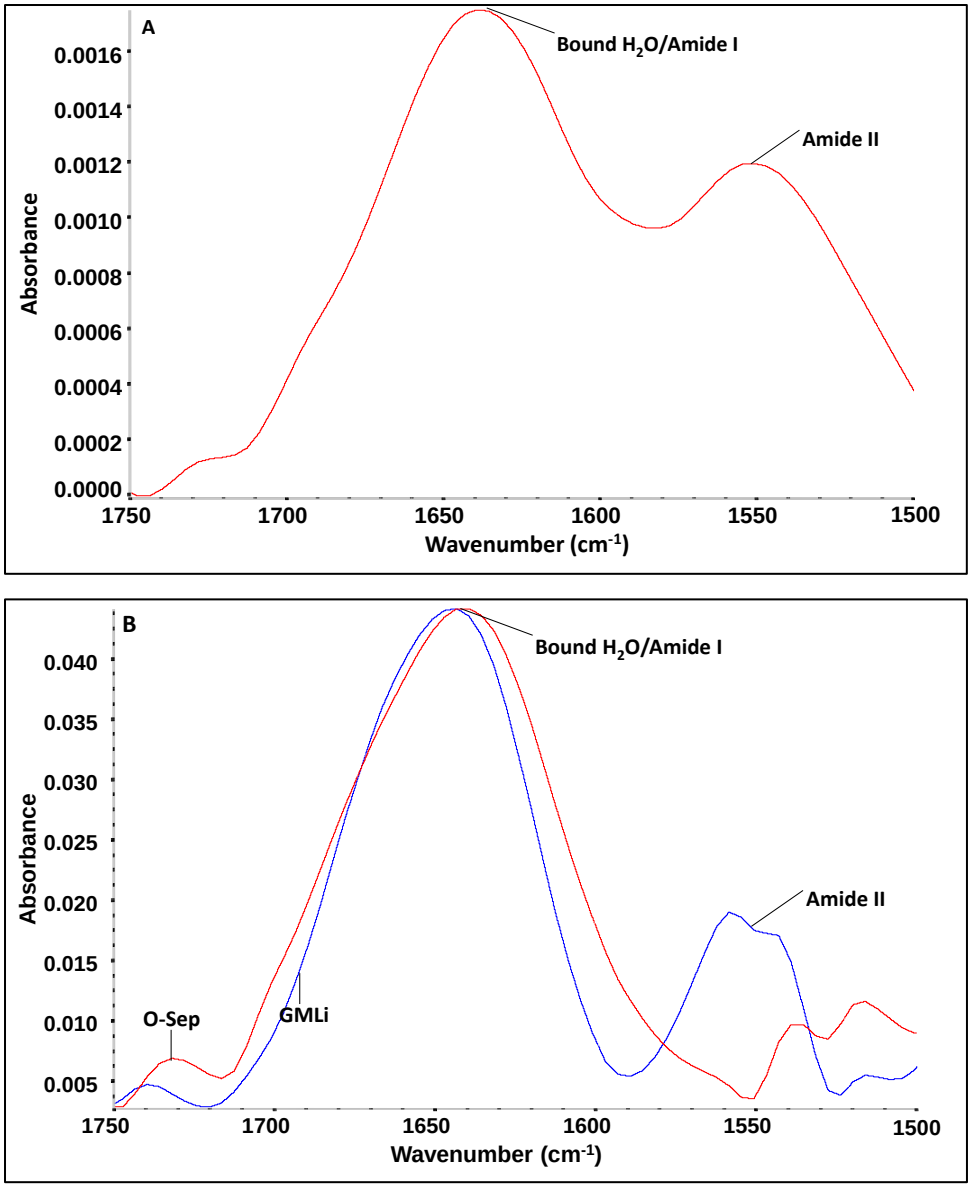

Fig. 9.4: **A)** ATR-FTIR differential spectrum of GML showing the amide I and amide II bands. **B)** ATR-FTIR spectrum of GML*i* after o-Sep subtraction, and o-Sep resin.

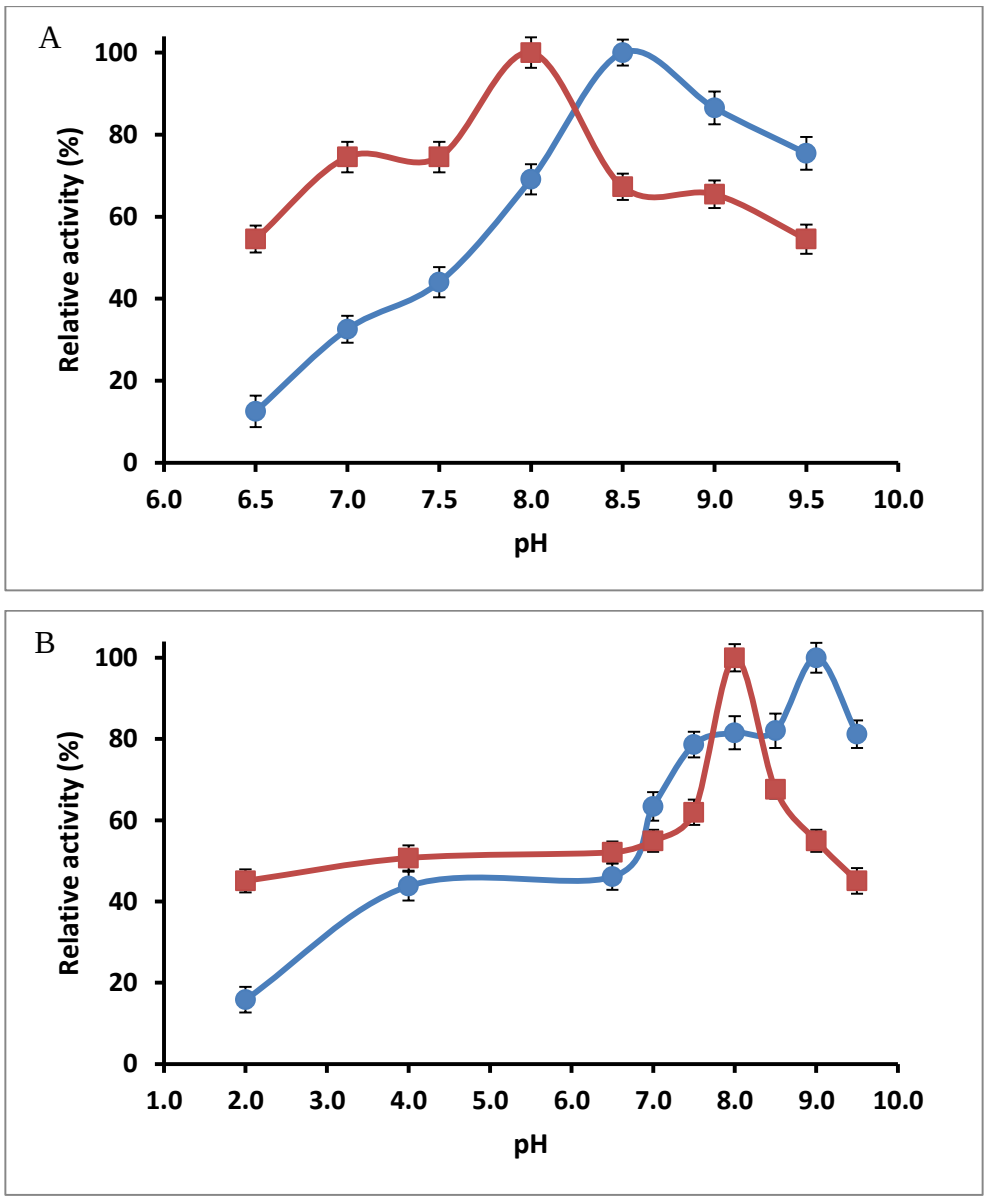

Fig. 9.5: Effect of pH on the **A)** activity and **B)** stability of GML (■) and GML*i* (●). Activities are relative to the respective pH of GML and GML*i* showing the highest activity.

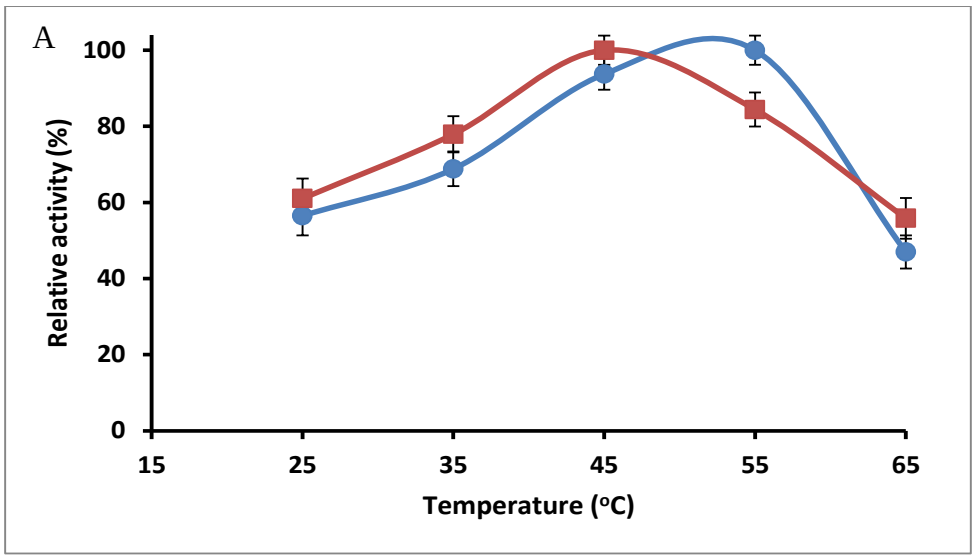

Fig. 9.6A: Effect of temperature on the activity of GML (■) and GML*i* (●). Activities are relative to the respective temperature of GML and GML*i* showing the highest activity.

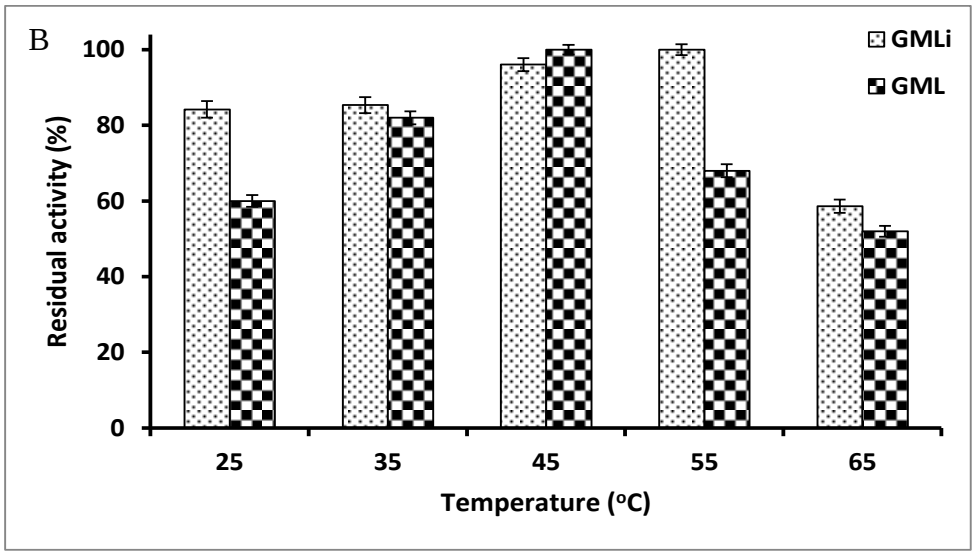

Fig. 9.6B: Thermal stability of GML and GML*i*. Residual activities are relative to the respective temperature of GML and GML*i* showing the highest activity.

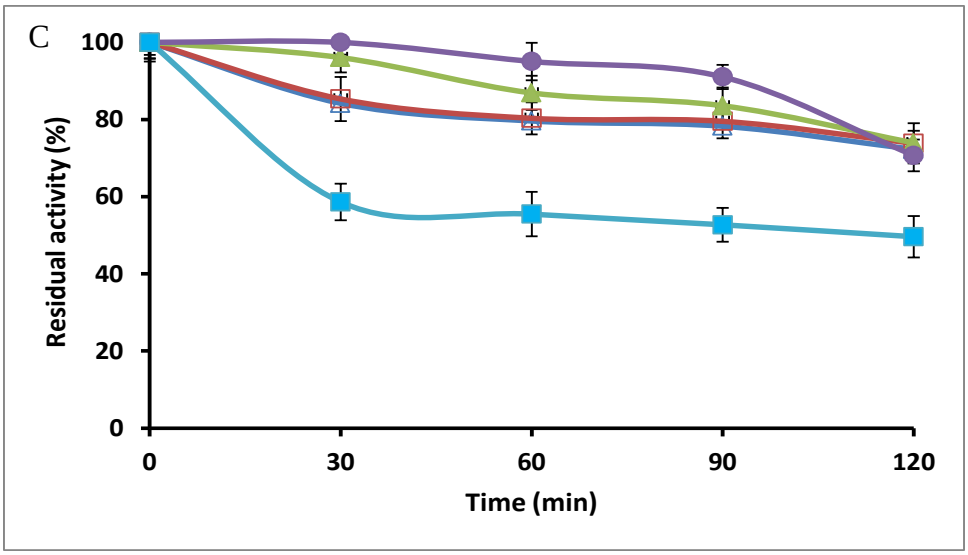

Fig. 9.6C: Effect of temperature at 25°C (Δ), 35°C (□), 45°C (▲), 55°C (●), and 65°C (■) on the stability of GML*i* after 30, 60, 90, and 120 min of incubation. Residual activities are relative to the un-incubated activity of GML*i*.

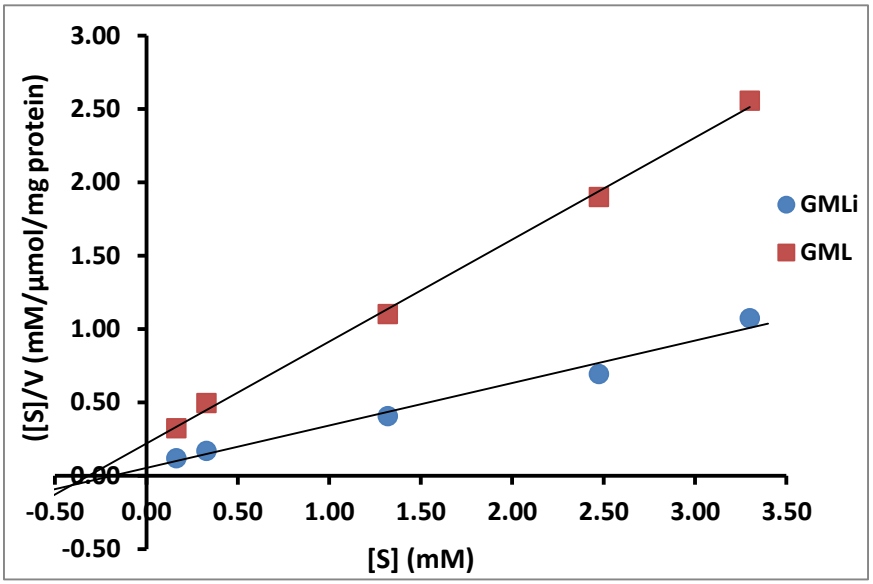

Fig. 9.7: The Hanes-Woolf plot of GML (■) and GML*i* (●).

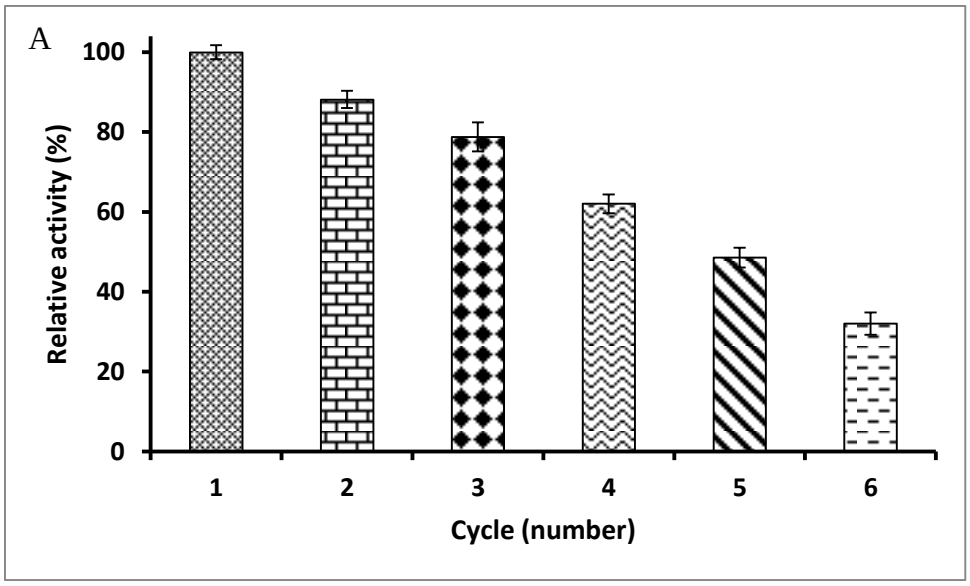

Fig. 9.8A: Operational stability of GML*i*. Activities are relative to the cycle showing the highest activity.

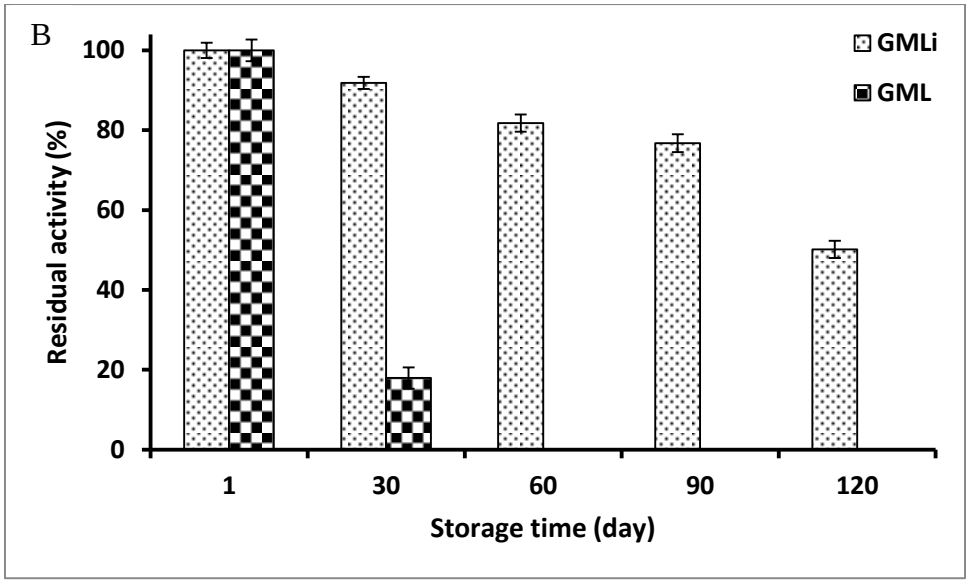

Fig. 9.8B: Stability of GML and GML*i* stored at -18°C after 1, 30, 60, 90, and 180 days. Activities are relative to the storage time showing the highest activity.

Solvent	Log *P*[a]	Type	Residual Activity (%)[b]	$R_{O/A}$[c]
Control[d]	-	Aqueous	-	-
Methanol	-0.76	Alcohol	15.58	0.11
Iso-propanol	-0.28	Alcohol	28.71	0.20
Ethanol	-0.24	Alcohol	42.02	0.29
Acetone	-0.23	Ketone	74.08	0.51
Butanol	0.84	Alcohol	50.62	0.35
Petroleum Ether	3.50	Ether	89.40	0.61
Hexane	3.76	Hydrocarbon	100	0.69
Iso-octane	4.50	Hydrocarbon	-	-

Table 9.1: Stability of GML*i* in organic solvents.

[a]Log *P* is the logarithm of partition coefficient of a solvent between 1-octanol and water.

[b]Residual activity (%) is relative to the activity of hexane.

[c]$R_{O/A}$ is the ratio of activity in organic media to that in aqueous media.

[d]Control standard assay.

CHAPTER X

GENERAL CONCLUSION, CONTRIBUTION TO KNOWLEDGE AND RECOMMENDATION FOR FUTURE WORK

10.1. General Conclusion

An efficient oil recovery scheme for salmon skin was presented and the salmon skin oil (SSO) obtained was identified as an alternative non-food biodiesel (BD) feedstock. The yield of SSO was dependent on solvent type, extraction time and temperature. At 25^{o}C, neither hexane nor petroleum ether was a good solvent for SSO extraction but at their respective boiling point their efficiency to extract SSO increased appreciably. Three hours was an adequate extraction time with the Soxhlet technique to obtain high yields of SSO, while longer extraction times led to no significant increase in the amount of SSO recovered. The results from the Soxtec extraction technique indicated significant reduction in extraction time, and solvent volume, with higher oil yields compared to the Soxhlet technique. Higher amount of oil was recovered when hexane was used as solvent. It was also safer to handle and cheaper in comparison with petroleum ether.

To monitor the stability of SSO intended for use as BD feedstock, the effects of storage temperature and time were studied to devise ways to minimize oxidation, and quality loss, and extend its shelf life. Storage temperature and time affected the hydrolysis and oxidation of SSO as indicated by the changes in free fatty acid (FFA), peroxide value (PV), and thiobarbituric acid reactive substances (TBARS). FFA, PV, and TBARS generally increased with storage temperature and time, while the fatty acid (FA) composition was not significantly affected. Recovered SSO could be stored for up to 45 days at either -18^{o}C or -80^{o}C with minimal increases and variations in FFA, PV, and TBARS.

The sodium hydrogen cyanamide (NaHNCN)-based Fourier Transform infrared (FTIR) spectroscopic method was adapted and enhanced through modifications for a rapid and environmentally friendly approach to measure FFA content in crude fish oil. This study demonstrated that the method, originally developed to measure the low FFA levels in refined edible oils, can readily be modified to cover a wider range of FFA

content such as in unrefined fish oil. A linear increase from 0.6% to 4.5% in FFA content was observed when SSO was stored over 120 days. The method is a flexible and viable alternative to the American Oil Chemists' Society (AOCS) titrimetric method.

The potential of processing waste from fishery (SSO) and animal origin (fat from poultry, pork, and beef-Rothsay composite) as feedstocks to produce fatty acid alkyl esters (FAAE) for use as BD was demonstrated. In this preliminary three-factor mixed analysis study, significant main effects of the experimental trial and all interactions were revealed. The result also indicated that FAEE yield significantly differs throughout the course of the reaction and is dependent on temperature, oil:alcohol molar ratio, and reaction time. The highest BD yield was *ca* 50% after 96 h of the transesterification reaction at 25^{o}C, using an oil:alcohol molar ratio of 1:4, and an enzyme load of 21.7 U.

Based on the results from the preliminary FAEE production study, the process was optimized by the application of response surface methodology (RSM) using central composite rotatable design (CCRD). CCRD proved to be an effective experimental design to model the effects of temperature, enzyme load, and oil:alcohol molar ratio on FAEE yield. The optima were relatively easily located and a large amount of the variation was explained by the fitted response surface models. The second-order polynomial function provided a good description of the relationships between the reaction variables and the response. The three independent variables studied had varying effects on FAEE yield, depending on reaction time. SSO-BD yield increase by *ca* 38% after optimization, using an enzyme load of 39.06 U, oil:alcohol ratio of 1:2 after 48 h of reaction at 50^{o}C. The methodology also allowed yield prediction by interpolation over the experimental domain. With a reduced number of combinations of values of the independent variables in the CCRD, as much information as in a full factorial design was concisely generated.

A reliable and fast methodology to provide both qualitative and quantitative information about the BD and the other reaction components were achieved in a single high performance liquid chromatography (HPLC) run and monitored on a refractive index (RI) detector. The HPLC method for the analysis of triacylglycerols (TAG), monoacylglycerols (MAG), diacylglycerols (DAG), FAAE, and alcohol, identified each analyte by their retention time. The retention time was determined by the analysis of

known reference standards. Reference standards were also used to generate a five-level calibration curve which related the RI response to mg/ml concentration in the analytes. The calibration curves presented demonstrated excellent linearity with a $R^2 > 0.99$ for each analyte. This demonstrated a simple and practical HPLC technique for unambiguous identification and quantitation of reaction components as well as residuals such as partial glycerides and alcohol, unreacted TAG, and FFA, which were produced during the transesterification reaction and have tendencies to contaminate the fuel.

Process-related fuel testing of quality determinants of the transesterified samples revealed various proportions of total and bound glycerol as well as residual TAG, MAG, and DAG. The high glycerol content is indicative of an incomplete reaction and unwashed transesterified BD. Although some of the ASTM D6751 specifications for fuel were not met, the BD made could be blended with petrodiesel or used in other applications such in boiler or as home heating oil where they are permitted.

In line with the goal of maximizing the use of processing waste and develop alternative sources of biomaterials, lipase was recovered from the viscera of grey mullet and immobilized on octyl-Sepharose CL-4B (o-Sep) to enhance its usability in catalysis. Using an alternative combination of solvents, grey mullet lipase was not inactivated during delipidation. Lipase was selectively adsorbed on o-Sep achieving a concerted single step purification-immobilization-stabilization-activation. Using this protocol strongly bound but non-distorted immobilized lipase was obtained which was suitable for application under a wide range of conditions (pH, temperature, and organic solvents), and reusable without significant risk of enzyme desorption. Immobilization enhanced the tolerance of the enzyme in both water- miscible and immiscible solvents, pH and temperature, and resulted in a more thermodynamically efficient catalyst. The enhanced activity and stability, and reusability of GML*i* were attributed to an efficient hydrophobic interaction between the enzyme and o-Sep, a favorable microenvironment provided by resin, and interfacial activation.

10.2. Contribution to Knowledge

1. A comparative study on the effects of various solvent systems and extraction variables on the recovery of salmon skin oil was carried out for the first time.

2. A new mid-infrared (MIR) FTIR method was developed by the modification of an existing method and applied for the first time to determine the wide range of FFA content in unrefined fish oil.
3. A study on Lipozyme®-IM-catalyzed transesterification of solvent-recovered salmon skin oil and a commercial blend of yellow grease and rendered animal fat to biodiesel, and detailed assessment of reaction intermediates and residuals were carried out for the first time.
4. This is the first study on the optimization of the lipase-catalyzed transesterification of salmon skin oil, and a commercial blend of yellow grease and rendered animal fat to biodiesel.
5. Fuel testing of lipase-catalyzed transesterified salmon skin oil and a commercial blend of yellow grease and rendered animal fat was carried out for the first time.
6. An alternative combination of polar and non-polar solvents was described and used to defat isolated grey mullet viscera without inactivating the proteins. Lipase from grey mullet viscera was immobilized and characterized for the first time and its hydrolytic activity and stability in both aqueous and organic solvents as well as thermal properties were also determined for the first time.

10.3. Recommendation for Future Work

1. Modification of the transesterification reaction protocol to reduce residual contents of TAG, DAG, and MAG in the final product, and incorporate a nominal washing step to remove the alcohol and free glycerol to meet the specifications of ASTM D6751.
2. Based on the stability GML*i* in organic solvents, further studies are required to optimize GMLi-catalyzed transesterification reactions to BD by varying parameters like; alcohol type, oil:alcohol molar ratio, solvent, reaction temperature and time.
3. A search for a cheaper and alternative support. Although o-Sep proved to be an excellent support for immobilizing GML, it is very expensive, thus large scale production of GML*i* will be expected to be proportionally costly. Further research is justified on alternative supports which provide similar microenvironment as o-Sep for enzyme-support interaction, activation, and catalytic efficiency.

REFERENCES

Abdulkarim, S. M., Lai, O. M., Muhammad, S. K. S., Long, K. and Ghazali, H. M. (2006). Use of enzymes to enhance oil recovery during aqueous extraction of *Moringa oleifera* seed oil. J. Food Lipids 13(2): 113-130.

Abigor, R., Uadia, P., Foglia, T., Haas, M., Jones, K., Okpefa, E., Obibuzor, J. and Bafor, M. (2000). Lipase-catalysed production of biodiesel fuel from some Nigerian lauric oils. Biochem. Soc. Trans. 28: 979-981.

Adhikari, S., Fernando, S. D. and Haryanto, A. (2008). Hydrogen production from glycerin by steam reforming over nickel catalysts. Renew. Energ. 33(5): 1097-1100.

Adreani, P. (2007). Government policy and tax situations worldwide: South American perspective. International Congress on Biodiesel: The Science and the Technologies. Vienna, Austria, 5-7 November, 2007.

AIDEA 2002. Alaska Industrial Development and Export Authority, UniSea fish oil demonstration project yields positive results. (Accessed: 05/19/11). http://www.akenergyauthority.org/PDF%20files/Unisea.pdf.

Aidos, I., Lourenço, S., Van der Padt, A., Luten, J. B. and Boom, R. M. (2002). Stability of crude herring oil produced from fresh byproducts: Influence of temperature during storage. J. Food Sci. 67(9): 3314-3320.

Aidos, I., van derPadt, A., Boom, R. M. and Luten, J. B. (2001). Upgrading of maatjes herring byproducts: Production of crude fish oil. J. Agric. Food Chem. 49: 3697-3704.

Akaike, H. (1987). Factor analysis and AIC. Psychometrika 52: 317-320.

Akaranta, O. and Anusiem, A. C. I. (1996). A bioresource solvent for extraction of castor oil. Ind. Crops Prodt. 5: 273-277.

Akoh, C. C., Chang, S. W., Lee, G. C. and Shaw, J. F. (2007). Enzymatic approach to biodiesel production. J. Agric. Food Chem. 55(22): 8995-9005.

Al-Alawi, A., van de Voort, F. R. and Sedman, J. (2004). New FTIR method for the determination of FFA in oils. J. Am. Oil Chem. Soc. 81: 441-446.

Al-Alawi, A., van de Voort, F. R. and Sedman, J. (2006). Automated of FTIR analysis: Automated quantification of moisture or FFA in edible oils. J. Lab Autom. 11(2): 23-29.

Alcantara, R., Amores, J., Canoira, L., Fidalgo, E., Franco, M. J. and Navarro, A. (2000). Catalytic production of biodiesel from soy-bean oil, used frying oil and tallow. Biomass Bioenerg. 18: 515-527.

Allen, C. A. W., Watts, K. C., Ackman, R. G. and Pegg, M. J. (1999). Predicting the viscosity of biodiesel fuels from their fatty acid ester composition. Fuel 78(11): 1319-1326.

Amigun, B., Sigamoney, R. and von Blottnitz, H. (2008). Commercialisation of biofuel industry in Africa: A review. Renew. Sust. Energ. Rev. 12(3): 690-711.

Andersen, O. and Weinbach, J.-E. (2010). Residual animal fat and fish for biodiesel production: Potentials in Norway. Biomass Bioenerg. 34(8): 1183-1188.

Anonymous, (2002). Isohexane: Likely choice for crushers seeking to replace n-hexane, Inform 13(4): 282-286.

Antczak, M. S., Kubiak, A., Antczak, T. and Bielecki, S. (2009). Enzymatic biodiesel synthesis-key factors affecting efficiency of the process. Renew. Energ. 34: 1185-1194.

AOCS (Ca 2c-25, Ca 5a-40, Ca 8-53). Official methods and recommended practices of the American Oil Chemists' Society (5th ed.), AOCS press, Champaign.

AP 2008. http://www.msnbc.msn.com/id/23321510. Virgin Atlantic flies biofuel-powered jumbo jet. The Associated Press, Feb, 2008. (Accessed: 03/25/09).

Arthur, R., Baidoo, M. F. and Antwi, E. (2011). Biogas as a potential renewable energy source: A Ghanaian case study. Renew. Energ. 36(5): 1510-1516.

Aryee, A. N. A. and Simpson, B. K. (2009). Comparative studies on the yield and quality of solvent-extracted oil from salmon skin. J. Food Eng. 92(3): 353-358.

Aryee, A. N. A., Simpson, B. K. and Villalonga, R. (2007). Lipase fraction from the viscera of grey mullet (*Mugil cephalus*): Isolation, partial purification and some biochemical characteristics. Enzyme Microb. Tech. 40(3): 394-402.

Aryee, A. N. A., Simpson, B. K., Cue, R. I. and Phillip, L. E. (2011). Enzymatic transesterification of fats and oils from animal discards to fatty acid ethyl esters for potential fuel use. Biomass Bioenerg. 35(10): 4149-4157.

Aryee, A. N. A., van de Voort, F. R. and Simpson, B. K. (2009). FTIR determination of free fatty acids in fish oils intended for biodiesel production. Process Biochem. 44: 401-405.

Arzamendi, G., Arguiñarena, E., Campo, I. and Gandía, L. M. (2006). Monitoring of biodiesel production: Simultaneous analysis of the transesterification products using size-exclusion chromatography. Chem. Eng. J. 122(1-2): 31-40.

ASTM D6751-11. Standard specification for biodiesel fuel (B100) blend stock for distillate fuels. http://www.astm.org/Standards/D6751.htm (Accessed: 09/16/11).

Atadashi, I. M., Aroua, M. K., Abdul Aziz, A. R. and Sulaiman, N. M. N. (2011). Refining technologies for the purification of crude biodiesel. Appl. Energ. 88(12): 4239-4251.

Aursand, M., Bleivik, B., Rainuzzo, J. R., Jørgensen, L. and Mohr, V. (1994). Lipid distribution and composition of commercially farmed Atlantic salmon (*Salmo salar*). J. Sci. Food Agric. 64: 239-248.

Bastida, A., Sabuquillo, P., Armisen, P., Fernández-Lafuente, R., Huguet, J. and Guisán, J. M. (1998). A single step purification, immobilization, and hyperactivation of lipases via interfacial adsorption on strongly hydrophobic supports. Biotechnol. Bioeng. 58(5): 486-493.

Behçet, R. (2011). Performance and emission study of waste anchovy fish biodiesel in a diesel engine. Fuel Process. Technol. 92(6): 1187-1194.

Benjakul, S., Visessanguan, W. and Tanaka, M. (2005). Properties of phenoloxidase isolated from the cephalothorax of kuruma prawn (*Penaeus japonicus*). J. Food Biochem. 29: 470-485.

Berchmans, H. J. and Hirata, S. (2008). Biodiesel production from crude *Jatropha curcas* L. seed oil with a high content of free fatty acids. Bioresour. Technol. 99(6): 1716-1720.

Bernard, F. and Prieur, A. (2007). Biofuel market and carbon modeling to analyse French biofuel policy. Energ. Policy. 35(12): 5991-6002.

Bernardes, O. L., Bevilaqua, J. V., Leal, M. C. M., Freire, D. M. G. and Langone M. A. P. (2007). Biodiesel fuel production by the transesterification reaction of soybean oil using immobilized lipase. Appl. Biochem. Biotechnol. 137-140: 105-114.

Berrios, M. and Skelton, R. L. (2008). Comparison of purification methods for biodiesel. Chem. Eng. J. 144(3): 459-465.

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S. and Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta 76(5): 965-977.

BIOBUS 2003. BIOBUS project-final report. http://www.stm.info/english/info/a-biobus-final.pdf (Accessed: 05/25/11).

Bledsoe, G. E., Bledsoe, C. D. and Rasco, B. (2003). Caviars and fish roe products. Crit. Rev. Food Sci. Nutr. 43(3): 317-356.

Bligh, E. G. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Phys. 37(8): 911-917.

Blout, E. R., De Lozb, C. and Asadourian, A. (1961). The deuterium exchange of water-soluble polypeptides and proteins as measured by infrared spectroscopy. J. Am. Chem. Soc. 83(8): 1895-1900.

Boran, G., Karaçam, H. and Boran, M. (2006). Changes in the quality of fish oils due to storage temperature and time. Food Chem. 98: 693-698.

Bower, C. K., Hietala, K. A., Oliveira, A. C. M. and Wu, T. H. (2009). Stabilizing oils from smoked pink salmon (*Oncorhynchus gorbuscha*). Food Chem. 74: C248-C257.

Bradford, M. M. (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Brennan, L. and Owende, P. (2010). Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. Renew. Sust. Energ. Rev. 14(2): 557-577.

Campbell, P. K., Beer, T. and Batten, D. (2011). Life cycle assessment of biodiesel production from microalgae in ponds. Bioresour. Technol. 102(1): 50-56.

Cañada, M. J. A., Medina, A. R. and Lendl, B. (2001). Determination of free fatty acids in edible oils by continuous-flow analysis with FT-IR spectroscopic detection. Appl. Spectrosc. 55: 356-360.

Canakci, M. (2007). The potential of restaurant waste lipids as biodiesel feedstocks. Bioresour. Technol. 98(1): 183-190.

Canakci, M. and Gerpen, J. V. (2001). Biodiesel production from oils and fats with high free fatty acids. Trans ASAE 44(6): 1429-1436.

Canakci, M., Ozsezen, A. N., Arcaklioglu, E. and Erdil, A. (2009). Prediction of performance and exhaust emissions of a diesel engine fueled with biodiesel produced from waste frying palm oil. Expt. Syst. Appl. 36(5): 9268-9280.

Cantrell, K. B., Ducey, T., Ro, K. S. and Hunt, P. G. (2008). Livestock waste-to-bioenergy generation opportunities. Bioresour. Technol. 99: 7941-7953.

Catchpole, T. L., Frid, C. L. J. and Gray, T. S. (2005). Discards in North Sea fisheries: Causes, consequences and solutions. Mar. Policy 29(5): 421-430.

Chantachum, S., Benjakul, S. and Sriwirat, N. (2000). Separation and quality of fish oil from precooked and non-precooked tuna heads. Food Chem. 69(3): 289-294.

Chen, J. W. and Wu, W. T. (2003). Regeneration of immobilized *Candida antarctica* lipase for transesterification. J. Biosci. Bioeng. 95(5): 466-469.

Chongkhong, S., Tongurai, C., Chetpattananondh, P. and Bunyakan, C. (2007). Biodiesel production by esterification of palm fatty acid distillate. Biomass Bioenerg. 31(8): 563-568.

Christie, W. W. (1992). Preparation of fatty acid methyl esters. Inform 3: 1031-1034.

Cochran, W. G. and Cox, G. M. (1992). Experimental designs. John Wiley and Sons, New York.

Dahle, L. K., Hill, E. G. and Holman, R. (1962). The thiobarbituric acid reaction and the autoxidations of polyunsaturated fatty acid methyl esters. Arch. Biochem. Biophys. 98: 253-261.

Darnoko, D., Cheryan, M. and Perkins, E. G. (2000). Analysis of vegetable oil transesterification products by gel permeation chromatography. J. Liq. Chromatogr. Rel. Technol. 23(15): 2327-2335.

de Boer, J. (1988). Chlorobiphenyls in bound and non-bound lipids of fishes: Comparison of different extraction methods. Chemosphere 17: 1803-1810.

Delorme, V., Dhouib, R., Canaan, S., Fotiadu, F., Carrière, F. and Cavalier, J.-F. (2011). Effects of surfactants on lipase structure, activity, and inhibition. Pharm. Res. 28: 1831-1842.

Demirbas, A. (2003). Biodiesel fuels from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterification and other methods: A survey. Energ. Convers. Manage. 44: 2093-2109.

Demirbas, A. (2005). Biodiesel production from vegetable oils via catalytic and non-catalytic supercritical methanol transesterification methods. Prog. Energ. Combust. 31: 466-487.

Demirbas, A. (2007). Biodiesel from sunflower oil in supercritical methanol with calcium oxide. Energ. Convers. Manage. 48(3): 937-941.

Demirbas, A. (2008). Comparison of transesterification methods for production of biodiesel from vegetable oils and fats. Energ. Convers. Manage. 49(1): 125-130.

Demirbas, A. (2009). Progress and recent trends in biodiesel fuels. Energ. Convers. Manage. 50(9): 2239-2249.

Derewenda, U., Brozowski, A. M., Lawson, D. M. and Derewenda, Z. S. (1992). Catalysis at the interface: The anatomy of a conformational change in a triglyceride lipase. Biochem. 31: 1532-1541.

De-Simone, S. G., Correa-Netto, C., Antunes, O. A. C., De-Alencastro, R. B. and Silva, Jr. F. P. (2005). Biochemical and molecular modeling analysis of the ability of two *p*-aminobenzamidine-based sorbents to selectively purify serine proteases (fibrinogenases) from snake venoms. J. Chromatrogr. B. 822(1-2): 1-9.

DFO 2008. Department of Fisheries and Oceans Canada (DFO). (Accessed 11/12/10). http://www.dfo-mpo.gc.ca/stats/commercial/land-debarq/sea-maritimes/s2008pq-eng.htm. http://www.dfo-mpo.gc.ca/stats/aqua/aqua08-eng.htm.

Dizge, N. and Keskinlera, B. (2008). Enzymatic production of biodiesel from canola oil using immobilized lipase. Biomass Bioenerg. 32(12): 1274-1278.

Dizge, N., Aydiner, C., Imer, D. Y., Bayramoglu, M., Tanriseven, A. and Keskinler, B. (2009). Biodiesel production from sunflower, soybean, and waste cooking oils by

transesterification using lipase immobilized onto a novel microporous polymer. Bioresour. Technol. 100: 1983-1991.

Dossat, V., Combes, D. and Marty, A. (1999). Continuous enzymatic transesterification of high oleic sunflower oil in a packed bed reactor: Influence of the glycerol production. Enzyme Microb. Tech. 25: 194-200.

Du, W., Liu, D., Li, L. and Dai, L. (2007). Mechanism exploration during lipase-mediated methanolysis of renewable oils for biodiesel production in a *tert*-butanol system. Biotechnol. Progr. 23(5): 1087-1090.

Du, W., Wang, L. and Liu, D. (2007). Improved methanol tolerance during Novozym 435-mediated methanolysis of SODD for biodiesel production. Green Chem. 9(2): 173-176.

Du, W., Xu, Y., Liu, D. and Zeng, J. (2004). Comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors. J. Mol. Catal B: Enzym. 30(3-4): 125-129.

Dubé, M. A., Zheng, S., McLean, D. D. and Kates, M. A. (2004). Comparison of attenuated total reflectance-FTIR spectroscopy and GPC for monitoring biodiesel production. J. Am. Oil Chem. Soc. 81(6): 599-603.

Dubois, J., van de Voort, F. R., Sedman, J., Ismail, A. A. and Ramaswamy, H. (1996). Quantitative FTIR analysis of anisidine value and aldehydes in thermally stressed oils. J. Am. Oil Chem. Soc. 73: 788-794.

Duffy, J. E., Canuel, E. A., Adey, W. and Swaddle, J. P. (2009). Biofuels: Algae. Science 326: 1345.

Dunford, N. T., Temelli, F. and Leblanc, E. (1997). Supercritical CO_2 extraction of oil and residual proteins from Atlantic mackerel (*Scomber scombrus*) as affected by moisture content. J. Food Sci. 62(2): 289-294.

Dunn, R. O. (2005). Cold weather properties and performance of biodiesel. In: The biodiesel handbook. Knothe, G., Van Gerpen, J. and Krahl, J. (eds), AOCS press, Champaign, Pg. 76-80.

Dunn, R. O. (2005). Other alternative diesel fuels from vegetable oils. In: The biodiesel handbook. Knothe, G., Van Gerpen, J. and Krahl, J. (eds), AOCS press, Champaign, Pg. 241-263.

Ekanayake, P. M., Park, G. T., Lee, Y. D., Kim, S. J., Jeong, S. C. and Lee, J. (2005). Antioxidant potential of eel (*Anguilla japonica* and *Conger myriaster*) flesh and skin. J. Food Lipids 12: 34-47.

EN14214:2003. http://www.biodiesels.com.br/docs/biodiesel_for_europe.pdf (Accessed 10/26/10).

Erlanger, B. F., Kokowsky, N. and Colen, W. (1961). The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95: 271-278.

Esquível, M. M., Bandarra, N. M., Fontan, I., Bernardo-Gil, M. G., Batista, I., Nunes, M. L. and Empis, J. A. (1997). Supercritical carbon dioxide extraction of sardine (*Sardina pilchardus*) oil. Lebensm.-Wiss. u.-Technol. 30: 715-720.

Falch, E., Aursand, M. and Rustad, T. (2006a). By-product from gadiform species as raw materials for the production of marine lipids as ingredients in food or feed. Process Biochem. 41: 666-674.

Falch, E., Rustad, T., Jonsdottir, R., Shaw, N. B., Dumay, J., Berge, J. P., Arason, S., Kerry, J., Sandbakk, M. and Aursand, M. (2006b). Geographical and seasonal differences in lipid composition and relative weight of by-product from *Gadidae* species. J. Food Comp. Anal. 18(6-7): 727-736.

Fan, X., Burton, R. and Austic, G. (2010). Preparation and characterization of biodiesel produced from fish oil. Chem. Technol. Fuels Oils 46(5): 287-293.

FAO 2008. Highlights of special studies. (Accessed: 06/02/11). ftp://ftp.fao.org/docrep/fao/011/i0250e/i0250e03.pdf.

FAO 2010, FAOSTAT. Statistics and Information Service of the Fisheries and Aquaculture Department. FAO yearbook. Fishery and Aquaculture Statistics. 2008/FAO. Rome, FAO. 2010. 72p. (Accessed: 05/20/11). http://www.fao.org/docrep/013/i1890t/i1890t.pdf.

FAO 1986. Trends in capture fisheries production, Yearbook of Fishery Statistics, vol. 98/1, Capture Production 2004. FAO, Rome p. 560p.

FAO-Food and Agriculture Organization. The state of world fisheries and aquaculture. Rome; 2008. http://www.fao.org/docrep/011/i0250e/i0250e00.HTM (Accessed 06/28/10).

Fernandez-Lafuente, R. (2010). Lipase from *Thermomyces lanuginosus*: Uses and prospects as an industrial biocatalyst. J. Mol. Catal. B: Enzym. 62(3-4): 197-212.

Fernández-Lorente, G., Betancor, L., Carrascosa, A. V. and Guisán, J. M. (2011). Release of omega-3 fatty acids by the hydrolysis of fish oil catalyzed by lipases immobilized on hydrophobic supports. J. Am. Oil Chem. Soc. 88(8): 1173-1178.

Fernandez-Lorente, G., Palomo, J. M., Cabrera, Z., Fernandez-Lafuente, R., Guisáán, J. M. (2007). Improved catalytic properties of immobilized lipases by the presence of very low concentrations of detergents in the reaction medium. Biotechnol. Bioeng. 97(2): 242-250.

Fernando, S., Karra, P., Hernandez, R. and Jha, S. K. (2007). Effect of incompletely converted soybean oil on biodiesel quality. Energ. 32: 844-851.

Fillières, R., Benjelloun-Mlayah, B. and Delmas, M. (1995). Ethanolysis of rapeseed oil: quantitation of ethyl esters, mono-, di-, and triglycerides and glycerol by high-performance size-exclusion chromatography. J. Am. Oil Chem. Soc. 72: 427-432.

Fincan, M. and Dejmek, P. (2002). In situ visualization of the effect of a pulsed electric field on plant tissue. J. Food Eng. 55: 223-230.

Fjerbaek, L., Christensen, K. V. and Norddahl, B. (2009). A review of the current state of biodiesel production using enzymatic transesterification. Biotechnol. Bioeng. 102(5): 1298-1315.

Folch, J., Less, M. and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497-509.

Freedman, B., Pryde, E. H. and Mounts, T. L. (1984). Variables affecting the yields of fatty esters from transesterified vegetable oils. J. Am. Oil Chem. Soc. 61(10): 1638-1643.

Freitas, L., Da Rós, P. C. M., Santos, J. C. and de Castro, H. F. (2009). An integrated approach to produce biodiesel and monoglycerides by enzymatic interestification of babassu oil (*Orbinya sp*). Process Biochem. 44: 1068-1074.

Fukuda, H., Hama, S., Tamalampudi, S. and Noda, H. (2008). Whole-cell biocatalysts for biodiesel fuel production. Trends Biotechnol. 26: 668-673.

Fukuda, H., Kondo, A. and Noda, H. (2001). Biodiesel fuel production by transesterification of oils. J. Biosci. Bioeng. 92(5): 405-416.

Fukuda, H., Yamaji, H., Kondo, A., Noda, H. and Hama, S. (2007). Enzymatic biodiesel fuel production using whole-cell biocatalysts immobilized within biomass support particles. J. Biotechnol. 131(2, 1): S24-S25.

Gagnon, M. D. and Vasudevan, P. T. (2011). Effects of solvent and enzyme source on transesterification activity. Energ. Fuels 25(10): 4669-4674.

Gair, S., Cruden, A., McDonald, J., Hegarty, T. and Chesshire, M. (2006). Fuel cells for power generation and waste treatment. J. Power Sources 154(2): 472-478.

Garcia-Galan, C., Berenguer-Murcia, A., Fernandez-Lafuente, R. and Rodrigues, R. C. (2011). Potential of different enzyme immobilization strategies to improve enzyme performance. Adv. Synth. Catal. 353(16): 2885-2904.

Garcia-Sanda, E., Omil, F. and Lema, J. M. (2003). Clean production in fish canning industries: Recovery and reuse of selected wastes. Clean Technol. Environ. Policy 5(3-4): 289-294.

Garofalo, R. (2007). European Perspective: Overview of the Biodiesel Industry in Europe. European Biodiesel Board. International Congress on Biodiesel: The Science and the Technologies. Vienna, Austria, 5-7 November, 2007.

Gbogouri, G. A., Linder, M., Fanni, J. and Parmentier, M. (2006). Analysis of lipids extracted from salmon (*Salmo salar*) heads by commercial proteolytic enzymes. Eur. J. Lipid Sci. Technol. 108(9): 766-775.

Geller, D. P., Adams, T. T., Goodrum, J. W. and Pendergrass, J. (2008). Storage stability of poultry fat and diesel fuel mixtures: Specific gravity and viscosity. Fuel 87(1): 92-102.

Gildberg, A. and Stenberg, E. (2001). A new process for advanced utilisation of shrimp waste. Process Biochem. 36(8-9): 809-812.

Gildberg, F., Guimas, L. and Binet, A. (2002). Production of tuna waste hydrolysates by a commercial neutral protease preparation. J. Mol. Catal. B: Enzym. 11: 1051-1059.

Gjellesvik, D. R., Lombardo, D. and Walther, B. T. (1992). Pancreatic bile salt dependent lipase from cod (*Gadus morhua*): Purification and properties. Biochim. Biophys. Acta. 1124: 123-134.

Guderjan, M., Elez-Martínez, P. and Knorr, D. (2007). Application of pulsed electric fields at oil yield and content of functional food ingredients at the production of rapeseed oil. Innov. Food Sci. Emerging Technol. 8(1): 55-62.

Guerard, F. (2007). Enzymatic methods for marine by-products recovery. In: Maximising the value of marine by-products. Shahidi, F. (ed.), Woodhead, Cambridge, Pg. 107-143.

Gunnlaugsdottir, H. and Ackman, G. R. (1993). Three extraction methods for determination of lipids in fish meal: Evaluation of hexane/isopropanol method as an alternative to chloroform-based methods. J. Sci. Food Agric. 61(2): 235-240.

Gwyther, C. L., Williams, A. P., Golyshin, P. N., Edwards-Jones, G. and Jones, D. L. (2011). The environmental and biosecurity characteristics of livestock carcass disposal methods: A review. Waste Manage. 31(4): 767-778.

Haard, N. F., Simpson, B. K. and Sikorskiz, Z. E. (1994). Biotechnological applications of seafood proteins and other nitrogenous compounds. In: Seafood proteins. Sikorski Z. E., Sun Pan, B. and Shahidi, F. (eds.), Chapman & Hall, NY. Pg. 194-216.

Haas, M. J. and Foglia, T. A. (2005). Alternative feedstock and technologies for biodiesel production. In: The biodiesel handbook. Knothe, G., Van Gerpen, J. and Krahl, J. (eds), AOCS press, Champaign, Pg. 42-61.

Haas, M. J., McAloon, A. J., Yee, W. C. and Foglia, T. A. (2005). A process model to estimate biodiesel production costs. Bioresour. Technol. 97: 671-678.

Halim, S. F. A., Kamaruddin, A. H. and Fernando, W. J. N. (2009). Continuous biosynthesis of biodiesel from waste cooking palm oil in a packed bed reactor: Optimization using response surface methodology (RSM) and mass transfer studies. Bioresour. Technol. 100: 710-716.

Hamilton, C. R., Kirstein, D. and Breitmeyer, R. E. (2006). The rendering industry's biosecurity contribution to public and animal health. In: Essential rendering. Pg. 71-94.http://assets.nationalrenderers.org/essential_rendering_book.pdf (Accessed: 11/12/10).

Hammond, G. P., Kallu, S. and McManus, M. C. (2008). Development of biofuels for the UK automotive market. Appl. Energ. 85(6): 506-515.

Hemre, G.-I. and Sandnes, K. (1999). Effect of dietary lipid level on muscle composition in Atlantic salmon *Salmo salar*. Aquacult. Nutr. 5: 9-16.

Henke, J. M., Klepper, G. and Schmitz, N. (2005). Tax exemption for biofuels in Germany: Is bio-ethanol really an option for climate policy? Energ. 30(14): 2617-2635.

Hernández-Mártin, E. and Otero, C. (2008). Different enzyme requirements for the synthesis of biodiesel: Novozym® 435 and Lipozyme® TL IM. Bioresour. Technol. 99: 277-286.

Hirata, H., Saeki, H., Nonaka, M., Kawasaki, K., Ooizumi, T. and Motoe, K. (1993). Recovery of fish oil from the manufacturing process of highly nutritional fish meat for foodstuffs from sardine. Nippon Suisan Gakkaishi 59: 111-116.

Holčapek, M., Jandera, P., Fischer, J. and Prokes, B. (1999). Analytical monitoring of the production of biodiesel by high-performance liquid chromatography with various detection methods. J. Chromatogr. A. 858(1): 13-31.

Holčapek, M., Lísa, M., Jandera, P. and Kabátová, N. (2005). Quantitation of triacylglycerols in plant oils using HPLC with APCI-MS, evaporative light-scattering, and UV detection. J. Separ. Sci. 28(12): 1315-1333.

Hong, J., Xu, D., Gong, P., Yu, J., Ma, H. and Yao, S. (2008). Covalent-bonded immobilization of enzyme on hydrophilic polymer covering magnetic nanogels. Micropor. Mesopor. Mater. 109(1-3): 470-477.

Hu, A., Zhao, S., Liang, H., Qiu, T. and Chen, G. (2007). Ultrasound assisted supercritical fluid extraction of oil and coixenolide from adlay seed. Ultrason Sonochem. 14(2): 219-224.

Huang, J. and Sathivel, S. (2008). Thermal and rheological properties and the effects of temperature on the viscosity and oxidation rate of unpurified salmon oil. J. Food Eng. 89: 105-111.

IEO 2010. International Energy Outlook 2010. (Accessed: 05/28/11). http://www.eia.gov/oiaf/ieo/liquid_fuels.html.

Isaksson, T., Tøgersen, G., Iversen, A. and Hildrum, K. I. (1995). Non-destructive determination of fat, moisture and protein in salmon fillets by use of near-infrared diffuse spectroscopy. J. Agric. Food Chem. 69: 95-100.

Ismail, A. A., van de Voort, F. R. and Sedman, J. (1993). Rapid quantitative determination of free fatty acids in fats and oils by FTIR spectroscopy. J. Am. Oil Chem. Soc. 70: 335-341.

Iso, M., Chen, B., Eguchi, M., Kudo, T. and Shrestha, S. (2001). Production of biodiesel fuel from triglycerides and alcohol using immobilized lipase. J. Mol. Catal. B: Enzym. 16: 53-58.

Iverson, S. J., Lang, S. L. C. and Cooper, M. (2001). Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. Lipids 36(11): 1283-1287.

Jaeger, K.-E., Ransac, S., Dijkstra, B. W., Colson, C., van Heuvel, M. and Misset, O. (1994). Bacterial lipases. FEMS Microbiol. Rev. 15(1): 29-63.

Jie, M. L. K. (2007). Government policy and tax situations worldwide: One country two systems, one Far East three systems: Biodiesel in Asia. International Congress on Biodiesel: The Science and the Technologies. Vienna, Austria, 5-7 November, 2007.

Jobe, J. (2007). Biodiesel in the U.S. - Growing the fuel supply. International Congress on Biodiesel: The Science and the Technologies. Vienna, Austria, 5-7 November, 2007.

Jobling, M., Larsen, A. V., Andreassen, B., Sigholt, T. and Olsen, R. L. (2002) Influence of a dietary shift on temporal changes in fat deposition and fatty acid composition of Atlantic salmon post-smolt during the early phase of seawater rearing. Aquac. Res. 33(11): 875-889.

Jørgensen, E. H., Johansen, S. J. S. and Jobling, M. (1997). Seasonal patterns of growth, lipid deposition and lipid depletion in anadromous Arctic charr. J. Fish Biol. 51: 312-326.

Jung, W. K., Park, P. J., Byun, H. G., Moon, S. H. and Kim, S. K. (2005a). Preparation of hoki (*Johnius belengerii*) bone oligophsophopeptide with high affinity to calcium by carnivorous intestine crude proteinase. Food Chem. 91: 333-340.

Kaieda, M., Samukawa, T., Kondo, A. and Fukuda, H. (2001). Effect of methanol and water contents on production of biodiesel fuel from plant oil catalyzed by various lipases in a solvent-free system. J. Biosci. Bioeng. 91: 12-15.

Kalbasi-Ashtari, A., Schutz, M. M. and Auvermann, B. W. (2008). Carcass rendering systems for farm mortalities: A review. J. Environ. Eng. Sci. 7(3): 199-211.

Karmee, S. K. and Chadha, A. (2005). Preparation of biodiesel from crude oil of *Pongamia pinnata*. Bioresour. Technol. 96(13): 1425-1429.

Kerry, J. P. and Murphy, S. C. (2007). Physical and chemical properties of lipid by-products from seafood waste In: Maximising the value of marine by-products. Shahidi, F. (ed.), Woodhead, Cambridge, Pg. 22-46.

Kim, S.-K. and Mendis, E. (2006). Bioactive compounds from marine processing byproducts - A review. Food Res. Int. 39(4): 383-393.

Kittirattanapiboon, K. and Krisnangkura, K. (2008). Separation of acylglycerols, FAME and FFA in biodiesel by size exclusion chromatography. Eur. J. Lipid Sci. Technol. 110: 422-427.

Knothe, G. (2001). Analytical methods used in the production and fuel quality assessment of biodiesel. Trans ASAE 44: 193-200.

Knothe, G. (2005a). Fuel Properties. In: The biodiesel handbook. Knothe, G., Van Gerpen, J. and Krahl, J. (eds), AOCS press, Champaign, Pg. 76-80.

Knothe, G. (2005b). Introduction. In: The biodiesel handbook. Knothe, G., Van Gerpen, J. and Krahl, J. (eds), AOCS press, Champaign, Pg. 1-3.

Knothe, G. (2005c). The history of vegetable oil-based diesel fuels. In: The biodiesel handbook. Knothe, G., Van Gerpen, J. and Krahl, J. (eds), AOCS press, Champaign, Pg. 4-16.

Knothe, G. (2005d). Viscosity of biodiesel. In: The biodiesel handbook. Knothe, G., Van Gerpen, J. and Krahl, J. (eds), AOCS press, Champaign, Pg. 81-82.

Knothe, G. (2006). Analyzing biodiesel: Standards and other methods. J. Am. Oil Chem. Soc. 83(10): 823-833.

Knothe, G. (2011). Will biodiesel derived from algae perform? In: Proceedings of the 102nd American Oil Chemists' Society Annual Meeting & Expo, AOCS Press, Urbana, IL.

Knothe, G. H. and Steidley, K. R. (2005). Kinematic viscosity of biodiesel fuel components and related compounds. Influence of compound structure and comparison to petrodiesel fuel components. Fuel 84(9): 1059-1065.

Knothe, G., Sharp, C. A. and Ryan, T. W. (2006). Exhaust emissions of biodiesel, petrodiesel, neat methyl esters, and alkanes in a new technology engine. Energ. Fuels 20: 403-408.

Kordel, M., Hofmann, B., Schomburg, D. and Schmid, R. (1991). Extracellular lipase of *Pseudomonas* sp. Strain ATCC 21808: Purification, characterization, crystallization and preliminary X-ray diffraction data. J. Bacteriol. 173: 4836-4841.

Kumari, V., Shah, S. and Gupta, M. N. (2007). Preparation of biodiesel by lipase-catalyzed transesterification of high free fatty acid containing oil from *Madhuca indica*. Energ. Fuels 21(1): 368-372.

Kuraishi, C., Yamazaki, K. and Susa, Y. (2001). Transglutaminase: Its utilization in food industry. Food Rev. Intl. 17: 221-246.

Kurtovic, I., Marshall, S. N. and Zhao, X. (2011). Hydrophobic immobilization of a bile salt activated lipase from Chinook salmon (*Oncorhynchus tshawytscha*). J. Mol. Catal. B: Enzym. 72(3-4): 168-174.

Kusdiana, D. and Saka, S. (2004). Effects of water on biodiesel fuel production by supercritical methanol treatment. Bioresour. Technol. 91(3): 289-295.

Laane, C., Sjef, B., Kees, V. and Cees, V. (1987). Rules for optimization of biocatalysis in organic solvents. Biotechnol. Bioeng. 30(1): 81-87.

Lang, X., Dalai, A. K., Bakhshi, N. N., Reaney, M. J. and Hertz, P. B. (2001). Preparation and characterization of bio-diesels from various bio-oils. Bioresour. Technol. 80(1): 53-62.

Lechner, M., Bauer-Plank, C. and Lorbeer, E. (1997). Determination of acylglycerols in vegetable oil methyl esters by on-line normal phase LC-GC. J. High Resol. Chromatogr. 20: 581-585.

Lee, D. H., Kim, J. M., Shin, H. Y., Kang, S. W. and Kim, S. W. (2006). Biodiesel production using a mixture of immobilized *Rhizopus oryzae* and *Candida rugosa* lipases. Biotechnol. Bioprocess. Eng. 11(6): 522-525.

Li, L., Du, W., Liu, D., Wang, L. and Li, Z. (2006). Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. J. Mol. Catal. B: Enzym. 43: 58-62.

Li, W., Du, W. and Liu, D. (2007). *Rhizopus oryzae* IFO 4697 whole cell catalyzed methanolysis of crude and acidified rapeseed oils for biodiesel production in *tert*-butanol system. Process Biochem. 42(11): 1481-1485.

Lima, V. M. G., Krieger, N., Mitchell, D. A. and Fontana, J. D. (2004). Activity and stability of a crude lipase from *Penicillium aurantiogriseum* in aqueous media and organic solvents. Biochem. Eng. J. 18: 65-71.

Lin, C.-Y. and Li, R.-J. (2009). Fuel properties of biodiesel produced from the crude fish oil from the soapstock of marine fish. Fuel Process. Technol. 90(1): 130-136.

Linder, M., Fanni, J. and Parmentier, M. (2005). Proteolytic extraction of salmon oil and PUFA concentration by lipases. Mar. Biotechnol. 15: 70-76.

Littell, R. C., Pendergast, J. and Natarajan, R. (2000). Modelling covariance structure in the analysis of repeated measures data. Stat. Med. 19(13): 1793-1819.

Lu, J., Chen, Y., Wang, F. and Tan, T. (2009). Effect of water on methanolysis of glycerol trioleate catalyzed by immobilized lipase *Candida sp*. in organic solvent system. J. Mol. Catal. B: Enzym. 56: 99-25.

Ma, F., and Hanna, M. A. (1999). Biodiesel Production: A review. Bioresour. Technol. 70: 1-15.

Mahamuni, N. N. and Adewuyi, Y. G. (2009). Fourier Transform Infrared spectroscopy (FTIR) method to monitor soy biodiesel and soybean oil in transesterification reactions, petrodiesel-biodiesel blends, and blend adulteration with soy oil. Energ. Fuels 23(7): 3773-3782.

Marty, A., Dossat, V. and Condoret, J. S. (1997). Continuous operation of lipase catalyzed reactions in non-aqueous solvents: Influence of hydrophilic compounds production. Biotechnol. Bioeng. 56: 232-237.

Maruyama, T., Nakajima, M., Uchikawa, S., Nabetani, H., Furusaki, S. and Seki, M. (2000). Oil-water interfacial activation of lipase for interesterification of triglyceride and fatty acid. J. Am. Oil Chem. Soc. 77(11): 1121-1127.

Matassoli, A. L. F., Corrêa, I. N. S., Portilho, M. F., Veloso, C. O. and Langone, M. A. P. (2009). Enzymatic synthesis of biodiesel via alcoholysis of palm oil. Appl. Biochem. Biotechnol. 155: 347-355.

Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M. and Fernandez-Lafuente, R. (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb. Tech. 40(6): 1451-1463.

Mead, R. (1988). The design of experiments: Statistical principles for practical application, Cambridge University Press, Cambridge.

Meeker, D. L. and Hamilton, C. R. (2006). An overview of the rendering industry. http://nationalrenderers.org/assets/essential_rendering_overview.pdf. (Accessed: 06/25/10).

Meher, L. C., Sagar, D. V. and Naik, S. N. (2006). Technical aspects of biodiesel production by transesterification-A review. Renew Sust. Energ. Rev. 10: 248-268.

Metcalfe, L. D., Schmitz, A. A. and Pelka, J. R. (1966). Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem. 38(3): 514-515.

Min, S., Evrendilek, G. A. and Zhang, H. Q. (2007). Pulsed electric fields: Processing system, microbial and enzyme inhibition, and shelf life extension of foods. IEEE Transactions on Plasma Science 35(1): 59-73.

Mittal, G. S. (2006). Treatment of wastewater from abattoirs before land application-A review. Bioresour. Technol. 97(9): 1119-1135.

Modi, M. K., Reddy, J. R. C., Rao, B. V. S. K. and Prasad, R. B. N. (2007). Lipase-mediated conversion of vegetable oils into biodiesel using ethyl acetate as acyl acceptor. Bioresour. Technol. 98(6): 1260-1264.

Moffat, C. F., McGill, A. S., Hardy, R. and Anderson, R. S. (1993). The production of fish oils enriched in polyunsaturated fatty acids-containing triglycerides. J. Am. Oil Chem. Soc. 70: 133-138.

Monteiro, M. R., Ambrozin, A. R. P., Lião, L. M. and Ferreira, A. G. (2008). Critical review on analytical methods for biodiesel characterization. Talanta 77(2): 593-605.

Murphy, J. D. and Power, N. M. (2008). How can we improve the energy balance of ethanol production from wheat? Fuel 87(10-11): 1799-1806.

Naranjo, J. C., Córdoba, A., Giraldo, L., García, V. S. and Moreno-Piraján, J. C. (2010). Lipase supported on granular activated carbon and activated carbon cloth as a catalyst in the synthesis of biodiesel fuel. J. Mol. Catal. B: Enzym. 66: 166-171.

Nelson, L. A., Foglia, T. A. and Marner, W. N. (1996). Lipase-catalyzed production of biodiesel. J. Am. Oil Chem. Soc. 73: 1191-1195.

Njinkoue, J. M., Barnathan, G., Miralles, J., Gaydou E.-M. and Samb, A. (2002). Lipids and fatty acids in muscle, liver and skin of three edible fish from Senegalese coast: *Sardinella maderensis*, Sar*dinella aurita* and *Cephalopholis taeniops*. Comp. Biochem. Physiol. Part B 131: 395-402.

Nomura, Y., Oohashi, K., Watanabe, M. and Kasugai, S. (2005). Increase in bone mineral density through oral administration of shark gelatin to ovariectomized rats. Nutrition 21: 1120-1126.

Noureddini, H., Gao, X. and Philkana, R. S. (2005). Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil. Bioresour. Technol. 96(7): 769-777.

Nurhan, T. D., Motonobu, G. and Feral, T. (1998). Modeling of oil extraction with supercritical CO_2 from Atlantic Mackerel (*scomber scombrus*) at different moisture contents. J. Supercrit. Fluids 13(1-3): 303-309.

Ockerman, H. W. and Hansen, C. L. (2000). Seafood by-products. In: Animal by-product processing and utilization. Ockerman, H. W. and Hansen, C. L. (eds), CRC Press LLC, USA, Pg. 393-438.

OECD/FAO 2010. OECD-FAO Agricultural Outlook 2010-2019. (Accessed: 05/26/11).http://www.oecd-ilibrary.org/agriculture-and-food/oecd-fao-agricultural-outlook-2010_agr_outlook-2010-en.

OECD/IEA 2010. Sustainable production of second-generation biofuels-Potential and perspectives in major economies and developing countries. OECD/IEA, Paris. http://www.oecd.org/dataoecd/17/12/44567743.pdf (Accessed: 05/26/11).

Ogawa, M., Portier, R. J., Moody, M. W., Bell, J., Schexnayder, M. A. and Losso, J. N. (2004). Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogonia cromis*) and sheepshead seabream (*Archosargus probatocephalus*). Food Chem. 88: 495-501.

Oliveira, L. S., Franca, A. S., Rodrigo, C. R. S. and Ferraz, V. P. (2008). Coffee oil as a potential feedstock for biodiesel production. Bioresour. Technol. 99: 3244-3250.

ONC 2004. Ocean Nutrition Canada: Fish to fuel, waste to resource. (Accessed: 05/24/10). http://eco-efficiency.management.dal.ca/Files/Success_Stories/OceanNutritionCanada2004SS.pdf.

Páez, B. C., Medina, A. R., Rubio, F. C., Moreno, P. G. and Grima, E. M. (2003). Modeling the effect of free water on enzyme activity in immobilized lipase-catalyzed reactions in organic solvents. Enzyme Microb. Tech. 33(6): 845-853.

Palomo, J. M., Ortiz, C., Fuentes, M., Fernandez-Lorente, G., Guisan, J. M. and Fernandez-Lafuente, R. (2004). Use of immobilized lipases for lipase purification via specific lipase-lipase interactions. J. Chromatogr. A. 1038: 267-273.

Patkie, S., Subramaniam, J., Kumar, R. and Patil, D. A. (2000). Environment management issues in an abattoir: A case study. Ind. J. Environ. Prot. 20: 821-828.

Pelton, J. T. and McLean, L. R. (2000). Spectroscopic methods for analysis of protein secondary structure. Anal. Biochem. 277(2): 167-176.

Pencreac'h, G., Leullier, M. and Baratti, J. C. (1997). Properties of free and immobilised lipase from *Burkholderia cepacia*. Biotechnol. Bioeng. 56: 181-189.

Pencreac'h, G. and Baratti, J. C. (2001). Comparison of hydrolytic activity in water and heptane for thirty-two commercial lipase preparations. Enzyme Microb. Tech. 28(4-5): 473-479.

Perretti, G., Motori, A., Bravi, E., Favati, F., Montanari, L. and Fantozzi, P. (2007). Supercritical carbon dioxide fractionation of fish oil fatty acid ethyl esters. J. Supercrit. Fluids 40(3): 349-353.

Pires-Cabral, P., da Fonseca, M. M. R. and Ferreira-Dias, S. (2010). Esterification activity and operational stability of *Candida rugosa* lipase immobilized in polyurethane foams in the production of ethyl butyrate. Biochem. Eng. J. 48(2): 246-252.

Plattner, R. D. (1981). High-performance liquid chromatography of triglycerides. Method Enzymol. 72: 21-34.

Pogorevc, M., Stecher, H. and Faber, K. (2002). A caveat for the use of log *P* values for the assessment of the biocompatibility of organic solvents. Biotechnol. Lett. 24: 857-860.

Pokorný, J., Schmidt, Š. and Parkányiová, J. (2005). Ultraviolet-Visible spectrophotometry in the analysis of lipid oxidation. In: Analysis of lipid oxidation. Kamal-Eldin, A. and Pokorný, J. (eds.), AOCS press, Champaign, Pg. 17-39.

Pousa, G. P. A. G., Santos, A. L. F. and Suarez, P. A. Z. (2007). History and policy of biodiesel in Brazil. Energ. Policy 35(11): 5393-5398.

Praporscic, I., Lebovka, N., Vorobiev, E. and Mietton-Peuchot, M. (2007). Pulsed electric field enhanced expression and juice quality of white grapes. Separ. Purif. Technol. 52(3): 520-526.

Pratt, R. G. (2008). Fungal population levels in soils of commercial swine waste disposal sites and relationships to soil nutrient concentrations. Appl. Soil Ecol. 38(3): 223-229.

Radin, N. S. (1981). Extraction of tissue lipid with a solvent of low-toxicity. Method Enzymol. 72: 5-9.

Rafflenbeul, W. (2001). Fish for a healthy heart. Eur. J. Lipid Sci. Technol. 103: 315-317.

Ramos, M. J., Fernandez, C. M., Casas, A., Rodriguez, L. and Perez, A. (2008). Influence of fatty acid composition of raw materials on biodiesel properties. Bioresour. Technol. 100(1): 261-268.

Ranz, A., Maier, E. and Lankmayr, E. (2010). Determination of fatty acid derivatives in fuel and diesel oil. Fuel 89(8): 2133-2139.

Rashid, U., Anwar, F. and Knothe, G. (2009). Evaluation of biodiesel obtained from cottonseed oil. Fuel Process. Technol. 90: 1157-1163.

Rathore, V. and Madras, G. (2007). Synthesis of biodiesel from edible and non-edible oils in supercritical alcohols and enzymatic synthesis in supercritical carbon dioxide. Fuel 86(17-18): 2650-2659.

Reuber, M. D. (1979). Carcinogenicity of chloroform. Environ. Health Persp. 31: 171-182.

Reyes, J. F. and Sepúlveda, M. A. (2006). PM-10 emissions and power of a diesel engine fueled with crude and refined biodiesel from salmon oil. Fuel 85: 1714-1719.

Rodrigues, R. C., Volpato, G., Wada, K. and Ayub, M. A. Z. (2009). Improved enzyme stability in lipase-catalyzed synthesis of fatty acid ethyl ester from soybean oil. Appl. Biochem. Biotechnol. 152: 394-404.

Rodriguez, J. A., Ben Ali, Y., Abdelkafi, S., Mendoza, L. D., Leclaire, J., Fotiadu, F., Buono, G., Carrière, F. and Abousalham, A. (2010). In vitro stereoselective hydrolysis of diacylglycerols by hormone-sensitive lipase. Biochim. Biophys. Acta. 1801(1): 77-83.

Roose, P. and Smedes, F. (1996). Evaluation of the results of the QUASIMEME lipid intercomparison: The Bligh and Dyer total lipid extraction method. Mar. Pollut. Bull. 32(8/9): 674-680.

Rothsay 2011. http://www.rothsaybiodiesel.ca/ (Accessed: 02/06/11).

Royon, D., Daz, M., Ellenrieder, G. and Locatelli, S. (2007). Enzymatic production of biodiesel from cotton seed oil using t-butanol as a solvent. Bioresour. Technol. 98: 648-653.

Rustad, T. (2007). Physical and chemical properties of protein seafood by-products. In: Maximising the value of marine by-products. Shahidi, F. (ed.), Woodhead, Cambridge, Pg. 3-21.

Ryckebosch, E., Drouillon, M. and Vervaeren, H. (2011). Techniques for transformation of biogas to biomethane. Biomass Bioenerg. 35(5): 1633-1645.

Ryczkowski, J. (2007). Spectroscopic evidences of EDTA interaction with inorganic supports during the preparation of supported metal catalysts. Vib. Spectrosc. 43(1): 203-209.

Salameh, M. G. (2003). Can renewable and unconventional energy sources bridge the global energy gap in the 21st century? Appl. Energ. 75(1-2): 33-42.

Salih, A. M., Smith, D. M., Price, J. F. and Dawson, L. E. (1987). Modified extraction 2-thiobarbituric acid method for measuring lipid oxidation in poultry. J. Poultry Sci. 66: 1483-1488.

Salis, A., Pinna, M., Monduzzi, M. and Solinas, V. (2005). Biodiesel production from triolein and short chain alcohols through biocatalysis. J. Biotechnol. 119: 291-299.

Samukawa, T., Kaieda, M., Matsumoto, T., Ban, K., Kondo, A., Shimada, Y., Noda, H. and Fukuda, H. (2000). Pretreatment of immobilized *Candida antarctica* lipase for biodiesel fuel production from plant oil. J. Biosci. Bioeng. 90(2): 180-183.

Santori, G., Arteconi, A., Di Nicola, G., Moglie, M. and Stryjek, R. (2009). Quantitation of compounds in biodiesel mixtures with reversed-phase liquid chromatography. Energ. Fuels 23(7): 3783-3789.

SAS 2010. The MIXED Procedure, SAS/STAT(R) 9.2 User's Guide, Second Edition, Cary, NC: SAS Institute Inc., 2010. (Accessed: 07/02/10) http://support.sas.com/documentation/cdl/en/statug/63033/HTML/default/viewer.htm#/documentation/cdl/en/statug/63033/HTML/default/mixed_toc.htm.

Sathivel, S., Bechtel, P., Babbitt, J., Prinyawiwatikul, W., Negulescu, I. I. and Reppond, K. D. (2004). Properties of protein powders from Arrowtooth flounder (*Atheresthes stomias*) and herring (*Clupea harengus*) byproducts. J. Agric. Food Chem. 52: 5040-5046.

Sawa, T. (1978). Information criteria for discriminating among alternative regression models. Econometrica 46: 1273-1282.

Sayyar, S., Abidin, Z. Z., Yunus, R. and Muhammad, A. (2009). Extraction of oil from jatropha seeds-Optimization and kinetics. Am. J. Appl. Sci. 6(7): 1390-1395.

Schober, S., Seidl, I., Meinitzer, A. and Mittelbach, M. (2009). A method for the determination of polyunsaturated fatty acid methyl esters in biodiesel: Results of an interlaboratory study. Eur. J. Lipid Sci. Technol. 111(8): 829-834.

Schumacher, L. (2005). Biodiesel lubricity. In: The biodiesel handbook. Knothe, G., Van Gerpen, J. and Krahl, J. (eds). AOCS Press, Champaign, Pg. 137-143.

Schwab, A. W., Bagby, M. O. and Freedman, B. (1987). Preparation and properties of diesel fuel from vegetable oils. Fuel 66: 1372-1378.

SDTC 2006. Sustainable Development Technology Canada. Sustainable development business case report. Renewable fuel-biofuels. (Accessed: 03/30/07). http://www.agwest.sk.ca/bioproducts/documents/RenewableFuel-Biofuels.pdf.

Shah, S. and Gupta, M. N. (2007). Lipase catalyzed preparation of biodiesel from *Jatropha* oil in a solvent free system. Process Biochem. 42(3): 409-414.

Shahidi, F. (2007). Marine oils from seafood waste. In: Maximising the value of marine by-products. Shahidi, F. (ed.), Woodhead, Cambridge, Pg. 258-278.

Shahidi, F. and Wanasundara, U. N. (1998). Omega-3 fatty acid concentrates: Nutritional aspects and production technologies. Trends Food Sci. Technol. 9: 230-240.

Sharrock, P., Fiallo, M., Nzihou, A. and Chkir, M. (2009). Hazardous animal waste carcasses transformation into slow release fertilizers. J. Hazard Mater. 167(1-3): 119-123.

Shimada, Y., Watanabe, Y., Sugihara, A. and Tominaga, Y. (2002). Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. J. Mol. Catal. B: Enzym. 17: 133-142.

Shimizu, M., Kudo, N., Nakajima, Y., Matsuo, N., Katsuragi, Y., Tokimitsu, I., Barceló, I., Mateu, C. and Barceló, F. (2008). Effect of lipase activity and specificity on the DAG content of olive oil from the shodoshima-produced olive fruits. J. Am. Oil Chem. Soc. 85(7): 629-633.

Siddiquee, M. N. and Rohani, S. (2011). Lipid extraction and biodiesel production from municipal sewage sludges: A review. Renew. Sust. Energ. Rev. 15(2): 1067-1072.

Sila, A., Nasri, R., Jridi, M., Balti, R., Nasri, M. and Bougatef, A. (2012). Characterisation of trypsin purified from the viscera of Tunisian barbel (*Barbus callensis*) and its application for recovery of carotenoproteins from shrimp wastes. Food Chem. 132(3): 1287-1295.

Silva, C. C. C. M., Ribeiro, N. F. P., Souza, M. M. V. M. and Aranda, D. A. G. (2010). Biodiesel production from soybean oil and methanol using hydrotalcites as catalyst. Fuel Process. Technol. 91(2): 205-210.

Sim, J. H., Kamaruddin, A. H. and Bhatia, S. (2010). The feasibility study of crude palm oil transesterification at 30^{o}C operation. Bioresour. Technol. 101(23): 8948-8954.

Simpson, B. K. and Haard, N. P. (1984). Trypsin from the Greenland cod (*Gadus ogac*). Isolation and comparative properties. Comp. Biochem. Physiol. 79B: 613-622.

Simpson, B. K., Gagne, N., Ashie, I. N. A. and Noroozi, E. (1997). Utilization of chitosan for preservation of raw shrimp (*Pandalus boresslis*). Food Biotechnol. 11: 25-44.

Smedes, F. and Askland, T. K. (1999). Revisiting the development of the Bligh and Dyer total lipid determination method. Mar. Pollut. Bull. 38(3): 193-201.

Smedes, F. and Thomasen, T. K. (1996). Evaluation of the Bligh and Dyer lipid determination method. Mar. Pollut. Bull. 32(8/9): 681-688.

SNV 2010. Domestic biogas newsletter SNV. Issue 2-January 2010 http://www.snvworld.org/en/ourwork/Documents/SNV_Domestic_Biogas_Newsletter_-_issue_2_-_January_2010.pdf (Accessed: 05/28/11).

Sörensen, M. H., Ng, J. B. S., Bergström, L. and Alberius. P. C. A. (2010). Improved enzymatic activity of *Thermomyces lanuginosus* lipase immobilized in a hydrophobic particulate mesoporous carrier. J. Colloid Interf. Sci. 343: 359-365.

Soumanou, M. M. and Bornscheuer, U. T. (2003). Improvement in lipase-catalyzed synthesis of fatty acid methyl esters from sunflower oil. Enzyme Microb. Tech. 33: 97-103.

Soumanou, M. M. and Bornscheuer, U. T. (2003). Lipase-catalyzed alcoholysis of vegetable oils. Eur. J. Lipid Sci. Technol. 105(11): 656-660.

Souza, M. S., Aguieiras, E. C. G., da Silva, M. A. P. and Langone, M. A. P. (2009). Biodiesel synthesis via esterification of feedstock with high content of free fatty acids. Appl. Biochem. Biotechnol. 154: 253-267.

Srivastava, A. and Prasad, R. (2000). Triglycerides-based diesel fuels. Renew. Sust. Energ. Rev. 4: 111-133.

Stallard, B. R. (1997). Near-IR versus Mid-IR: Separability of three classes of organic compounds. Appl. Spectrosc. 51: 625-630.

Stein, K. (2007). Food vs. Biofuel. J. Am. Dietetic Assoc. 107(11): 1870, 1872-1876, 1878.

Steubing, B., Zah, R. and Ludwig, C. (2011). Life cycle assessment of SNG from wood for heating, electricity, and transportation. Biomass Bioenerg. 35(7): 2950-2960.

Stevenson, D. E., Stanley, R. A. and Furneaux, R. H. (1994). Near-quantitative production of fatty acid alkyl esters by lipase-catalyzed alcoholysis of fats and oils with adsorption of glycerol by silica gel. Enzyme Microb. Tech. 16: 478-484.

Stobutzki, I., Miller, M. and Brewer, D. (2001a). Sustainability of fishery bycatch: A process for assessing highly diverse and numerous bycatch. Environ. Conserv. 28: 167-181.

Svenskbiogas 2005. Biogas train-environmentally friendly and cost effective. http://www.svenskbiogas.se/sb/docs/english/Biogastrain_produktblad_2005.pdf (Accessed: 05/28/11).

Swisher, K. J. (2006). The global market for rendered products. In: Essential Rendering. Pg. 213-228. http://assets.nationalrenderers.org/essential_rendering_book.pdf.

Tamalampudi, S., Talukder, M. R., Hama, S., Numata, T., Kondo, A. and Fukuda, H. (2008). Enzymatic production of biodiesel from Jatropha oil: A comparative study of immobilized-whole cell and commercial lipases as a biocatalyst. Biochem. Eng. J. 39(1): 185-189.

Thassitou, P. K. and Arvanitoyannis, I. S. (2001). Bioremediation: A novel approach to food waste management. Trends in Food Sci. Technol. 12(5-6): 185-196.

Tilman, D., Socolow, R., Foley, J. A., Hill, J., Larson, E., Lynd, L., Pacala, S., Reilly, J., Searchinger, T., Somerville, C. and Williams, R. (2009). Beneficial biofuels-the food, energy, and environment trilemma. Science 325: 270-271.

Tischer, W. and Wedekind, F. (1999). Immobilized enzymes: Methods and applications. Top. Curr. Chem. 200: 95-126.

Tiss, A., Frédéric Carrière, F. and Verger, R. (2001). Effects of gum Arabic on lipase interfacial binding and activity. Anal. Biochem. 294(1): 36-43.

Tongboriboon, K., Cheirsilp, B. and H-Kittikun, A. (2010). Mixed lipases for efficient enzymatic synthesis of biodiesel from used palm oil and ethanol in a solvent-free system. J. Mol. Catal. B: Enzym. 67(1-2): 52-59.

Tschersich, P. and Choudhury, G. S. (1998). Arrowtooth flounder (*Atheresthes stomias*) protease as a processing aid. J. Aquat. Product Technol. 7: 77-89.

Tufvesson, P., Törnvall, U., Carvalho, J., Karlsson, A. J. and Hatti-Kaul, R. (2011). Towards a cost-effective immobilized lipase for the synthesis of specialty chemicals. J. Mol. Catal. B: Enzym. 68(2): 200-205.

Türkan, A. and Kalay, S. (2006). Monitoring lipase-catalyzed methanolysis of sunflower oil by reversed-phase high-performance liquid chromatography: Elucidation of the mechanisms of lipases. J. Chromatogr. A. 127: 34-44.

Undeland, I., Härrödand, M. and Lingnert, H. (1998). Comparison between methods using low-toxicity solvents for the extraction of lipids from herring (*Clupea harengus*). Food Chem. 61(3): 355-365.

USDA 2009. Honduras Biofuels Annual Report 2009. (Accessed: 05/24/10). http://gain.fas.usda.gov/Recent%20GAIN%20Publications/General%20Report_Tegucigalpa_Honduras_5-28-2009.pdf.

van de Voort, F. R., Sedman, J. and Russin, T. (2001). Lipid analysis by vibrational spectroscopy. Eur. J. Lipid Sci. Technol. 103: 815-826.

van de Voort, F. R., Sedman, J., Yaylayan, V. and Saint Laurent, C. (2003). Determination of acid number and base number in lubricants by Fourier transform infrared spectroscopy. Appl. Spectrosc. 57: 1425-1431.

Van Gerpen, J. (2005). The basics of diesel engines and diesel fuels. In: The biodiesel handbook. Knothe, G., Van Gerpen, J. and Krahl, J. (eds), AOCS press, Champaign, Pg. 17-25.

van Rantwijk, F., Lau, R. M. and Sheldon, R. A. (2003). Biocatalytic transformations in ionic liquids. Trends Biotechnol. 21: 131-138.

Veljković, V. B., Lakicevic, S. H., Stamenkovic, O. S., Todorovic, Z. B. and Lazic, K. L. (2006). Biodiesel production from tobacco (*Nicotiana tabacum L.*) seed oil with a high content of free fatty acids. Fuel 85: 2671-2675.

Verger, R. (1997). Interfacial activation of lipases: Facts and artifacts. Trends Biotechnol. 15: 32-38.

Verleyen, T., Verhe, R., Cano, A., Huyghebaert, A. and De Greyt, W. (2001). Influence of triacylglycerol characteristics on the determination of free fatty acids in vegetable oils by Fourier transform infrared spectroscopy. J. Am. Oil Chem. Soc. 78: 981-984.

Vicente, G., Martínez, M. and Aracil, J. (2004). Integrated biodiesel production: A comparison of different homogeneous catalysts systems. Bioresour. Technol. 92(3): 297-305.

Warabi, Y., Kusdiana, D. and Saka, S. (2004). Reactivity of triglycerides and fatty acids of rapeseed oil in supercritical alcohols. Bioresour. Technol. 91: 283-287.

Watanabe, Y., Shimada, Y., Sugihara, A. and Tominaga, Y. (2002). Conversion of degummed soybean oil to biodiesel fuel with immobilized *Candida antarctica* lipase. J. Mol. Catal. B: Enzym. 17: 151-155.

Watanabe, Y., Shimada, Y., Sugihara, A., Noda, H., Fukuda, H. and Tominaga, Y. (2000). Continuous production of biodiesel fuel from vegetable oil using immobilized *Candida antarctica* lipase. J. Am. Oil Chem. Soc. 77: 355-360.

Weber, J. A. (2009). Feedstock supplies for U. S. biodiesel production. (Accessed: 05/24/11). http://www.biodieselsustainability.com/gao.pdf.

Weetall, H. H. (1976). Covalent coupling methods for inorganic support materials. Method Enzymol. 44: 134-148.

WHH 2007. Wilson home heating: Biofuels (Accessed: 05/24/10). http://homeheating.wilsons.ca/services/index.php?id=53.

Wu, T. H. and Bechtel, P. J. (2008). Salmon by-product storage and oil extraction. Food Chem. 111: 868-871.

Wu, T. H. and Bechtel, P. J. (2009). Quality of crude oil extracted from aging walleye pollock (*Theragra chalcogramma*) byproducts. J. Am. Oil Chem. Soc. 86(9): 903-908.

Wyatt, V. T., Hess, M. A., Dunn, R. O., Foglia, T. A., Haas, M. J. and Marmer, W. N. (2005). Fuel properties and nitrogen oxide emission levels of biodiesel produced from animal fats. J. Am. Oil Chem. Soc. 82: 585-591.

Xu, Y., Du, W. and Liu, D. (2005). Study on the kinetics of enzymatic interesterification of triglycerides for biodiesel production with methyl acetate as the acyl acceptor. J. Mol. Catal. B: Enzym. 32(5-6): 241-245.

Xu, Y., Du, W., Zeng, J. and Liu, D. (2004). Conversion of soybean oil to biodiesel fuel using lipozyme TL IM in a solvent free medium. Biocatal. Biotransfor. 22: 45-48.

Yagiz, F., Kazan, D. and Akin, A. N. (2007). Biodiesel production from waste oils by using lipase immobilized on hydrotalcite and zeolites. Chem. Eng. J. 134(1-3): 262-267.

Yang, F., Su, W.-J., Lu, B.-J., Wu, T., Sun, L.-C., Hara, K. and Cao, M.-J. (2009). Purification and characterization of chymotrypsins from the hepatopancreas of crucian carp (*Carassius auratus*). Food Chem. 116(4): 860-866.

Yin, J.-Z., Xiao, M. and Song, J.-B. (2008). Biodiesel from soybean oil in supercritical methanol with co-solvent. Energ. Convers. Manage. 49(5): 908-912.

Young, F. V. K. (1985). The refining and hydrogenation of fish oil. International Association of fish meal manufacturers. Fish Bull. 17: 27.

Zaks, A. and Klibanov, A. M. (1985). Enzyme-catalyzed processes in organic solvents. Proc. Natl. Acad. Sci. 82: 3192-3196.

Zhang, H.-Z. and Lee, T.-C. (1997). Rapid near-infrared spectroscopic method for the determination of free fatty acid in fish and its application in fish quality assessment. J. Agric. Food Chem. 45: 3515-3521.

Zhang, S. B., Wang, Z. and Xu, S. Y. (2007). Optimization of the aqueous enzymatic extraction of rapeseed oil and protein hydrolysates. J. Am. Oil Chem. Soc. 84(1): 97-105.

Zhang, Y., Dubé, M. A., McLean, D. D. and Kates, M. (2003). Biodiesel production from waste cooking oil: 1. Process design and technological assessment. Bioresour. Technol. 89(1): 1-16.

Zhou, S. and Ackman, R. G. (1996). Interference of polar lipids with the alkalimetric determination of free fatty acid in fish lipids. J. Am. Oil Chem. Soc. 73(8): 1019-1023.

Zuta, C. P., Simpson, B. K., Chan, H. M. and Phillip, L. (2003). Concentrating PUFA from mackerel processing waste. J. Am. Oil Chem. Soc. 80(9): 933-936.

Zuta, C. P., Simpson, B. K., Zhao, X. and Leclerc, L. (2007). The effect of α-tocopherol on the oxidation of mackerel oil. Food Chem. 100(2): 800-807.