ENZYMATIC TRANSESTERIFICATION OF FISH OIL FOR THE PRODUCTION OF BIODIESEL

by

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ABSTRACT

The production of biodiesel from fish oil was carried out using enzymatic transesterification. The effects of the oil: alcohol molar ratio (1:1, 1:2, 1:3, 1:4 and 1:5), alcohol type (methanol and 2-butanol), reaction temperature (35, 40, 45 and 50ºC), reaction time (4, 8, 12 and 16 h) and solvent type (solvent and solvent-free) on the biodiesel conversion yield using *Candida antarctica* (Novozyme 435) and experimental enzyme (NS88001) were investigated. The biodiesel conversion yield increased when the reaction temperature was increased from 35 to 40ºC and then decreased when the reaction temperature was further increased to 45 and 50ºC, respectively. Increasing the oil: alcohol molar ratio from 1:1 to 1:4 increased the biodiesel conversion yield which then decreased when the oil: alcohol molar ratio was further increased to 1:5. The highest biodiesel conversion yield was obtained after 16 h at the oil: alcohol molar ratio of 1:4 and the reaction temperature of 40ºC using Novozyme 435 and NS88001 individually and in combination. No reaction was observed at the oil: alcohol molar ratio of 1:1 for Novozyme 435 enzyme and at 1:1 and 1:2 oil: alcohol molar ratios for NS88001 enzyme in a solvent- free system. The highest biodiesel conversion yield from Novozyme 435 (80.24%) was observed with 2-butanol, the highest biodiesel conversion yield from NS88001 (74.34%) was observed with methanol and the highest biodiesel conversion yield from the combination of enzymes (Novozyme 435 and NS88001) lipase (82.37%) was observed with 2-butanol. The stability of the enzymes Novozyme 435 and NS88001 individually and in combination slightly decreased after 10 cycles and completely stopped after 20-30 cycles.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ANOVA	Analysis of Variance
AOCS	American Oil Chemist's Society
ASTM	American Society for Testing and Materials
B	Base Catalyst
B5	Biodiesel 5%
B20	Biodiesel 20%
B100	Biodiesel 100%
BSPs	Biomass Support Particles
BaO	Barium Oxide
BSTFA	Bistrimethylsilyltrifluoroacetamide
BF_3	Boron Trifluoride
CEN	Central European Norm
D	Density
D2	Diesel Fuel
DG	Diglycerides
DHA	Docosahexaenoic Acid (22:6n-3)
E	Ester Concentration
EPA	Eicosapentaenoic Acid (20:5n-3)
FA	Fatty Acid
FAs	Fatty Acids
FAAE	Fatty Acid Alkyl Ester
FAAEs	Fatty Acid Alkyl Esters
FAME	Fatty Acid Methyl Ester
FFA	Free Fatty Acid
FID	Flame Ionization Detectors
GC	Gas Chromatography
HC	Hydrocarbon
HHV	Higher Heating Value
K	Kelvin
MCM	Mobile Crystal Material

MG	Monoglyceride
MgO	Magnesium Oxide
MSTFA	Methyl N-trimethylsilyl- trifluoroacetamide
MPa	Magnitude Pascal
OMA	Official Methods of Analysis
PUFA	Polyunsaturated Fatty Acid(s)
TAG	Triacylglycerol
TG	Triglycerides
USA	United States of America

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CHAPTER 1. INTRODUCTION

The use of alternative fuels instead of conventional fossil fuels is becoming increasingly significant due to decreasing petroleum reserves and increasing greenhouse gases, all of which lead to global warming, ozone depletion and political and health concerns (Fukuda et al., 2001; Akoh et al., 2007; Ghaly et al., 2010). Plant oils have been used as alternative fuels for many years, since they are renewable and readily available. However, these oils cannot be used directly as fuel sources in diesel engines due to: (a) high viscosity which leads to poor fuel atomization during the injection process, (b) low volatility and (c) polymerization which results in deposit formation, incompletion combustion and poor emissions (Ma and Hanna, 1999; Meher et al., 2006). To overcome these disadvantages, oils can be converted into fatty acid methyl esters (FAME) which are also known as biodiesel. Biodiesel is an alternative fuel that is non-toxic, completely biodegradable and renewable and can be adapted easily without any modification to diesel engines.

Numerous vegetable oils derived from plants (including canola, palm, soybean, sunflower, rapeseed, coconut and groundnut) have been converted into biodiesel (Srivastava and Prasad, 2000; Fukuda et al., 2001; Akoh et al., 2007). However, plant-derived oils are used for food, livestock feed and in the oleochemical industries which increases crop demand (McNeff et al., 2008). Thus, their use as a feedstock for biodiesel would have a severe negative impact, especially in developing countries. There would also be a 70% increase in greenhouse gas emissions when heavy crops are used as biodiesel feedstock, due to the intensive use of energy and fertilizers (Li et al., 2007; Jegannathan et al., 2008). Therefore, alternative feedstocks such as waste cooking oils, fats (lard and tallow), fish waste, and microalgae oils have been considered for biodiesel production (Marachetti et al., 2008; Ranganathan et al., 2008). However, waste from fish processing is a critical problem for the fish processing industries. Fish processing wastes are rich in oil, valuable minerals, enzymes, pigments and flavours which can be used to produce renewable energy and some valuable by-products, such as proteins, fertilizer, animal feed, omega-3-fatty acids (EPA and DHA), amino acids and enzymes.

Several processes have been developed for biodiesel production, such as pyrolysis, microemulsification and transesterification. The chemical change of the products from the reactants caused by the thermal energy in the presence of air or nitrogen sparging is called a pyrolytic process. These products are similar to the petroleum-derived fuel. However, during the pyrolysis process, the removal of oxygen leads to reduce the environmental benefits (Ma and Hanna, 1999). The problem of the high viscosity of the substrates has been investigated using microemulsions with solvents (methanol, ethanol and 1-butanol) to meet the international standards of petroleum-derived fuels. However, an increase of lubricating oil viscosity, irregular injector needle sticking, incomplete combustion and heavy carbon deposits were reported in the laboratory screening endurance test (Ziejewski et al., 1984). Therefore, transesterification process plays a vital role, in order to overcome these disadvantages.

The process of displacing alcohol from an ester to form another ester is called transesterification. Transesterification is the most simple and efficient method to produce biodiesel by using acids, alkalis, or enzymes as catalysts. Triglycerides with high free fatty acid and water contents are not essential for a biodiesel conversion process using an acid catalyst. However, the reaction rates are slower than those of the alkali catalytic process (Freedman et al., 1986). The alkali-catalysis transesterification process has been widely used in the biodiesel industry, because it gives a high yield of conversion of fatty acid methyl esters from triglycerides at low temperatures and pressures in a relatively short reaction time of 4-10 hours (Alcantara et al., 2000; Kaieda et al., 1999; Srivastava and Prasad, 2000). However, it has several drawbacks including product separation, soap formation and negative environmental impacts such as greenhouse gas, CO, hydrocarbons, NOx and particles in exhaust emissions (Jegannathan et al., 2008; Nielsen et al., 2008).

To overcome some of the problems with chemical transesterification, enzymes are used as catalysts. The use of enzymes has several advantages: (a) less downstream processing with no difficulties with product separation, (b) no alkaline wastewater generation and (c) a high degree of product purity especially with the use of lipases as catalysts (Ghaly et al., 2010; Ma and Hanna, 1999). To reduce the cost, immobilized enzymes are used in the production process as they can be reused for a longer time (Du et al., 2004; Huang et al., 2010).

However, the production of enzymatic biodiesel requires optimization of factors such as oil: alcohol molar ratio, temperature, alcohol, organic solvents, and reaction time. Thus, the aim of this research is to investigate the potential of producing biodiesel from fish oil using lipase as a catalyst.

CHAPTER 2. OBJECTIVES

The aim of this study was to investigate the potential of producing biodiesel from fish oil by enzymatic transesterification. The specific objectives were:

1. To evaluate the effectiveness of the lipases *Candida antarctica* (Novozyme 435) and experimental enzyme (NS88001) individually and in combination.
2. To study the effects of the following parameters on biodiesel conversion yield:
    a) Oil: alcohol molar ratio (1:1, 1:2, 1:3, 1:4 and 1:5).
    b) Alcohol type (Methanol and 2-butanol).
    c) Reaction temperature (35, 40, 45 and 50ºC).
    d) Reaction time (4, 8, 12 and 16 h).
3. To evaluate the effectiveness of solvent and solvent-free systems.
4. To determine the reusability factor for the lipase enzymes (Novozyme 435 and NS88001) in the transesterification process.

CHAPTER 3. LITERATURE REVIEW

3.1. Biodiesel

The recovery of energy from biomass sources is becoming more attractive due to the rising cost of fossil fuels and increasing emission of greenhouse gases (Sensoz et al., 2000). Biomass is a renewable source of energy that can be converted into energy products like biofuels. Interest in biomass use has grown because it is a carbon neutral source of energy (Dowaki et al., 2007). In order to meet the increased demand for energy and to replace petroleum products, alternative biofuels such as biodiesel and bioethanol can be used. Moreover, they have greater efficiency than gasoline and have a higher heating value (HHV).

The current energy demand is met by conventional resources such as coal, petroleum, and natural gas. However, gas and oil reserves will be sufficient for only a few more decades. The demand for energy will continue to rise and an alternative fuel source such as the biofuels can help meet this demand (Demirbas, 2006). In early 1900, Dr Rudolf Diesel utilized peanut oil to ignite one of his engines and demonstrated its potential as an alternative fuel in the future (Nitschke and Wilson, 1965). Currently, biofuel can be easily produced and used in the transportation sector (Demirbas, 2006). It is technically feasible, efficient, more environmentally sustainable and economically competitive. Other features include portability, renewability, biodegradability, higher heat content, availability, less aromatic content and minimal sulphur content. Biodiesel plants are commonly supported by several countries in the Europe and many vehicles are running successfully on these vegetable oils (Demirbas, 2008).

Biodiesel can be defined as a monoalkyl ester, which is derived from resources of biological products like vegetable oil, animal fat, marine oil and cooking oil (Ma and Hanna, 1999; Fjerbaek et al., 2009). It can be chemically called a Fatty Acid Methyl Ester (FAME) due to its composition and it is technically derived from triglycerides (Leung et al., 2010). In the transesterification process, the triglycerides are converted into fatty esters with glycerol as a by-product; the process is achieved with an alcohol in the presence of a catalyst (Fuduka et al., 2001; Diwani et al., 2009).

Homogeneous or heterogeneous catalysts are widely used. Recently, the approach of using an enzymatic catalyst has been widely encouraged in order to reduce pollution and downstream separation problems (Shimada et al., 2002). The high cost of enzyme catalysts as a barrier for large scale biodiesel production and the stability of biodiesel created using enzyme catalysts have been reported (Watanabe et al., 2002; Chen and Wu, 2003). The conversion of less viscous long-chain monoesters from more viscous triglycerides allows for several noteworthy characteristics of biodiesel such as a high flashing point, better combustion efficiency, biodegradability and good lubricity in comparison with petro-diesel fuel (Zheng et al., 2009).

Biodiesel can be either blended with petroleum- based fuel or used in its pure state. B100 is a pure biodiesel which has zero net emission of carbon dioxide (Vasudevan and Briggs, 2008). Harmful substances such as carbon monoxide (CO), hydrocarbons (HC) and particulate matter (PM) have been greatly reduced using B100 (U.S. Department of Energy, 2004). B20 is a combination of 20% biodiesel and 80% petroleum-based blend fuel which reduces the net emission of carbon dioxide by 15.66% (Fukuda et al., 2001; Vasudevan and Briggs, 2008). Thus, biodiesel can be used as an alternative fuel in order to reduce these emission levels.

3.2. Raw Materials for Biodiesel Production

The basic materials used in biodiesel production are plant-derived oils, waste oils and fats and microbial oils (Akoh et al., 2007). There are 300 oil-bearing plants and trees that can be used as feedstock for biodiesel production (Subramanian et al., 2005). Vegetable oils (such as castor, corn, jatropha, peanut, soybean, sunflower, canola, palm, cotton seed, and rapeseed), animal fats (including beef tallow; poultry fat and lard, and omega-3 fatty acids from fish oil), hemp oil, and microalgae oil are also good resources as feedstock because they are effective and have environmental benefits (Ranganatha et al., 2008; Antczak et al., 2009). Yellow greases are a combination of vegetable oils and animal fats which can also be used (Knothe, 2005). Chemically, these vegetable oils and animal fats contain triglyceride molecules with three fatty acids that are attached to one glycerol (Sonntag, 1979). Table 3.1 shows the world consumption of vegetable and marine oils for the period of 1998-2003.

Table 3.1. World consumption of vegetable and marine oils (million metric tons) (Demirbas, 2008).

Oil	1998	1999	2000	2001	2002	2003
Soybean	23.5	24.5	26.0	26.6	27.2	27.9
Palm	18.5	21.2	23.5	24.8	26..3	27.8
Rapeseed	12.5	13.3	13.1	12.8	12.5	12.1
Sunflower seed	9.2	9.5	8.6	8.4	8.2	8.0
Peanut	4.5	4.3	4.2	4.7	5.3	5.8
Cottonseed	3.7	3.7	3.6	4.0	4.4	4.9
Coconut	3.2	3.2	3.3	3.5	3.7	3.9
Palm kernel	2.3	2.6	2.7	3.1	3.5	3.7
Olive	2.2	2.4	2.5	2.6	2.7	2.8
Fish	1.2	1.2	1.2	1.3	1.3	1.4
Total	80.7	85.7	88.4	91.8	95.1	98.3

However, plant-derived fats and oils are not ideal for use in biodiesel production because the crops can also be used as food, in the oleochemcial industries and as livestock feed (Li et al., 2007; Jegannathan et al., 2008). Biodiesel industries would compete with chemical, food and livestock feed industries for plant sources (McNeff et al., 2008). An increased demand for these plants could increase fertilizer use, contributing to increased greenhouse gas emissions which are also a serious environmental concern. For example, there is a 70% increase in greenhouse gas emission due to biodiesel production from heavily fertilized plants (Jegannathan et al., 2008).

The feedstock choice plays an important role, as biodiesel production must meet the ASTM standards including iodine value, cetane number and saponification number of FAMEs of the oil (Sharma and Singh, 2010). The best feedstock suited for biodiesel production is defined by a high level of oleic acid: a chain 18 carbons long with a single double bond (Knothe, 2005). In the choice of feedstock, a balance between the unsaturation and length of FA chain should be maintained (Robles-Medina et al., 2009).

3.3. Fats and Oils

Fats and oils are members of the lipid family. “Oil” refers to a lipid that is liquid at room temperature whereas “fat” refers to a lipid that is solid or semi-solid at room temperature. Lipids consist of esters of glycerol such as monoglycerides (MG), diglycerides (DG) and triglycerides (TG) and low to moderate contents of carboxylic acids or free fatty acids (FFA). FFAs contain 4 to 24 carbon atoms with some degree of unsaturation (Suwannakarn, 2008). Other compounds such as phospholipids, polypeptides, sterols, water, odorants and other impurities can be found in crude oils and fats. The chemical structure of MG, DG and TG consist of a back bone glycerol as shown in Figure 3.1. The fatty acid composition of fat or oil is also an important factor in the production of biodiesel. Table 3.2 shows the fatty acid profile of many fats and oils used in biodiesel production.

In cold conditions, oils containing lower levels of unsaturated fatty acids than saturated fatty acids may solidify and clog the fuel line (Akoh et al., 2007; Demirbas, 2008). Biodiesel with a high level of unsaturated fatty acids is suitable for cold and warm weather conditions

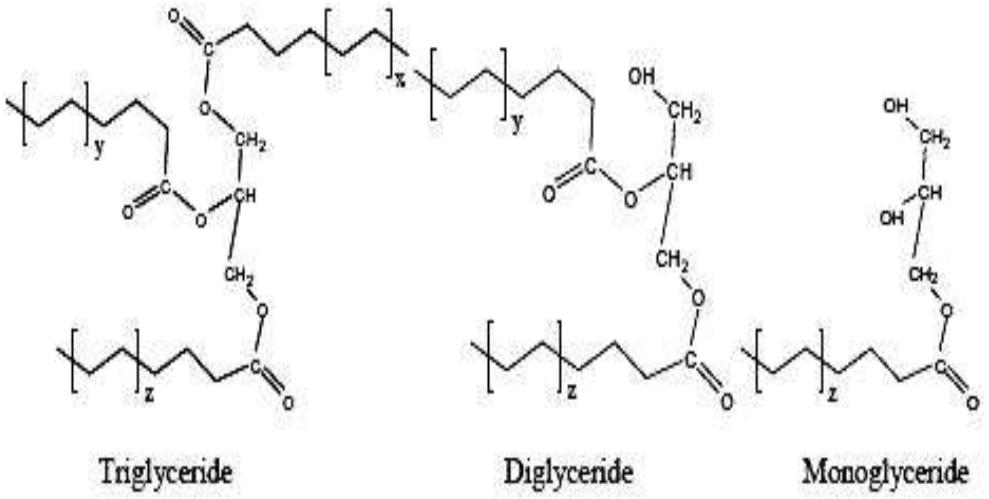


Figure 3.1. The chemical structures of oils and fats (Suwannakarn, 2008).

Table 3.2. Fatty acids profile of oils and fats used for biodiesel production (Akoh et al., 2007; Marchetti et al., 2007).

Oil/fat	arachidic (20:0)	behemic (22:0)	gadoleic/gondoic (20:1)	lignoceric (24:0)	linoleic (18:2)	linolenic (18:3)	Oleic (18:1)	palmitic (16:0)	palmitoleic (16:1)	stearic (18:0)	other
Canola					22.3	8.2	64.4	3.5		0.9	0.7
Coconut							6.0	5.0		3.0	86.0
Cotton seed					57.5		13.3	28.3		0.9	
Groundnut					26.0		51.6	8.5		6.0	7.9
Jatropha	0.2				36.2		37.0	16.4	1.0	6.2	3.0
Karanj	1.6	5.4	1.2	1.4	17.7	3.6	51.8	10.2		7.0	0.1
Microalgae					2.2	0.9	1.3	15.5	17.3	0.3	62.5
Olive	0.4		0.3		8.5	0.7	74.2	11.8	1.5	2.6	
Palm Oil					10.1	0.2	40.5	42.6	0.3	4.4	1.9
Peanut	1.3	2.5		1.2	32.0	0.9	48.3	11.4		2.4	
Rapeseed					22.3	8.2	64.4	3.5		0.9	0.7
Safflower Seed					77.0		13.5	7.3	0.1	1.9	0.2
Soybean	0.3				53.8	9.3	20.8	11.4		4.4	
Sunflower	0.3				62.4		25.5	7.1		4.7	
Tallow							44.5	29.0		24.5	2.0

due to lower viscosity and higher cloud and pour points. However, the quality of biodiesel can be reduced due to the processing of oils with lower combustion temperatures and cetane indexes. Oils with large-chain FAs produce biodiesel with a high combustion temperature and cetane index, greater viscosity, and low pour and cloud points. Thus, oils from vegetables are widely used in commercial biodiesel production because they contain lower FFA levels (Liu et al., 2007).

3.4. Methods for Biodiesel Production

The direct use of feedstock in diesel engines is unsatisfactory and not practical for direct and indirect diesel engines. It can cause serious problems including, gum formation due to polymerization and oxidation during storage, scuffing of the engine liner, thickening of the lubricating oil because of its low volatility and high viscosity, high cloud and pour points and high carbon deposits (Ma et al., 1999; Murugesan et al., 2009). Table 3.3 shows the problem and probable cause for using direct oil in a diesel engine (Harwood, 1984). To prevent those problems, the feedstock is chemically altered to produce biodiesel (Fukuda et al., 2001). Oil containing FFA and TGs are reduced to fatty acid alkyl esters (FAAEs) (Fjerbaek et al., 2009). Biodiesel production methods are mostly classified into three types: pyrolysis, microemulsion and transesterification (Murugesan et al., 2009; Ma and Hanna, 1999).

3.4.1. Pyrolysis

Pyrolysis is a process of heat applied to TGs under anaerobic conditions to cleave the chemical bonds and produce different products such as aromatics, alkenes, carboxylic acids, small quantity of gaseous products, alkadienes, and alkanes (Schwab et al., 1988; Ma and Hanna, 1999). The pyrolytic chemistry is very difficult to characterize due to the variety of reaction paths and reaction products that occur during the chemical reaction. Vegetable oils, animal fats, fatty acids and fatty acid methyl esters have been used as pyrolyzed material. The pyrolytic fat process has been investigated for more than ten decades (Sonntag, 1979). The compressed organic material is separated to produce biogas and biodiesel fuels. These biofuel fractions have the same chemical composition as fossil fuels and they are simple, renewable, effective and environmentally friendly and create no waste water or air pollution (Pioch et al., 1993). Chang and Wan (1947), first reported the pyrolysis of tung oil calcium

Table 3.3. The problem and probable cause for using direct oil in diesels engine (Harwood, 1984).

Term	Problem	Probable cause	Potential solution
Short-term	Cold weather starting	High viscosity, low cetane and low flash point of vegetable oils.	Preheat fuel prior to injection. Chemically alter the fuel to an ester
	Plugging and gumming of filters, lines, and injectors	Natural gums (Phosphatides) in vegetable oil. Other ash	Partially refine the oil to remove gums. Filter to 4-microns
	Engine knocking	Very low cetane of some oils. Improper injection timing.	Adjust injection timing. Use higher compression engines. Preheat fuel prior to injection. Chemically alter fuel to an ester
Long-term	Coking of injectors on piston and head of engine	High viscosity of vegetable oil, incomplete combustion of fuel.	Heat fuel prior to injection. Switch engine to diesel fuel when operations at part load. Chemically alter the vegetable oil to an ester
	Carbon deposit on piston and head of engine	Poor combustion at part loads with vegetable oils.	
	Excessive engine wear	High viscosity of vegetable oil, incomplete combustion of fuel.	Heat fuel prior to injection. Switch engine to diesel fuel when operations at part load. Chemically alter the vegetable oil to an ester. Increase motor oil changes. Motor oil additives to inhibit oxidation.
		Poor combustion at part loads with vegetable oils.	
		Possibly free fatty acids in vegetable oil.	
		Dilution of engine lubricating oil due to blow-by of vegetable oil	
	Failure of engine lubricating oil to polymerization	Collection of polyunsaturated vegetable oil blow-by in crankcase to the point where polymerization occurs	

soap on a large scale. Tung oil was initially reacted with lime to form soap and then extreme heat was applied to produce crude oil. This was then refined to produce fuel, gasoline and a small amount of kerosene. 50 liters of crude oil was obtained from 68 kilograms of soap by saponification of Tung oil. The effect of temperature on the saponified products from heated glycerides was studied by Grossley et al. (1962). Metallic salts have been used largely as catalysts to the reactants in order to obtain products such as paraffins and olefins.

Soybean oil was decomposed thermally and then distilled by a standard distillation apparatus (Niehaus et al., 1986). Safflower oil was used as control because of its high oleic oil content. These soybean and high oleic safflower oils were distilled and the total identified hydrocarbon were 73-77% and 80-88%, respectively. Alkanes and alkenes were the main components accounting for 60% of the total weight, followed by carboxylic acids with 9.6 - 16.1% (Schwab et al., 1988). Copra and palm oil stearins were cracked at 450°C by using catalyst SiO_2/Al_2O_3 to produce low molecular weight products (such as solids, liquid and gases) and a conversion rate of 74% and 84% was obtained, respectively (Pioch et al., 1993). A mixture of methyl esters was produced from rapeseed oil using the pyrolytic method in a tubular reactor using nitrogen and a high temperature between 500 and 850°C by Billaud et al. (1995). The results showed that the conversion of methyl esters was increased with an increasing of the temperature.

Palm oil can be pyrolyzed and then converted into hydrocarbons in the presence of zeolite catalysts. In addition, palm oil can be converted into diesel, kerosene, coke, and water with a 70% recovery yield, and gasoline range hydrocarbons can alone be converted with a maximum yield of 40% of the total products (Leng et al., 1999). Palm oil was converted into gasoline in the presence of a composite micro-mesoporous zeolite catalyst in which a maximum yield of 48% of conversion yield was obtained. The yield was increased with an increase in the temperature and catalyst to molar ratio (Sang et al., 2003). Animal tallow was pyrolyzed at 775 K and converted into liquid products with a maximum yield of 77.1%. In pyrolytic liquid products, the degree of repolymerization increased with increasing temperature (Demirbas, 2008).

Pyrolytic and thermal cracking equipment are relatively quite expensive, produce low value products and use more gasoline than diesel fuel. During thermal processing, the

removal of oxygen also eliminates some environmental benefits of using an oxygenated fuel. However, the product from these methods is chemically similar to petroleum based diesel fuel (Ma and Hanna, 1999).

3.4.2. Microemulsions

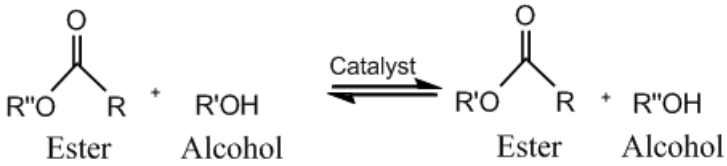
A microemulsion can be defined as what forms when two immiscible liquids and one or more ionic or non-ionic amphiphiles are collided with each other at equilibrium to spontaneously form an optically isotropic fluid microstructure (Schwab et al., 1987). It can be used to reduce the viscosity of oils physically with solvents such as methanol, ethanol and 1-butanol. Explosive vaporization of the low boiling constituents can be used to improve spray characteristics in the micelles (Pryde, 1984). Soybean oil blends in both ionic and non-ionic microemulsions of ethanol are efficient with lower cetane and energy content in short term performances similar to that of No. 2 diesel (Goering et al., 1982b). Alkali-refined and winterized sunflower oil (53%) was emulsified with ethanol (13.3%) and 1-butano1 (33.4%) which resulted in a viscosity of 6.31cSt at 40°C (Ziejewski et al., 1984).

In laboratory screening tests conducted with oil of a low viscosity and lasting 200 h, there were no significant quality losses in the performance of engine but irregular injector needle sticking, heavy carbon deposits, incomplete combustion and an increase of lubricating oil viscosity were observed. Similarly, a 200 hour EMA screening test with shipp non-ionic fuel containing No. 2 diesel fuel (50%), degummed and alkali-refined soybean oil (25%), ethanol (5%) and 1-butanol (20%) and showed some major problems such as carbon and lacquer deposits on the injector tips, in-take valves and tops of the cylinder liners. The shipp non-ionic fuel performed better than a 25% blend of sunflower oil in diesel oil (Goering and Fry, 1984a).

The engine performance of a microemulsion with 53% sunflower oil was found to be comparable to a 25% blend of sunflower oil in diesel (Ziejewski et al., 1984). Similarly, a microemulsion of soybean oil with methanol and 2-octanol was examined and found to be effective (Goering, 1984b). All microemulsions with solvents like butanol, hexanol and octanol met the maximum viscosity requirement for No. 2 diesel (Jain and Sharma, 2010).

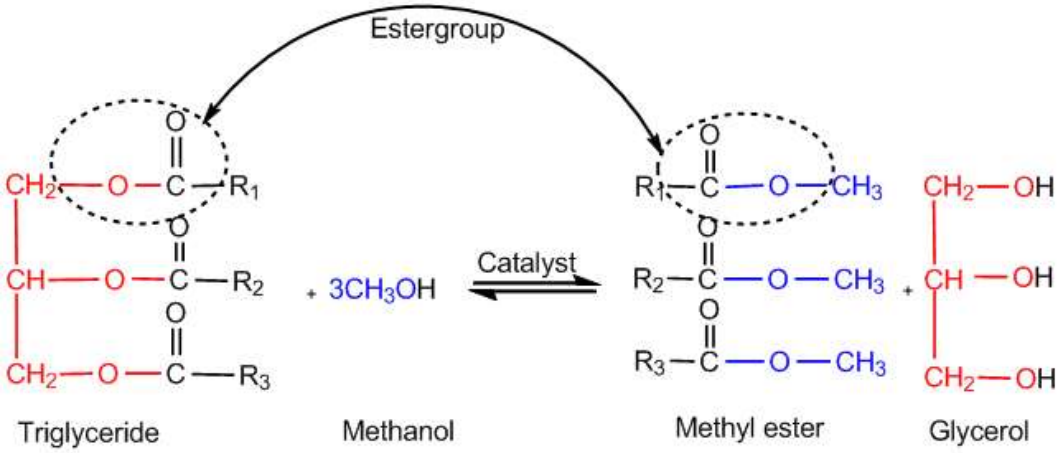
3.4.3. Transesterification

Transesterification is the process of exchanging acyl groups between an ester and an alcohol, to produce biodiesel and glycerine (Ranganathan et al., 2008; Akoh et al., 2007). The sequence of the transesterification process is shown in Figure 3.2. The initial step of pretreatment is followed by transesterification and purification. The fatty ester is released simultaneously with the reformation of the hydroxyl (OH) group in glycerol. The general transesterification equation is follows:



(3.1)

Group R is a fatty acid, R' is the length of the acyl acceptor, and R" is the triglyceride molecule. The overall reaction is controlled by chemical equilibrium as follows:



(3.2)

The overall reaction occurs in sequence of three continuous stages as shown in Figure 3.3. (a) conversion of TGs to DGs, (b) conversion of DGs to MGs and (c) conversion of MGs to glycerin. R represents FA; R1, R2 and R3 represent the hydrocarbon chains of the fatty acid alkyl groups of the triglyceride (Ghaly et al., 2010; Marchetti et al., 2008). For each molecule of triglyceride, three molecules of alcohol are needed to produce three molecules of fatty esters. The transesterification reaction of acyl-acceptors can involve carboxylic acids, in which case the reaction would be known as acidolysis, or alcohol, with alcoholysis, or another ester with interestification. These reactions, alcoholysis and interesterification,

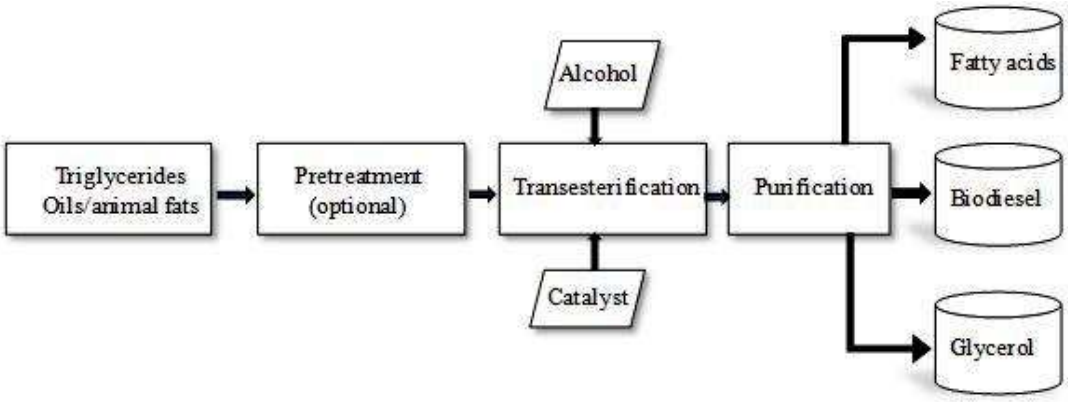
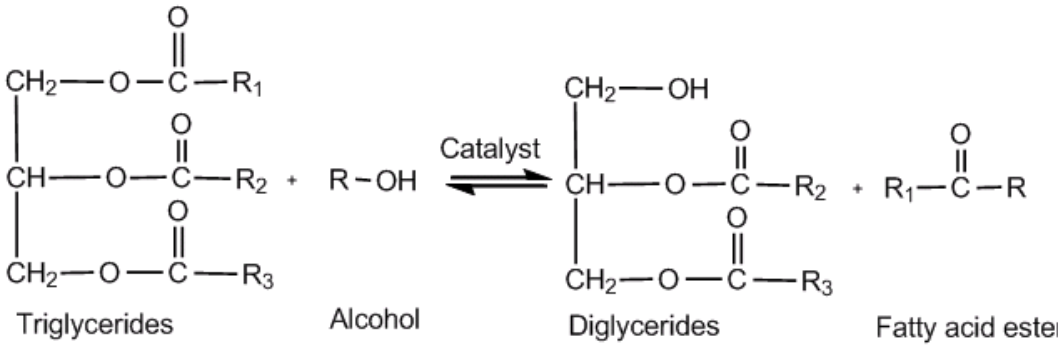


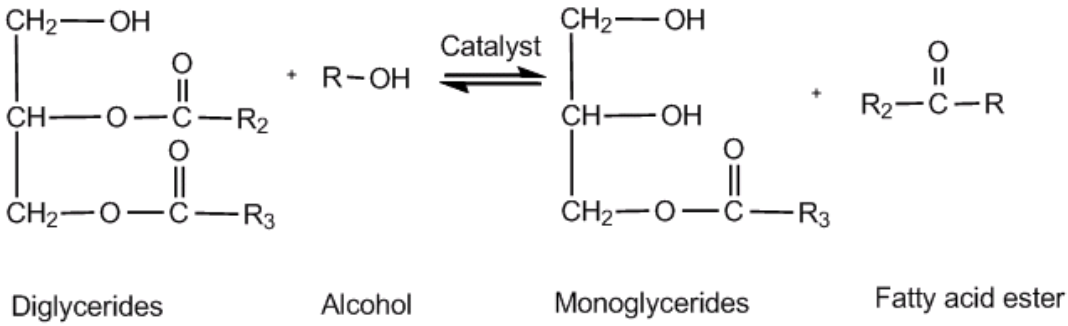
Figure 3.2. Sequence of transesterification process (Ghaly et al., 2010).

(a) Conversion of TGs to DGs:



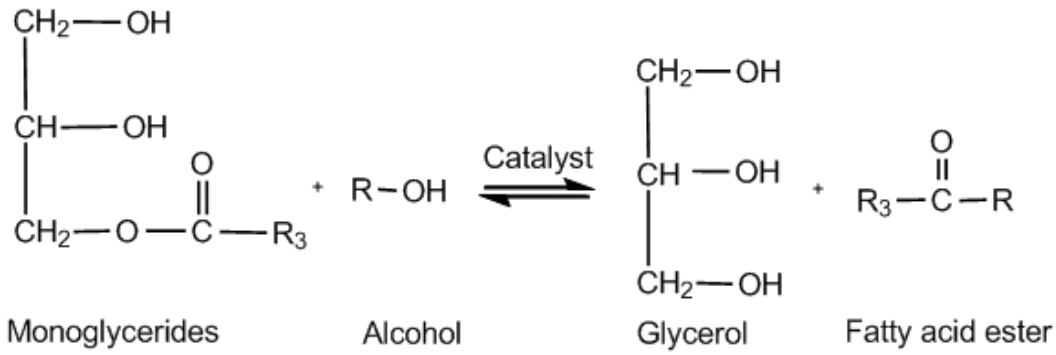
(3.3)

(b) Conversion of DGs to MGs:



(3.4)

(c) Conversion of MGs to glycerin molecules:



(3.5)

Figure 3.3. Three continuous stages of the transesterification reaction (Ghaly et al., 2010).

produce the fatty acid methyl esters to make biodiesel (Robles-Medina et al., 2009). The most commonly used acyl-acceptors for the transesterification are alcohols. There are many alcohols used for biodiesel production, including methanol, ethanol, propanol (Fukuda et al., 2001), isopropanol (Shaw et al., 1991), branched chain alcohols, butanol (Nelson et al., 1996), octanol (Marchetti et al., 2007), t-butanol (Li et al., 2006) and ethyl or methyl esters (Modi et al., 2007).

Methanol is the most widely used alcohol for FAME production and the reaction is known as methanolysis. Ethanol is also used but it is relatively expensive, less volatile and less reactive, renewable and eco-friendly as it is produced from agricultural products when compared to methanol. Glycerol, a by-product of the transesterification process by means of an alcohol, can be widely used in the pharmaceutical industry (Bacovsky et al., 2007). The high FFA content of the feedstock may lead to soap formation and affect the ester yield, so the FFAs are separated from the glycerol molecule (Leung et al., 2010).

Feedstocks can be pretreated before the transesterification process, when the FFA content is more than 2.5% wt. (ISTC, 2007). This can be done by three different pretreatment methods: acid esterification, ion exchange resins and extraction with alcohol method (Banerjee and Chakraborty, 2009; Ozbay et al., 2008). In general, transesterification is the process of mixing the reactants, but catalysts are needed to accelerate the transesterification reaction by one of the following methods: (a) acids, (b) alkaline and (c) enzymes (Murugesan et al., 2009; Leung et al., 2010). Heat can be applied to increase the speed of the reaction. However, transesterification reactions occurring in temperatures below 350°C and above 400°C may lead to obtaining a lower ester yield and degrading the ester bonds (Ranganathan et al., 2008). Commonly, to speed up the reaction process, transesterification can be done just above alcohol's boiling point or around 71–72°C. Alcohol to oil molar ratio, mixing intensity, temperature and the concentration of the catalyst are the parameters that affect the transesterification reaction (Marchetti et al., 2007).

3.5. Chemical Transesterification

In chemical transesterification, a catalyst (acid or alkali) is widely used to increase the reaction rate and yield. Because the reaction is reversible, excess alcohol is used to shift the

equilibrium to the product side (Leung et al., 2010). Transesterification processes that involve chemical catalysts are energy demanding and require much downstream processing to purify the end products (Xu and Wu, 2003). The multistep purification processes after transesterification are: (a) glycerol separation by centrifugation, (b) catalyst neutralization, (c) deodorization and (d) pigment removal (Banerjee and Chakraborty, 2009). The problems associated with the chemical catalyst can be eliminated by the use of enzyme catalysts. Ultimately, the enzymatic transesterification method for the production of biodiesel is an interesting one (Jegannathan et al., 2008). A typical alkali transesterification process for fish oil is shown in Figure 3.4.

3.5.1. Alkali Catalyst Transesterification

For alkali catalysis, either KOH or NaOH is used with an alcohol (methanol or ethanol) to convert oil into biodiesel. These catalysts are used industrially due to their availability and low cost. Methoxide ions are obtained by both acid and base catalysts by active species as shown in equations 3.6 and 3.7.

a) $CH_3O^-Na^+ \longrightarrow CH_3O^- + Na^+$ (3.6)

b) $NaOH + CH_3OH \rightleftharpoons CH_3O^- + H_2O + Na^+$ (3.7)

In step (a), methoxide ions are formed by dissociation of methoxide salts. In step (b), methoxide ions are formed when hydroxyl ions from alkaline hydroxides react with methanol. Later, these methoxide ions become strong nucleophiles and target the carbonyl moiety of a glycerol molecule in order to produce fatty acid methyl esters.

Transesterification of base catalysis can be technically feasible, but the major problem associated with this method is feedstock, catalyst and alcohol specification. If the free fatty acid (FFA) content is more than 0.5 wt% in a chemical reaction, the formation of soap occurs when it reacts with a metal hydroxide catalyst which leads to increased viscosity, formation of gels and increased overall production cost. Thus, the downstream recovery and purification of the product is very difficult and expensive when using base catalysis (Ma and Hanna, 1999). The formation of soap using a base catalyst and the formation of FFAs

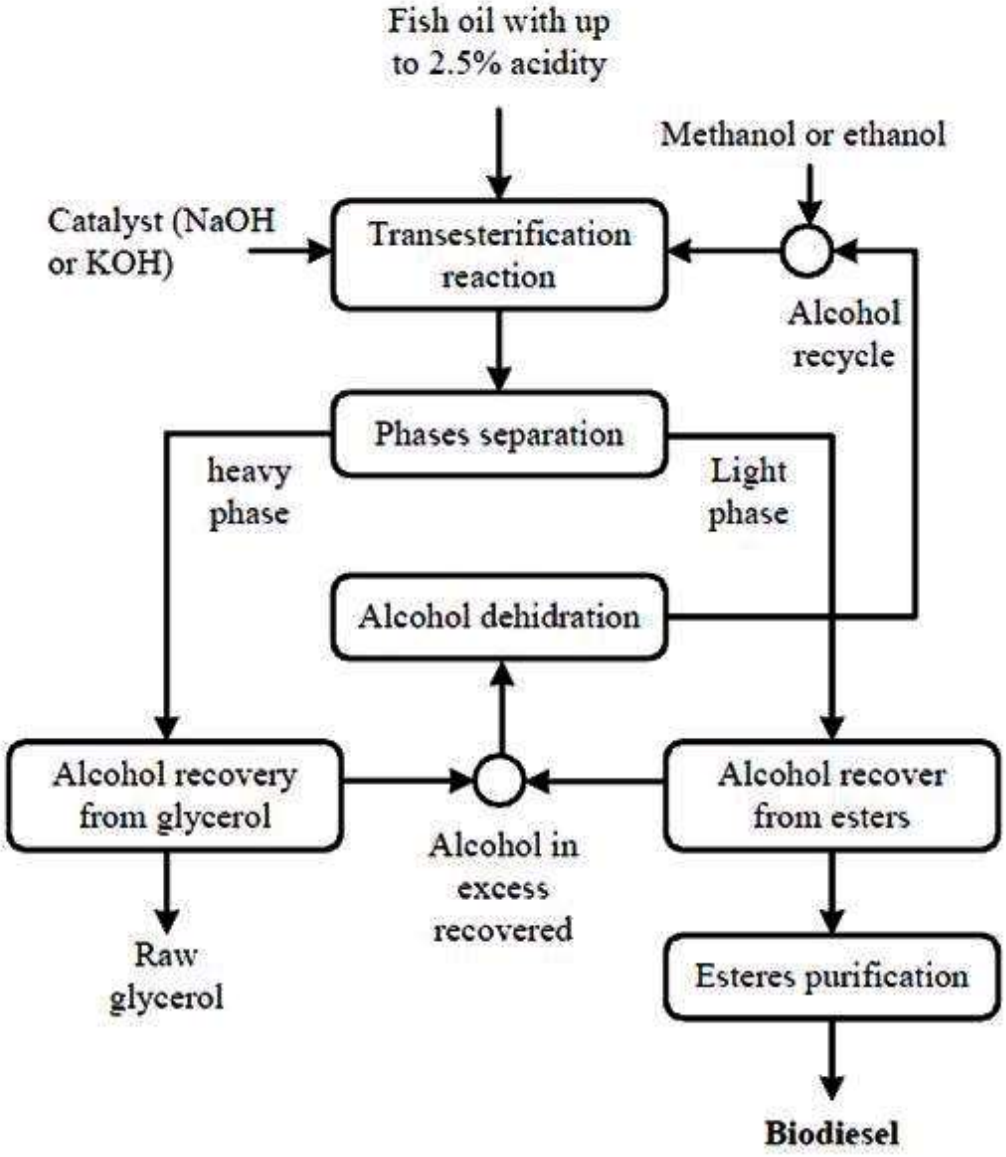
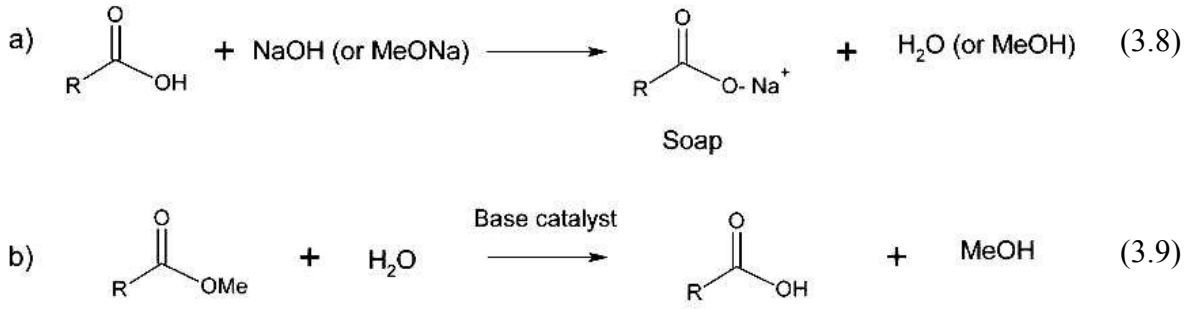


Figure 3.4. Process flow schematic for production of biodiesel from fish oil by alkali process (Viela et al., 2010).

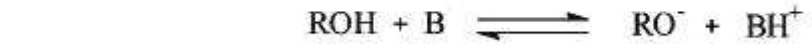
by a water molecule is shown in equations 3.8 and 3.9. Eventually, the alcohol and catalyst are essentially anhydrous with less than 0.1-0.3 wt% and it is assumed that the FFAs hydrolyze the alkyl esters due to the presence of water.



The molar ratio of oil to alcohol may vary from 1:1 to 1:6. The molar ratio of catalyst may vary from 0.5% to 1%w/w (Srivastava and Prasad, 2000) and from 0.005% to 0.35% w/w when added to a reactor (Ma and Hanna, 1999). Standard temperature (60°C) is very important but it depends on the type of catalyst used. The temperature range could be from 25 to 120°C for different degrees of catalyst conversion (Fukuda et al., 2001).

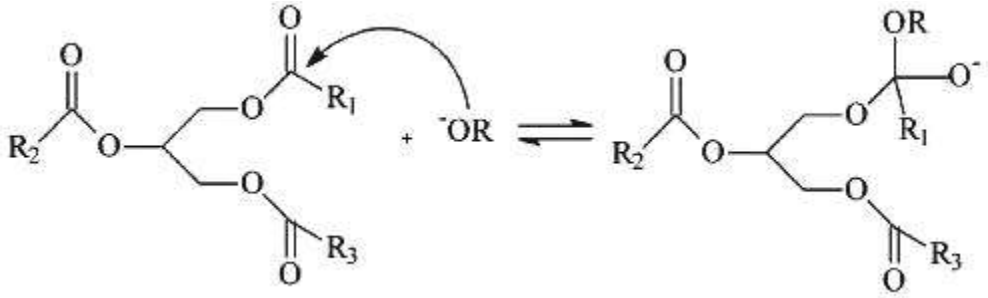
The homogeneous base catalyst mechanism described by Lotero (2006) is shown in Figure 3.5. Srivastava and Prasad (2000) stated that the reactions that are homogeneous alkali-catalyzed is almost 4000 times faster than the homogeneous acid catalyzed. Alkoxide group synthesis is a general step for basic heterogeneous catalyst (Lopez et al., 2007). The possibilities of using alkoxides have been tested, in which metal hydroxide and oxides are used to catalyze the transesterification with methanol at the reflux temperature. NaOH is more active; calcium methoxide is medium active and barium hydroxide is less active (Gryglewicz, 1999). Gryglewicz (1999) reported that when calcium oxide and magnesium oxide powder were used as catalysts, the reaction rate was low and there was no catalytic activity.

Portnoff et al. (2006) stated that using microwave energy can increase the rate of transesterification because the selective catalyst energizes the interaction with the reactants. Transesterification of vegetable oil using ZnO loaded Sr $(NO_3)_2$ catalyst gave a good yield by calcination for 5 hours at 873 K (Yang et al., 2007). Similarly, transesterification of oils with



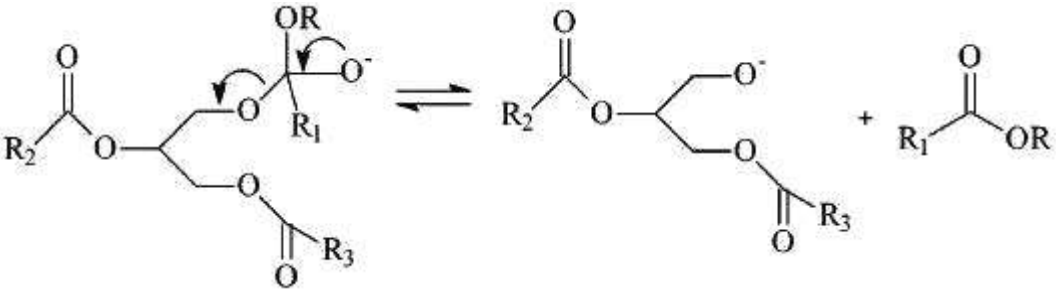
(3.10)

(a) Production of the active species RO.



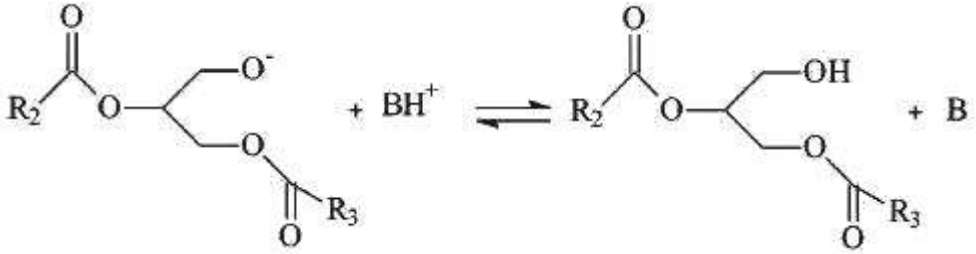
(3.11)

(b) Nucleophilic attack of RO^- to carbonyl group on TG, forming of a tetrahedral intermediate.



(3.12)

(c) Intermediate breakdown.



(3.13)

(d) Regeneration of the RO^- active species.

B- Base catalyst
R_1, R_2, R_3 – carbon chain of the fatty acids
R_4 - alkyl group of the alcohol

Figure 3.5. Mechanism of Homogeneous Base Catalyst(Lotero et al., 2006).

sodium silicate as catalyst gave a good yield of biodiesel at moderate temperature (60-120°C) but the catalyst could not be reused (Lopez et al., 2007).

In basic catalysis, the resin stability is based on the alcohol washing before the resin is used. However, exchange of most OH^- groups with MeO^- groups cannot give the product of carboxylic acid with the deactivation of catalyst elimination (Liu et al., 2007). Kim et al. (2004) tested with Na/NaOH/γ-Al_2O_3 as the alkaline catalyst to synthesis biodiesel by transesterification of vegetable oil using methanol and co-solvent hexane. A good result (more than 90% conversion yield) was obtained using mixed oxides as the catalyst which includes the molar ratio of oil to methanol (1:9) at 60°C for 2 hours (Monteiro and cruz, 2004).

Monteiro and Cruz (2004) tested three catalysts (a) Na_2O- SiO_2, BaO (10%) - MCM-41, CO_2O_3- SiO_2, La_2O_3 (10%)-MCM-41, (b) MgO, ZrO_2- SiO_2, MO_2O_5- SiO_2, KOH/ZrO_2-SiO_2, MgO (10%)- MCM-41 and (c) CaO. Na_2O- SiO_2, CaO and La_2O_3 (10%)-MCM-41 the results showed good yield, converting 76%, 67% and 81% of oils into biodiesel, respectively.

3.5.2. Acid Catalyst Transesterification

Acid catalysts are the second most commercially used catalysts. The following acids are most frequently utilized in the biodiesel production process: sulfuric acid, sulfonic acid, hydrochloric acid and organic-sulfuric acids (Freedman et al., 1984). However, acid catalysts are corrosive in nature and lead to a slower reaction rate and lower ester yield (Srivastava and Prasad, 2000). Marchetti et al.(2008) reported that vegetable oils esterified with acid catalysts gave a high yield without formation of soap but these acids required high temperature (55–80°C) and high molar ratios of substrates (30:1) with 0.5–1.0 mol% catalyst concentration in order to get 99% conversion yield in 50 hours. Freedman et al. (1986) reported that when one mol% of sulphuric acid reacts with thirty mol% of oil with a ratio of 30:1 at 65°C, a conversion yield of 99% was obtained in 50 hours. In contrast, ethanolysis needs 18 hours at 78°C and butanolysis needs 3 hours at 117°C, respectively. When the samples have high free fatty acid (FFA) content it is easy to produce biodiesel with acid transesterification.

In acid catalyzed transesterification, alcohols such as methanol, amyl alcohol, propanol, ethanol and butanol have been used. In particular, ethanol and methanol are widely used in

the biodiesel industry and research laboratories (Fukuda et al., 2001). Methanol is usually the first choice as a solvent due to its low cost. However, ethanol is synthesized from agricultural products and is eco-friendly. Therefore, biodiesel synthesized from ethanol is a completely biodegradable fuel (Demirbas, 2003). Nye et al. (1983) identified butanol is the most suitable alcohol for the synthesis of biodiesel using 0.1% H_2SO_4 catalyst in waste cooking oil when comparing with methanol, ethanol and propanol.

Canakci and Van Gerpen (1999) stated that in an acid catalyzed reaction, the presence of water reduces the reaction rates and so the conversion to ester was approximately 70%. Sridharan and Mathai (1974) noticed that the small ester from the process is disabled by the presence of water compounds. Liu (1994) reported that a strong Lewis acid (BF3) is adequate to esterify the FFAs in short duration of time. In practice, esterification is subjected to thermodynamic limitations. Therefore, water removal is necessary in order to obtain good conversion rates and also to complete the reaction.

Good selectivity and reactivity of the methyl ester can be obtained through the thermostable catalyst. In order to produce an effective product with these materials, a high molar alcohol to oil ratio (60:1) and high temperatures (200°C) are needed. Waghoo et al. (1999) tested the transesterification of ethyl acetate with various alcohols in the presence of a hydrous tin oxide to achieve high conversion of ester. They also tested this process with aromatic and linear alcohols at 170-210°C.

Srivastava and Prasad (2000) stated that the homogeneous acid-catalyst is almost 4000 times slower than the homogeneous base-catalyst. However, the advantage of the homogenous acid catalyst is that presence of free fatty acids in the feedstock does not strongly affect the acid catalyst. Moreover, esterification and transesterification are catalyzed simultaneously. Therefore, the acid catalysts can directly synthesize biodiesel from low cost feedstocks like grease and used cooking oil which has FFAs $\geq$ 6%. Zhang et al. (2003) stated that acid catalysts may compete with base catalysts to produce biodiesel economically from a low cost feedstock like virgin oil.

The chemical pathway of homogeneous acid-catalyzed transesterification is shown in Figure 3.6. The protonation of carbonyl oxygen is catalyzed by substrate interaction to

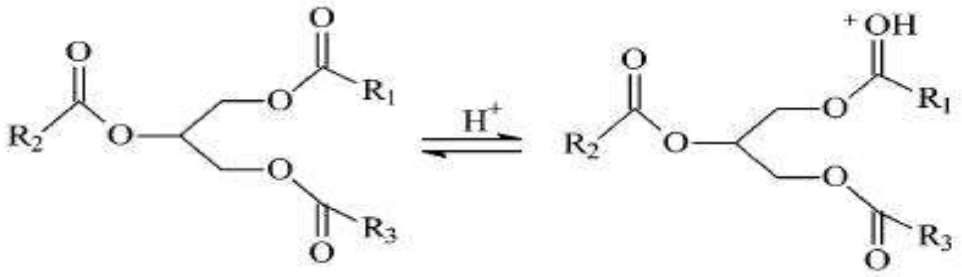
increase carbon atom by adjoining electrophilicity for the nucleophilic attack. The overall reaction has been categorized by the rate limiting step versus time, with 3 regimes observed time reaction. At first, mass transfer controls are identified by the reaction and concluded from the low miscibility of the chemical and catalyst. Secondly, the ester acts as emulsifier and creates a sudden change in product formation. Finally, equilibrium is achieved in the last regime to complete the reaction (Lotero et al., 2006).

Although a homogeneous acid catalyst is effective in converting fats into fuels, the problem with this system is contamination of the product and the use of separation and purification techniques which lead to high production costs. In order to maintain a viable cost, a continuous flow processing system was introduced for the production of biodiesel by Lotero et al. (2006). This system has some reaction steps in which the separation process is limited and uses a strong solid heterogeneous catalyst. A packed bed continuous flow reactor has the potential to make it easier for the solid catalyst to reduce the generated waste and facilitate easier separation to increase the purification of the product.

Mittelbach et al. (1995) investigated the functions of a continuous layered aluminosilicate with the transesterification of triglycerides with alcohol in the presence of a solid sulphuric acid catalyst. They used a molar ratio of alcohol to oil (30:1) and 5% wt catalyst. Kaita et al. (2002) used molar ratios (1:3 to 1:0.01) aluminium phosphate designed with a different metaltophosphoric acid and methanol.

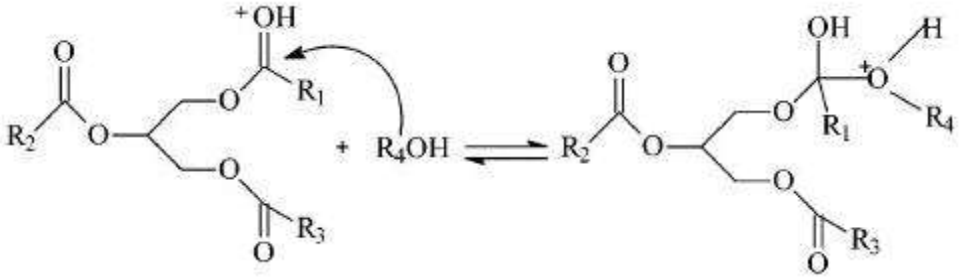
Bronzed solid acids were used for transesterification of â-ketoesters for the production of pheromones (Madje et al., 2004; Balaji et al., 1998). Other catalysts were also used including Envirocat EPZG (Bandgar et al., 2001), zeolites (Sasidharan et al., 2004), kaolinite clay (Bandgar et al., 2001), Amberlyst (Liu et al., 1994), sulfated SnO_2 (Chavan et al., 1996) and B_2O_3/ZrO_2 (Madje et al., 2004). The transesterification of β-ketoesters is shown in equation 3.17.

Esterification of carboxylic acids with a solid acid catalyst is considered to be the most significant due to the low cost of feedstock and the high concentrations of free fatty acids. In particular, ion exchange resins (Nafion and Amberlyst-15) have been used during



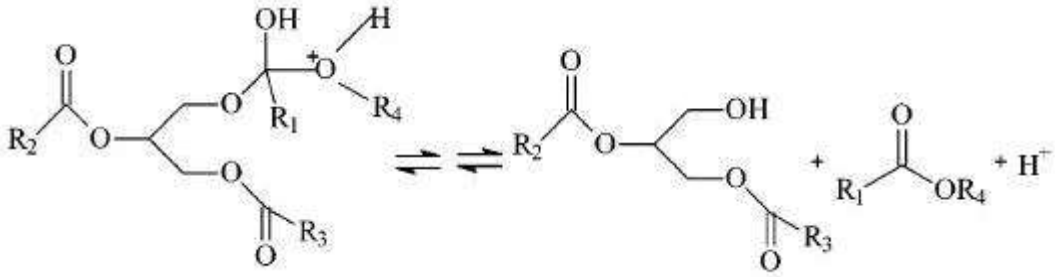
(3.14)

(a) Protonation of the carbonyl group by the acid catalyst.



(3.15)

(b) Nucleophilic attack of the alcohol, forming a tetrahedral intermediate.



(3.16)

(c) Proton migration and breakdown of the intermediate.

R_1, R_2, R_3 – carbon chain of the fatty acids
R_4 - alkyl group of the alcohol

Figure 3.6. Mechanism of Homogeneous Acid Catalyst (Lotero et al., 2006).

esterification (Chen et al., 1999). Generally, when using organic resins, the catalytic activity depends on the swelling properties which affect the overall reactivity. The resin pores are

(3.17)

macro pores with huge molecules containing long hydrocarbon chains without any diffusion limitations and the acid sites can be readily used (Zhang et al., 2001). However, some of the ion exchange resins are unstable above 140°C, so in this case an inorganic acid catalyst is generally used.

Zeolites are the most popular inorganic acid catalyst used to produce ester. They are very active catalysts for large carboxylic acids esterification. However, the reaction is slow. For fatty acid esterification, large pore zeolites can be used but they also give some undesirable byproducts (Corma et al., 1994). Sulphated zirconia (SO_4/ZrO_2) has been recently used in acid catalysis (Yadav and Nair, 1999). Sulphated zirconia is active due to its acid strength, but it deactivates because of sulphate leaching followed by hydrolysis (Omota et al., 2003). Chlorosulfonic acid precursor is used to prepare the above catalyst which does not dissolve in sulphuric acid but in an organic solvent.

3.5.3. Supercritical Alcohol Transesterification

There are many methods for the synthesis of biodiesel through alkyl esterification in which supercritical alcohol plays a major role. This process produces the biodiesel from triglyceride (TG) with a fast reaction rate (Kusdiana et al., 2001). With the use of this type of alcohol, the FFAs can be completely converted into biodiesel esters (Marchetti et al., 2007). MgO catalyst is used to promote transesterification and increase the reaction rate in supercritical conditions (Tateno et al., 2004). Supercritical methanol has been used to convert FFAs by methyl esterification and transesterification of TG using non-catalytic transesterification method (Demirbas, 2002). The general idea of using supercritical alcohol is basically a connection between temperature and pressure which is based upon the

thermophysical properties of solvents such as viscosity, dielectric constant, polarity and specific weight. The critical temperature and critical pressure of various alcohols is shown in Table 3.4. A favorable ester conversion is based on increasing the supercritical temperature (Demirbas, 2002). The molar ratio of transesterified vegetable oils to alcohol under supercritical conditions is in the range of 1:40-1:6 under catalytic conditions and the conversion yield varies from 50 to 95% as vegetable oils, animal fats and alcohols usually contain water. The influence of the presence of water on MgO and calcined hydrotalcite performances have also been studied (Di Serio et al., 2006). In the conventional transesterification method, water and free fatty acids causes negative effects such as formation of soap and decreased catalyst effectiveness in the synthesis of biodiesel (Wright et al., 1994). The transesterification process may occur at different temperatures depending upon the feedstock oil used (Demirbas, 2003).

3.6. Enzymatic Transesterification

Enzymes are bio-catalysts that allow many chemical reactions to occur within a living system. Enzymes have been used in the production of products such as drinks, baking, detergents, textiles, pulp and paper, leather, starch hydrolysis and fructose, semi synthetic penicillins in large scale industries (Kudli-Shrinivas, 2007) as well as in the production of drug intermediates, biosurfactants and designer fats (Shah et al., 2003). In addition, enzymes have been effectively used in the biodiesel production process, though this approach has not been commercialized except in China (Du et al., 2004).

The advantages of using enzyme catalysts over the chemical catalysts are: (a) no formation of soap, (b) the esterifying ability for both FFA's and TG's in a single step without the post treatment step, (c) production of a high level of glycerol as a by-product, (d) the ability to manage with different qualities of raw materials and (e) utilization of less energy in reactions under mild conditions compared with the alkali and acid catalysts (Ghaly et al., 2010; Fjerbaek et al., 2009). However, this enzymatic process has several disadvantages: (a) high reaction time (b) a highly concentrated catalyst is required to complete the reaction and (c) the cost of production is very high. In order to reduce the cost, immobilized enzymes have been used. However, the activity of the enzymes is reduced within 100 days of application (Fjerbaek et al., 2009). A comparison of biodiesel production by different

Table 3.4. Critical temperatures and critical pressures of various alcohols (Demirbas, 2002).

Alcohol	Critical temperature (K)	Critical pressure (MPa)
Methanol	512.2	8.1
Ethanol	516.2	6.4
1- Propanol	537.2	5.1
1-Butanol	560.2	4.9

techniques is presented in Table 3.5.

3.6.1. Mechanism of Biodiesel Production Using Enzymes

A two-step mechanism is involved in the production of a single ester bond from a TAG with the addition of alcohol by enzymatic transesterification. Hydrolysis takes place in the first step to break ester bonds and produce alcohol, followed by the second step in which esterification takes place with the second substrate (Xu, 2000). The equations are as follows:

$$E + ESs \leftrightarrow E.ESs \leftrightarrow F.Bp \leftrightarrow F + Bp \quad (3.18)$$

$$F + As \leftrightarrow F.As \leftrightarrow E.ESp \leftrightarrow E + ESp \quad (3.19)$$

The substrate and product are indicated by the subscripts s and p, respectively. For ester, alcohol substrate (A_s), product with alcohol moiety (B_p), free enzymes (E), ester substrate (ES_s), fatty acid alkyl esters (FAAE) (ES_p) and (F) fatty acid (Paiva et al., 2000).

Generally, enzymes have an active 3D structure in an aqueous environment with exposed polar groups and buried non-polar groups inside. A lipolytic reaction using lipases, unlike other enzymes, is naturally complicated, because lipids are water insoluble (Akoh et al., 2007; Sellappan and Akoh, 2005). Therefore, water is required in the reaction media heterogeneously to maintain an active lipase, (and the immiscibility of the lipids) by forming a liquid-liquid interface to catalyze the reaction. The activity of lipase can be influenced naturally by the interface, interfacial area and interfacial properties.

Enzymes are activated through the interface by adsorption that helps to open the catalytic site (Sellappan and Akoh, 2004; Shaw et al., 1990). Interfaces can be classified into three types: solid–liquid, liquid–liquid, and liquid–gas. These interfaces can be activated by influencing the interfacial hydrophobicity. In a lipid- water system, the activity of the enzyme increases by increasing an interfacial area due to the amount of enzyme adsorbed (Akoh et al., 2007). Enzyme adsorption at the interface leads to activation and substrate binding before the catalysis is achieved completely. The reaction products accumulate on the interface, which reduces the interfacial pressure that leads to high surface energy (Akoh et al., 2007).

Table 3.5. Comparison of biodiesel production by different techniques (Marchetti et al., 2007).

Variable	Alkali catalysis	Lipase catalysis	Supercritical alcohol	Acid catalysis
Reaction temperature (°C)	60-70	30-40	239-385	55-80
FFA in raw materials	Saponified products	Methyl esters	Esters	Esters
Water in raw materials	Interference with reaction	No influence	-	Interference with reaction
Yield of methyl esters	Normal	Higher	Good	Normal
Recovery of glycerol	Difficult	Easy	-	Difficult
Purification of methyl esters	Repeated washing	None	-	Repeated washing
Production cost of catalyst	Cheap	Relatively expensive	Medium	Cheap

3.6.2. Enzyme Choice

Enzymatic biodiesel production from TAG requires a non-stereospecific lipase to convert all TG, DG, and MG into FAAE and simultaneously esterify FFA (Fjerbaek et al., 2009). Biodiesel production using lipases is easy and uses less reaction temperature compared with chemical catalyst. High ester yield, lower inhibition of product and alcohol are the main advantages of using enzymes.

Bacteria and fungi are the two major micro-organisms that are used to synthesize lipases for the enzymatic transesterification process. Depending on the source of lipase and alcohol used, some of the enzymes are capable of converting more than 90% of raw material into biodiesel with a variant reaction temperature of 30-50°C and reaction time of 8-90 hours. In particular, immobilized *Pseudomonas cepacia* and free enzymes were used to transesterified jatropha oil with ethanol and soybean oil with methanol for 8 h and 90 h, respectively (Fjerbaek et al., 2009). Therefore, the conversion yield does not depend on the lipase origin alone but also on the reaction time and temperature, activity of water, alcohol choice, free or immobilized enzymes, molar ratios of oil to alcohol, and the life time of the enzyme (Ma et al., 1999; Shah et al., 2003; Demirbas, 2009; Fjerbaek et al., 2009; Ghaly et al., 2010).

3.6.3. Microbial Lipases

Lipases are identified in all living organisms and classified on the basis of sources: microorganism, animal and plant. Lipases can be produced at a high yield from microorganisms such as bacteria and fungi. There are 38 distinct bacteria used to produce the lipase for biodiesel production. They are: *Aspergillus niger, Bacillus thermoleovorans, Burkholderia cepacia, Candida antarctica, Candida cylindracea, Candida rugosa, Chromobacterium viscosum, Fusarium heterosporum, Fusarium oxysporum, Getrichum candidum Humicola lanuginose, Oospora lactis, Penicillium cyclopium, Penicillium roqueforti, Pseudomonas aeruginosa, Pseudomonas cepacia, Pseudomonas fluorescens, Pseudomonas putida, Rhizomucor miehei, Rhizopus arrhizus, Rhizopus chinensis, Rhizopus circinans, Rhizopus delemr, Rhizopus fusiformis, Rhizopus japonicus NR400, Rhizopus oryzae, Rhizopus stolonifer NRRL1478, Rhodotorula rubra, Saccharomyces cerevisiae, Staphylococcus hyicus* and *Thermomyces lanuginose* (Ghaly et al., 2010; Fjerbaek et al.,

2009). However, the most effective lipases that are produced for transesterification processes are *Candida antarctica, Candida rugosa, Pseudomonas cepacia, Pseudomonas fluorescens, Rhizomucor miehei, Rhizopus chinensis, Rhizopus oryzae* and *Thermomyces lanuginose* (Vasudevan and Briggs, 2008).

Mittelbach (1990) reported that methanolysis and ethanolysis with *Candida antarctica* achieved 90 and 82% without using a solvent. Rodrigues et al. (2008) stated that the conversion yield decreases with the increased carbon chain of the alcohol. Li et al. (2006) and Salis et al. (2005) reported that *Candida antarctica* with tert-butanol solvent and a solvent-free medium of methanolysis gave 90 and 45% conversion yield, respectively. The use of *Pseudomonas cepacia* for methanolysis and ethanolysis (Noureddini et al., 2005) and butanolysis (Salis et al., 2005) was examined in the absence of a solvent and resulted in 67, 65 and 100% conversion yield, respectively. Rodrigues et al. (2008) reported that the highest conversion yield was achieved using *Rhizomucor miehei* for butanolysis of short chain alcohols. Salis et al. (2005) reported that using *Rhizomucor miehei* for butanolysis in a solvent free system gave a 99% yield conversion. Rodrigues et al. (2008) found no significant variations when *Thermomyces lanuginose* reacted with ethanol, propanol and butanol but the methanolysis reaction with *Thermomyces lanuginosa* gave a higher conversion rate. Methanolysis with solvent *tert*-butanol reached a conversion rate of 85% (Li et al., 2006). A combination of *Candida antarctica* and *Thermomyces lanuginose* in methanolysis using *tert*-butanol solvent gave 95% conversion yield (Li et al., 2006).

Rapeseed oil with 2-ethyl-1-hexanol in addition to *Candida rugosa* lipase gave a 97% ester conversion. The ester of fatty alcohol (C_4-$C_{18:1}$) in a solvent free system with an immobilized Lipozyme IM-20 lipase from *Mucor miehei* was investigated by De et al. (1999). Triglycerides of grease, sunflower oil and fish oil with ethanol and lipases from *Candida antarctica* (Breivik et al., 1997), *Mucor miehei* (Selmi and Thomas, 1998) and *Pseudomonas cepacia* (Wu et al., 1999) were investigated and more than 80% conversion yield was obtained. The most significant conversions of TG were obtained using *Mucor miehei* and *Candida antarctica* lipases.

A high conversion of palm kernel oil (72%) was achieved with ethanol using *Pseudomonas cepacia* lipase compared to 15% with methanol (Abigor et al., 2000).

Methanolysis can also be achieved without using an organic solvent in water containing a system using 1(3)-regiospecific (Kaieda et al., 1999) and non-regiospecific (Kaieda et al., 2001) lipases in soybean oil. The catalytic ability is greater when non-regiospecific lipases such as *P.fluorescens, P.cepacia* and *C. Rugosaare* used. The *R.oryzae* lipase showed effective methanolysis with soybean oil and exhibits in 1(3) – regiospecificity (Scheib et al., 1998). Various lipases have been tested and the immobilized Novozyme 435 (*Candida antarctica*) was the most efficient lipase when using the methanolysis reaction (Shimada et al., 1999). Extracellular and intracellular lipases are produced by the microorganism and can be used in different application levels. These lipases can be immobilized on a solid support.

3.6.3.1. Intracellular Lipases: Intracellular lipase refers to the lipase utilized within the cells (Robles-Medina et al., 2009). Intracellular lipases are generally produced by microbes using solid state or submerged fermentation followed by separation and purification (Figure 3.7). Intracellular lipases are used as a whole cell biocatalyst in the bioconversion process in order to eliminate the purification step because it is very expensive. Whole cell biocatalysts have been used directly in the production of biodiesel and polyesters (Liu et al., 2000). Several microbes have the ability to be immobilized on certain supports spontaneously which eliminates the cost of purification step (Fukuda et al., 2001).

Intracellular lipases are relatively stable and increase the conversion rate, but the transesterification process is much slower than that with extracellular lipases (Ranganathan et al., 2008). Certain microbes (*Candida antarctica, Rhizopus chinensis, Rhizopus oryzae* and *Saccharomyces cerevisiae)* are used as whole cell biocatalysts (Fukuda et al., 2009). Examples of enzymatic biodiesel production using immobilized and free enzymes are shown in Tables 3.6 and 3.7.

3.6.3.2. Extracellular Lipases: The use of extra cellular lipase in the methanolysis reaction is very effective. The methanolysis of lipase production using an extracellular lipase is shown in Figure 3.8. Various lipases have been tested, of which the immobilized Novozyme 435 *(Candida antarctica)* was the most efficient. The lipase can be inactivated by the addition of 1.5 molar of methanol and shaken against oil. Watanabe et al. (2000) reported that the excess methanol formed droplets and dissolved in the oil. Lipase inactivation can be

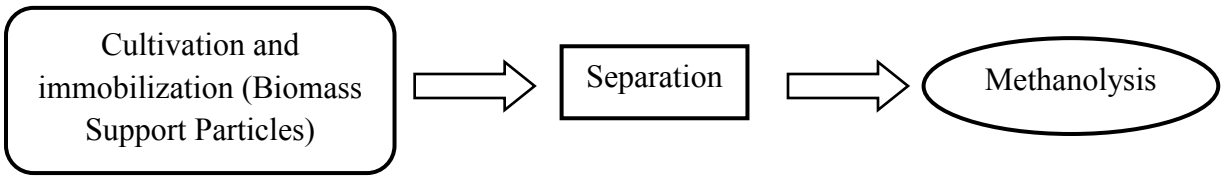


Figure 3.7. Methanolysis of lipase production using intracellular lipase (Fukuda et al., 2001).

Table 3.6. Enzymatic production of biodiesel using immobilized lipases.

Feed stock	Lipase	Acyl - acceptor	Alcohol: Substrate	Solvent	Temperature (°C)	Other conditions	Yield (%)	Authors
Rapeseed oil	*Candida sp.99-125*	methanol	3:1 molar ratio added in three steps	petroleum ether	40	36 hrs, 180 rpm, batch stirred reactor	83	Deng et al. (2005); Nie et al. (2006); Tan et al. (2006)
Salad oil	*Candida sp.99-125*	methanol	-	n-hexane	40	30 hrs, 180 rpm, batch stirred reactor	95	Deng et al. (2005); Nie et al. (2006); Tan et al. (2006)
Waste oil	*Candida sp.99-125*	methanol	-	petroleum ether	40	22 hrs, 180 rpm, three packed bed reactors	92	Deng et al. (2005); Nie et al. (2006); Tan et al. (2006)
Vegetable Oil	*Candida sp.99-125*	methanol	-	petroleum ether	40	30 hours, 180 rpm, batch stirred reactor	96	Deng et al. (2005); Nie et al. (2006)
Sunflower oil	*Candida antarctica*	methanol	-	None	-	-	3	Mittelbach (1990)
Sunflower oil	*Candida antarctica*	methanol	4:1 molar ratio added continuously	-	50	12 hrs, 130 rpm	97	Belafi-Bako et al. (2002)
Sunflower oil	*Candida antarctica*	methanol	3:1 molar ratio added in four steps	propanol	-	24 hrs	93.20	Deng et al. (2005)
Sunflower oil	*Candida antarctica*	methyl acetate	12:1	none	40	10 hrs	92	Xu et al. (2003)
Ethanol	*Candida antarctica*	ethanol	-	none	-	-	82	Mittelbach (1990)
Soybean oil	*Candida antarctica*	methanol	- -	-	-	preincubated in ethyl oleate for 0.5 hours	97	Samukawa et al. (2000)
Soybean oil	*Candida antarctica*	methanol	-	none	-	Stepwise addition of methanol	93.80	Watanabe et al. (2002)
Soybean oil	*Candida antarctica*		4:1	ionic liqid [Emim][TfO]	40	12 hrs	80	Ha et al. (2007)
Soybean oil	*Candida antarctica*	methyl acetate	12:1	none	40	14 hrs, 150 rpm	92	Du et al. (2004)
Tallow	*Candida antarctica*	methanol	3:1	-	30°C	72 hrs, 200rpm	74	Lee et al. (2002)

Table 3.6 Continued.

Feed stock	Lipase	Acyl - acceptor	Alcohol: Substrate	Solvent	Temperature (°C)	Other conditions	Yield (%)	Authors
Rapeseed Oil	*Candida antarctica*	methanol	-	*tert*-butanol	-	-	95	Li et al. (2006)
Cottonseed Oil	*Candida antarctica*	methanol	-	*tert*-butanol	-	-	97	Royon et al. (2007)
Jatropha oil	*Candida antarctica*	2-propanol	4:1	hexane	50°C	8 hrs, 150 rpm	92.8-93.4	Modi et al. (2006)
Soybean oil	*Pseudomonas fluoresces*	methanol	-	*n*-heptane	-	use of recombinant lip b68	92	Lou et al. (2006)
Sunflower Oil	*Pseudomonas fluoresces*	methanol	4.5:1 molar ratio added in three steps	none	40	24 hours, 200 rpm	>95	Soumanou and Bornscheuer (2003)
Sunflower Oil	*Pseudomonas fluoresces*	Isobutanol	3:1 molar ratio added in four steps	-	40	24 hrs	45.30	Deng et al. (2005)
Sunflower Oil	*Pseudomonas cepacia*	1-butanol	3:1 molar ratio added in four steps	-	40	24 hrs	88.40	Deng et al. (2005)
Soybean oil	*Pseudomonas cepacia*	methanol	-	none	-	-	67	Noureddini et al. (2005)
Soybean oil	*Pseudomonas cepacia*	ethanol	-	none	-	-	65	Noureddini et al. (2005)
Palm kernel Oil	*Pseudomonas cepacia*	ethanol	-	none	-	-	72	Abigor et al. (2000)
Palm kernel	*Pseudomonas cepacia*	t-butanol	-	none	-	-	62	Abigor et al. (2000)
Oil	*Pseudomonas cepacia*	*n*-propanol	-	none	-	-	42	Abigor et al. (2000)
Mahua oil	*Pseudomonas cepacia*	ethanol	4:1 molar ratio	-	40	6 hrs,200 rpm	96	Kumari et al. (2007)
Jatropha oil	*Pseudomonas cepacia*	ethanol	4:1 molar ratio	-	50	8 hrs, 200 rpm	98	Shah and Gupta (2007)
Jatropha oil	Whole cell *Rhizopusoryzae*	methanol	3:1	-	30	60 hrs, glutaraldehyde treatment	80	Tamalampudi et al. (2008)

Table 3.6 Continued.

Feed stock	Lipase	Acyl - acceptor	Alcohol: Substrate	Solvent	Temperature (°C)	Other conditions	Yield (%)	Authors
Soybean oil	*Rhizopus oryzae*	methanol		-	37	165 hrs, 150 rpm	71	Matsumoto et al. (2001)
Soybean oil	*Rhizopus oryzae*	methanol	-	none	-	-	80-90	Kaieda et al. (2001)
Soybean oil	*Rhizopus oryzae*	methanol	-	-	-	Stepwise addition of methanol, glutaraldehyde treatment	90	Ban et al. (2001)
Sunflower Oil	*Rhizomucor miehei*	methanol	3:1 molar ratio added in three steps	*n*-hexane	40	30 hrs, 200 rpm	>80	Soumanou and Bornscheuer (2003)
Sunflower Oil	*Rhizomucor miehei*	ethanol	3:1 molar ratio added in four steps	*n*-hexane	40	24 hrs	79.10	Soumanou and Bornscheuer (2003)
Soybean oil	*Rhizomucor miehei*	methanol	-	*n*-hexane	-	-	92.20	Shieh et al. (2003)
Sunflower Oil	*Thermomyces lanuginose*	methanol	3:1 molar ratio added in three steps	*n*-hexane	40	30 hrs, 200 rpm	>60	Soumanou and Bornscheuer (2003)
Sunflower Oil	*Thermomyces lanuginose*	1-propanol	3:1 molar ratio added in four steps	-	40	24 hrs	89.80	Deng et al. (2005)
Sunflower Oil	*Thermomyces lanuginose*	2-propanol	3:1 molar ratio added in four steps	-	40	24 hrs	72.80	Deng et al. (2005)
Rapeseed oil	*Thermomyces lanuginose*	methanol	4:1	*tert* butanol	35	12 hrs130 rpm	95	Li et al. (2006)
Jatropha oil	*Chromobacterium viscosum*	ethanol	4:1	none	40	8 hrs, 200 rpm, addition of 0.5% (w v-1) water	92	Shah et al. (2004)
Sunflower oil	*Mucor miehei*	ethanol	3:1	none	30	5 hrs	83	Selmi and Thomas (1998)

Table 3.7. Enzymatic production of biodiesel using free lipases.

Feed stock	Lipase Form	Acyl - acceptor	Alcohol: Substrate	Solvent	Temperature (°C)	Other conditions	Yield (%)	Authors
Soybean oil	*Pseudomonas fluoresces*	methanol	3:1 molar ratio added in three steps	none	35	90 hrs, 150 rpm	90	Kaieda et al. (2001)
Soybean oil	*Pseudomonas cepacia*	methanol	3:1 molar ratio added in three steps	-	35	90 hrs, 150 rpm	>80	Kaieda et al. (2001)
Jatropha oil	*Chromobacterium viscosum*	ethanol	4:1	none	40	8 hrs, 200 rpm, addition of 1% (w/v) water	73	Shah et al. (2004)
Sunflower oil	*Mucor miehei*	ethanol	3.6:1	petroleum ether	45	5 hrs	82	Mittelbach (1990)
Tallow	*Mucor miehei*	methanol	3:1	hexane	45	8 hrs, 200 rpm	94.8	Nelson et al.(1996)
Rapeseed oil	*Mucor miehei*	methanol	3:1	hexane	45	5 hrs, 200 rpm	77.3	Nelson et al. (1996)
		ethanol	3:1	hexane	45	5 hrs, 200 rpm	98.2	Nelson et al. (1996)
Soybean oil	*Mucor miehei*	methanol	3:1	hexane	45	5 hrs, 200 rpm	75.4	Nelson et al. (1996)
		ethanol	3:1	hexane	45	5 hrs, 200 rpm	97.4	Nelson et al. (1996)

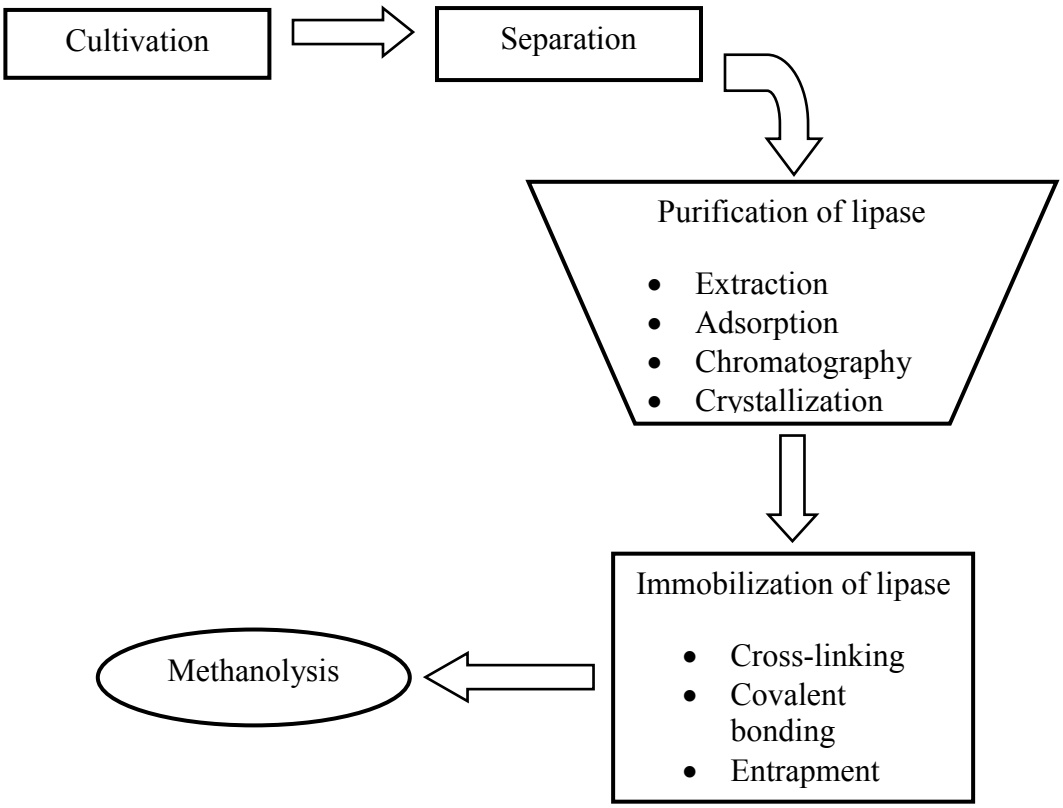


Figure 3.8. Methanolysis of lipase production using extracellular lipase (Fukuda et al., 2001).

avoided by adding methanol in a step by step fashion. After 50 cycles, a 95% conversion yield of ester was achieved using Novozyme 435. Finally, 90-93% of methyl ester yield was obtained without any heavy reduction in the conversion, after using the lipase for at least 100 days.

The methanolysis for the production of biodiesel using Novozyme 435 pretreatment was investigated by Samukawa et al. (2000). When Novozyme 435 was preincubated for half an hour in methyl oleate and 12 hour in soybean oil, the methanolysis progressed faster. A conversion yield of about 97% was reached with the addition of 0.33 molar methanol (added stepwise in 0.25-0.4 hour intervals).

Methanolysis can also be achieved without using organic solvent in water containing a system of 1(3)-regiospecific (Kaieda et al., 1999) and non-regiospecific (Kaieda et al., 2001) lipases in soybean oil. The catalytic ability is greater when non-regiospecific lipases *(P. fluorescens, P. cepacia and C. rugosa)* were used (Kaieda et al., 2001). Also, *R.oryzae* lipase shows effective methanolysis with soybean oil and exhibits in 1(3) – regiospecificity (Scheib et al., 1998).

3.6.4. Immobilization

Lipases can be evaluated as biocatalysts with the help of soluble enzymes in an aqueous media. Most of the lipases have been used with organic media and require favorable protocols in order to use and reuse immobilized lipase in an industrial setting. There are several immobilization techniques that have been used and examined, such as adsorption, cross linking, entrapment, and encapsulation to form Biomass Support Particles (BSPs). The methods of enzyme immobilization are shown in Figure 3.9.

The Biomass Support Particles (BSPs) have several advantages over other techniques: (a) chemical additives are not required, (b) preproduction of cells is not required, (c) no need to handle aseptic particles, (d) the mass transfer rate is rapid within BSPs, (e) BSPs are reusable, (f) particles resist mechanical shear, (g) easy to scale up the bioreactor and (h) less expense (Atkinson et al., 1979). Due to these merits, the BSPs technique has been widely used with various cell systems such as plant (Pak et al., 1990), animal (Yamaji and Fukuda, 1997), microbial (Liu et al., 1999) and insect (Yamaji et al., 2000). Also, during batch

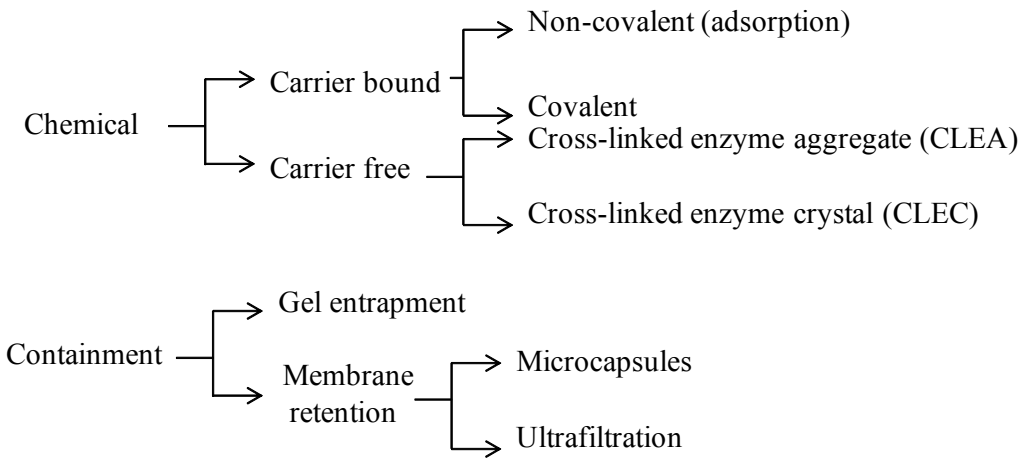


Figure 3.9. Methods of enzyme immobilization (Illanes et al., 2008).

cultivation, the immobilization can be performed immediately without requiring any purification with the use of a whole cell biocatalyst.

For lipase production, the culture conditions, cell pretreatment effects, and methanolysis reaction with water content have been investigated using immobilized cells (*R. oryzae*) as a whole catalyst within BSPs (Ban et al., 2001). During batch cultivation, the immobilized cells were efficiently immobilized within a BSPs-polyurethane foam (support). Without glucose, the intracellular activity was supported with the addition of oleic acid or olive oil as substrate-related compounds for the culture medium. About 80-90% conversion to methyl ester was achieved in the presence of water (10-20%) with stepwise methanol addition using immobilized cells (BSPs) without pretreatment of the solvent. Methyl esters can also be achieved by this same process by exchanging extracellular lipase for intracellular lipase (Kaieda et al., 1999).

The adsorption technique is simple, non-toxic and less expensive. However, attachment is by weak forces. Numerous carriers have been investigated and utilized to immobilize the lipases. Some of them are polypropylene EP 100, toyonite 200-M, celite, diatomaceous earth, anion resin and accurel (Shah etal., 2004). Ester yields of 76-98% were achieved from vegetable oils and waste oils using immobilized enzymes. Non-polar solvents have more adsorbing features than polar solvents (Yang et al., 2006). Loss of enzyme activity is possible because of leaching either by van der Waals forces or dispersion forces but not due to enzyme deactivation (Yadhav and Jadhav, 2005). Therefore, the adsorption technique has not been utilized widely by industries due to the low ability to reuse enzymes.

Cao et al. (2003) reported that the transesterification rate increased using a cross linking technique. It linked enzymes using multifunctional and bi-functional reagents such as hexamethylene diisocynate and glutaraldehyde. However, the separation process was difficult due to the enzyme and substrate having particle sizes less than 10μm. Ban et al. (2002) reported that a cross linking treatment is necessary to stabilize immobilized cells (*R. oryzae*) using a solution of 0.1% glutaraldehyde. Thus, the activity of lipase can be obtained without any consequential decrease during batch cycles reaching 70-83% of methyl ester within 72 hours. However, the lipase activity can be reduced without the addition of

glutaraldehyde. The whole cell biocatalysts can be utilized as an industrial application due to its lipase production simplicity and long period of lipase stability (Ban et al., 2001).

The Encapsulation method involves bounding of the enzyme within the capsules. However, the conversion yield is low due to the limited lipase activity on TGs (Vicente et al., 1994). Also, the membrane can be clogged which prohibits the reaction and decreases the enzyme activity (Fjerbaek et al., 2009).

The entrapment technique involves the capture of microbial cells within the inner cavities of a polymer matrix (gel). Lipases that are immobilized by the entrapment technique are more stable and show better activities than cross-linking, adsorption and encapsulation (Kennedy et al., 1990). This entrapment technique is relatively simple, robust and easy to recover during continuous process (Meter et al., 2007). The major disadvantage of this method is the mass transfer limitation which results in a lower conversion yield when compared with adsorption and cross linking techniques (Jegannathan et al., 2008).

3.6.5. Lipase Reusability and Recovery

The cost of lipases is the most common problem associated with the industrial biodiesel production process. Therefore, immobilized enzymes have been generally used to reduce the cost. This results in a biocatalyst that is easy to handle and enables easy recycling and recovery (Fjerbaek et al., 2009). The longevity and durability of the lipase can be decided by the immobilization matrix strength and cultivation method. However, mass transfer limitations are caused both internally and externally due to immobilization of huge molecules (Fjerbaek et al., 2009).

Acyl acceptors have been used to produce biodiesel in the presence of reused immobilized enzymes, which reduce yield with lower alcohols like methanol and ethanol. A stepwise addition of alcohol can be used to reduce the lipase deactivation. In a study by Lee et al. (2002), transesterification of olive oil with stepwise addition of methanol allowed the enzyme to be reused repeatedly, with over 85% conversion maintained after eighty cycles. Also, methyl acetate was found more significant with 92% conversion and without any loss in lipase activity after 50 batches (Du et al., 2004). Immobilized enzymes such as

Novozyme435 and Lipozyme TLIM were used to produce biodiesel and reused twenty times achieving a 97.2% yield conversion (Huang et al., 2010).

To increase the stability of the enzyme, addition of solvents in the production system has been suggested (Li et al., 2007). The longevity of the enzymes increased the yield to 70% over many cycles by the pretreatment of gluteraldehyde. However, the yield decreased to 50% after six cycles. Another technique is to wash the lipase with *tert*-butanol, which results in no loss in the fatty esters even after 200 cycles of use (Li et al., 2007). The reuse of enzymes with the use of isopropanol achieved over 80% yield conversion, even after the enzyme was reused after five cycles (Lee et al., 2002). Lipase reusability has also been demonstrated by washing with hexane between cycles, though the enzymes were sufficiently active for not more than three cycles (Salah et al., 2007). The stability of the *Carica papaya* lipase (CPL) has been investigated with and without using a *tert*-butanol solvent and it was found that the conversion yield unchanged even after the lipase was reused for thirty cycles without any loss (Pinyaphong et al., 2011).

3.7. Factors Affecting the Transesterification Reaction

The transesterification process of biodiesel production can be affected by several factors and conditions used. If the parameters are not optimized, the reaction becomes either incomplete or the yield is reduced by a significant extent. The process parameters are: (a) type of alcohol, (b) type of solvent, (c) molar ratio of alcohol: oil, (d) lipase pre-treatment and (e) reaction temperature.

3.7.1. Alcohol Type

There are various different compounds that have been considered as acceptable acyl acceptors for the transesterification process as they give good ester yield and polyester as biodegradable products. According to Modi et al. (2007), methyl and ethyl acetate have been seen as more efficient acyl acceptors. But, the cost of these acceptors is relatively higher than the commonly used alcohols (Vasudevan and Briggs, 2008). Also, these two acyl acceptors produce different byproducts, excluding glycerol (Xu and Wu, 2003).

Coggon et al. (2007) reported that the primary, secondary and longer chain alcohols and straight and branched chain alcohols were used in the transesterification process. However, longer chain alcohols gave a lower yield. Iso et al. (2001) reported that the most commonly used alcohols are: methanol, ethanol, propanol, iso-propanol, 2-propanol, n-butanol and iso-butanol. Salis et al. (2005) investigated various alcohols without solvent using *Pseudomonas cepacia* lipase in triolein and found that this combination gave a 40% conversion yield in methanol, 83% conversion yield in 2-butanol, 93% conversion yield in ethanol and 99% conversion yield of propanol, 2-methyl-1-propanol, and a combination of pentanol isomers. Salis et al. (2005) stated that lipases are deactivated due to the addition of insoluble methanol to the reaction system which affects the yield.

Methanol and ethanol are the two most economically feasible alcohols used in the biodiesel production process. However, these alcohols inhibit and deactivate the enzymes. In particular, methanol was found to be the most deactivating alcohol (Chen and Wu, 2003). Transesterification using lipases in ethanol is comparatively better than in methanol because the rate of transesterification increases when the length of carbon chain increases (Antczak et al., 2009). In addition, ethanol is produced from a renewable source whereas methanol is produced from fossil fuels. Commercially, methanol is potentially a good acyl source for enzymatic transesterification for biodiesel production with respect to time (Fjerbaek et al., 2009). Ranganathan et al. (2008) suggested that ethanol is the best acyl acceptor for the biodiesel production, since there is an increase in the ethanol production all over the world which decreases the cost of ethanol.

Lower chained alcohols have inhibiting effects that can be overcome by either the stepwise addition of alcohols or the use of solvents in the reaction medium (Watanabe et al., 2002; Modi et al., 2007). Usually, methanol is added in stepwise rather than ethanol and it showed no deactivation of lipases. The introduction of methanol using stepwise and direct addition into a reaction system containing olive oil resulted in 98.92 and 65.00% conversion yields, respectively (Lee et al., 2008). Large amounts of enzyme can be used to prevent inhibition which leads to an increase in the production cost (Fjerbaek et al., 2009).

3.7.2. Solvents

Solvents are generally used in the transesterification process in order to protect the enzyme from denaturation by the alcohol, as the use of solvent increases alcohol solubility (Kumari et al., 2009). On the other hand, the solvent may also increase the glycerol solubility by coating the enzyme which inhibits the performance (Royon et al., 2007). The use of solvent on the reactants and products reduces enzyme inhibition and also ensures a homogenous mixture (Ranganathan et al., 2008). The reaction rate is significantly increased with the solvent system in comparison to solvent-free systems (Vasudevan and Briggs, 2008).

The most common solvents used in the transesterification process are: cyclohexane, hexane, *n*-heptane, isooctane, petroleum ether, 2-butanol and *tert*-butanol (Nelson et al., 1996; Coggon et al., 2007; Soumanou and Bornscheuer, 2003). *Tert*-butanol is the most popular solvent among all the solvents because it is slightly polar, has an enzyme-stabilizing effect and is not influenced by any reactants or products and solvents based on their polarity (Li et al., 2006; Fjerbaek et al., 2009).

Solvents, especially *tert*-butanol and 2-butanol, are proposed to facilitate the regeneration of deactivated lipase (Robles-Medina et al., 2009). A methanolysis reaction using *Candida antarctica* with *tert*-butanol solvent showed an overall increase in the yield conversion (Royon et al., 2007). Two instances of methanolysis of the *Thermomyces lanuginose* lipase without solvent and with *tert*-butanol solvent gave 10 and 75% conversion yields, respectively (Li et al., 2006). Several solvents have been examined in which *n*-heptane gave a good conversion yield when methanolysis of *Rhizopus chinensis* lipase in soybean oil was used (Qin et al., 2008).

The transesterification of biodiesel production without solvent has also been investigated. Refined cotton oil has been transesterified with primary and secondary alcohols in the absence of solvent using *Candida antarctica* lipase resulting in 72 and 94% conversion yields, respectively (Kose et al., 2002). Similarly, sunflower oil used in a reaction medium containing *Mucor miehei* lipase and ethanol without adding solvent gave an 83% conversion yield (Selmi and Thomas, 1998). Using solvents in biodiesel production has a great

advantage in decreasing inhibitory effects when lower-chained alcohols are used. However, the solvent has to be removed from the final product as they are volatile, hazardous and expensive. The addition of solvents increases the reactor volume, which basically leads to an increase in biodiesel production costs (Ranganathan et al., 2008; Vasudevan and Briggs, 2008; Fjerbaek et al., 2009).

3.7.3. Molar Ratio of Alcohol to Oil

The molar ratio of alcohol to oil is one of the most significant factors that affect the ester yield. The optimum molar oil to alcohol ratio is generally based on the reaction system, feedstock, enzyme and alcohol used. However, the transesterification process requires three moles of alcohol and one mole of TG to convert to three moles of FAME and one mole of glycerol. The ester yield is reduced due to presence of glycerine in the solution (Antczak et al., 2009).

Enciner et al. (2002) investigated molar ratios from 3:1 to 15:1 of cynara oil to ethanol and found that the ratio of 9:1 gave a high conversion yield. Enciner et al. (2002) reported that when the molar ratio increased, the yield of ester also increased and the process was incomplete when molar ratio of less than 6:1 was used. However, glycerin separation interferes with a high ratio of alcohol to oil due to increased solubility. In a reaction medium, an excess of alcohol forms droplets that deactivate the enzyme.

The solubility of alcohol plays a critical role in the methanolysis of enzyme transesterification in a solvent free system because alcohol concentration decreases enzyme activity (Iso et al., 2001).The enzymes are generally inhibited by the solubility of the alcohols when they have less than three carbon atoms stoichiometrically. In addition, alcohols with more than three carbons do not inhibit the enzymes because they dissolve in the feedstock (Shimada et al., 2002).

A solvent system requires an excess of alcohol in order to achieve a good yield. For better yield, the molar ratio of methanol to oil can be in between 3:1 and 6:1 stoichiometrically (Matassoli et al., 2009). Stepwise addition of alcohol is more efficient because it minimizes the inhibition level in a solvent free system (Kose et al., 2002). On the

other hand, using ethanol in biodiesel production resulted in lower inhibition of enzymes by using a higher molar ratio of alcohol to oil.

Ethanolysis of fish oil has been tested using a lipoprotein lipase in a solvent free system and a higher molar ratio of alcohol to oil ratio (over 11:1) had a significant inhibitory effect (Robles Medina et al., 2009). Butanolysis of triolein using the *Pseudomonas cepacia* lipase was examined with four different ratios 3:1, 6:1, 9:1, and 12:1 of which all gave 100% conversion yield but with different reaction times (Salis et al., 2005). Using *Candida antarctica* in the reaction medium, the effect of alcohol to oil ratios from 1:1 to 6:1 was tested and it was observed that the conversion yield was higher in the ratio between 2:1 and 5:1 (Jeong and Park, 2008).

3.7.4. Lipase Pretreatment

Enzymes can be deactivated when low chain alcohols are used in the reaction medium. To prevent this enzyme deactivation, lipases can be pretreated by soaking them in a medium prior to being used in the transesterification process (Ranganthan et al., 2008). Methyl oleate, *tert*-butanol and isopropanol have been employed as pre-treatment media for the transesterification reaction process (Fjerbaek et al., 2009).

Immobilized *Candida antarctica* lipases were used in both the reaction medium with and without pretreatment in isopropanol and an increase in ester conversion yield was observed when the reaction was pretreated (Jegannathan et al., 2008). The deactivation of the lipases was reduced, resulting in 97% conversion yield by pre-incubating immobilized *Candida antarctica* in methyl oleate and soybean oil for 30 minutes and 12 hours, respectively (Fukuda et al., 2001). The methyl ester yield reached 97% with the stepwise addition of 0.33 molar ratio of methanol from 0.25 to 0.4 hour (Samukawa et al., 2000). In another experiment, 0.1% gluteraldehyde treatment was used to stabilize the *Rhizopus oryzae* cells, the conversion yield was more than 70% even after six cycles compared to 50% without the treatment of gluteraldehyde (Ranganathan et al., 2008). This pretreatment method is greatly significant in small scale, but increases overall production cost if it is used in a large scale process.

3.7.5. Reaction Temperature

The conversion rate of transesterification increases with reacting time which generally depends on the reaction temperature (between 20°C and 70°C). Enzymes are known to have a large thermal stability (Marchetti et al., 2007). However, the optimal temperatures for most of the lipases are between 30 and 60°C (Fjerbaek et al., 2009). Overall, the optimum reaction temperature is basically dependent on the alcohol to oil molar ratio, enzyme stability and the type of solvents (Antczak et al., 2009).

Candida antarctica lipase was investigated with methanol at a temperature in the range of 25-55°C and the optimum temperature was found to be 40°C (Jeong and Park, 2008). The optimal temperature was found to be 45°C when a combination of two lipases *(Rhizopus oryzae* and *Candida rugosa)* was used in the methanolysis reaction (Lee et al., 2008). *Rhizopus chinensis* lipase was tested using methanol at a temperature in the range of 20°C-60°C and it was observed that the optimal temperature was 30°C (Qin et al., 2008). The *Pseudomonas cepacia* lipase was tested in butanol at different temperatures between (20°C-70°C) and the optimum temperature was found to be 50°C after 1 hour and 40°C after 2 hours (Salis et al., 2005).

CHAPTER 4. MATERIALS AND METHODS

4.1. Glassware

The glassware used for the research included test tubes, beakers, reagent bottles, Pyrex bottles and pipettes. All glassware was washed using soap and hot water and rinsed with distilled water prior to use for the experiments.

4.2. Chemicals

Methanol, 2-butanol, hexane, tertrahydrofuran, N, O - Bis (Trimethylsilyl)-Trifluroacetamide (BSTFA), FAME standards such as methyl myristate, methyl pentadecanote, methyl cis-11-eicosenoate, methyl all-cis-5,8,11,14,17- eicosapentaenoate (EPA), methyl erucate, methyl all-cis-7,10,13,16,19-docosapentaenoate (DPA), and methyl all-cis-4,7,10,13,16,19-docosahexenoate (DHA) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate were purchased from Alltech Associates,Inc., (Deerfield, Illinois, USA). Methyl stearidonate was purchased from Cayman chemical (Ann Arbor, Michigan, USA).

4.3. Equipment

The equipment used in the experiments included: Hewlett-Packard gas chromatograph, coupled with flame ionization detector (FID) (HP5890 Series II, Agilent Technologies, Mississauga, Ontario, Canada), HELIFLEX Capillary Column30m x 0.32mm x 0.25µm, (AT-FAME, Alltech Associates, Inc., Deerfield, Illinois, USA), microprocessor controlled water bath (Precision 280 Series, Thermo Scientific, Ohio, USA), reciprocal shaking water bath (Precision 2870 Series, Thermo Scientific, Ohio, USA), micro pipette (Eppendorf Research Plus, Fisher Scientific, Toronto, Ontario, Canada). GC Crimp vials (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada) and digital balance (AE 200 Scale -Mettler Toledo International Inc., Mississauga, Ontario, Canada).

4.4. Fish Oil

Crude fish oil XM 1812 C (Mackerel oil) was obtained from Ocean Nutrition Canada, Mulgrave, Nova Scotia, Canada. It was stored in a cool, dry place at a temperature of 15°C. The characterization of Crude fish oil XM 1812 C (Mackerel oil) is shown in Table 4.1.

4.5. Enzyme

The immobilized lipase *Candida antarctica* (Novozyme 435) was purchased from Sigma Aldrich (St. Louis, Missouri, USA) and an experimental immobilized lipase enzyme (NS88001) was obtained from Novozymes North America Inc (Franklinton, North Carolina, USA).

4.6. Experimental Design

The experimental work performed in the laboratory to produce biodiesel from fish oil by transesterification was carried out in three stages.

In the first stage, the optimization of the transesterification process was carried out by investigating the effectiveness of two lipases [(*Candida antarctica* (Novozyme 435) and an experimental enzyme (NS 88001)] and the effects of the oil: alcohol molar ratio (1:1, 1:2, 1:3, 1:4 and 1:5), alcohol type (methanol and 2-butanol), reaction temperature (35, 40, 45 and 50°C), solvent system (solvent and solvent-free) and reaction time (4, 8, 12 and 16 hours) on the biodiesel production as shown in Table 4.2.

In the second stage, the transesterification process was carried out to investigate the effects of alcohol (methanol and 2-butanol), reaction time (4, 8, 12 and 16 hour) and solvent system (solvent and solvent-free) at constant reaction temperature of 40°C and oil: alcohol molar ratio (1:4) on the effectiveness of combined lipases (Novozyme 435 and NS88001) for transesterification of fish oil to biodiesel as shown in Table 4.3.

In the third stage, the reusability factor for the lipase enzymes (Novozyme 435 and NS88001) individually and in combination for the transesterification process were also carried out by investigating the number of cycles used as shown in Table 4.4.

Table 4.1. The Characterization of Crude fish oil XM 1812 C (Mackerel oil).

Parameter	Specification
Appearance	Clear yellow oil
Odor	Fishy
Acid value (mg KOH/g)	0.37
Peroxide value (meq/kg)	0.30
p-Anisidine value	6.87
Free fatty acid (%)	0.19
Moisture content (%)	0.05

Table 4.2. Optimization parameters of transesterification process.

Factors	Parameters
Enzymes	*Candida antarctica* (Novozyme 435) and an experimental enzyme (NS88001)
Oil: alcohol molar ratio	1:1, 1:2, 1:3, 1:4 and 1:5
Alcohol	Methanol and 2-butanol
Reaction Time	4, 8, 12 and 16 h
Reaction Temperature	35, 40, 45 and 50°C
System	Solvent and solvent-free

No. of replicates = 2
Total no. of samples = 1280

Table 4.3. Enzymatic transesterification using a combination of enzymes.

Factors	Parameters
Enzymes	Combination of *Candida antarctica* (Novozyme 435) and experimental enzyme (NS88001)
Alcohol	Methanol and 2-butanol
Oil: alcohol molar ratio	1:4
Reaction temperature	40°C
Reaction time	4, 8, 12 and 16 hours
System	Solvent and solvent-free

No. of replicates = 2
Total no. of samples = 32

Table 4.4. Lipase reusability condition.

Factors	Parameters
Enzymes	*Candida antarctica* (Novozyme 435), experimental enzyme (NS88001) and Combination of both enzymes
Oil: alcohol molar ratio	1:4
Alcohol	Methanol and 2-butanol
Reaction Time	16 h
Reaction Temperature	40°C
System	Solvent and solvent-free

No. of cycles =50

4.7. Production of the Biodiesel from the Fish Oil using Individual Enzyme

The experimental procedure is shown in Figure 4.1. A 2 g sample of crude Mackerel oil was transferred into a 50 ml conical flask using a micro pipette (Eppendorf Research Plus, Fisher Scientific, Toronto, Ontario, Canada). The conical flask assembly was placed in a reciprocal shaking water bath (Precision 2870 Series, Fisher Scientific, Toronto, Ontario, Canada) and heated to the desired temperature. The enzyme Novozyme 435 was evaluated at different oil: alcohol molar ratios (1:1, 1:2, 1:3, 1:4 and 1:5), reaction temperatures (35, 40, 45 and 50°C) and solvent systems (with and without solvent). First, the oil: alcohol molar ratio of 1:1, reaction temperature of 35°C and solvent were selected. A measured amount of the alcohol methanol and the enzyme catalyst (25% wt based on oil % wt) were added individually to the conical flask. The reciprocal shaking bath was turned on at 200 rpm. An aliquot of 100 µl of reaction medium was taken at 4, 8, 12 and 16 hours and then dried with nitrogen gas. 100 µl tertrahydrofuran and 200 µl of BSTFA were added for gaschromatography (GC) analysis. The same procedure was followed for all oil: alcohol molar ratios, solvent systems, reaction temperature and reaction times. The entire procedure was repeated with the enzyme NS88001.

4.8. Production of the Biodiesel from the Fish Oil using Combination Enzymes

The experimental procedure is shown in Figure 4.2. A 2 g sample of crude Mackerel oil was transferred into a 50 ml conical flask using a micro pipette (Eppendorf Research Plus, Fisher Scientific, Toronto, Ontario, Canada). The conical flask assembly was placed in a reciprocal shaking water bath (Precision 2870 Series, Fisher Scientific, Toronto, Ontario, Canada) and heated to the desired temperature of 40°C. The combination of the two enzymes was evaluated at different reaction times (4, 8, 12 and 16 hours) and solvent systems (with and without solvent). A measured amount of the alcohol methanol (1:4 oil: alcohol molar ratio) and the enzyme catalyst (12.5% wt of each enzyme based on oil % wt) were added to the conical flask. The reciprocal shaking bath was turned on at 200 rpm. An aliquot of 100 µl of reaction medium was taken at 4, 8, 12 and 16 hours and then dried with nitrogen gas. 100µl tertrahydrofuran and 200 µl of BSTFA were added for gas chromatography (GC) analysis. The same procedure was followed for all the solvent systems and alcohols.

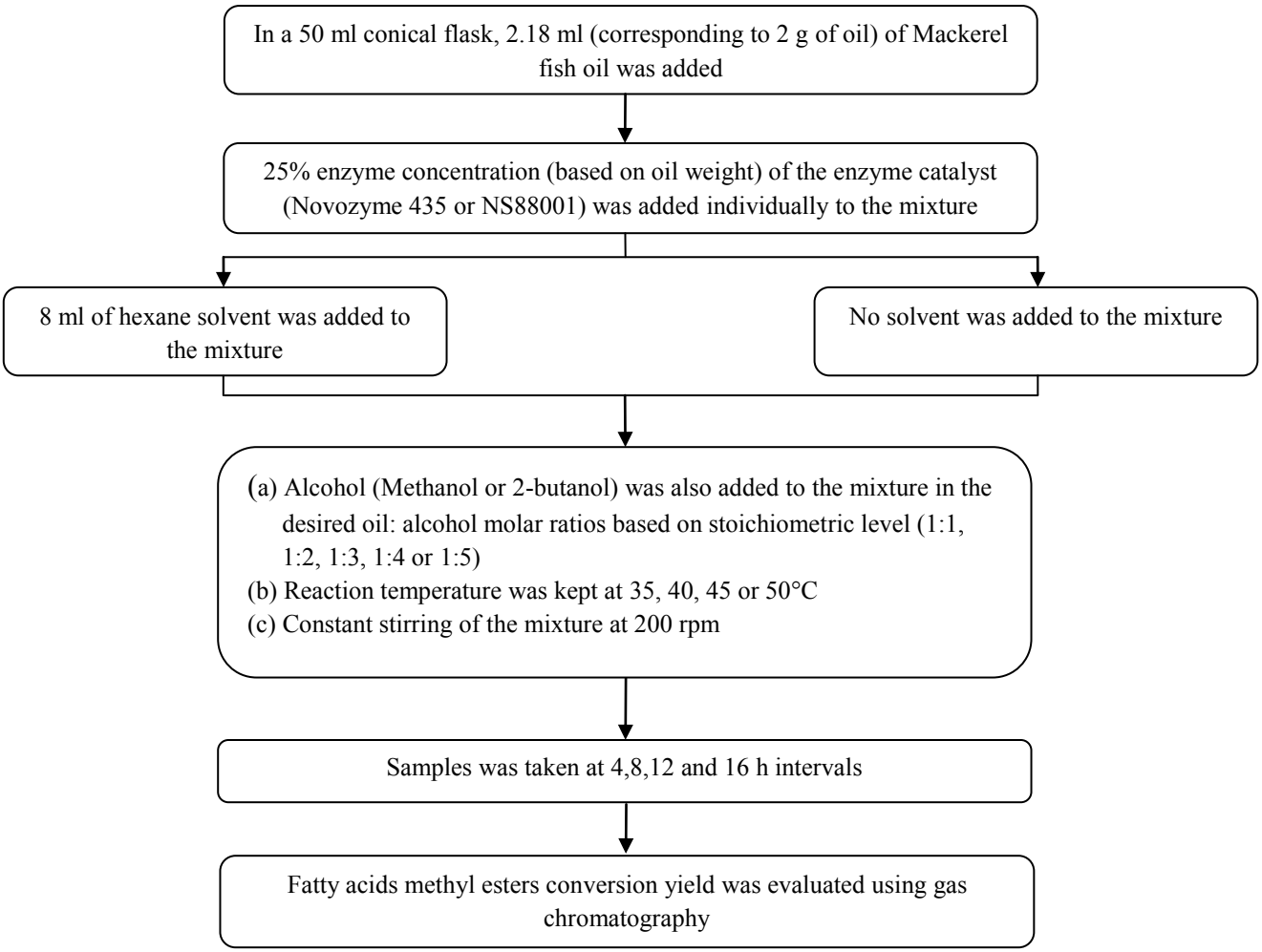


Figure 4.1. Enzymatic transesterification process of individual enzyme catalyst by solvent and solvent free systems.

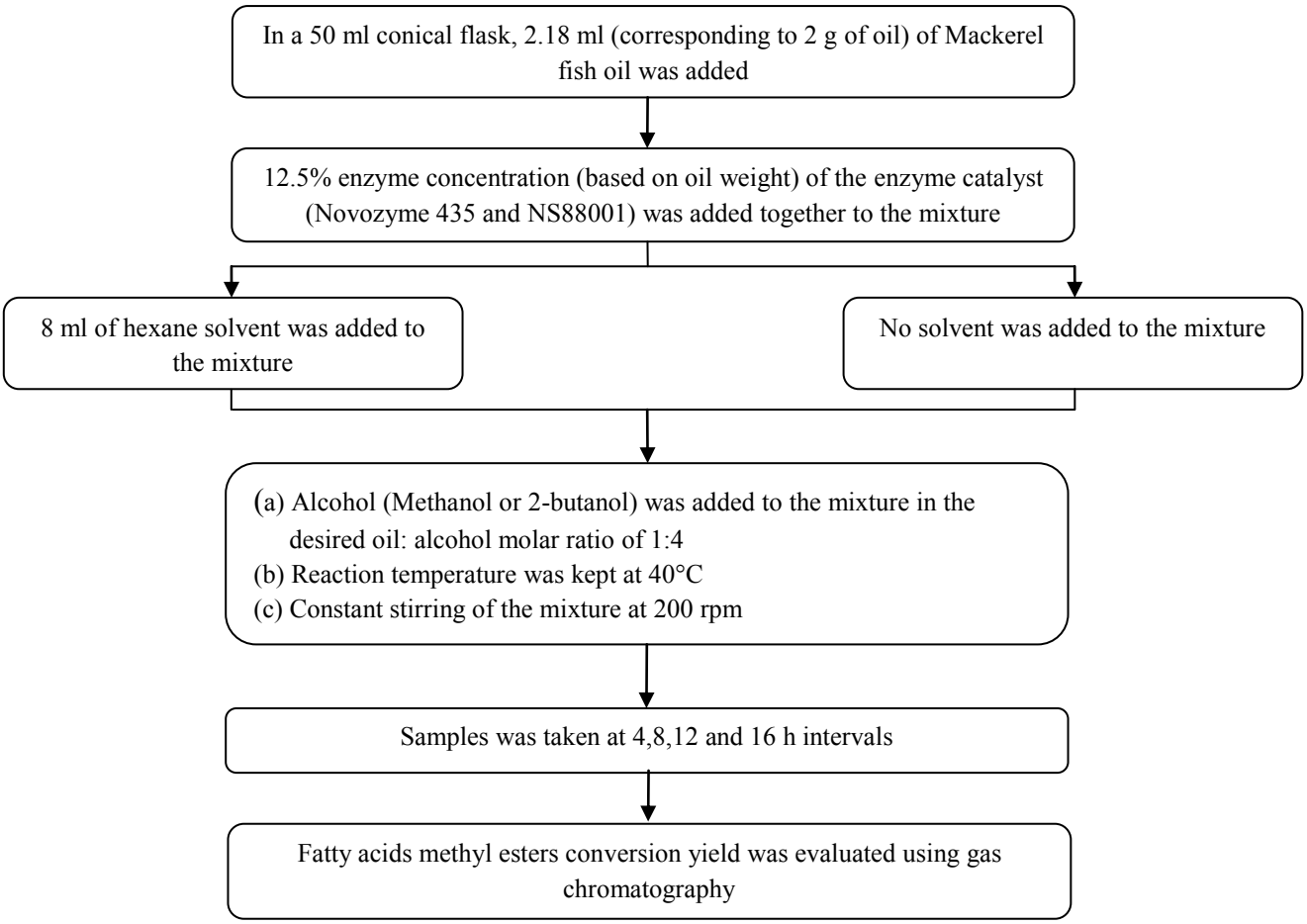


Figure 4.2. Enzymatic transesterification process of combination enzyme catalyst by solvent and solvent free systems.

4.9. Reusability of Lipase

The experimental procedure is shown in Figure 4.3. A 2 g sample of crude Mackerel oil was transferred into a 50 ml conical flask using a micro pipette (Eppendorf Research Plus, Fisher Scientific, Toronto, Ontario, Canada). The conical flask assembly was placed in a reciprocal shaking water bath (Precision 2870 Series, Fisher Scientific, Toronto, Ontario, Canada) and heated to the desired temperature of 40°C. The measured amount of alcohol (1:4 oil: alcohol molar ratio) and enzyme catalyst (25% wt individually or 12.5% wt in combination based on oil) were added to the conical flask. The reciprocal shaking bath was turned on at 200 rpm. An aliquot of 100 µl of reaction medium was taken at each cycle after 16 h and then dried with nitrogen gas. 100µl tertrahydrofuran and 200 µl of BSTFA were added for gas chromatography (GC) analysis. The enzymes were washed with hexane three times before each cycle and re-introduced into the system with new substrate and alcohol without any treatment. The same procedure was followed for 50 cycles.

4.10. FAME Analysis

FAMEs were analyzed by the procedure reported by Nelson et al. (1996). A 100 µL aliquot was taken from the transesterification process at selected time intervals (4, 8, 12 and 16 hours) and flushed with nitrogen at 45°C in order to evaporate the hexane for the analysis of FAME. A 10mg of the residue was dissolved in 100 µL of tertrahydrofuran and 200 µL of BSTFA. Then, the mixture was heated in a microprocessor-controlled water bath (280 series, Fisher Scientific, Toronto, Ontario, Canada) at 90-95°C for 15minutes. The sample was then cooled to room temperature for few minutes. A volume of 5mL of hexane was added. An aliquot of 1.5mL mixture was transferred to the GC crimp vials and capped tightly for further analysis using GC.

An aliquot of 10 µL of the mixture was separated by fatty acid class (methyl ester, MAG, DAG and TAG) based on the carbon atom by gas chromatography, coupled with flame ionization detector (FID) (HP5890 Series II, Agilent Technologies, Mississauga, Ontario, Canada). A HELIFLEX Capillary Column 30m in length, 0.32mm of internal diameter and 0.25µm film thickness (AT-FAME, Alltech Associates, Inc., Deerfield, Illinois, USA) was used for analyses. The separated samples were injected directly into the column

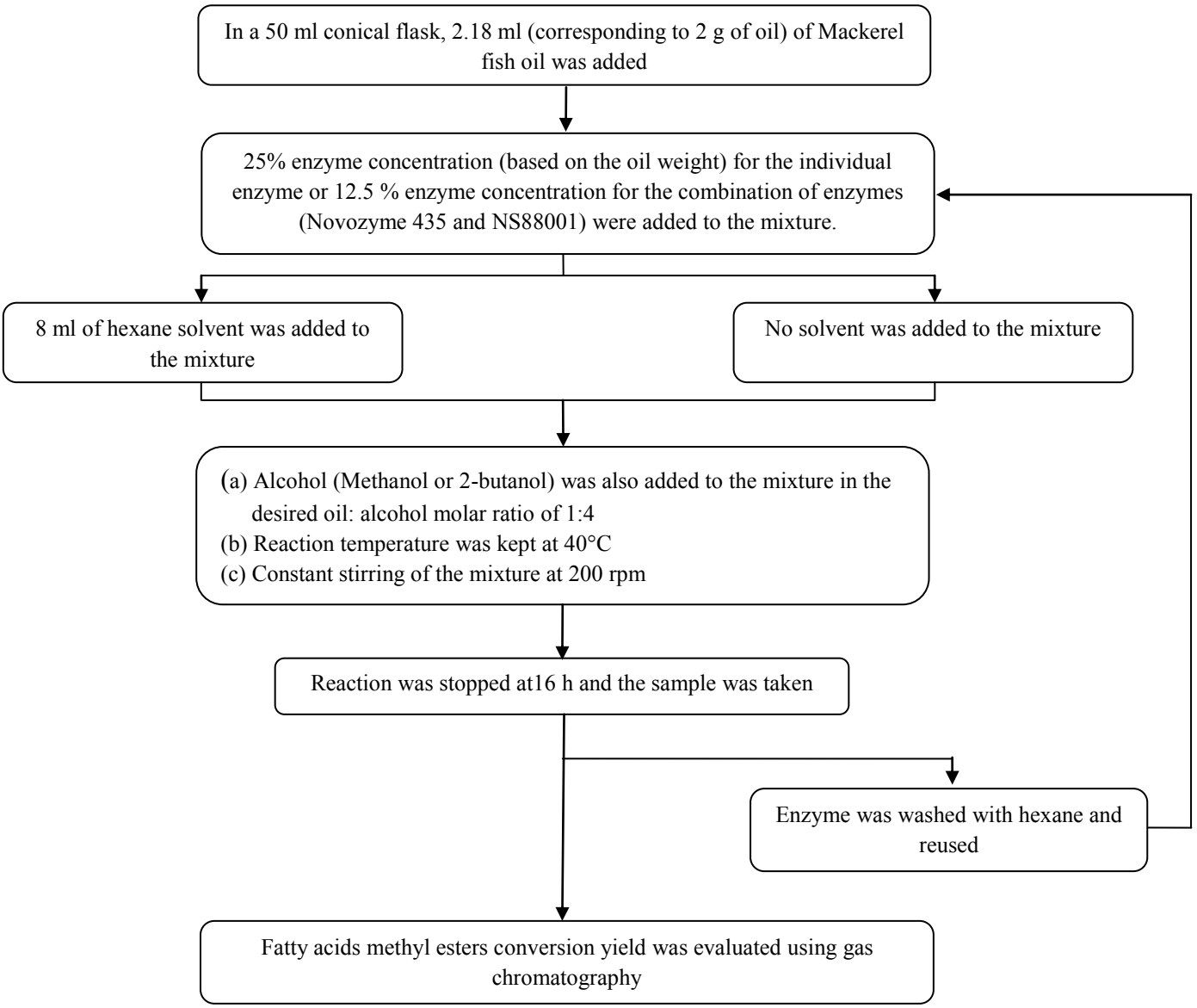


Figure 4.3. Reusability of the enzyme catalyst by solvent and solvent free systems.

with the initial oven temperature of 60°C (0.10 min), followed by a final temperature of 280°C (20°C/min) which was held for 10 minutes. The detection system was equipped with a flame ionization detector (FID) operating at 275°C with helium as a carrier gas at a flow rate of 0.6 mL/min. The total run time was 40 minutes. Peaks in the chromatograms were identified by comparing the retention time and area count with the internal standards of known compositions. The procedure and sample calculation is shown in Figures 4.4 and 4.5.

4.11. Recovery FAMEs Yield

The FAMEs (%) recovery yield was quantified using the peak area and the retention response of internal standards - methyl myristate, methyl pentadecanote, methyl cis-11-eicosenoate, methyl all-cis-5,8,11,14,17- eicosapentaenoate (EPA), methyl erucate, methyl all-cis-7,10,13,16,19-docosapentaenoate (DPA) and methyl all-cis-4,7,10,13,16,19-docosahexenoate (DHA) from Sigma Aldrich (St. Louis, Missouri, USA). Methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate from Alltech Associates, Inc. (Deerfield, Illinois, USA) and methyl stearidonate from Cayman chemical (Ann Arbor, Michigan, USA).

100 mg of each sample was mixed with 5 mL of hexane (100 mg/5 mL hexane). An aliquot of 1.5mL of the standard mixture was transferred to the GC crimp vials and capped. Then, 10µL of a sample was injected in the GC. The peak areas were integrated and noted. The recovery yield in terms of the percentage of fatty acid methyl ester (%FAME) was calculated as:

$$\%\text{FAMEs} = \frac{\text{PeakAreaA}}{\Sigma(\text{PeakAreaA} + \text{PeakAreaB} + \cdots + \text{PeakAreaN})} * 100 \qquad (4.1)$$

4.12. Statistical Analysis

The data for the conversion yields were collected and the effects of lipase, oil: alcohol molar ratio, reaction temperature, solvent system, alcohol type, reaction time on the biodiesel production were evaluated using Minitab Statistics Software (version16.2.2, Minitab Inc, State college, Pennsylvania, USA. Both the Analysis of Variance (ANOVA) and Tukey's Grouping were performed on the data to test the effects of individual parameters and the differences among the level of each parameter.

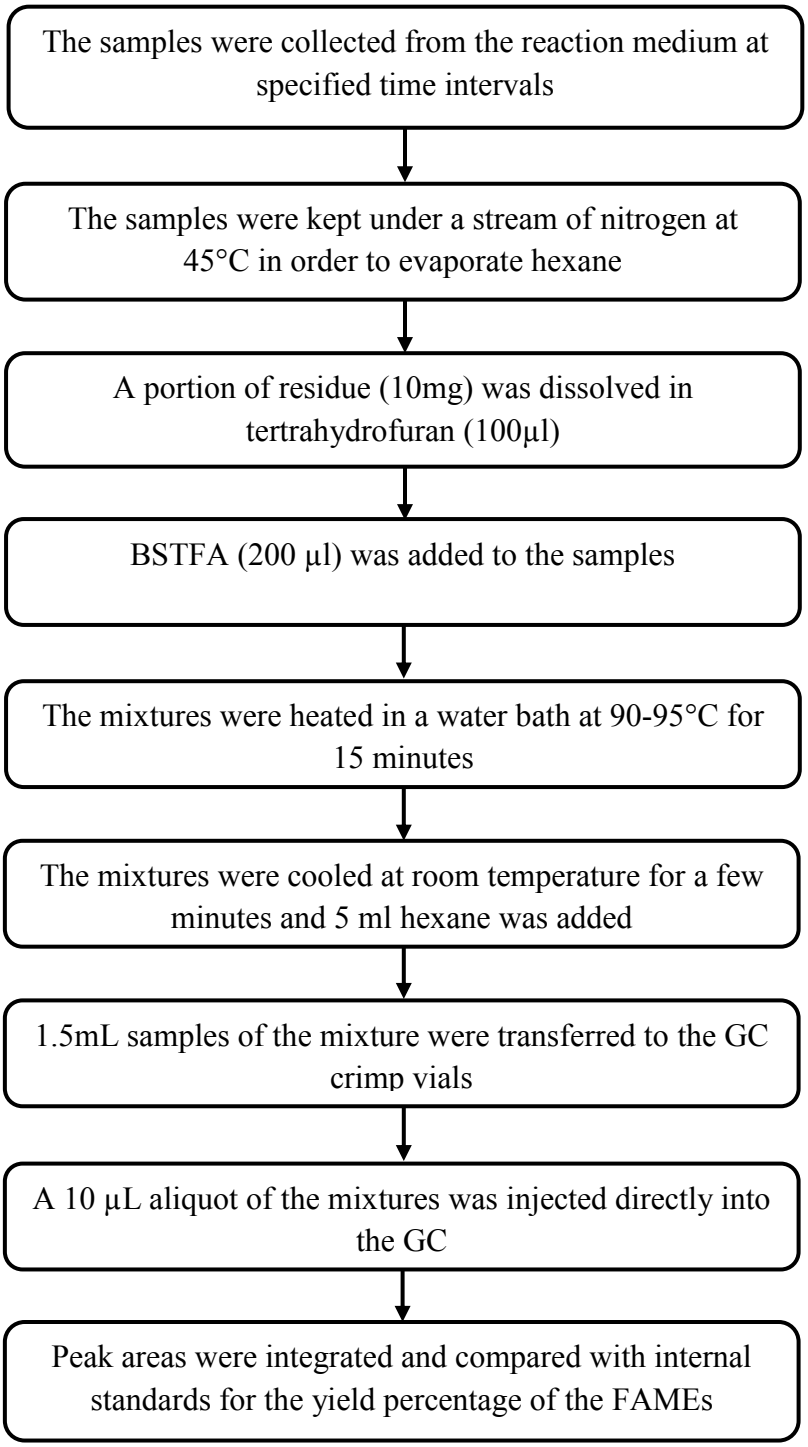


Figure 4.4. Yield evaluation for FAME using gas chromatography.

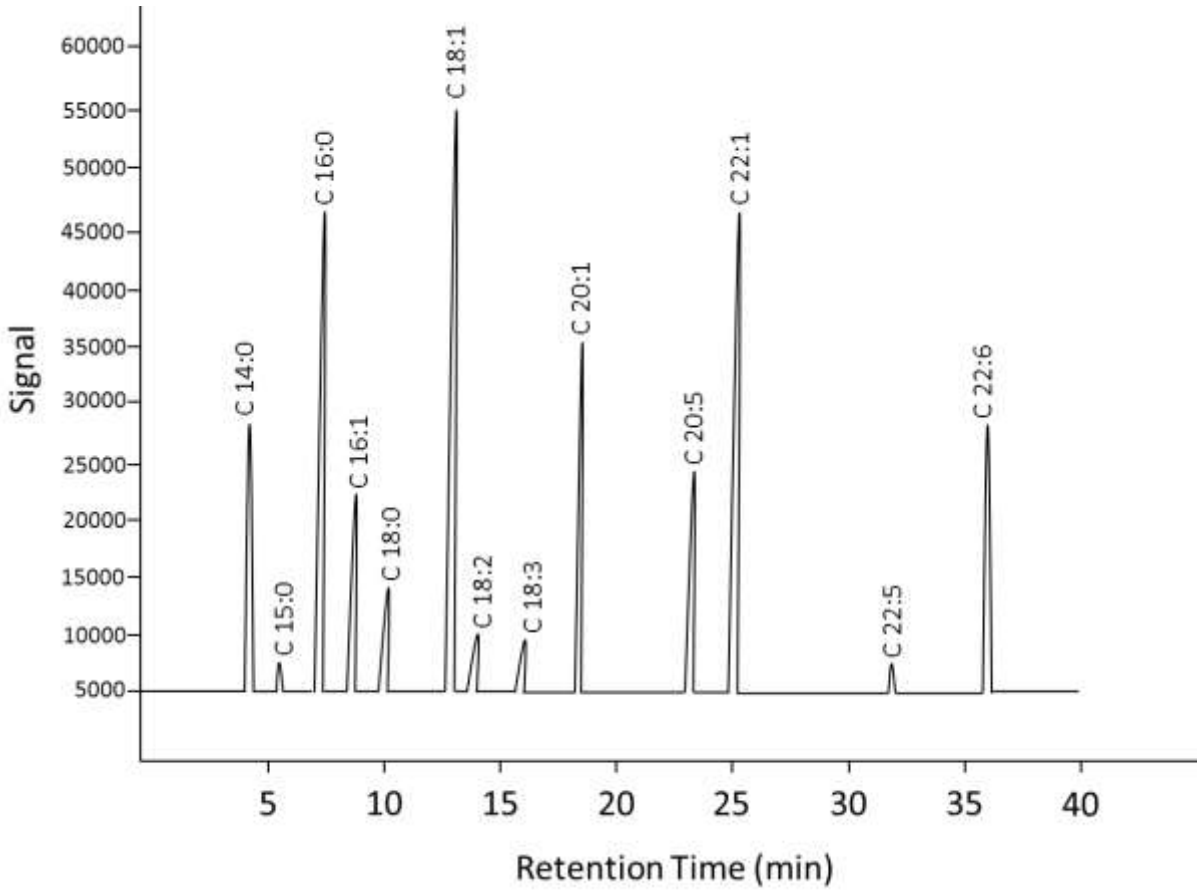


Figure 4.5. Sample chromatogram for FAME.

CHAPTER 5. RESULTS

5.1. Biodiesel Production by Individual Enzyme Catalysts

Biodiesel was produced from the fish oil (Mackerel) by enzymatic transesterification using two lipases individually [(*Candida antarctica* (Novozyme 435) and an experimental enzyme (NS88001)]. The experiments were carried out to investigate the effects of reaction time (4, 8, 12 and 16 h), oil: alcohol molar ratio (1:1, 1:2, 1:3. 1:4 and 1:5), temperature (35, 40, 45 and 50°C), alcohol type (Methanol and 2-butanol) and solvent system (with solvent and solvent-free). The biodiesel conversion yield was determined after the transesterification process using gas chromatography. The results are shown in Tables 5.1- 5.8.

Analysis of variance (ANOVA) and Tukey's grouping were performed on the biodiesel conversion yield data using Minitab Statistics Software (version 16.2.2, Minitab Inc, State College, Pennsylvania, USA). The results are shown in Tables 5.9 and 5.10. The effects of enzyme type, oil: alcohol molar ratio, alcohol type, solvent system, reaction temperature and reaction time were highly significant at the 0.001 level. There also appeared to be significant two way, three way, four way and five way interactions between the parameters at the 0.001 level. The results obtained from Tukey's grouping test indicated that the two enzymes were significantly different from each other at the 0.05 level. The highest average biodiesel conversion yield of 39.94% was achieved with the Novozyme 435. The oil: alcohol molar ratios1:1, 1:2, 1:3 and 1:4 were significantly different from each other but the oil: alcohol molar ratio of 1:3 and 1:5 were not significantly different from each other at the 0.05 level. The highest average biodiesel conversion yield of 48.04% was achieved with the oil: alcohol molar ratios of 1:4. All reaction times were significantly different from each other at the 0.05 level. The highest average biodiesel conversion yield of 41.10% was achieved at the reaction time of 16 h. The reaction temperatures of 35, 40 and 45ºC were significantly different from each other but the reaction temperatures of 35 and 50ºC were not significantly different from each other at the 0.05 level. The highest average biodiesel conversion yield of 40.87% was achieved with the reaction temperature of 40ºC. The two solvent systems were significantly different from each other at the 0.05 level. The highest average biodiesel conversion yield of 38.89% was achieved with hexane as a solvent system. The two alcohol types were

Table 5.1. Biodiesel yield (% wt) from fish oil using 0.5 grams of *Candida antarctica* (Novozyme 435) with methanol as alcohol and hexane as solvent at different reaction times, oil: alcohol molar ratios and reaction temperatures.

Time (h)	Oil: Alcohol Molar Ratio	Reaction Temperature (°C)			
		35	40	45	50
4	1:1	16.44 ± 0.78	20.39 ± 0.84	17.34 ± 0.59	12.98 ± 1.14
	1:2	31.08 ± 1.19	38.13 ± 0.97	36.42 ± 0.88	30.24 ± 0.95
	1:3	35.58 ± 1.45	40.06 ± 1.30	37.14 ± 0.76	34.73 ± 1.02
	1:4	36.92 ± 1.03	42.88 ± 0.84	39.92 ± 1.06	37.07 ± 1.21
	1:5	34.68 ± 0.99	41.41 ± 0.77	39.12 ± 0.81	32.89 ± 1.10
8	1:1	19.45 ± 0.74	24.94 ± 1.12	22.71 ± 0.90	20.43 ± 0.88
	1:2	33.23 ± 1.15	40.08 ± 1.47	36.97 ± 1.04	34.90 ± 1.07
	1:3	36.67 ± 1.29	47.98 ± 0.95	45.98 ± 0.61	37.21 ± 0.98
	1:4	40.18 ± 1.00	51.42 ± 1.06	50.13 ± 0.73	39.33 ± 0.86
	1:5	38.34 ± 1.17	47.04 ± 0.86	46.74 ± 1.09	36.29 ± 1.19
12	1:1	21.52 ± 1.47	29.08 ± 1.47	26.91 ± 0.69	21.76 ± 0.97
	1:2	37.66 ± 1.18	45.58 ± 1.17	41.30 ± 0.52	36.02 ± 0.81
	1:3	40.25 ± 0.90	59.18 ± 1.03	57.97 ± 0.80	41.67 ± 0.87
	1:4	43.19 ± 1.30	62.43 ± 0.83	61.02 ± 0.88	44.06 ± 0.93
	1:5	41.08 ± 0.98	60.99 ± 1.24	60.54 ± 0.92	40.69 ± 0.76
16	1:1	25.65 ± 1.09	31.23 ± 1.12	28.43 ± 1.05	24.22 ± 1.27
	1:2	41.32 ± 1.26	50.70 ± 1.02	48.59 ± 0.81	39.60 ± 1.07
	1:3	45.99 ± 0.98	62.40 ± 0.79	61.96 ± 0.70	42.06 ± 0.91
	1:4	48.26 ± 1.29	65.86 ± 1.20	62.53 ± 1.08	45.97 ± 1.17
	1:5	43.51 ± 0.99	63.92 ± 1.07	61.75 ± 0.81	41.56 ± 0.86

Table 5.2. Biodiesel yield (% wt) from fish oil using 0.5 grams of *Candida antarctica* (Novozyme 435) with methanol as alcohol and without solvent at different reaction times, oil: alcohol molar ratios and reaction temperatures.

Time (h)	Oil: Alcohol Molar Ratio	Reaction Temperature (°C)			
		35	40	45	50
4	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	34.75 ± 0.95	41.20 ± 0.93	38.22 ± 1.08	27.19 ± 0.94
	1:3	38.04 ± 0.83	44.41 ± 0.77	41.29 ± 0.61	28.96 ± 1.44
	1:4	40.43 ± 1.22	47.26 ± 0.87	43.96 ± 0.83	31.06 ± 1.11
	1:5	36.43 ± 0.77	44.65 ± 0.81	41.66 ± 0.64	24.29 ± 0.88
8	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	37.03 ± 1.26	44.70 ± 1.01	42.46 ± 0.83	29.14 ± 0.98
	1:3	39.76 ± 1.39	50.01 ± 1.24	48.10 ± 0.94	31.03 ± 0.91
	1:4	42.22 ± 0.91	54.10 ± 0.92	51.43 ± 0.73	34.20 ± 0.97
	1:5	39.60 ± 0.64	48.25 ± 1.37	46.95 ± 0.89	28.32 ± 0.88
12	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	41.02 ± 1.03	48.38 ± 0.75	46.06 ± 1.10	33.25 ± 1.03
	1:3	44.05 ± 1.26	63.02 ± 1.05	60.01 ± 0.61	35.10 ± 1.15
	1:4	47.18 ± 0.67	67.30 ± 0.76	63.42 ± 0.71	40.06 ± 0.82
	1:5	44.99 ± 0.78	63.79 ± 0.99	58.60 ± 0.69	31.28 ± 0.87
16	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	43.86 ± 0.81	55.24 ± 0.82	52.39 ± 0.73	35.80 ± 1.46
	1:3	48.22 ± 0.98	66.28 ± 0.74	63.14 ± 0.86	36.54 ± 0.85
	1:4	50.74 ± 0.77	71.39 ± 0.80	66.48 ± 0.93	42.91 ± 1.17
	1:5	47.86 ± 1.29	67.74 ± 1.37	63.82 ± 1.08	36.78 ± 0.79

Table 5.3. Biodiesel yield (% wt) from fish oil using 0.5 grams of *Candida antarctica* (Novozyme 435) with 2-butanol as alcohol and hexane as solvent at different reaction times, oil: alcohol molar ratios and reaction temperatures.

Time (h)	Oil: Alcohol Molar Ratio	Reaction Temperature (°C)			
		35	40	45	50
4	1:1	18.32 ± 0.89	23.76 ± 1.19	20.09 ± 0.87	17.88 ± 1.05
	1:2	33.24 ± 1.27	39.21 ± 0.97	36.21 ± 0.92	34.69 ± 1.00
	1:3	34.99 ± 1.12	40.42 ± 1.10	37.90 ± 0.86	36.41 ± 1.10
	1:4	40.29± 0.98	49.08 ± 0.99	48.20 ± 1.05	44.33 ± 0.93
	1:5	40.16± 0.86	41.63 ± 0.82	42.17 ± 0.98	39.71 ± 0.98
8	1:1	20.32 ± 1.27	26.87 ± 1.06	21.95 ± 1.01	18.85 ± 1.03
	1:2	35.44 ± 1.43	42.40 ± 0.73	38.79 ± 0.87	36.20 ± 0.92
	1:3	41.20 ± 0.91	49.32 ± 1.11	45.68 ± 0.79	40.55 ±1.31
	1:4	46.96 ± 0.89	60.22 ± 0.77	58.01 ± 0.86	47.31 ± 0.84
	1:5	42.05 ± 0.61	48.81 ± 0.83	47.51 ± 0.74	44.24 ± 0.91
12	1:1	23.78 ± 1.22	31.44 ± 1.07	26.61 ± 0.98	21.03 ± 0.86
	1:2	39.21 ± 0.81	47.82 ± 0.72	42.03 ± 0.96	39.44 ± 0.98
	1:3	44.15 ± 1.00	56.55 ± 0.84	48.00 ± 0.85	45.96 ± 1.07
	1:4	50.88 ± 0.85	74.61 ± 1.03	72.07 ± 0.91	51.14 ±1.01
	1:5	45.71 ± 1.14	62.68 ± 0.81	58.58 ± 0.80	46.76 ± 0.92
16	1:1	28.69 ± 0.96	37.62 ± 1.28	34.90 ± 1.00	24.74 ± 1.29
	1:2	41.92 ± 1.04	51.11 ± 0.74	44.93 ± 0.78	42.01 ± 0.95
	1:3	50.47 ± 0.98	60.88 ± 0.93	55.74 ± 0.86	53.29 ± 1.17
	1:4	56.30 ± 0.81	76.66 ± 0.88	74.42 ± 0.71	54.55 ± 1.10
	1:5	49.22 ± 0.86	69.48 ± 0.91	68.33 ± 0.66	50.78 ± 0.91

Table 5.4. Biodiesel yield (% wt) from fish oil using 0.5 grams of *Candida antarctica* (Novozyme 435) with 2-butanol as alcohol and without solvent at different reaction times, oil: alcohol molar ratios and reaction temperatures.

Time (h)	Oil: Alcohol Molar Ratio	Reaction Temperature (°C)			
		35	40	45	50
4	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	36.16 ± 1.02	41.21 ± 0.87	38.18 ± 0.83	31.90 ± 1.39
	1:3	38.04 ± 1.05	44.06 ± 1.12	43.27 ± 0.91	33.13 ± 0.82
	1:4	43.51 ± 0.86	52.13 ± 0.96	50.82 ± 0.86	37.18 ± 1.07
	1:5	35.85 ± 1.13	47.92 ± 0.83	45.36 ± 0.78	34.71 ± 1.34
8	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	39.10 ± 0.77	45.26 ± 0.79	43.27 ± 0.88	33.21 ± 0.89
	1:3	43.04 ± 1.05	53.15 ± 0.69	50.01 ± 1.09	37.22 ± 1.03
	1:4	49.37 ± 0.78	62.88 ± 1.33	61.01 ± 0.74	43.26 ± 0.75
	1:5	40.42 ± 0.66	56.99 ± 1.09	55.73 ± 0.92	35.85 ± 0.90
12	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	44.05 ± 1.07	50.06 ± 1.18	47.37 ± 0.78	34.94 ± 1.43
	1:3	48.18 ± 1.15	58.88 ± 1.35	53.16 ± 0.87	42.30 ± 0.76
	1:4	53.34 ± 0.76	75.30 ± 0.69	72.11 ± 1.00	49.05 ± 0.93
	1:5	47.14 ± 0.84	69.05 ± 0.86	63.86 ± 0.89	37.28 ± 0.91
16	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	47.41 ± 0.64	54.72 ± 1.13	52.98 ± 1.08	36.28 ± 0.98
	1:3	54.00 ± 1.01	61.00 ± 1.05	58.13 ± 0.94	45.30 ± 0.87
	1:4	59.23 ± 0.93	80.24 ± 0.97	77.37 ± 0.73	55.91 ± 1.03
	1:5	53.28 ± 0.88	71.98 ± 1.26	70.18 ± 0.87	46.34 ± 0.86

Table 5.5. Biodiesel yield (% wt) from fish oil using 0.5 grams of NS88001 with methanol as alcohol and hexane as solvent at different reaction times, oil: alcohol molar ratios and reaction temperatures.

Time (h)	Oil: Alcohol Molar Ratio	Reaction Temperature (°C)			
		35	40	45	50
4	1:1	20.78 ± 1.48	23.29 ± 1.37	20.13 ± 0.90	18.95 ± 1.19
	1:2	24.85 ± 1.01	31.26 ± 0.99	29.37 ± 1.10	26.47 ± 1.48
	1:3	28.22 ± 0.79	32.98 ± 1.18	31.27 ± 0.74	28.14 ± 1.39
	1:4	30.68 ± 0.62	41.41 ± 0.93	32.02 ± 0.93	28.94 ± 1.00
	1:5	27.09 ± 0.95	37.62 ± 0.68	29.16 ± 1.23	26.72 ± 1.24
8	1:1	23.53 ± 0.82	31.41 ± 1.23	28.87 ± 1.00	27.03 ± 1.15
	1:2	30.32 ± 0.99	39.05 ± 0.83	37.17 ± 1.03	29.86 ± 1.29
	1:3	34.54 ± 0.93	45.78 ± 1.05	43.46 ± 0.94	39.72 ± 0.84
	1:4	36.04 ± 1.29	55.16 ± 0.95	52.48 ± 0.87	44.14 ± 1.24
	1:5	32.92 ± 1.09	47.42 ± 1.18	43.72 ± 0.95	30.20 ± 1.39
12	1:1	24.87 ± 0.78	33.14 ± 1.38	30.06 ± 0.79	28.81 ± 1.34
	1:2	33.58 ± 1.44	41.35 ± 0.86	39.64 ± 1.00	36.27 ± 0.88
	1:3	36.46 ± 0.91	55.92 ± 1.17	48.63 ± 1.02	42.58 ± 1.08
	1:4	48.85 ± 0.81	65.94 ± 0.88	58.81 ± 0.76	47.44 ± 1.20
	1:5	33.90 ± 1.24	54.75 ± 0.72	45.55 ± 0.67	37.83 ± 1.01
16	1:1	28.50 ± 0.98	36.14 ± 1.39	34.85 ± 0.76	31.57 ± 1.19
	1:2	35.90 ± 1.11	47.77 ± 1.24	45.02 ± 1.07	37.41 ± 1.03
	1:3	39.81 ± 1.03	58.82 ± 0.90	52.32 ± 0.69	44.78 ± 1.14
	1:4	51.73 ± 0.84	70.38 ± 1.33	59.20 ± 0.96	52.44 ± 0.96
	1:5	38.04 ± 1.02	58.90 ± 1.15	48.68 ± 0.74	39.62 ± 0.66

Table 5.6. Biodiesel yield (% wt) from fish oil using 0.5 grams of NS88001 with methanol as alcohol and without solvent at different reaction times, oil: alcohol molar ratios and reaction temperatures.

Time (h)	Oil: Alcohol Molar Ratio	Reaction Temperature (°C)			
		35	40	45	50
4	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable	Not extractable
	1:3	29.86 ± 0.98	37.03 ± 1.26	34.07 ± 1.12	26.93 ± 1.12
	1:4	32.01 ± 1.32	41.96 ± 1.07	37.35 ± 0.81	32.13 ± 0.98
	1:5	26.68 ± 0.80	38.91 ± 1.32	33.67 ± 1.22	29.07 ± 1.20
8	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable	Not extractable
	1:3	37.35 ± 0.91	48.10 ± 0.90	45.64 ± 0.87	32.13 ± 1.06
	1:4	43.08 ± 1.12	60.13 ± 1.19	56.07 ± 1.10	35.95 ± 1.19
	1:5	35.98 ± 0.75	56.37 ± 0.81	50.23 ± 0.98	33.02 ± 1.36
12	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable	Not extractable
	1:3	38.90 ± 0.93	60.59 ± 1.16	53.01 ± 0.80	38.64 ± 1.11
	1:4	51.31 ± 0.67	71.02 ± 1.05	61.81 ± 1.05	43.17 ± 0.93
	1:5	36.36 ± 0.88	69.10 ± 1.12	54.02 ± 1.00	35.96 ± 1.16
16	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable	Not extractable
	1:3	44.29 ± 0.89	63.03 ± 1.25	56.31 ± 0.95	40.09 ± 1.22
	1:4	53.96 ± 1.07	74.34 ± 0.64	65.03 ± 0.87	46.80 ± 1.29
	1:5	41.64 ± 1.49	72.14 ± 1.07	62.34 ± 0.80	45.89 ± 1.34

Table 5.7. Biodiesel yield (% wt) from fish oil using 0.5 grams of NS88001 with 2-butanol as alcohol and hexane as solvent at different reaction times, oil: alcohol molar ratios and reaction temperatures.

Time (h)	Oil: Alcohol Molar Ratio	Reaction Temperature (°C)			
		35	40	45	50
4	1:1	17.22 ± 1.36	24.02 ± 0.80	22.46 ± 0.95	19.21 ± 1.27
	1:2	19.41 ± 1.03	29.11 ± 1.23	27.78 ± 0.63	24.40 ± 1.36
	1:3	23.13 ± 0.83	32.45 ± 1.11	30.30 ± 0.73	28.47 ± 1.29
	1:4	24.98 ± 0.99	34.39 ± 0.83	32.46 ± 0.65	28.91 ± 1.46
	1:5	23.68 ± 0.89	26.74 ± 0.86	25.09 ± 0.61	23.31 ± 0.83
8	1:1	20.04 ± 1.32	26.98 ± 1.47	24.76 ± 1.05	22.41 ± 1.17
	1:2	22.62 ± 0.76	34.73 ± 1.34	32.09 ± 0.64	28.84 ± 1.34
	1:3	27.55 ± 0.91	36.69 ± 1.16	34.46 ± 0.95	32.21 ± 0.99
	1:4	31.71 ± 1.16	42.98 ± 1.41	39.07 ± 0.88	34.75 ± 0.89
	1:5	26.93 ± 1.11	41.16 ± 1.34	34.25 ± 0.61	32.55 ± 0.73
12	1:1	24.84 ± 1.49	30.87 ± 1.29	28.38 ± 0.88	26.11 ± 1.12
	1:2	29.60 ± 1.05	37.39 ± 1.32	35.67 ± 0.62	31.83 ± 0.98
	1:3	34.88 ± 1.28	46.24 ± 1.24	39.95 ± 0.74	38.20 ± 1.25
	1:4	36.24 ± 1.52	47.68 ± 1.14	43.10 ± 1.07	41.15 ± 0.81
	1:5	34.05 ± 1.17	43.74 ± 1.12	37.87 ± 1.00	34.66 ± 0.92
16	1:1	25.94 ± 0.83	31.11 ± 1.17	29.35 ± 0.82	26.30 ± 1.31
	1:2	33.13 ± 1.32	42.47 ± 1.35	37.93 ± 0.74	32.26 ± 0.84
	1:3	38.25 ± 0.86	49.68 ± 0.75	44.06 ± 0.95	40.85 ± 1.23
	1:4	42.90 ± 1.29	54.35 ± 0.83	48.54 ± 0.88	42.11 ± 1.17
	1:5	38.71 ± 0.83	49.72 ± 1.30	41.15 ± 0.92	35.88 ± 1.27

Table 5.8. Biodiesel yield (% wt) from fish oil using 0.5 grams of NS88001 with 2-butanol as alcohol and without solvent at different reaction times, oil: alcohol molar ratios and reaction temperatures.

Time (h)	Oil: Alcohol Molar Ratio	Reaction Temperature (°C)			
		35	40	45	50
4	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable	Not extractable
	1:3	21.40 ± 0.78	29.19 ± 0.88	27.33 ± 0.79	19.83 ± 1.23
	1:4	25.05 ± 0.88	30.99 ± 1.07	29.28 ± 0.90	22.17 ± 1.01
	1:5	22.34 ± 0.81	25.74 ± 0.98	26.24 ± 1.17	16.72 ± 1.17
8	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable	Not extractable
	1:3	24.70 ± 1.05	33.06 ± 1.03	30.81 ± 0.88	24.16 ± 0.84
	1:4	28.75 ± 1.22	37.33 ± 0.87	34.12 ± 0.93	26.16 ± 1.33
	1:5	25.00 ± 1.10	33.91 ± 1.05	30.12 ± 1.16	19.20 ± 1.01
12	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable	Not extractable
	1:3	28.25 ± 1.02	38.32 ± 0.80	36.58 ± 1.03	28.36 ± 1.31
	1:4	32.30 ± 1.15	42.46 ± 0.93	38.87 ± 0.86	33.32 ± 0.86
	1:5	27.29 ± 0.76	36.95 ± 1.02	35.29 ± 0.98	24.70 ± 1.17
16	1:1	Not extractable	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable	Not extractable
	1:3	33.16 ± 0.95	41.28 ± 1.09	39.56 ± 0.92	30.59 ± 0.92
	1:4	37.28 ± 0.86	49.29 ± 0.86	46.56 ± 1.08	35.82 ± 1.12
	1:5	31.08 ± 0.88	47.09 ± 0.95	40.83 ± 0.98	29.60 ± 1.17

Table 5.9. Analysis of variance for biodiesel yield (individual enzymes).

Source	DF	SS	MS	F	P
Total	1279	448623			
Model					
EN	1	34321	34320.7	19528.7	0.001
SS	1	21826	21826.3	12419.3	0.001
MR	4	211026	52756.4	30018.7	0.001
TI	3	33224	11074.8	6301.6	0.001
TE	3	28303	9434.3	5368.2	0.001
AT	1	1161	1160.6	660.4	0.001
EN*SS	1	6179	6179.3	3516.1	0.001
EN*MR	4	20562	5140.4	2924.9	0.001
EN*TI	3	217	72.3	41.1	0.001
EN*TE	3	1252	417.4	237.5	0.001
EN*AT	1	8875	8875.5	5050.2	0.001
SS*MR	4	34059	8514.9	4845.0	0.001
SS*TI	3	491	163.5	93.0	0.001
SS*TE	3	1046	348.5	198.3	0.001
SS*AT	1	224	223.7	127.3	0.001
MR*TI	12	6175	514.6	292.8	0.001
MR*TE	12	6493	541.1	307.9	0.001
MR*AT	4	694	173.6	98.8	0.001
TI*TE	9	2060	228.9	130.2	0.001
TI*AT	3	222	74.1	42.2	0.001
TE*AT	3	628	209.4	119.1	0.001
EN*SS*MR	4	13971	3492.9	1987.5	0.001
EN*SS*TI	3	111	37.1	21.1	0.001
EN*SS*TE	3	215	71.8	40.9	0.001
EN*SS*AT	1	203	203.4	115.7	0.001
EN*MR*TI	12	241	20.1	11.4	0.001
EN*MR*TE	12	495	41.3	23.5	0.001
EN*MR*AT	4	4001	1000.3	569.2	0.001
EN*TI*TE	9	292	32.4	18.4	0.001
EN*TI*AT	3	425	141.6	80.6	0.001
EN*TE*AT	3	172	57.4	32.6	0.001
SS*MR*TI	12	780	65.0	37.0	0.001
SS*MR*TE	12	1492	124.3	70.7	0.001
SS*MR*AT	4	477	119.1	67.8	0.001
SS*TI*TE	9	53	5.9	3.4	0.001
SS*TI*AT	3	14	4.7	2.7	0.001

Table 5.9. Continued.

Source	DF	SS	MS	F	P
MR*TI*TE	36	947	26.3	15.0	0.001
MR*TE*AT	12	517	43.1	24.5	0.001
TI*TE*AT	9	193	21.5	12.2	0.001
EN*SS*MR*TI	12	327	27.3	15.5	0.001
EN*SS*MR*TE	12	527	43.9	25.0	0.001
EN*SS*MR*AT	4	796	199.0	113.2	0.001
EN*SS*TI*TE	9	74	8.2	4.7	0.001
EN*SS*TI*AT	3	27	9.0	5.1	0.001
EN*MR*TI*TE	36	230	6.4	3.6	0.001
EN*MR*TI*AT	12	368	30.7	17.4	0.001
SS*MR*TI*TE	36	113	3.1	1.8	0.001
SS*MR*TI*AT	12	79	6.6	3.7	0.001
SS*TI*TE*AT	9	44	4.8	2.8	0.001
MR*TI*TE*AT	36	301	8.3	4.8	0.001
EN*TI*TE*AT	9	107	11.9	6.8	0.001
EN*SS*MR*TI*TE	36	179	5.0	2.8	0.001
EN*SS*MR*TI*AT	12	91	7.6	4.3	0.001
EN*MR*TI*TE*AT	36	146	4.1	2.3	0.001
SS*MR*TI*TE*AT	36	127	3.5	2.0	0.001
EN*SS*TI*TE*AT	9	49	5.5	3.1	0.001
EN*SS*MR*TE*AT	12	94	7.8	4.4	0.001
EN*SS*MR*TI*TE*AT	36	109	3.0	1.7	0.001
Error	682	1199	1.8		

DF : Degree of freedom
SS : Sum of square
MS : Mean of square
EN = Enzyme
MR = Oil: Alcohol molar ratio
AT = Alcohol type
TI = Reaction time
TE = Reaction temperature
SS = Solvent system
R^2 : 0. 9973

Table 5.10. Tukey's grouping for biodiesel yield (individual enzymes).

Factors	Level	N	Mean (%)	Tukey Grouping
Enzyme Type	Novozyme 435	640	39.94	A
	NS88001	640	29.58	B
Oil : Alcohol Molar Ratio	1:1	256	12.52	A
	1:2	256	28.58	B
	1:3	256	42.05	C
	1:4	256	48.04	D
	1:5	256	42.63	C
Reaction Time (hour)	4	320	27.58	A
	8	320	32.61	B
	12	320	37.61	C
	16	320	41.10	D
Reaction Temperature (°C)	35	320	30.96	A
	40	320	40.87	B
	45	320	37.73	C
	50	320	29.48	A
Solvent System	Hexane	640	38.89	A
	Without Hexane	640	30.63	B
Alcohol Type	Methanol	640	35.71	A
	2-Butanol	640	33.81	B

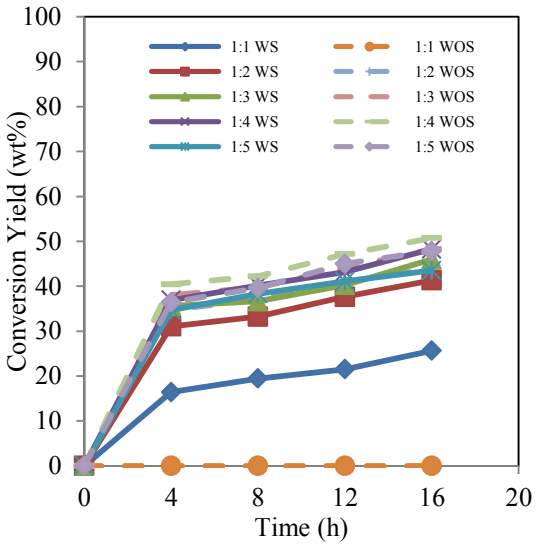
Treatments with the same letter are not significantly different at 0.05 level.

significantly different from each other at the 0.05 level. The highest average biodiesel conversion yield of 35.71% was achieved with methanol.

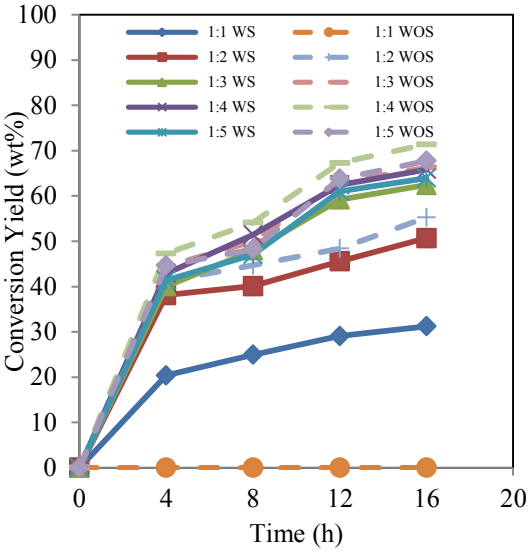
5.1.1. Effect of Reaction Time

The effects of reaction time on the biodiesel conversion yield using different enzymes at different oil: alcohol molar ratios, reaction temperature, solvent system and alcohol type are shown in Figures 5.1 - 5.4. The results showed that there was an initial rapid biodiesel conversion in the first 4 h followed by a slow gradual increase thereafter until the end of the experiment (16 h) for all reaction temperatures (35, 40, 45 and 50°C), oil: alcohol molar ratios (1:1, 1:2, 1:3, 1:4 and 1:5), with the two alcohols (methanol and 2-butanol) and with and without solvent.

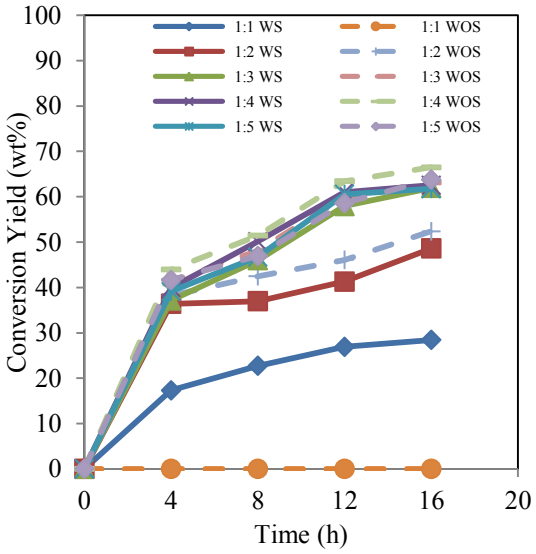
Figure 5.1 shows the effect of reaction time on the biodiesel conversion yield using Novozyme 435 and methanol with and without solvent. The biodiesel conversion yield in the solvent system at the reaction temperature of 35°C reached 16.44%, 31.08%, 35.58%, 36.92% and 34.68% after 4 h for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. When the reaction time was further increased from 4 to 16 h, the biodiesel conversion yield increased gradually from 16.44 to 25.65% (56.02%), from 31.08 to 41.32% (32.94%), from 35.58 to 45.99% (29.25%), from 36.92 to 48.26% (30.71%) and from 34.68 to 43.51% (25.46%) for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, in the solvent-free system, the biodiesel conversion yield at the reaction temperature of 35°C reached 34.75%, 38.04%, 40.43% and 36.43% after 4 h for the oil: alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. No reaction was observed for the oil: alcohol molar ratio of 1:1. When the reaction time was further increased from 4 to 16 h, the biodiesel conversion yield increased gradually from 34.75 to 43.86% (26.21%), from 38.04 to 48.22% (26.76%), from 40.43 to 50.74% (25.50%) and from 36.43 to 47.86% (31.37%) for the oil: alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. Similar trends were observed at the reaction temperatures of 40, 45 and 50°C and the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5 with and without solvent but no reaction was observed at the 1:1 oil: alcohol molar ratio for the reaction temperatures of 40, 45 and 50°C in the solvent-free system.



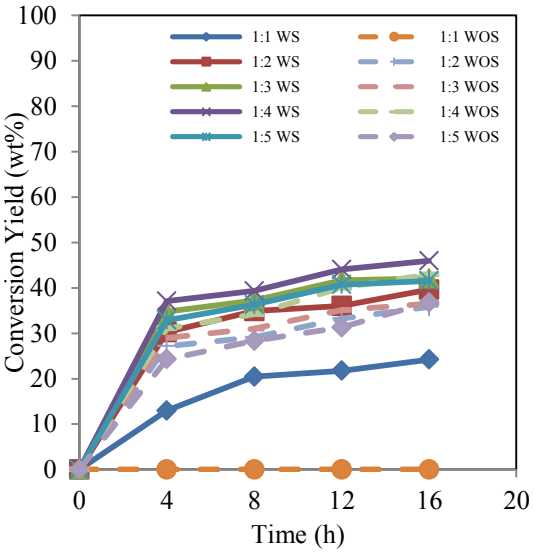
(a) 35ºC



(b) 40ºC



(c) 45ºC



(d) 50ºC

Figure 5.1. Effect of reaction time on the conversion yield using Novozyme 435 lipase with methanol at different reaction temperatures and oil: alcohol molar ratios with and without solvent (WS= with solvent and WOS= without solvent).

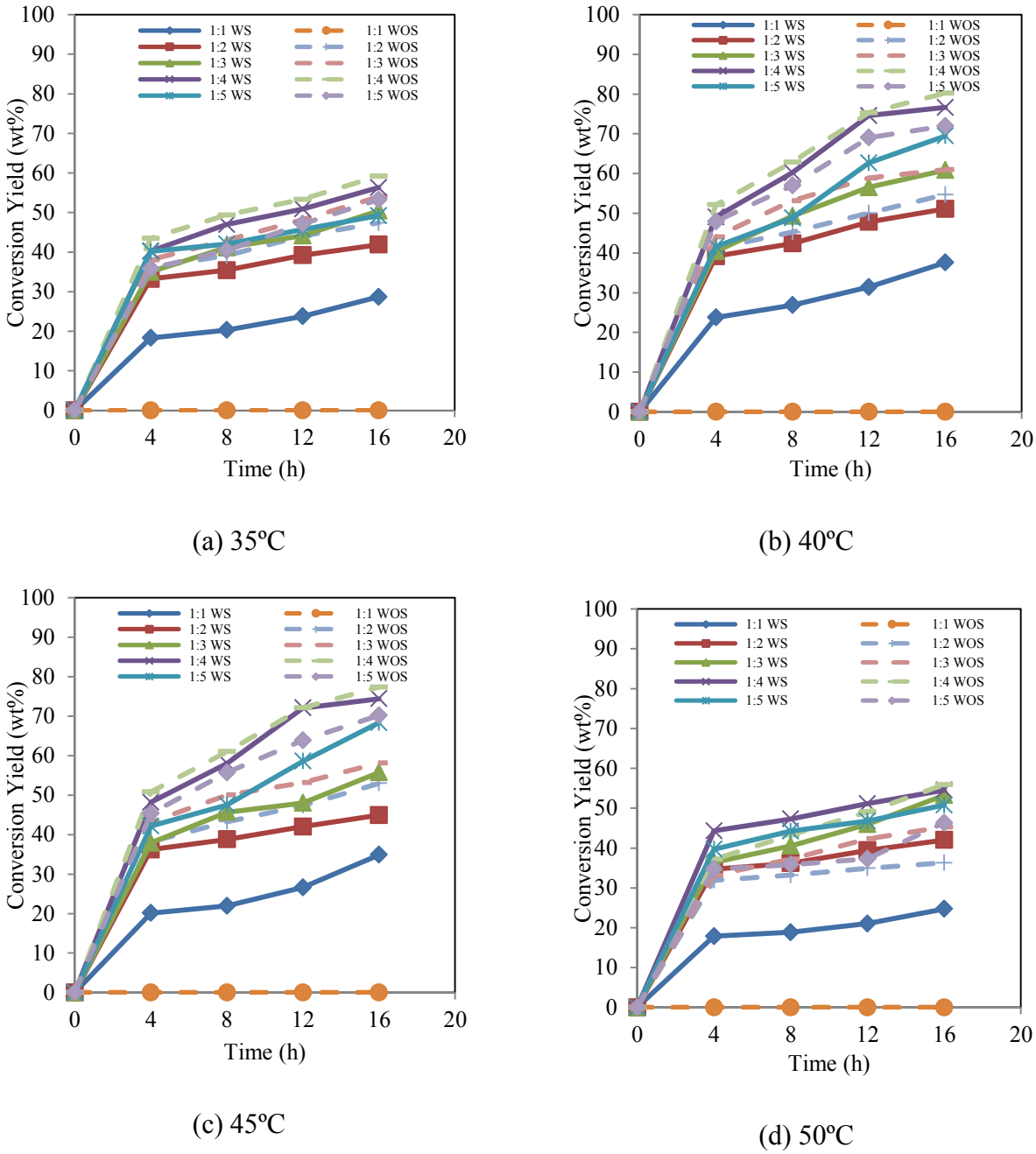
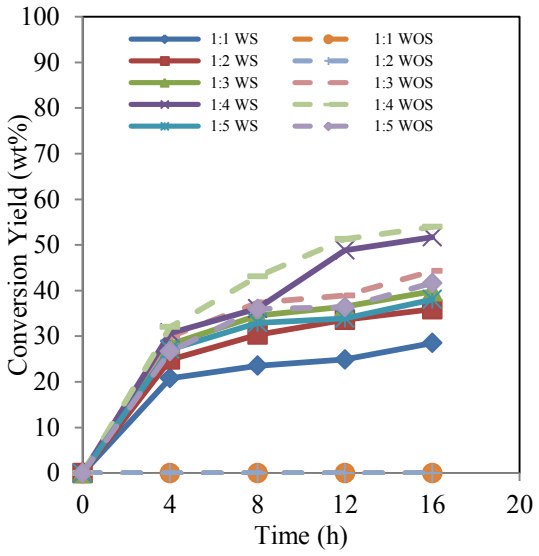
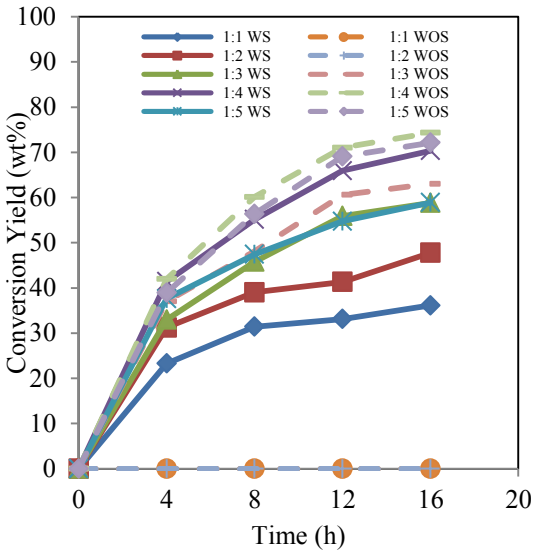


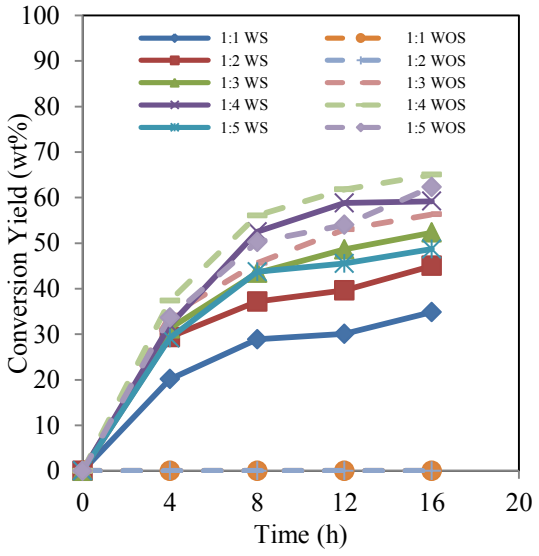
Figure 5.2. Effect of reaction time on the conversion yield using Novozyme 435 lipase with 2-butanol at different reaction temperatures and oil: alcohol molar ratios with and without solvent (WS= with solvent and WOS= without solvent).



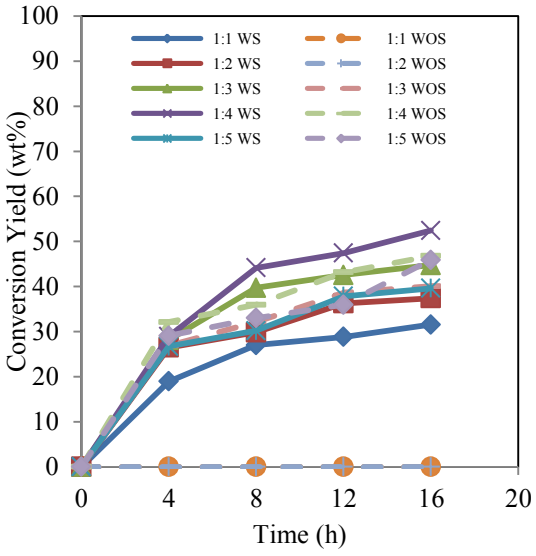
(a) 35ºC



(b) 40ºC



(c) 45ºC



(d) 50ºC

Figure 5.3. Effect of reaction time on the conversion yield using NS88001 lipase with methanol at different reaction temperatures and oil: alcohol molar ratios with and without solvent (WS= with solvent and WOS= without solvent).

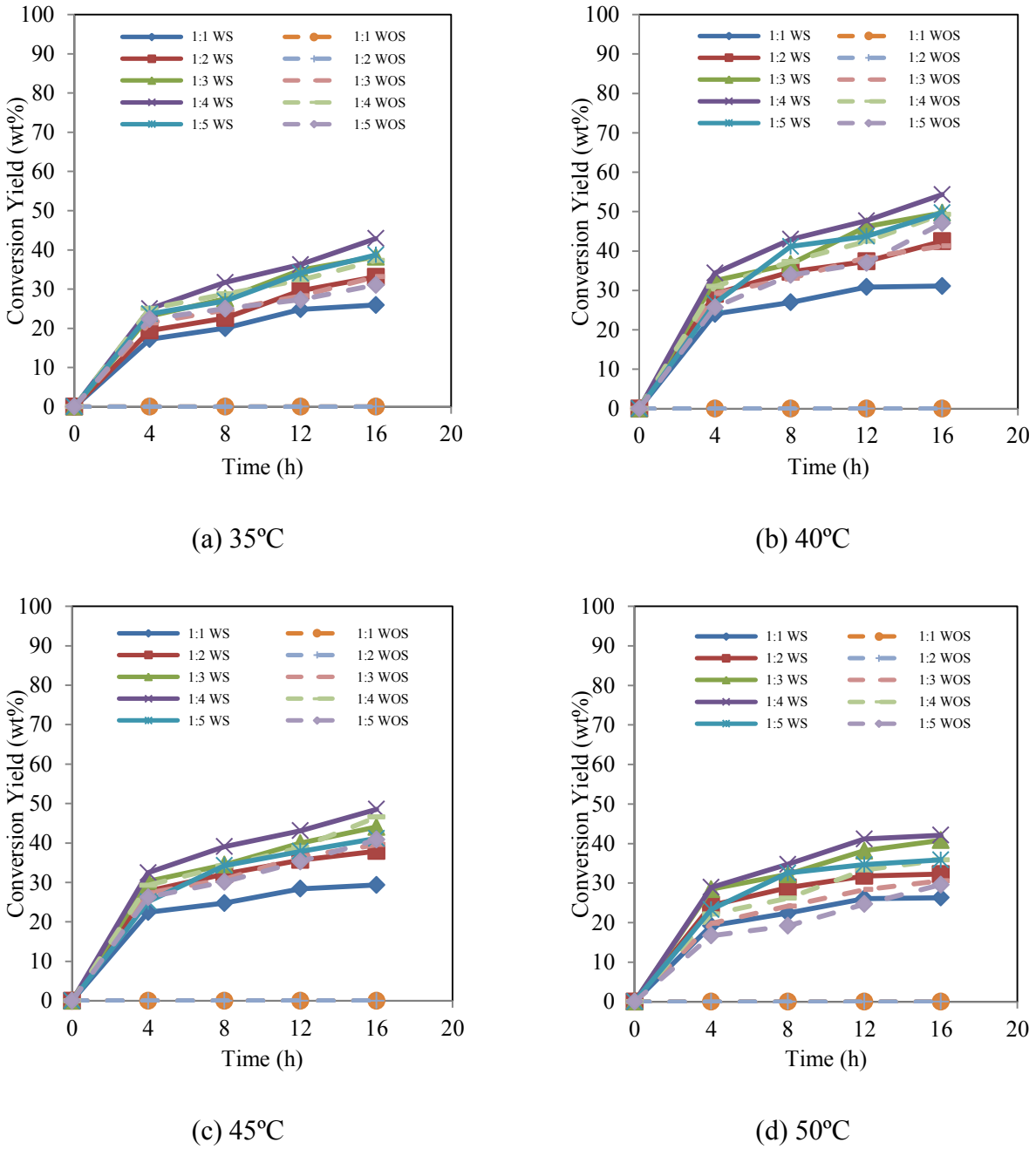


Figure 5.4. Effect of reaction time on the conversion yield using NS88001 lipase with 2-butanol at different reaction temperatures and oil: alcohol molar ratios with and without solvent (WS= with solvent and WOS= without solvent).

Similar trends were observed using the enzyme Novozyme 435 with 2-butanol as shown in Figure 5.2. The biodiesel conversion yield in the solvent system at the reaction temperature of 35°C reached 18.32%, 33.24%, 34.99%, 40.29% and 40.16% after 4 h for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. When the reaction time was further increased from 4 to 16 h, the biodiesel conversion yield increased gradually from 18.32 to 28.69% (56.60%), from 33.24 to 41.92% (26.11%), from 34.99 to 50.47% (44.24%), from 40.29 to 56.30% (39.73%) and from 40.16 to 49.22% (22.55%) for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, in the solvent-free system, the biodiesel conversion yield at the reaction temperature of 35°C reached 36.16%, 38.04%, 43.51% and 35.85% after 4 h for the oil: alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. No reaction was observed for the oil: alcohol molar ratio of 1:1. When the reaction time was further increased from 4 to 16 h, the biodiesel conversion yield increased gradually from 36.16 to 47.41% (31.11%), from 38.04 to 54.00% (41.95%), from 43.51 to 59.23% (36.12%) and from 35.85 to 53.28% (48.61%) for the oil: alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. Similar trends were observed at the reaction temperatures of 40, 45 and 50°C and the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5 with and without solvent but no reaction was observed at the 1:1 oil: alcohol molar ratio for the reaction temperatures of 40, 45 and 50°C in the solvent-free system.

Figure 5.3 shows the effect of reaction time on the biodiesel conversion yield using NS88001 with methanol. The biodiesel conversion yield in the solvent system at the reaction temperature of 35°C reached 20.78%, 24.85%, 28.22%, 30.68% and 27.06% after 4 h for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. When the reaction time was further increased from 4 to 16 h, the biodiesel conversion yield increased gradually from 20.78 to 28.50% (37.15%), from 24.85 to 35.90% (44.46%), from 28.22 to 39.81% (41.07%), from 30.68 to 51.73% (68.61%) and from 27.09 to 38.04% (40.42%) for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, in the solvent-free system, the biodiesel conversion yield at the reaction temperature of 35°C reached 29.86%, 32.01% and 26.68% after 4 h for the oil: alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. No reaction was observed for the oil: alcohol molar ratio of 1:1 and 1:2. When the reaction time was further increased from 4 to 16 h, the biodiesel conversion yield increased gradually from 29.86 to 44.29% (48.32%), from 32.01 to 53.96% (68.57%) and from 26.68 to 41.64%

(56.07%) for the oil: alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. Similar trends were observed at the reaction temperatures of 40, 45 and 50°C and the oil: alcohol molar ratios of 1:3, 1:4 and 1:5 with and without solvent but no reaction was observed at the 1:1 and 1:2 oil : alcohol molar ratio for the reaction temperatures of 40, 45 and 50°C in the solvent-free system.

Similar trends were observed using the enzyme NS88001 with 2-butanol as shown in Figure 5.4. The biodiesel conversion yield in the solvent system at the reaction temperature of 35°C reached 17.22%, 19.41%, 23.13%, 24.98% and 23.68% after 4 h for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. When the reaction time was further increased from 4 to 16 h, the biodiesel conversion yield increased gradually from 17.22 to 25.94% (50.63%), from 19.41 to 33.13% (70.68%), from 23.13 to 38.25% (65.36%), from 24.98 to 42.90% (71.73%) and from 23.68 to 38.71% (63.47%) for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, in the solvent-free system, the biodiesel conversion yield at the reaction temperature of 35°C reached 21.40%, 25.05% and 22.34% after 4 h for the oil: alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. No reaction was observed for the oil: alcohol molar ratios of 1:1 and 1:2. When the reaction time was further increased from 4 to 16 h, the biodiesel conversion yield increased gradually from 21.40 to 33.16% (54.95%), from 25.05 to 37.28% (48.82%) and from 22.34 to 31.08% (39.12%) for the oil: alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. Similar trends were observed at the reaction temperatures of 40, 45 and 50°C and the oil: alcohol molar ratios of 1:3, 1:4 and 1:5 with and without solvent but no reaction was observed at the 1:1 and 1:2 oil: alcohol molar ratio for the reaction temperatures of 40, 45 and 50°C in solvent-free system.

5.1.2. Effect of Oil: Alcohol Molar Ratio

The effect of oil: alcohol molar ratio on the biodiesel conversion yield using different enzymes at different reaction times and alcohol types with and without solvent are shown in Figures 5.5 - 5.8. The results showed that there was an increase in the biodiesel conversion yield when the oil: alcohol molar ratios increased from 1:1 to 1:4 followed by a decrease in the biodiesel conversion yield when the oil: alcohol molar ratios were further increased to 1:5

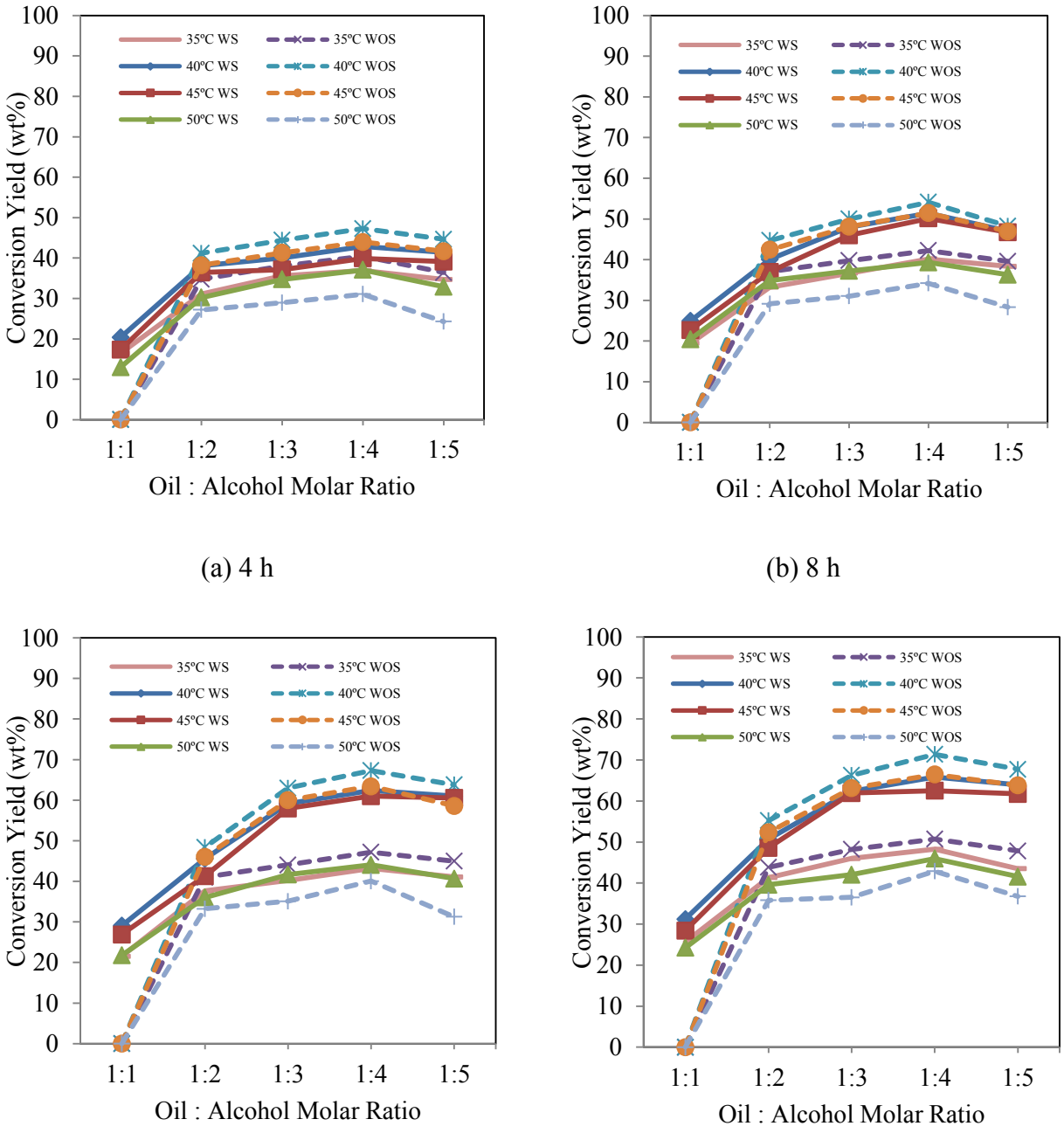


Figure 5.5. Effect of oil: alcohol molar ratio on the conversion yield using *Candida antarctica* (Novozyme 435) with methanol at different reaction temperatures and reaction times with and without solvent (WS= with solvent, WOS= without solvent).

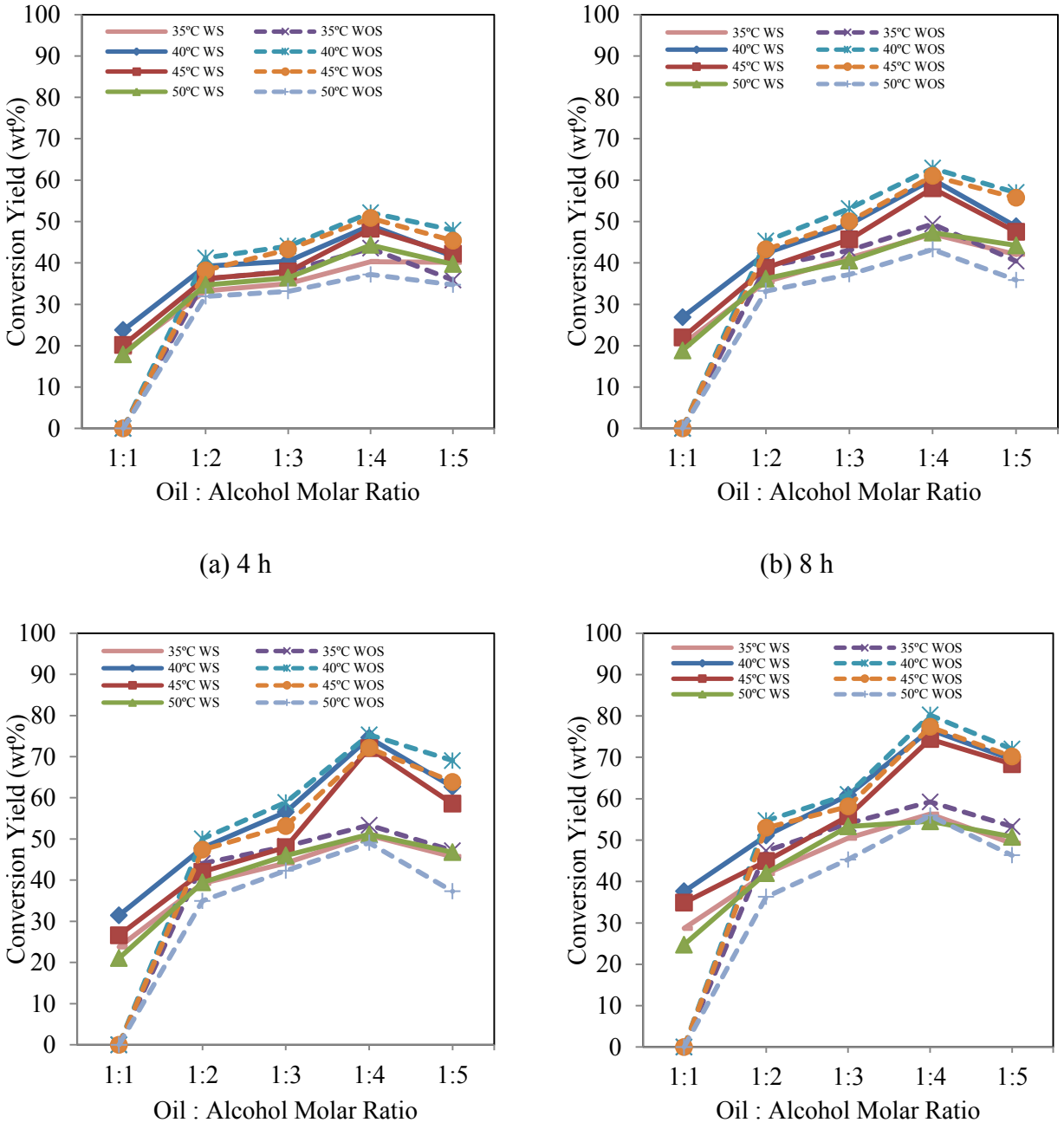


Figure 5.6. Effect of oil: alcohol molar ratio on the conversion yield using *Candida antarctica* (Novozyme 435) with 2-butanol at different reaction temperatures and reaction times with and without solvent (WS= with solvent, WOS= without solvent).

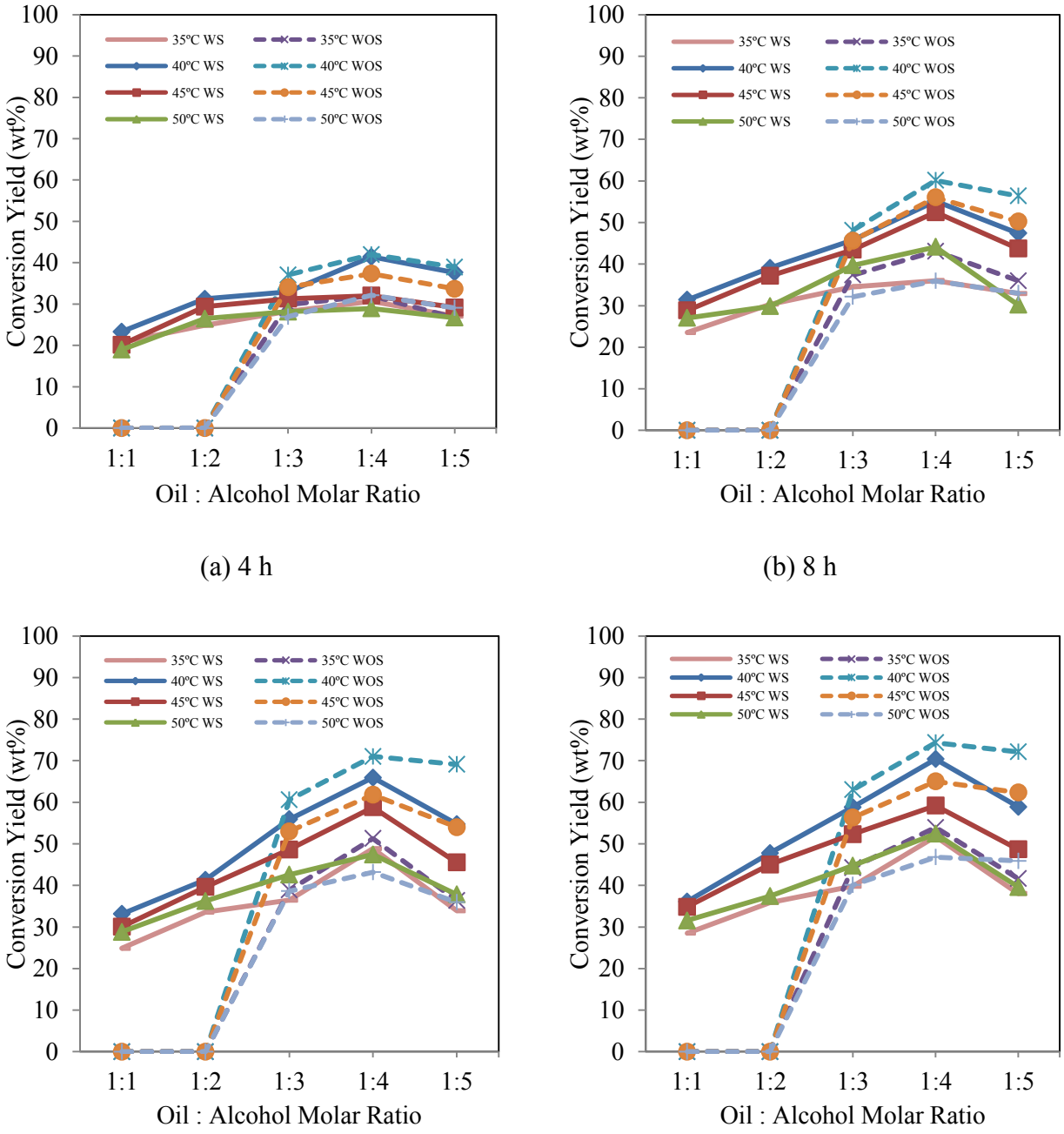


Figure 5.7. Effect of oil: alcohol molar ratio on the conversion yield using an experimental lipase (NS88001) with methanol at different reaction temperatures and reaction times with and without solvent (WS= with solvent, WOS= without solvent).

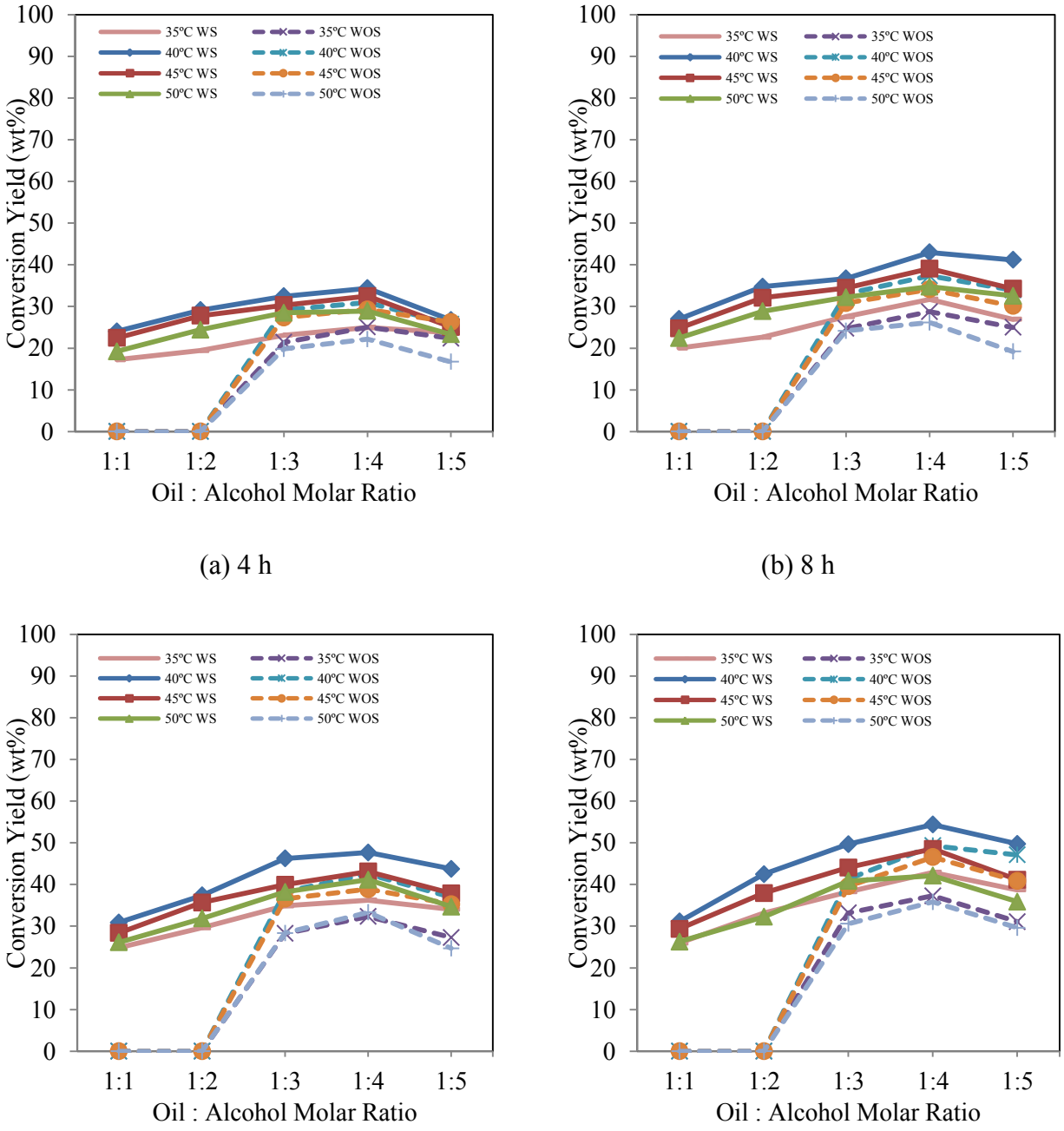


Figure 5.8. Effect of oil: alcohol molar ratio on the conversion yield using an experimental lipase (NS88001) with 2-butanol at different reaction temperatures and reaction times with and without solvent (WS= with solvent, WOS= without solvent).

for all the reaction times (4, 8 12 and 16 h) and reaction temperatures (35, 40, 45 and 50°C) with and without solvent.

Increasing the oil: alcohol molar ratio from 1:1 to 1:4, while using the enzyme Novozyme 435 with methanol and hexane as a solvent, increased the biodiesel conversion yield from 16.44 to 36.92% (124.57%), from 20.39 to 42.88% (110.29%), from 17.34 to 39.92% (130.21%) and from 12.98 to 37.07% (185.59%) at the 4 h reaction time for the reaction temperatures of 35, 40, 45 and 50ºC, respectively. When the oil: alcohol molar ratio was further increased from 1:4 to 1:5, the biodiesel conversion yield decreased from 36.92 to 34.68% (6.06%), from 42.88 to 41.41% (3.42%), from 39.92 to 39.12% (2.00%) and from 37.07 to 32.89% (11.27%) for the reaction temperatures of 35, 40, 45 and 50°C, respectively. However, increasing the oil: alcohol molar ratio from 1:2 to 1:4, while using the enzyme Novozyme 435 with methanol in the solvent-free system, increased the biodiesel conversion yield from 34.75 to 40.43% (16.34%), from 41.20 to 47.26% (14.70%), from 38.22 to 43.96% (15.01%) and from 27.19 to 31.06% (14.23%) at the 4 h reaction time for the reaction temperatures of 35, 40, 45 and 50°C, respectively. No reaction was observed in the solvent - free system at the 1:1 oil: alcohol molar ratio. When the oil: alcohol molar ratio was further increased from 1:4 to 1:5, the biodiesel conversion yield decreased from 40.43 to 36.43% (9.89%), from 47.26 to 44.65% (5.52%), from 43.96 to 41.66% (5.23%) and from 31.06 to 24.29% (21.79%) for the reaction temperatures of 35, 40, 45 and 50°C, respectively. Similar trends were observed at the 8, 12 and 16 h and all the reaction temperatures (35, 40, 45 and 50°C) with and without solvent.

Figure 5.6 shows the effect of oil: alcohol molar ratio on the biodiesel conversion yield using Novozyme 435 and 2-butanol with and without solvent. When the oil: alcohol molar ratio was increased from 1:1 to 1:4, while using the enzyme Novozyme 435 with 2-butanol and hexane as a solvent, the biodiesel conversion yield increased from 18.32 to 40.29% (119.92%), from 23.76 to 49.08% (106.56%), from 20.09 to 48.20% (139.92%) and from 17.88 to 44.33% (147.93%) at the 4 h reaction time for the reaction temperatures of 35, 40, 45 and 50ºC, respectively. When the oil: alcohol molar ratio was further increased from 1:4 to 1:5, the biodiesel conversion yield decreased from 40.29 to 40.16% (0.32%), from 49.08

to 41.63% (15.17%), from 48.20 to 42.17% (12.51%) and from 44.33 to 39.71% (10.42%) for the reaction temperatures of 35, 40, 45 and 50°C, respectively. However, when the oil: alcohol molar ratio was increased from 1:2 to 1:4, while using the enzyme Novozyme 435 with 2-butanol in the solvent-free system, the biodiesel conversion yield increased from 36.16 to 43.51% (20.32%), from 41.21 to 52.13% (26.49%), from 38.18 to 50.82% (33.10%) and from 31.90 to 37.18% (16.55%) at the 4 h reaction time for the reaction temperatures of 35, 40, 45 and 50°C, respectively. No reaction was observed in the solvent -free system at the 1:1 oil: alcohol molar ratio. When the oil: alcohol molar ratio was further increased from 1:4 to 1:5, the biodiesel conversion yield decreased from 43.51 to 35.85% (17.60%), from 52.13 to 47.92% (8.07%), from 50.82 to 45.36% (10.74%) and from 37.18 to 34.71% (6.64%) for the reaction temperatures of 35, 40, 45 and 50°C, respectively. Similar trends were observed with the 8, 12 and 16 h and all the reaction temperatures (35, 40, 45 and 50°C) with and without solvent.

Figure 5.7 shows the effect of oil: alcohol molar ratio on the biodiesel conversion yield using NS88001 and methanol with and without solvent. Increasing the oil: alcohol molar ratio from 1:1 to 1:4, while using the enzyme NS88001 with methanol and hexane as a solvent, increased the biodiesel conversion yield from 20.78 to 30.68% (47.64%), from 23.29 to 41.41% (77.80%), from 20.13 to 32.02% (59.06%) and from 18.95 to 28.94 % (52.71%) at the 4 h reaction time for the reaction temperatures of 35, 40, 45 and 50ºC, respectively. When the oil: alcohol molar ratio was further increased from 1:4 to 1:5, the biodiesel conversion yield decreased from 30.68 to 27.09% (11.70%), from 41.41 to 37.62% (9.15%), from 32.02 to 29.16% (8.93%) and from 28.94 to 26.72% (7.67%) for the reaction temperatures of 35, 40, 45 and 50°C, respectively. However, increasing the oil: alcohol molar ratio from 1:3 to 1:4, while using the enzyme NS88001 and methanol in the solvent-free system, increased the biodiesel conversion yield from 29.86 to 32.01% (7.20%), from 37.03 to 41.96% (13.31%), from 34.07 to 37.35% (9.62%) and from 26.93 to 32.13% (19.30%) at the 4 h reaction time for the reaction temperatures of 35, 40, 45 and 50°C, respectively. No reaction was observed in the solvent -free system at the 1:1 and 1:2 oil: alcohol molar ratios. When the oil: alcohol molar ratio was further increased from 1:4 to 1:5, the biodiesel conversion yield decreased from 32.01 to 26.68% (16.65%), from 41.96 to 38.91% (7.26%), from 37.35 to 33.67% (9.85%) and from 32.13 to 29.07% (9.52%) for the reaction temperatures of 35, 40, 45 and

50°C, respectively. Similar trends were observed with the 8, 12 and 16 h and all the reaction temperatures (35, 40, 45 and 50°C) with and without solvent.

Figure 5.8 shows the effect of oil: alcohol molar ratio on the biodiesel conversion yield using NS88001 and 2-butanol with and without solvent. Increasing the oil: alcohol molar ratio from 1:1 to 1:4, while using the enzyme NS88001 with 2-butanol and hexane as a solvent, increased the biodiesel conversion yield from 17.22 to 24.98% (45.06%), from 24.02 to 34.39% (43.17%), from 22.46 to 32.46% (44.52%) and from 19.21 to 28.91 % (50.49%) at the 4 h reaction time for the reaction temperatures of 35, 40, 45 and 50ºC, respectively. When the oil: alcohol molar ratio was further increased from 1:4 to 1:5, the biodiesel conversion yield decreased from 24.98 to 23.68% (5.20%), from 34.39 to 26.74% (22.24%), from 32.46 to 25.09% (22.70%) and from 28.91 to 23.31% (19.37%) for the reaction temperatures of 35, 40, 45 and 50°C, respectively. However, increasing the oil: alcohol molar ratio from 1:3 to 1:4, while using the enzyme NS88001 with 2-butanol in the solvent-free system, increased the biodiesel conversion yield from 21.40 to 25.05% (17.05%), from 29.19 to 30.99% (6.16%), from 27.33 to 29.28% (7.13%) and from 19.83 to 22.17% (11.80%) at the 4 h reaction time for the reaction temperatures of 35, 40, 45 and 50°C, respectively. No reaction was observed in the solvent -free system at the 1:1 and 1:2 oil: alcohol molar ratios. When the oil: alcohol molar ratio was further increased from 1:4 to 1:5, the biodiesel conversion yield decreased from 25.05 to 22.34% (10.81%), from 30.99 to 25.74% (16.94%), from 29.28 to 26.24% (10.38%) and from 22.17 to 16.72% (24.58%) for the reaction temperatures of 35, 40, 45 and 50°C, respectively. Similar trends were observed with the 8, 12 and 16 h and all the reaction temperatures (35, 40, 45 and 50°C) with and without solvent.

5.1.3. Effect of Reaction Temperature

The effect of reaction temperature on the biodiesel conversion yield using different enzymes at different oil: alcohol molar ratios, reaction times, solvent systems and alcohol types are shown in Figures 5.9 - 5.12. The results showed that there was an increase in the biodiesel conversion yield when the reaction temperature was increased from 35 to 40°C followed by a decrease when the reaction temperature were further increased from 40 to 50°C for all the reaction times (4, 8 12 and 16 h), oil: alcohol molar ratio (1:1, 1:2, 1:3, 1:4 and 1:5), alcohols (methanol and 2-butanol) with and without solvent.

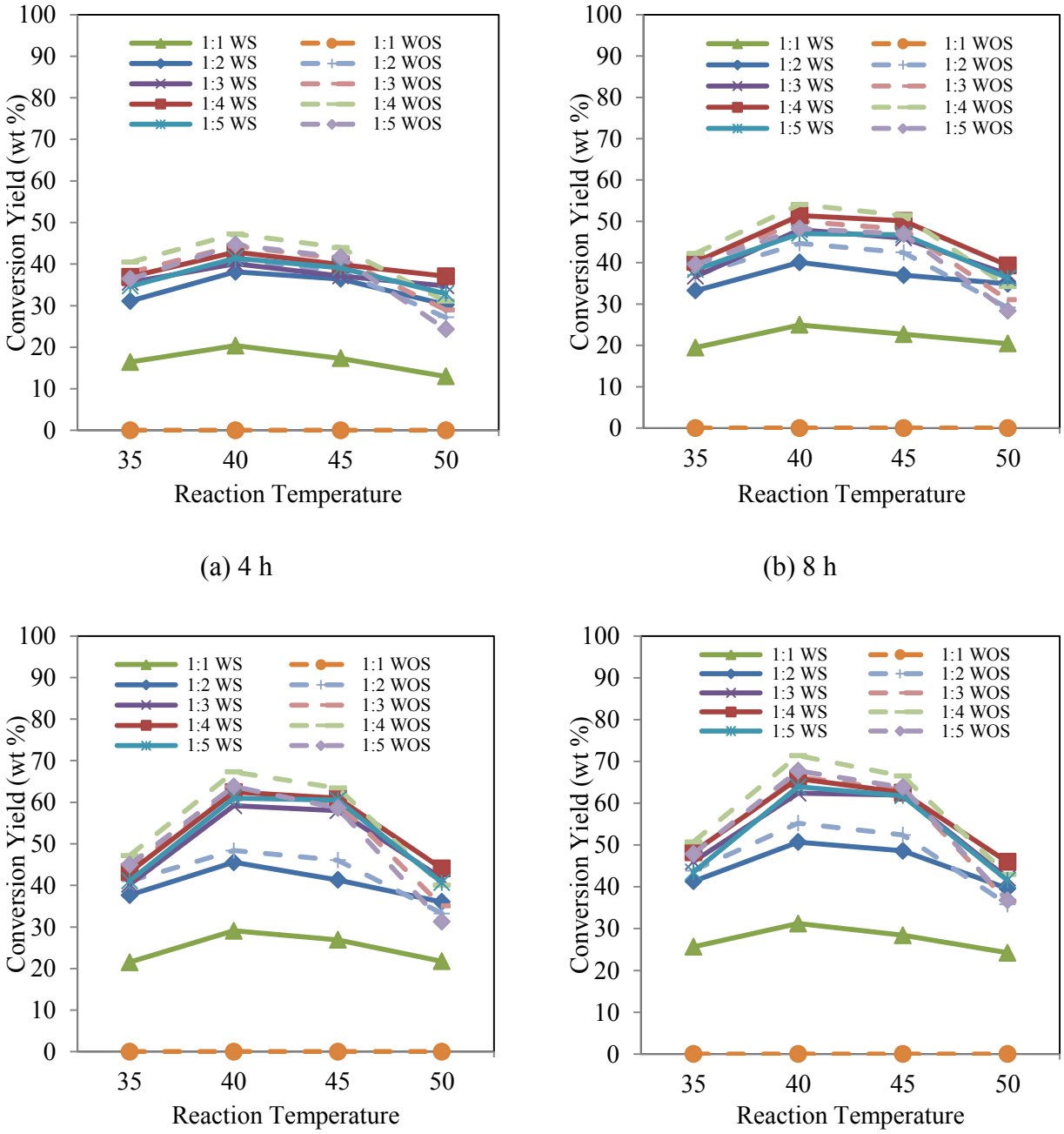


Figure 5.9. Effect of reaction temperature on the conversion yield using *Candida antarctica* (Novozyme 435) with methanol at different oil: alcohol molar ratios and reaction times with and without solvent (WS= with solvent, WOS= without solvent).

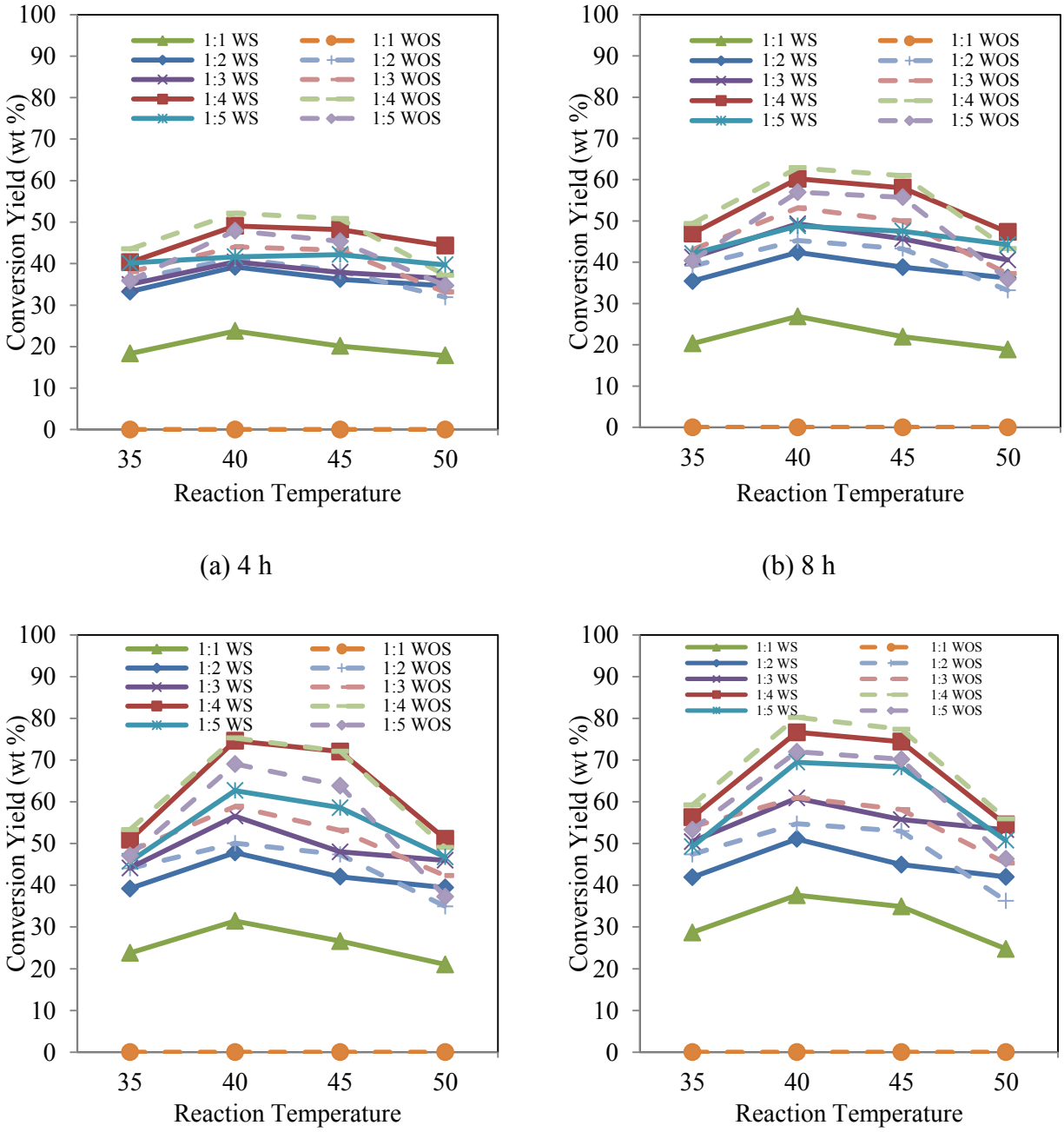


Figure 5.10. Effect of reaction temperature on the conversion yield using *Candida antarctica* (Novozyme 435) with 2-butanol at different oil: alcohol molar ratios and reaction times with and without solvent (WS= with solvent, WOS= without solvent).

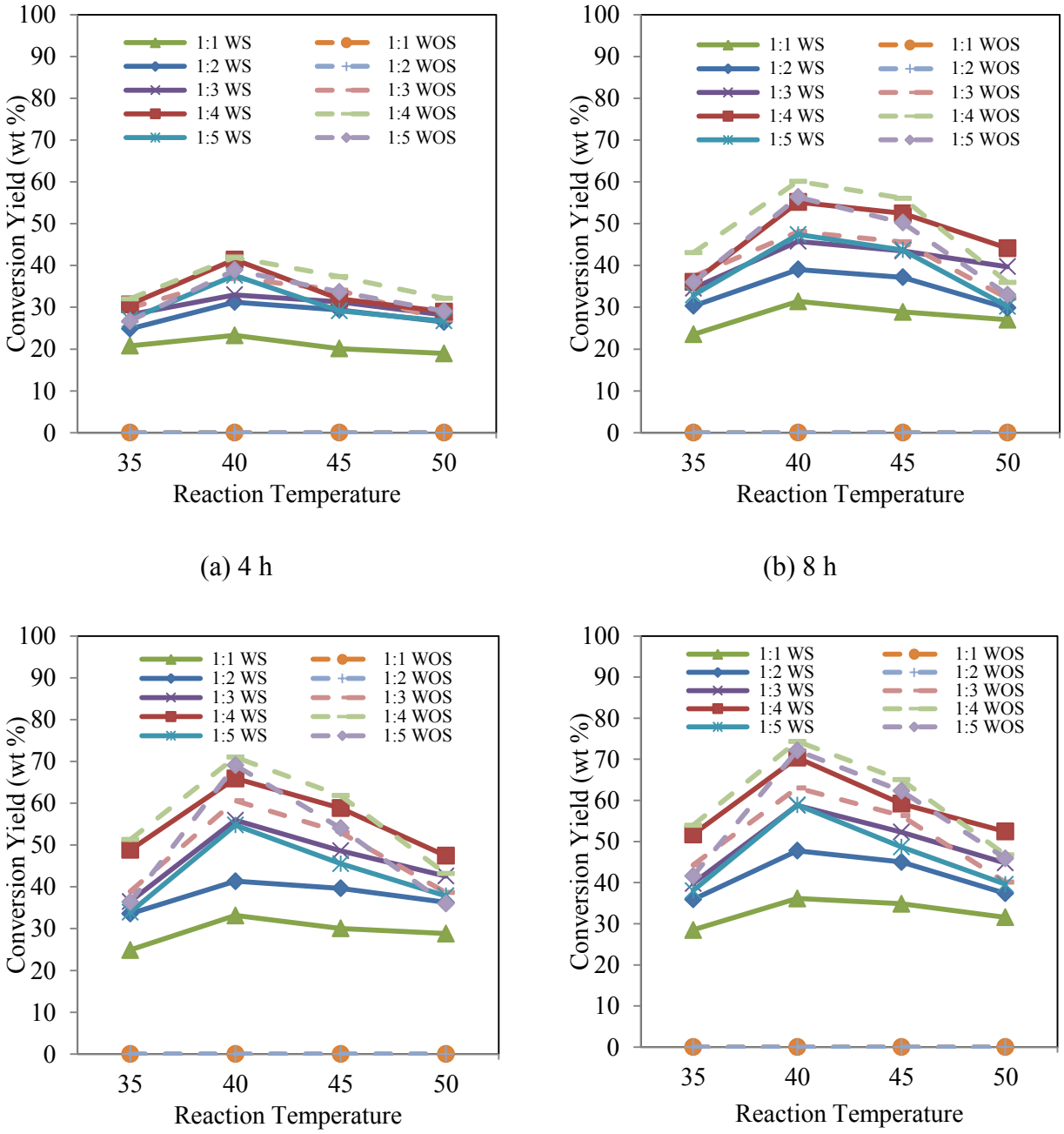


Figure 5.11. Effect of reaction temperature on the conversion yield using an experimental lipase (NS88001) with methanol at different oil: alcohol molar ratios and reaction times with and without solvent (WS= with solvent, WOS= without solvent).

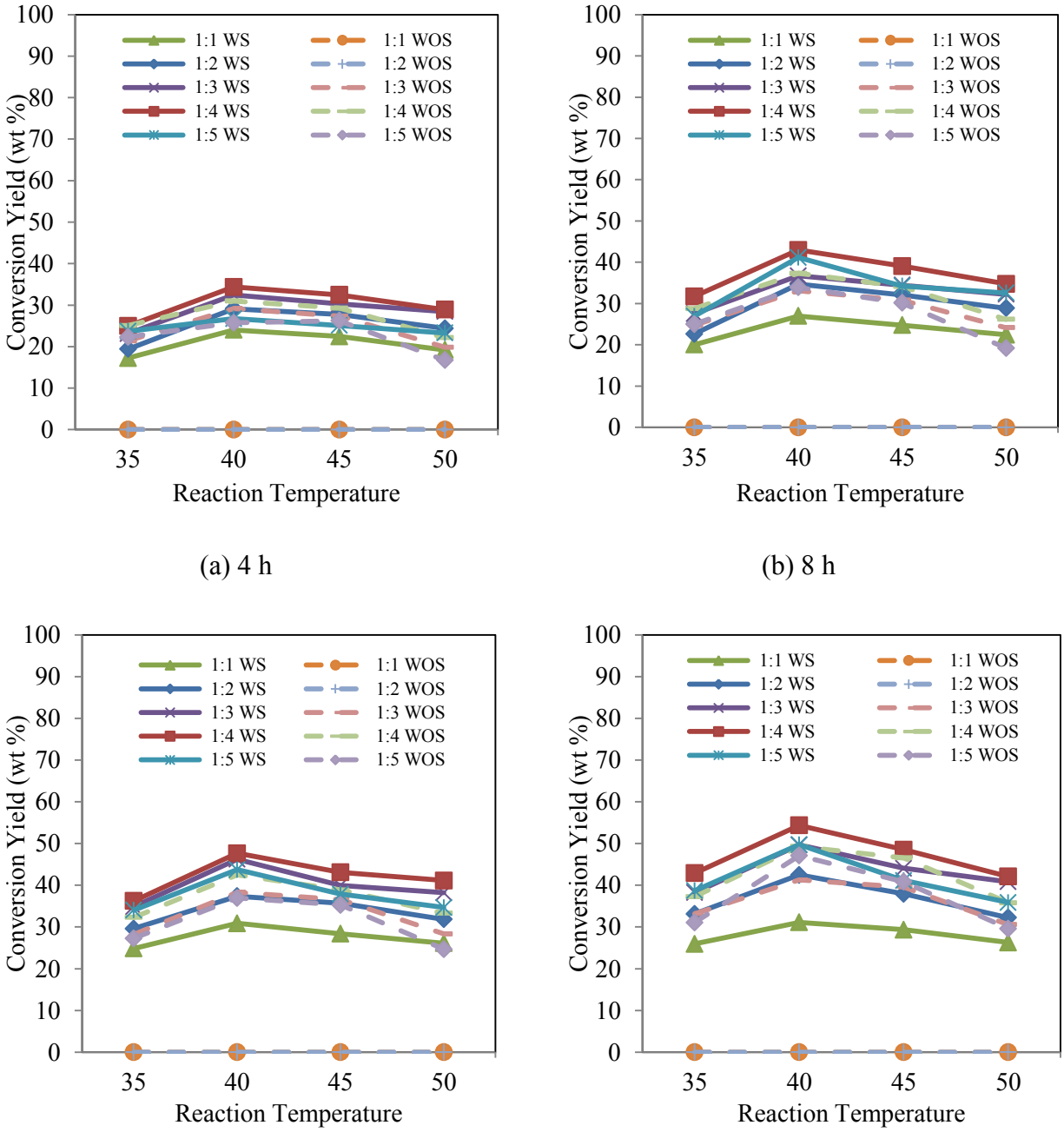


Figure 5.12. Effect of reaction temperature on the conversion yield using an experimental lipase (NS88001) with 2-butanol at different oil: alcohol molar ratios and reaction times with and without solvent (WS= with solvent, WOS= without solvent).

Increasing the reaction temperature from 35 to 40°C, while using the enzyme Novozyme 435 with methanol and hexane as a solvent, increased the biodiesel conversion yield from 16.44 to 20.39% (24.02%), from 31.08 to 38.13% (22.68%), from 35.58 to 40.06% (12.59%), from 36.92 to 42.88% (16.14%) and from 34.68 to 41.41% (19.40%) at the 4 h reaction time for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. When the reaction temperature was further increased from 40 to 50°C, the biodiesel conversion yield decreased from 20.39 to 12.98% (36.34%), from 38.13 to 30.24% (20.69%), from 40.06 to 34.73% (13.30%), from 42.88 to 37.07% (13.54%) and from 41.41 to 32.89% (20.57%) for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, increasing the reaction temperature from 35 to 40°C,while using the enzyme Novozyme 435 with methanol in the solvent-free system, increased the biodiesel conversion yield from 34.75 to 41.20% (18.56%), from 38.04 to 44.41% (16.74%), from 40.43 to 47.26% (16.89%) and from 36.43 to 44.65% (22.56%) at 4 h reaction time for the oil: alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. No reaction was observed in the solvent –free system at the 1:1 oil: alcohol molar ratio. When the reaction temperature was further increased from 40 to 50°C, the biodiesel conversion yield decreased from 41.20 to 27.19% (34.00%), from 44.41 to 28.96% (34.78%), from 47.26 to 31.06% (34.27%) and from 44.65 to 24.29% (45.59%) for the oil: alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. Similar trends were observed with the 8, 12 and 16 h reaction time and the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5 with solvent and at the oil: alcohol molar ratios of 1:2, 1:3 1:4 and 1:5 without solvent.

Figure 5.10 shows the effect of reaction temperature on the biodiesel conversion yield using Novozyme 435 and 2-butanol with and without solvent. Increasing the reaction temperature from 35 to 40°C, while using the enzyme Novozyme 435 with 2-butanol and hexane as a solvent, increased the biodiesel conversion yield from 18.32 to 23.76% (29.69%), from 33.24 to 39.21% (17.96%), from 34.99 to 40.42% (15.51%), from 40.29 to 49.08% (21.81%) and from 40.16 to 41.63% (3.66%) at the 4 h reaction time for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. When the reaction temperature was further increased from 40 to 50°C, the biodiesel conversion yield decreased from 23.76 to 17.88% (24.74%), from 39.21 to 34.69% (11.52%), from 40.42 to 36.41% (9.92%), from 49.08 to 44.33% (9.67%) and from 41.63 to 39.71% (4.61%) for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, increasing the reaction temperature from

35 to 40°C, while using the enzyme Novozyme 435 with 2-butanol in the solvent-free system, increased the biodiesel conversion yield from 36.16 to 41.21% (13.96%), from 38.04 to 44.06% (15.82%), from 43.51 to 52.13% (19.81%) and from 35.85 to 47.92% (33.66%) at the 4 h reaction time for the oil: alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. No reaction was observed in the solvent-free system at the 1:1 oil: alcohol molar ratio. When the reaction temperature was further increased from 40 to 50°C, the biodiesel conversion yield decreased from 41.21 to 31.90% (22.59%), from 44.06 to 33.13% (24.80%), from 52.13 to 37.18% (28.67%) and from 47.92 to 34.71% (27.56%) for the oil: alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. Similar trends were observed at the 8, 12 and 16 h reaction times and the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5 with solvent and the oil: alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5 without solvent.

Figure 5.11 shows the effect of reaction temperature on the biodiesel conversion yield by NS88001 and methanol with and without solvent. Increasing the reaction temperature from 35 to 40°C, while using the enzyme NS88001 with methanol and hexane as a solvent, increased the biodiesel conversion yield from 20.78 to 23.29% (12.07%), from 24.85 to 31.26% (25.79%), from 28.22 to 32.98% (16.86%), from 30.68 to 41.41% (34.97%) and from 27.09 to 37.62% (38.87%) at the 4 h reaction time for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. When the reaction temperature was further increased from 40 to 50°C, the biodiesel conversion yield decreased from 23.29 to 18.95% (18.63%), from 31.26 to 26.47% (15.32%), from 32.98 to 28.14% (14.67%), from 41.41 to 28.94% (30.11%) and from 27.09 to 26.72% (1.36%) for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, increasing the reaction temperature from 35 to 40°C, while using the enzyme NS88001 with methanol in the solvent-free system, increased the biodiesel conversion yield from 29.86 to 37.03% (24.01%), from 32.01 to 41.96% (31.08%) and from 26.68 to 38.91% (45.83%) at the 4 h reaction time for the oil: alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. No reaction was observed in the solvent -free system at the 1:1 and 1:2 oil: alcohol molar ratios. When the reaction temperature was further increased from 40 to 50°C, the biodiesel conversion yield decreased from 37.03 to 26.93% (27.27%), from 41.96 to 32.13% (23.42%), and from 38.91 to 29.07% (25.28%) for the oil: alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. Similar trends were observed at the 8, 12 and

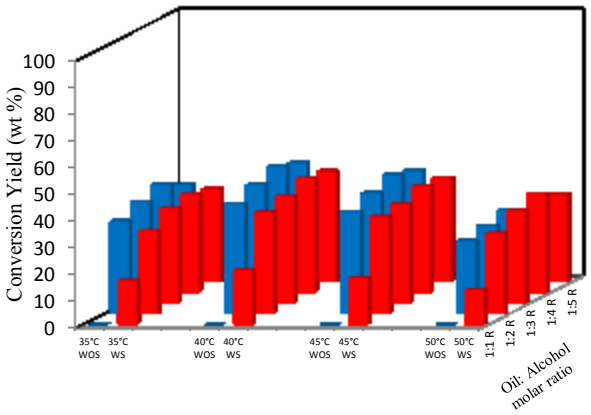
16 h reaction time and the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5 with solvent and the oil: alcohol molar ratios of 1:3, 1:4 and 1:5 without solvent.

Figure 5.12 shows the effect of reaction temperature on the biodiesel conversion yield using NS88001 and 2-butanol with and without solvent. Increasing the reaction temperature from 35 to 40°C, while using the enzyme NS88001 with 2-butanol and hexane as a solvent, increased the biodiesel conversion yield from 17.22 to 24.02% (39.48%), from 19.41 to 29.11% (49.97%), from 23.13 to 32.45% (40.29%) from 24.98 to 34.39 % (37.67%) and from 23.68 to 26.74% (12.92%) at the 4 h reaction time for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. When the reaction temperature was further increased from 40 to 50°C, the biodiesel conversion yield decreased from 24.02 to 19.21% (20.02%), from 29.11 to 24.40% (16.18%), from 32.45 to 28.47% (12.26%), from 34.39 to 28.91% (15.93%) and from 26.74 to 23.31% (12.82%) for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, increasing the reaction temperature from 35 to 40°C, while using the enzyme NS88001 with 2-butanol in the solvent-free system, increased the biodiesel conversion yield from 21.40 to 29.19% (36.40%), from 25.05 to 30.99% (23.71%) and from 22.34 to 25.74% (15.21%) at the 4 h reaction time for the oil: alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. No reaction was observed in the solvent -free system at the 1:1 and 1:2 oil: alcohol molar ratios. When the reaction temperature was further increased from 40 to 50°C, the biodiesel conversion yield decreased from 29.19 to 19.83% (32.06%), from 30.99 to 22.17% (28.46%) and from 25.74 to 16.72% (35.04%) for the oil: alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. Similar trends were observed at the 8, 12 and 16 h reaction times and the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5 with solvent and the oil: alcohol molar ratios of 1:3, 1:4 and 1:5 without solvent.

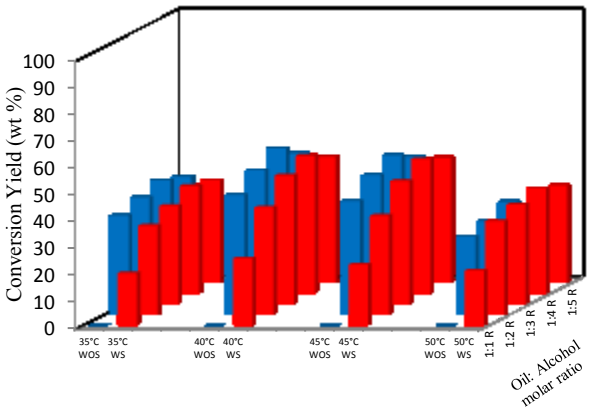
5.1.4. Effect of Solvent

The effect of solvent on the biodiesel conversion yield using different enzymes and alcohols, at different oil: alcohol molar ratios, reaction times and reaction temperatures are shown in Figures 5.13 – 5.16.

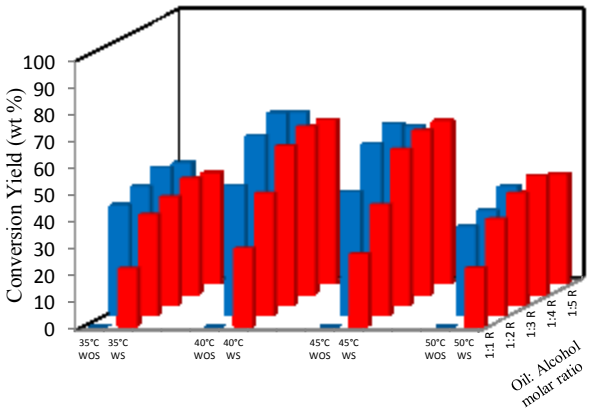
Figure 5.13 shows the effect of solvent on the biodiesel conversion yield using Novozyme 435 lipase with methanol at different oil: alcohol molar ratios, reaction



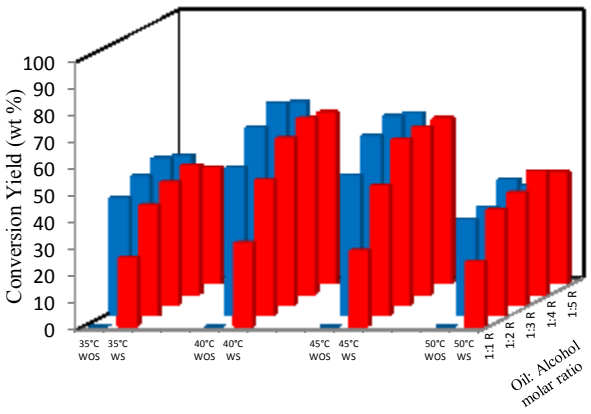
(a) 4 h



(b) 8 h

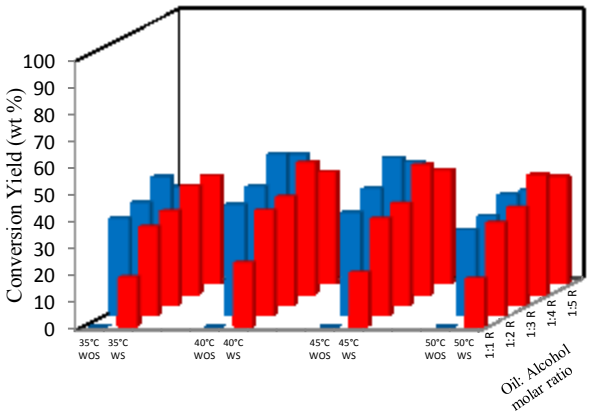


(c) 12 h

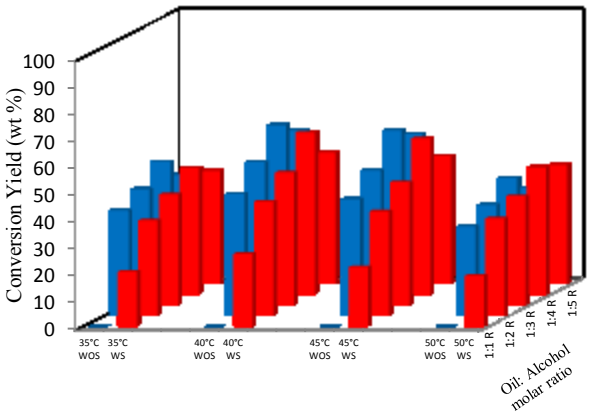


(d) 16 h

Figure 5.13. Effect of solvent system on the conversion yield using *Candida antarctica* (Novozyme 435) with methanol at different oil: alcohol molar ratios and reaction times with and without solvent (WS= with solvent, WOS= without solvent).



(a) 4 h



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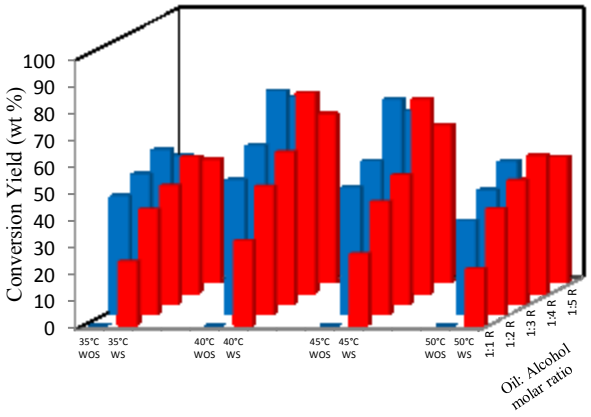
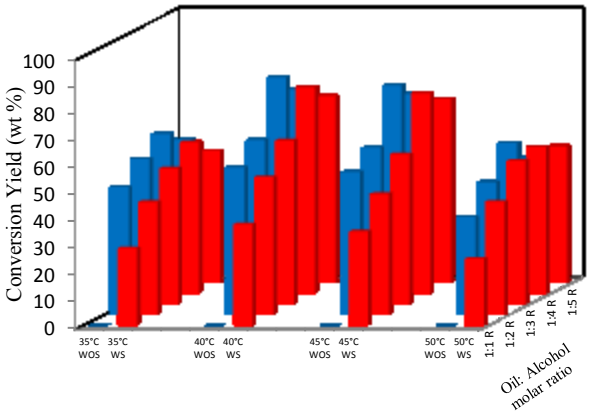
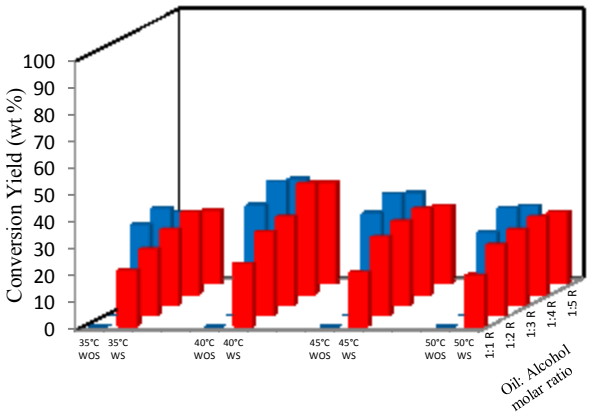


(c) 12 h

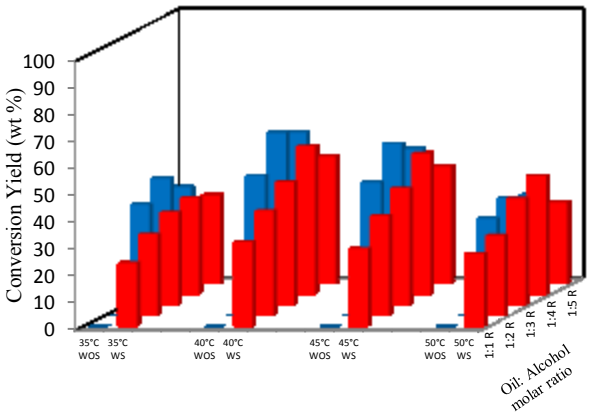


(d) 16 h

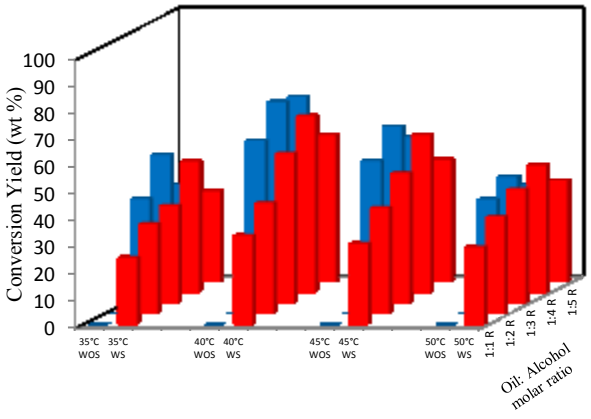
Figure 5.14. Effect of solvent system on the conversion yield using *Candida antarctica* (Novozyme 435) with 2-butanol at different oil: alcohol molar ratios and reaction times with and without solvent (WS= with solvent, WOS= without solvent).



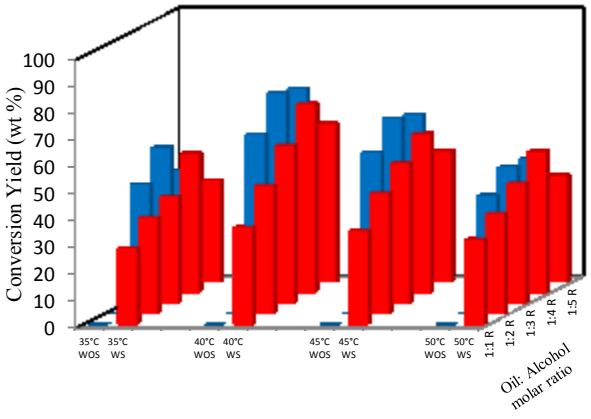
(a) 4 h



(b) 8 h

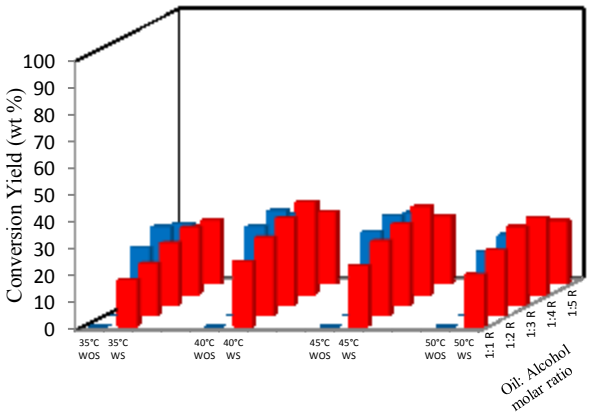


(c) 12 h

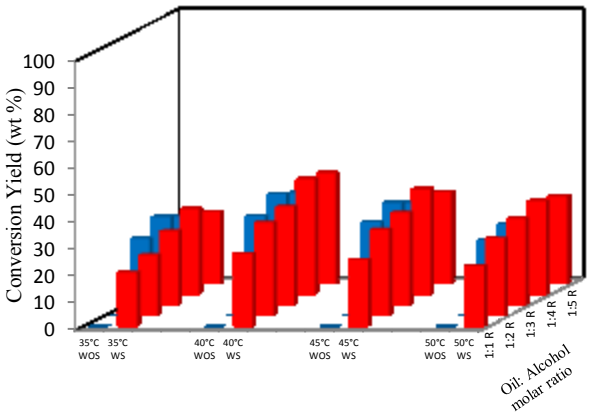


(d) 16 h

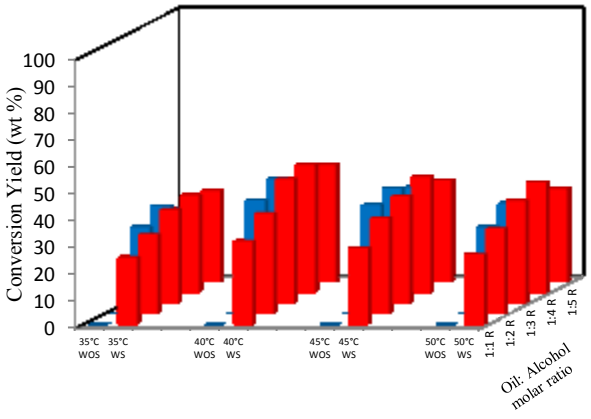
Figure 5.15. Effect of solvent system on the conversion yield using an experimental lipase (NS88001) with methanol at different oil: alcohol molar ratios and reaction times with and without solvent (WS= with solvent, WOS= without solvent).



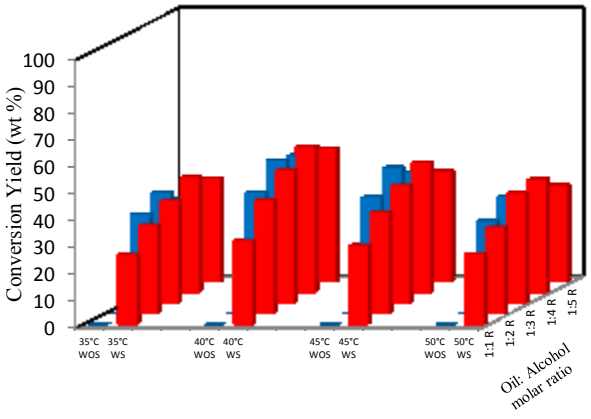
(a) 4 h



(b) 8 h



(c) 12 h



(d) 16 h

Figure 5.16. Effect of solvent system on the conversion yield using an experimental lipase (NS88001) with 2-butanol at different oil: alcohol molar ratios and reaction times with and without solvent (WS= with solvent, WOS= without solvent).

temperatures and reaction times. No reaction was observed at the 1:1 oil: alcohol molar ratio without solvent. The solvent free- system achieved higher biodiesel conversion yield at all oil: alcohol molar ratios, reaction times and the reaction temperatures of 35, 40 and 45ºC. However, the solvent system achieved higher biodiesel conversion yield at the reaction temperature of 50ºC for all oil: alcohol molar ratios. Similar trends were observed using Novozyme 435 lipase and 2-butanol with and without solvent as shown in Figure 5.14.

Figure 5.15 shows the effect of solvent on the biodiesel conversion yield using NS88001 lipase with methanol at different oil: alcohol molar ratios, reaction temperatures and reaction times. No reaction was observed at the 1:1 and 1:2 oil: alcohol molar ratio without solvent. The solvent free- system achieved higher biodiesel conversion yield at all oil: alcohol molar ratios, reaction times and the reaction temperatures of 35, 40 and 45ºC. However, the solvent system achieved higher biodiesel conversion yield at the reaction temperature of 50ºC for all the oil: alcohol molar ratios.

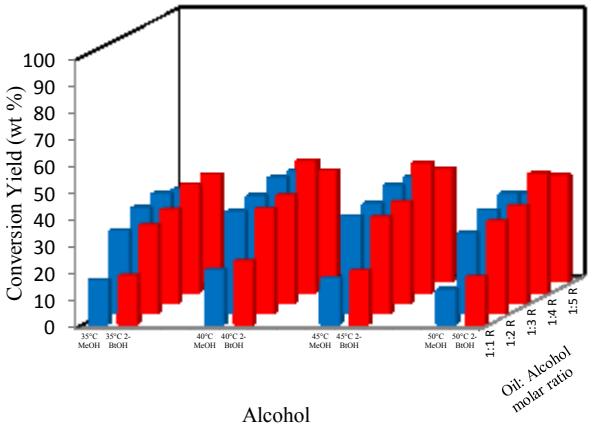
Figure 5.16 shows the effect of solvent on the biodiesel conversion yield using NS88001 lipase and 2-butanol at different oil: alcohol molar ratios, reaction temperatures and reaction times. No reaction was observed at the 1:1 and 1:2 oil: alcohol molar ratios without solvent. The solvent system achieved high biodiesel conversion yield at all the oil: alcohol molar ratios, reaction times and reaction temperatures.

5.1.5. Effect of Alcohol

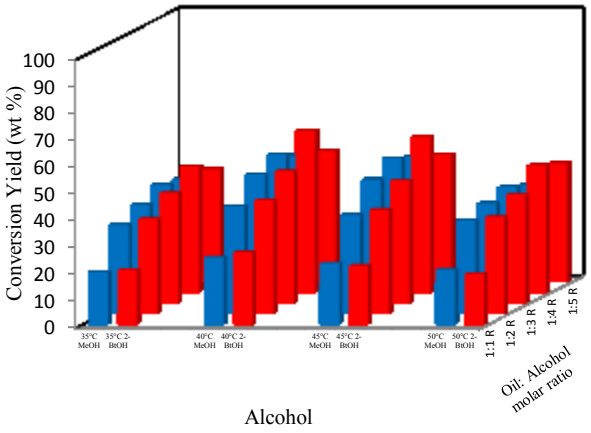
The effect of alcohol on the biodiesel conversion yield using different enzymes and solvent systems, at different oil: alcohol molar ratios, reaction times and reaction temperatures are shown in Figures 5.17 – 5.20.

Figure 5.17 shows the effect of alcohol type on the biodiesel conversion yield using Novozyme 435 lipase and hexane as a solvent at different oil: alcohol molar ratios, reaction temperatures and reaction times. Higher biodiesel conversion yield was achieved using 2-butanol at the oil: alcohol molar ratios, reaction times and reaction temperatures.

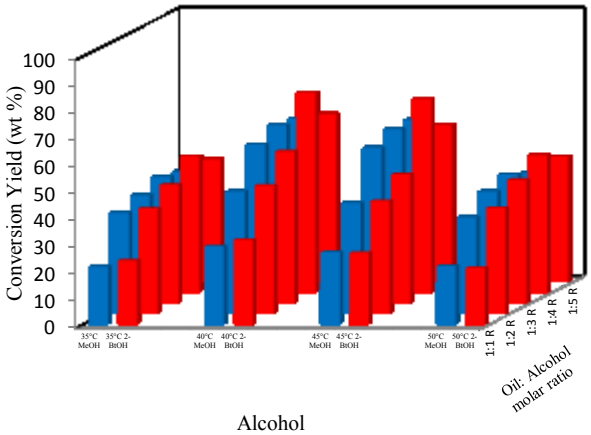
Figure 5.18 shows the effect of alcohol type on the biodiesel conversion yield using Novozyme 435 lipase in a solvent- free system at different oil: alcohol molar ratios, reaction



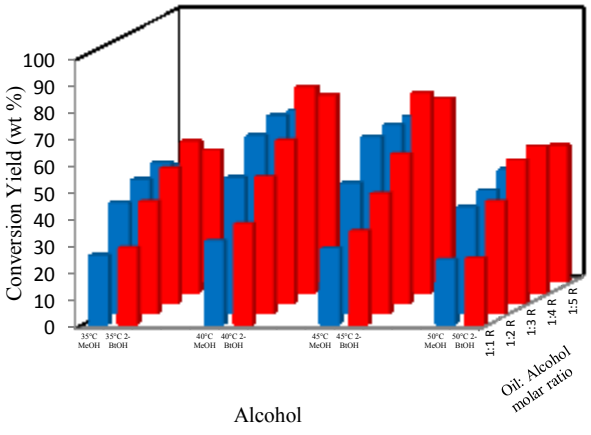
(a) 4 h



(b) 8 h

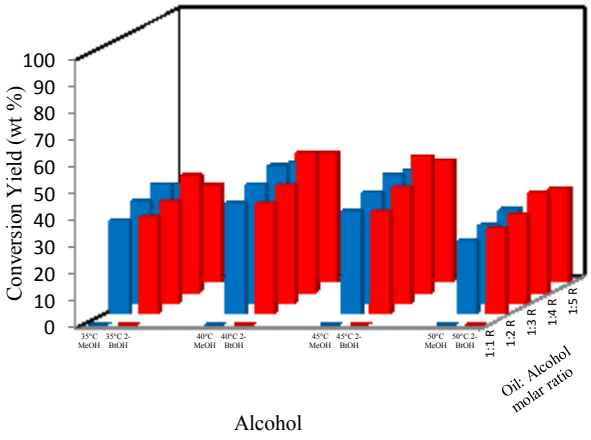


(c) 12 h

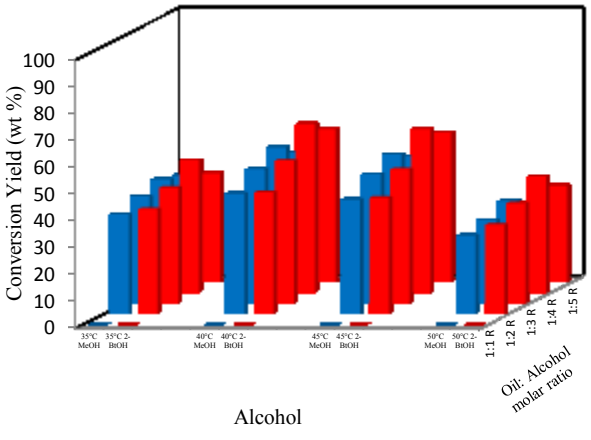


(d) 16 h

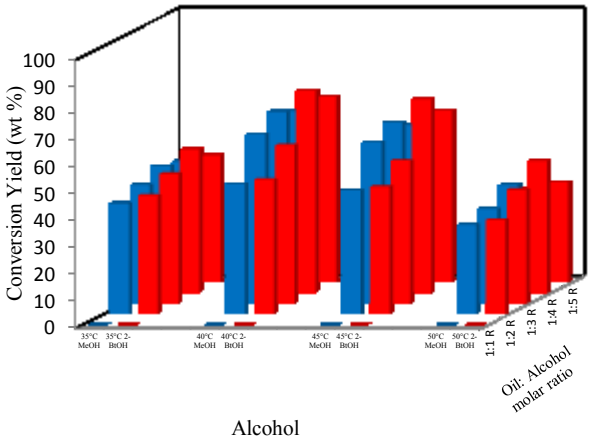
Figure 5.17. Effect of alcohol on the conversion yield using *Candida antarctica* (Novozyme 435) with hexane as a solvent at different oil: alcohol molar ratios and reaction times.



(a) 4 h



(b) 8 h



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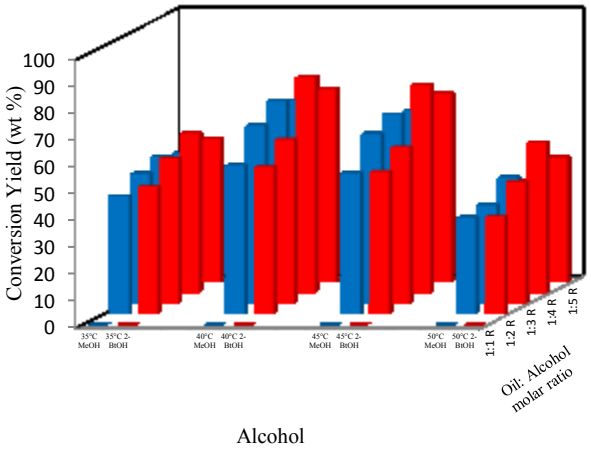
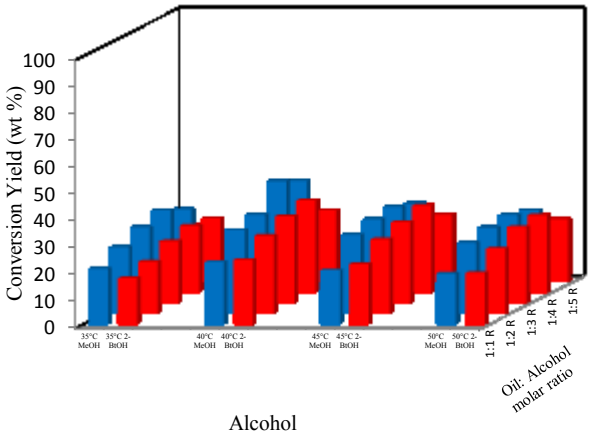
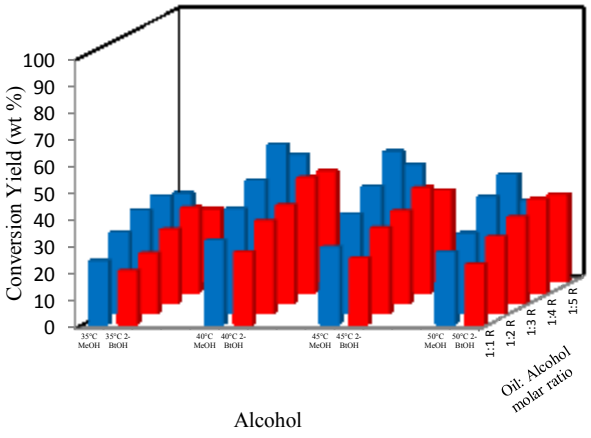


(d) 16 h

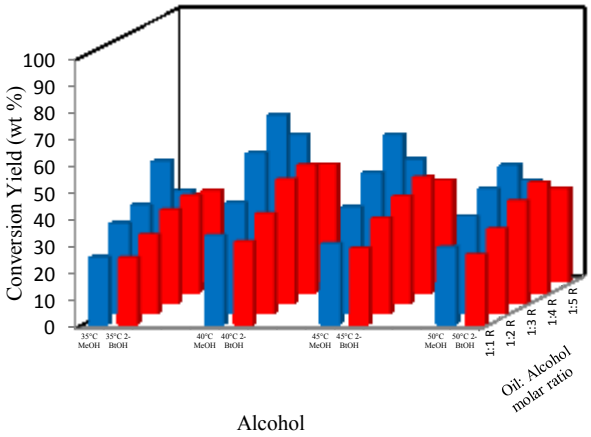
Figure 5.18. Effect of alcohol on the conversion yield using *Candida antarctica* (Novozyme 435) without solvent at different oil: alcohol molar ratios and reaction times.



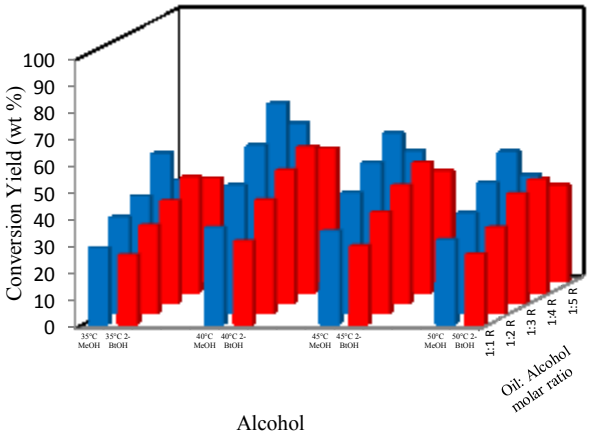
(a) 4 h



(b) 8 h

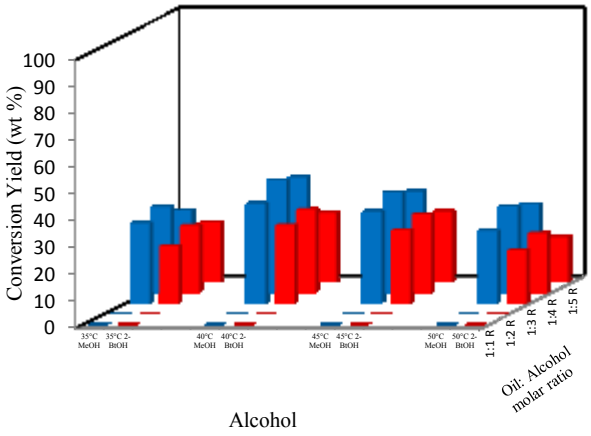


(c) 12 h

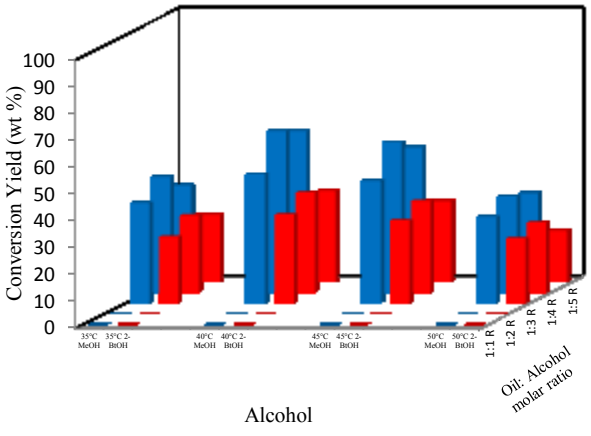


(d) 16 h

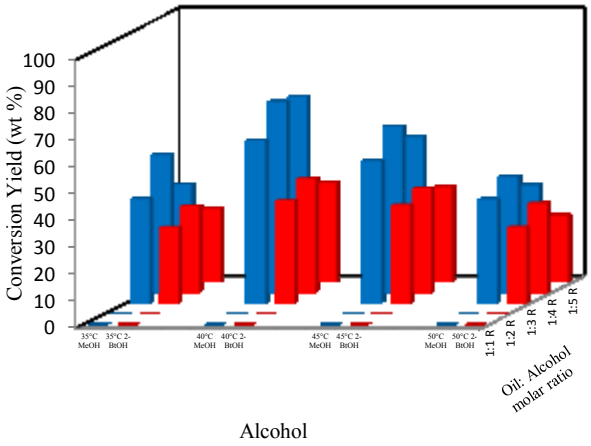
Figure 5.19. Effect of alcohol on the conversion yield using an experimental lipase (NS88001) with hexane as a solvent at different oil: alcohol molar ratios and reaction times.



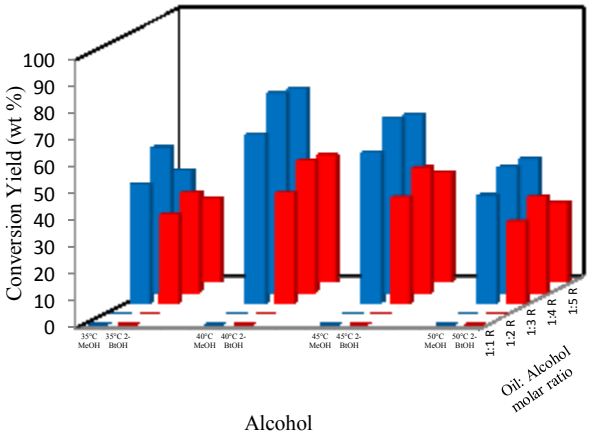
(a) 4 h



(b) 8 h



(c) 12 h



(d) 16 h

Figure 5.20. Effect of alcohol on the conversion yield using an experimental lipase (NS88001) without solvent at different oil: alcohol molar ratios and reaction times.

temperatures and reaction times. No reaction was observed at the 1:1 oil: alcohol molar ratio. Higher biodiesel conversion yield were achieved using 2-butanol at all the oil: alcohol molar ratios, reaction times and reaction temperatures.

Figure 5.19 shows the effect of alcohol type on the biodiesel conversion yield using NS88001 lipase and hexane as a solvent at different oil: alcohol molar ratios, reaction temperatures and reaction times. Higher biodiesel conversion yield were achieved using methanol at all oil: alcohol molar ratios, reaction times and reaction temperatures. Similar trends were observed using NS88001 lipase and methanol in a solvent- free system at different oil: alcohol molar ratios, reaction temperatures and reaction times as shown in Figure 5.20. No reaction was observed at the oil: alcohol molar ratios of 1:1 and 1:2.

5.2. Biodiesel Production by a Combination of Enzyme Catalysts

The transesterification process results of the individual enzymes showed that the reaction temperature of 40ºC and the oil: alcohol molar ratio of 1:4 were the optimum. Therefore, the enzymatic transesterification by a combination of the two enzymes was carried out at the optimum reaction temperature of 40ºC and the oil: alcohol molar ratio of 1:4 to investigate the effects of reaction time (4, 8, 12 and 16 h), alcohol type (methanol and 2-butanol) and solvent system (solvent and solvent–free) on the biodiesel conversion yield. The results are shown in Table 5.11.

Analysis of variance (ANOVA) and Tukey's grouping were performed on the biodiesel conversion yield data using Minitab Statistics Software (version 16.2.2, Minitab Inc, State College, Pennsylvania, USA).The results are shown in Tables 5.12 and 5.13. The effects of solvent system, alcohol type and reaction time were highly significant at the 0.001 level. All interactions between these parameters were also significant at the 0.001 level. The results obtained from Tukey's grouping test indicated that the two solvent systems were significantly different from each other at the 0.05 level. The highest average biodiesel conversion yield of 63.24% was achieved with hexane as a solvent. The two alcohols were also significantly different from each other at the 0.05 level. The highest average biodiesel conversion yield of 62.16% was achieved with methanol. The reaction times of 4, 8, and 16 h were significantly different from each other at the 0.05 level but the reaction time of 12 h was

Table 5.11. Biodiesel yield (wt%) from fish oil using combined Novozyme 435and NS88001 with different alcohols, different system at a reaction temperature of 40ºC and 1:4 oil: alcohol molar ratio.

Time (h)	Biodiesel Conversion Yield (wt %)			
	Solvent System		Without Solvent	
	Methanol	2-Butanol	Methanol	2-Butanol
4	44.47 ± 0.97	46.17 ± 1.05	50.93 ± 0.88	34.38 ± 1.09
8	54.71 ± 1.27	65.42 ± 0.74	62.47 ± 1.03	43.16 ± 1.16
12	68.39 ± 0.76	73.21 ± 0.97	70.17 ± 0.97	51.47 ± 1.14
16	71.18 ± 0.91	82.37 ± 0.85	74.99± 1.22	57.39 ± 0.94

Table 5.12. Analysis of variance for biodiesel yield (Combination of Enzyme).

Source	DF	SS	MS	F	P
Total	31	5612.77			
Model					
SS	1	464.54	464.54	244.106	0.001
AT	1	239.13	239.13	125.660	0.001
TI	3	3467.00	1155.67	607.282	0.001
SS*AT	1	1264.83	1264.83	664.646	0.001
SS*TI	3	78.21	26.07	13.699	0.001
AT*TI	3	25.00	8.33	4.379	0.001
SS*AT*TI	3	43.61	14.54	7.639	0.001
Error	16	30.45	1.90		

DF : Degree of freedom
SS : Sum of square
MS : Mean of square
R^2 : 99.46%
TI : Reaction time
SS : Solvent system
AT : Alcohol type

Table 5.13. Tukey's grouping for biodiesel yield (Combination of enzymes).

Factors	Level	N	Mean (%)	Tukey Grouping
Solvent System	Hexane	16	63.24	A
	Without Hexane	16	55.62	B
Alcohol	Methanol	16	62.16	A
	2-Butanol	16	56.69	B
Reaction Time (hour)	4	8	43.98	A
	8	8	56.44	B
	12	8	65.81	BC
	16	8	71.48	C

Groups with the same letter are not significantly different at the 0.05 level.

not significantly different from the reaction times of 8 and 16 h at the 0.05 level. The highest average biodiesel conversion yield of 71.48% was achieved at the reaction time of 16 h.

5.2.1. Effect of Reaction Time

The effect of reaction time on the biodiesel conversion yield using a combination of the two enzymes (Novozyme 435 and NS88001) is shown in Figures 5.21. The results showed that there was an initial rapid biodiesel conversion in the first 4 h followed by a slow gradual increase thereafter until the end of the experiment (16 h) for the two alcohols (methanol and 2-butanol) with and without solvent.

The biodiesel conversion yield using a combination of Novozyme 435 and NS88001 enzyme in a solvent system, reached 44.47% for methanol and 46.17% for 2-butanol at the 4 h reaction time. When the reaction time was further increased from 4 to 16 h, the biodiesel conversion yield increased gradually from 44.47 to 71.18% (60.06%) for methanol and from 46.17 to 82.37% (78.40%) for 2-butanol. Similar trends were achieved in the solvent - free system. The biodiesel conversion yield reached 50.93% for methanol and 34.38% for 2-butanol at the 4 h reaction time. When the reaction time was further increased from 4 to 16 h, the biodiesel conversion yield increased gradually from 50.93 to 74.99% (47.24%) for methanol and from 34.38 to 57.39% (66.92%) for 2-butanol.

5.2.2. Effect of Solvent

Figure 5.22 shows the effect of solvent system on the biodiesel conversion yield using a combination of Novozyme 435 and NS88001 at different alcohol types and reaction times. The solvent systems with 2-butanol achieved higher biodiesel conversion yield at all the reaction times. However, the solvent- free system achieved the higher biodiesel conversion yield with methanol at all the reaction times.

5.2.3. Effect of Alcohol

Figure 5.23 shows the effect of alcohol type on the biodiesel conversion yield using a combination of Novozyme 435 and NS88001 at different solvent system type and reaction times. Higher biodiesel conversion yields were achieved using methanol in a solvent- free

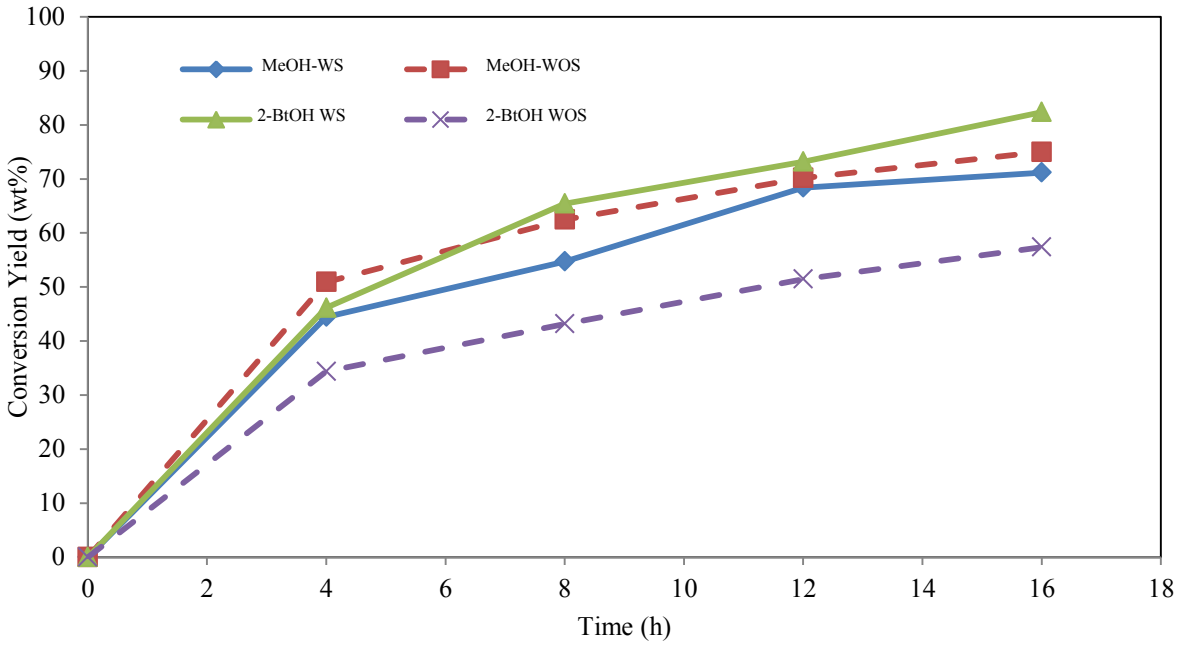


Figure 5.21. Effect of reaction time on the conversion yield of biodiesel by a combination of Novozyme 435 and NS88001 using different alcohols, with and without solvent at 1:4 oil: alcohol molar ratio and 40ºC.

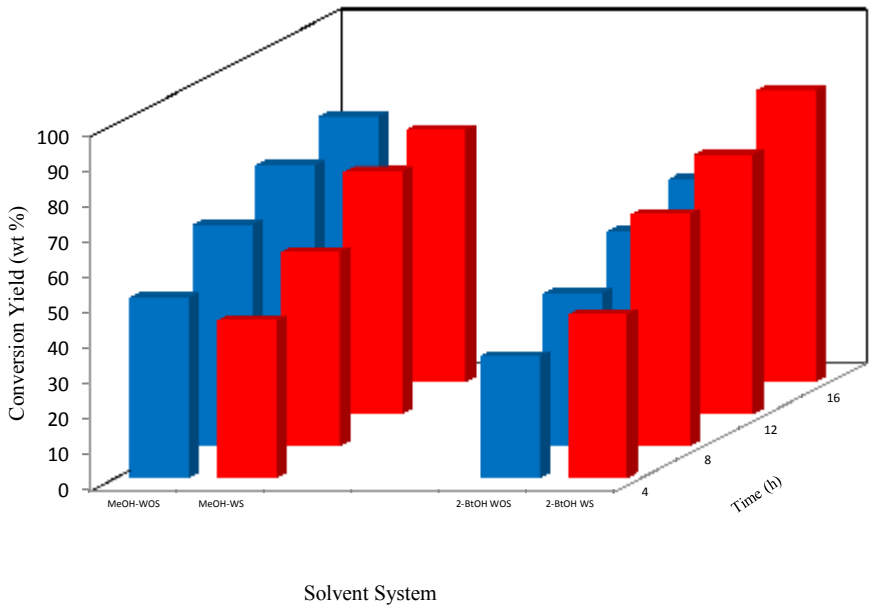


Figure 5.22. Effect of solvent system on the conversion yield using a combination of Novozyme 435 and NS88001 at different alcohols at 1:4 oil: alcohol molar ratio and 40ºC.

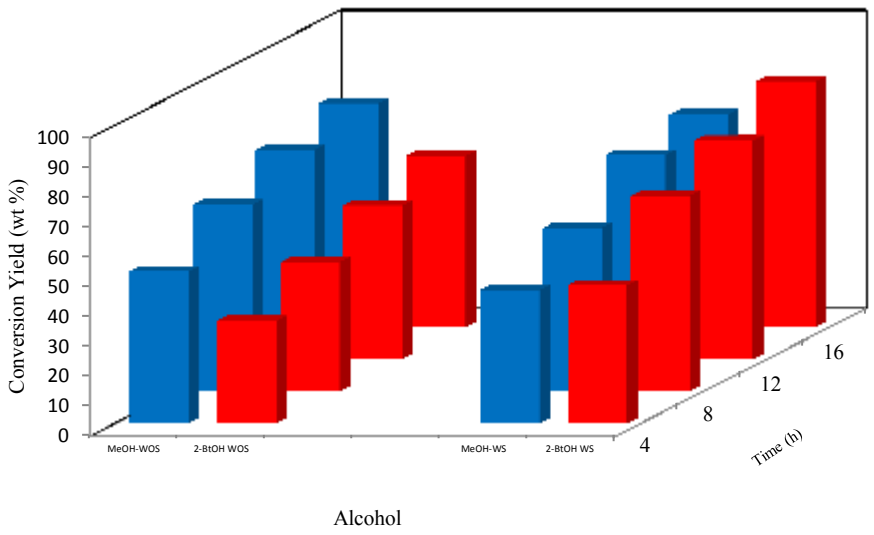


Figure 5.23. Effect of alcohol on the conversion yield using a combination of Novozyme 435 and NS88001 with and without solvent at 1:4 oil: alcohol molar ratio and 40ºC.

system at all the reaction times. However, higher biodiesel conversion yields were achieved using 2-butanol in a solvent system at all the reaction times.

5.3. Reusability of Enzyme

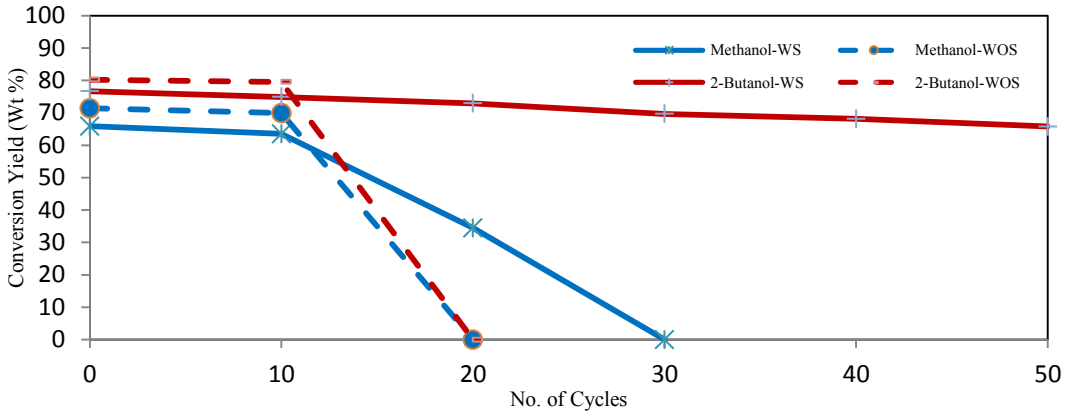
The effect of enzyme reusability on the biodiesel conversion yield using different enzymes with different alcohols at the optimum conditions (40°C reaction temperature, 1:4 oil: alcohol molar ratio and 16 h reaction time) is shown in Table 5.14 and Figure 5.24.

When the enzyme Novozyme 435 was used with methanol and hexane as a solvent, the biodiesel conversion yield decreased slightly from 65.86 to 63.48% (3.61%) after 10 cycles and then decreased rapidly reaching 0.00% after 30 cycles. However, when the same enzyme was used with methanol in the solvent free system, the biodiesel conversion yield also decreased slightly from 71.39 to 69.91% (2.07%) after 10 cycles and then decreased much rapidly reaching 0.00% after 20 cycles. On the other hand, when the enzyme Novozyme 435 was used with 2-butanol and hexane as a solvent, the biodiesel conversion yield decreased gradually from 76.66 to 65.77% (14.20%) after 50 cycles. However, when the same enzyme was used with2-butanol in the solvent-free system, the biodiesel conversion yield showed insignificant decrease of 0.89% (from 80.24 to 79.52%) after 10 cycles and then decreased very rapidly reaching 0.00% after 20 cycles.

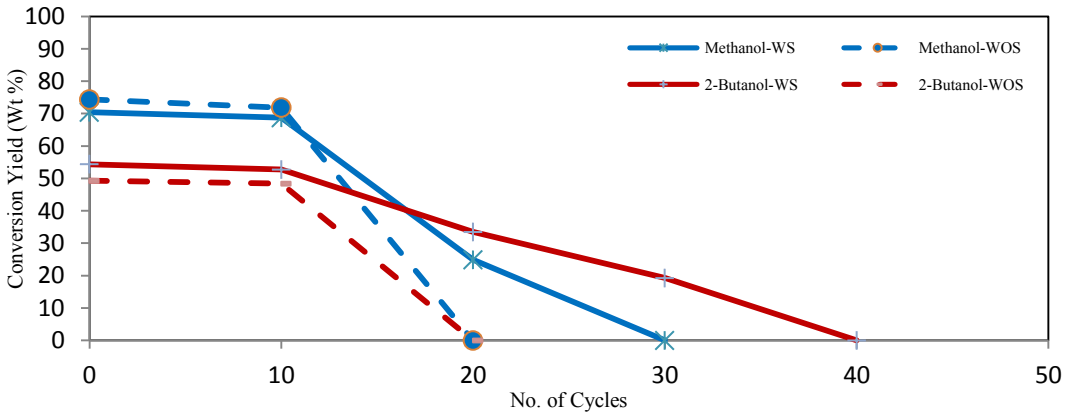
When the enzyme NS88001was used with methanol and hexane as a solvent, the biodiesel conversion yield decreased slightly from 70.38 to 68.74% (2.33%) after 10 cycles and then decreased rapidly reaching 0.00%after 30 cycles. However, when the same enzyme was used with methanol in the solvent-free system, the biodiesel conversion yield also decreased slightly from 74.38 to 71.81% (3.45%) after 10 cycles and then decreased very rapidly reaching 0.00% after 20 cycles. On the other hand, when the enzyme NS88001 was used with2-butanoland hexane as a solvent, the biodiesel conversion yield decreased slightly from 54.35 to 52.72% (2.99%) after 10 cycles and then decreased rapidly reaching 0.00% after 40 cycles. However, when the same enzyme was used with 2-butanolin the solvent-free system, the biodiesel conversion yield also decreased slightly from 49.29 to 48.36% (1.88%) after 10 cycles and then decreased very rapidly reaching 0.00% after 20 cycles.

Table 5.14. Biodiesel conversion yield of Novozyme 435, NS88001 and Combination of enzymes (Novozyme 435 and NS88001) at different alcohols with and without solvent system as affected by the number of cycles.

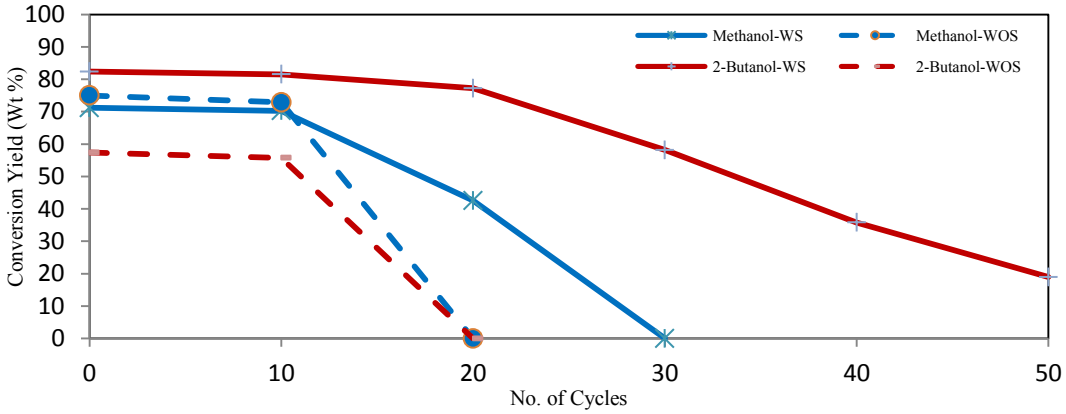
Enzymes	Solvent System	Alcohol	Number of Cycles					
			0	10	20	30	40	50
Novozyme 435	With	Methanol	65.86	63.48	34.47	-	-	-
		2-Butanol	76.66	74.87	72.91	69.68	68.14	65.77
	Without	Methanol	71.39	69.91	-	-	-	-
		2-Butanol	80.24	79.52	-	-	-	-
NS88001	With	Methanol	70.38	68.74	24.89	-	-	-
		2-Butanol	54.35	52.72	33.46	19.24	-	-
	Without	Methanol	74.38	71.81	-	-	-	-
		2-Butanol	49.29	48.36	-	-	-	-
Combination	With	Methanol	71.18	70.23	42.54	-	-	-
		2-Butanol	82.37	81.51	77.23	58.14	35.80	18.96
	Without	Methanol	74.99	72.85	-	-	-	-
		2-Butanol	57.39	55.74	-	-	-	-



(a) Novozyme 435



(b) NS88001



(c) Combination

Figure 5.24. Biodiesel yield of Novozyme 435, NS88001 and Combination of enzymes (Novozyme 435 and NS88001) at different alcohols with and without solvent system as affected by the number of cycles.

When the combination of the two enzymes (Novozyme 435 and NS88001) was used with methanol and hexane as a solvent, the biodiesel conversion yield decreased slightly from 71.18 to 70.23% (1.33%) after 10 cycles and then decreased very rapidly reaching 0.00% after 30 cycles. However, when the same combination of the two enzymes was used with methanol in the solvent-free system, the biodiesel conversion yield decreased slightly from 74.99 to 72.85% (2.85%) after 10 cycles and then decreased very rapidly reaching 0.00%after 20 cycles. On the other hand, when the combination of the two enzymes was used with 2-butanol and hexane as a solvent, the biodiesel conversion yield decreased from 82.37 to 77.23% (6.24%) after 20 cycles and then decreased rapidly reaching 18.96% after 50 cycles. However, when the same combination of the two enzymes was used with 2-butanol in the solvent-free system, the biodiesel conversion yield decreased slightly from 57.39 to 55.74% (2.87%) after 10 cycles and then decreased very rapidly reaching 0.00%after 20 cycles.

CHAPTER 6. DISCUSSION

6.1. Biodiesel Extraction by Individual Enzymes Catalyst

The selection of enzyme plays an important role in the biodiesel process. Enzyme catalysts (lipases) are very competitive in comparison to chemical catalysts because of their high catalyzing ability towards a variety of TAG substrates to produce biodiesel (Mittelbach, 1990; Nelson et al., 1996; Ma et al., 1999; Du et al., 2004; Shah et al., 2004; Watanabe et al., 2006; Shah and Gupta, 2007; Gog et al., 2012). However, few lipases have been commercialized in which Novozyme 435 is commonly used for producing biodiesel (Al-Zuhair, 2007; Xu et al., 2012). In this study, the enzymatic transesterification of fish oil was carried out using *Candida antarctica* (Novozyme 435) and the experimental enzyme (NS88001) for the production of biodiesel. The effects of reaction time (4, 8, 12 and 16 h), oil: alcohol molar ratio (1:1, 1:2, 1:3 1:4 and 1:5), reaction temperature (35, 40, 45 and 50ºC), solvent system (with solvent and solvent- free) and alcohol type (methanol and 2-butanol) on the biodiesel conversion yield from fish oil were investigated.

6.1.1. Effect of Reaction Time

Increasing the reaction time increased the biodiesel conversion yield. All the curves obtained at different oil: alcohol molar ratios (1:1, 1:2, 1:3, 1:4 and 1:5), different alcohols (methanol and 2-butanol), different enzymes (Novozyme 435 and NS88001), different reaction temperatures (35, 40, 45 and 50ºC) and different reaction times (4, 8, 12 and 16 h) tend to have an initial rapid phase during the first 4 h followed by a gradual increase thereafter until the end of the experiment (16 h). Increasing the reaction time from 4 to 16 h at the reaction temperature of 35ºC, increased the biodiesel conversion yield using Novozyme 435 with methanol by 56.02, 32.94, 29.25, 30.71 and 25.46% for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. Similar trends were seen with both enzymes and both alcohols for all oil: alcohol molar ratios, reaction temperatures with and without solvent.

In this study, the maximum biodiesel conversion yield of 76.66% was obtained using Novozyme 435 and 2-butanol with hexane as solvent, a molar ratio of 1:4 and a reaction time of 16 h. Only 65.86% biodiesel conversion yield was achieved under the same condition with

methanol in the solvent system. The formation of esters from the oil increases with increasing reaction time (Freedman et al., 1984). Several authors observed that the initial reaction was rapid due to the initial mixing and dispersion of the alcohol and oil (Leung et al., 2006; Meher et al., 2006). The alcohol has a positive effect on the reaction kinetics by facilitating formation of homogeneous suspension of the reactants and the catalyst (Martin and Otero, 2008).

Both lipases did not completely convert the oil into esters under any of the reaction conditions used. For Novozyme 435 lipase, the oil: alcohol molar ratio was increased to 1:4 to provide sufficient liquid to maintain a uniform suspension of the lipase using 2-butanol. However, failure to achieve quantitative conversions was not the result of a lack of excess alcohol, but rather due to increased oil: alcohol molar ratio. On the other hand, the biodiesel conversion yield of 74.42% was obtained using Novozyme 435 lipase and 2-butanol with hexane as solvent, a 1:4 oil: alcohol molar ratio, a reaction temperature of 45ºC and a reaction time of 16 h. Under the same conditions, Nelson et al. (1996) obtained a biodiesel conversion yield of 83.8% using tallow as a substrate. The difference in the biodiesel conversion yield is mainly due to the polyunsaturated fatty acids which are present in the substrate (oil). Kose et al. (2002) reported that the optimum reaction time was 7 h because the conversion yield did not change when the reaction time was increased till 24 h. In this study the optimum reaction time was 16 h.

Pinyaphong et al. (2011) reported a maximum biodiesel conversion yield of 83% obtained with a 1:4 oil: alcohol molar ratio using fish oil and 20% *Carica papaya* lipase enzyme (based on oil weight) with t-butanol as solvent at a reaction time of 18 h. Nelson et al. (1996) reported a maximum biodiesel conversion yield of 83.8% with a 1:3 oil: alcohol molar ratio using tallow and 25% *Candida antarctica* (SP 435) with hexane as solvent at a reaction time of 16 h. Watanabe et al. (1999) reported a maximum biodiesel conversion of 95% with a 1:3 oil: alcohol molar ratio using tuna oil and 4% *Candida antarctica* (Novozyme 435) lipase with stepwise addition of alcohol at a reaction time of 24 - 48 h. Chen et al. (2006) reported a maximum biodiesel conversion yield of 89% was achieved with a 1:4 oil: alcohol molar ratio using waste cooking oil with methanol and 30% *Rhizopus oryzae* lipase at a reaction time of 9 h. Martin and Otero (2008) obtained biodiesel

conversion yield of 85% with a 1:4.5 oil: alcohol molar ratio using vegetable oil and 50% Novozyme 435 lipase at a reaction time of 7 h. Du et al. (2004) obtained the biodiesel conversion yield of 92% with a 1:12 oil: alcohol molar ratio using soybean oil Novozyme 435 lipase at the reaction time of 14 h. Xu et al. (2004) reported a biodiesel conversion yield of 98% with three step addition of a 1:1 oil: alcohol molar ratio using soybean oil and 10% Novozyme 435 lipase at the reaction time of 12 h. Modi et al. (2006) reported a biodiesel conversion yields of 92.8, 91.7 and 93.4% were achieved at a 1:4 oil: alcohol molar ratio using 10% Novozyme 435 lipase at the reaction time of 8 h using three different oils crude jatropha, karanj and sunflower oils.

6.1.2. Effect of oil: alcohol molar ratio

Increasing the oil: alcohol molar ratio from 1:1 to 1:4 at the 4 h reaction time, increased the biodiesel conversion yield when using Novozyme 435 with methanol by 124.57, 110.29, 130.21 and 185.59% for the reaction temperatures of 35, 40, 45 and 50ºC, respectively. When the oil: alcohol molar ratio was further increased from 1:4 to 1:5 at the 4 h reaction time, the biodiesel conversion yield decreased by 6.06, 3.42, 2.00 and 11.27% for the reaction temperatures of 35, 40, 45 and 50ºC, respectively. Similar trends were obtained with both enzymes for all the reaction times and alcohols with and without solvent.

In this study, the highest biodiesel conversion yields of 76.66 and 70.38% were obtained using *Candida antarctica* (Novozyme 435) with 2-butanol and NS88001 with methanol using hexane as solvent at 25% enzyme concentration, a 1:4 oil: alcohol molar ratio and 16 h reaction time, respectively. Pinyaphong et al. (2011) reported a biodiesel conversion yield of 83% with a 1:4 oil: alcohol molar ratio using fish oil and 20% *Carica papaya* lipase enzyme (based on oil weight) with t-butanol as solvent. Nelson et al. (1996) reported a maximum biodiesel conversion of 83.8% with a 1:3 oil: alcohol molar ratio using tallow and 25% *Candida antarctica* (SP 435) with hexane as solvent. Watanabe et al. (1999) reported a maximum biodiesel conversion yield of 95% with a 1:3 oil: alcohol molar ratio using tuna oil and 4% *Candida antarctica* (Novozyme 435) lipase with stepwise addition of alcohol. Chen et al. (2006) reported a maximum biodiesel conversion yield of 89% with a 1:4 oil: alcohol molar ratio using waste cooking oil and 30% *Rhizopus oryzae* lipase. Martin and Otero

(2008) obtained a biodiesel conversion yield of 85% with a 1: 4.5 oil: alcohol molar ratio using vegetable oil and 50% Novozyme 435 lipase.

In this study, the results showed that the two lipases (Novozyme 435 and NS88001) differ in their catalytic activity. The reaction catalyzed by Novozyme 435 lipase was faster than that catalyzed by the experimental catalyst using both alcohols (methanol and 2-butanol). An increase in the number of moles of alcohol (with respect to the oil) from 1:1 to 1:4 at the 4 h reaction time increased the formation of esters using Novozyme 435 with methanol by 124.57, 110.29, 130.21 and 185.59% for the reaction temperatures of 35, 40, 45 and 50ºC, respectively. When the oil: alcohol molar ratio was further increased from 1:4 to 1:5 at the 4 h reaction time, the biodiesel conversion yield decreased by 6.06, 3.42, 2.00 and 11.27% for the reaction temperatures of 35, 40, 45 and 50ºC, respectively.

Based on the stoichiometeric reaction, the use of an amount of alcohol equal to the number of fatty acids residues is sufficient to complete conversion reaction. However, thermodynamic or kinetic constrains could prevent complete conversion to esters (Martin and Otero, 2008). Therefore, in order to maintain a uniform suspension of the biocatalyst, sufficient amount of alcohol is necessary. When Novozyme 435 lipase was used as catalyst with the higher oil: alcohol molar ratio of 1:5, it resulted in a lower conversion yield of oil to esters than the oil: alcohol molar ratio of 1:4. Several authors reported that an excess amount of alcohol was required in order to increase the reaction rates, to minimize the diffusion limitations and to retain the glycerol formed in the reaction solution. This can prevent the immobilized lipase from glycerol-mediated deactivation, since the glycerol blocks the catalytic pores which are liberated in the biodiesel production (Rodrigues et al., 2008; Noureddini et al., 2005; Martin and Otero, 2008).

However, an excess of alcohol to oil molar ratio leads to an increase in the polarity of the system and results in the inactivation of the biocatalyst (Rodrigues et al., 2008; Salis et al., 2005, Kose et al., 2002; Selmi and Thomas 1998). Pinyaphong et al. (2011) suggested that the decrease in the biodiesel conversion yield could be due to inactivation of the enzyme at high concentrations of alcohols. The lipase is tolerant to alcohol up to an oil: alcohol molar ratio of 1:4. Soumanou and Bornscheuer (2003) suggested that the excess methanol in the reaction system distorts the essential aqueous layer which stabilizes the immobilized lipase.

Noureddini et al. (2005) suggested that alcohol in excess of the stoichiometeric oil: alcohol molar ratio of 1:3 should be used to ensure higher reaction rates and to minimize the diffusion limitations. However, an excess alcohol may also inhibit the activity of the enzyme which leads to a decrease in its catalytic activity during the transesterification process.

Pinyaphong et al. (2011), Soumanou and Bornscheuer, (2003), Noureddini et al. (2005), Chen et al. (2006), Kumari et al. (2009), Nelson et al. (1996) found the optimum oil: alcohol molar ratio for the formation of esters to be 1:4. Pinyaphong et al. (2011) reported that the biodiesel conversion yield increased with increasing in the oil: alcohol molar ratio from 1:3 to 1:4 using fish oil and methanol. They stated that the lipase was tolerant to alcohol within this range of oil: alcohol molar ratio and it maintained its activity. Further increases in the oil: alcohol molar ratio decreased the biodiesel conversion yield due to inactivation of the enzyme at high concentrations of insoluble methanol. Chen et al. (2006) reported that the highest methyl ester yield was obtained with a 1:4 oil: alcohol molar ratio and further increases in the oil: alcohol molar ratio lead to decreases in the biodiesel conversion yield using waste cooking oil and methanol. This is due to the excess methanol in the reaction system distorting the essential aqueous layer which stabilizes the immobilized lipase (Soumanou and Bornscheuer, 2003). Noureddini et al. (2005) reported that alcohol in excess of the stoichiometeric oil: alcohol molar ratio of 1:3 was used to ensure the reaction rates were higher and to minimize the diffusion limitations. However, excess alcohol may also inhibit the activity of the enzyme which leads to a decrease in its catalytic activity during the transesterification process. Watanabe et al. (1999) reported that the biodiesel conversion yield decreased using *Candida antarctica* with tuna oil in the presence of more than 2/3 molar equivalent of alcohol (ethanol) as irreversible deactivation of lipase occurred. Xu et al. (2004) and Shimada et al. (2002) reported that the biodiesel conversion yield decreased dramatically when more than 1.5 molar equivalents of methanol were used in the reaction mixture using soybean oil. They attributed the decrease to lipase inactivation through contact with immiscible methanol which would appear as droplets in the oil.

6.1.3. Effect of Reaction Temperature

Increasing the reaction temperature from 35 to 40ºC at the 4 h reaction time, increased in the biodiesel conversion yield using Novozyme 435 and methanol increased by 24.02, 22.68,

12.59, 16.14 and 19.40% for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. When the reaction temperature was further increased from 40 to 50°C at the 4 h reaction time, the biodiesel conversion yield decreased by 36.34, 20.69, 13.30, 13.54 and 20.57% for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. Similar trends were obtained using both the enzymes (Novozyme 435 and NS88001) for all the reaction times and oil: alcohol molar ratios with and without solvent.

In this study, when the reaction temperature was increased from 35 to 40°C at the 4 h reaction time for Novozyme 435 lipase with hexane as solvent, the biodiesel conversion yield increased by 24.02, 22.68, 12.59, 16.14 and 19.40% for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. Similar trends were observed for the NS88001 lipase with and without solvent under the same reaction condition. However, increasing the reaction temperature increased the biodiesel conversion yield due to the reduction of substrate oil viscosity and enhanced mass transfer between substrate and enzyme catalyst. Antczak et al. (2009) and Kumari et al. (2009) reported that the interaction between the substrate and the surface of the enzymes, due to the hydrogen bonding and ionic interactions, depends on the reaction temperature which plays a vital role in maintaining the thermostability of lipase in the system.

When the reaction temperature was further increased from 40 to 50°C at the 4 h reaction time, the biodiesel conversion yield decreased by 36.34, 20.69, 13.30, 13.54 and 20.57% for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. A higher temperature may denature the specific structure of enzymes resulting in a decrease in the biodiesel conversion yield. Denaturation of enzyme support matrix may also cause the enzyme to leak from the outer layer of the support matrix. Kose et al. (2002) reported that increasing the reaction temperature over 50°C in a solvent free-system decreased the biodiesel conversion yield due to inhibition of enzyme activity by higher temperature. Nie et al. (2006) reported that the higher temperature increases the reaction rate faster but exceeding the optimum temperature may denature the enzyme. However, the optimum reaction temperature is dependent on other parameters involved in the reaction such as oil: alcohol molar ratio, enzyme activity and stability and type of system used.

Several researchers reported on the negative effect of higher temperature on the biodiesel yield. Pinyaphong et al. (2011) reported that the biodiesel conversion yield reached maximum (56%) when the reaction temperature was increased from 30 to 40°C and then decreased when the temperature was further decreased from 40 to 60°C using fish oil, enzyme *Carica papaya* and methanol. Kumari et al. (2009) reported that the biodiesel conversion yield reached the maximum (94%) when the reaction temperature was increased from 30 to 55°C using Jatropha oil, enzyme *Enterobacter aerogenes* and t-butanol. Chen et al (2006) reported that the biodiesel conversion yield increased and reached the maximum (87%) when the reaction temperature was increased from 30 to 40°C and then decreased when the reaction temperature was further increased from 40 to 70°C using waste cooking oil and enzyme Lipozyme RM IM. Royon et al. (2007) reported that the biodiesel conversion yield reached the maximum (97%) when the reaction temperature increased from 30 to 50°C using *Candida antarctica* lipase, cottonseed oil and methanol. Rodrigues et al. (2008) reported that a maximum biodiesel conversion yield of 53% was obtained at the reaction temperature of 35°C which then decreased with increases in reaction temperature above 35°C using soybean oil and enzyme Novozyme 435. Nie et al. (2006) reported that the biodiesel conversion yield reached a maximum of 90% at the reaction temperature of 40°C and then decreased when further increased in the reaction temperature.

6.1.4. Effect of Solvent

The highest biodiesel conversion yields of 80.24% and 74.34% were obtained by the Novozyme 435 with solvent and NS88001 lipase in solvent free-system at the reaction temperature of 40ºC and the oil: alcohol molar ratio of 1:4, respectively. Pinyaphong et al. (2011) reported a maximum biodiesel conversion yield of 83% by *Carica papaya* lipase enzyme using fish oil with 2-butanol as solvent. Mittelbach (1990) reported biodiesel conversion yields of 80 and 76% using *Pseudomonas* lipase and sunflower oil at a reaction time of 14 h and a reaction temperature of 50°C and ethanol with and without solvent, respectively. Kumari et al. (2009) reported a maximum biodiesel conversion yield of 94% using *Enterobacter aerogenes,* jatropha oil and methanol with t-butanol as solvent at a reaction temperature of 55°C and oil: alcohol molar ratio of 1:4. Royon et al. (2007) reported a biodiesel conversion yield of 97% using *Candida antarctica,* cotton seed oil and methanol

with t-butanol as solvent. Nelson et al. (1996) reported a maximum conversion of biodiesel of 83.8% using tallow and 25% *Candida antarctica* (SP 435) with hexane as solvent at 1:3 oil: alcohol molar ratio and a reaction time of 16 h. Soumanou and Bornscheuer (2003) reported a biodiesel conversion yields of 80 and 90% using immobilized Lipozyme TL IM and sunflower oil with methanol in a solvent and solvent- free systems. They stated that the solvent system significantly reduced the negative effects of methanol and glycerol in a reaction medium. Shimada et al. (2002) and Xu et al. (2003) reported that the decrease in the biodiesel conversion yield in a solvent- free system was due to the inactivation of lipase by the presence of insoluble methanol in reaction. Nie et al. (2006) reported that a maximum biodiesel conversion yield of 96% was obtained using *Candida* sp 99-125 with salad oil and n-hexane (non-polar solvent). However, the biodiesel conversion yield decreased to 40% when acetone (polar solvent) was used.

In this study, n-hexane was used as solvent. The addition of organic solvents (hexane) in the reaction medium was added to improve mutual solubility of triglycerides and alcohols which would protect enzymes from denaturation due to high alcohol concentration as reported by several authors (Nelson et al., 1996; Soumanou and Bornscheuer, 2003; Ranganathan et al., 2008; Lu et al., 2009; Fjerbaek et al., 2009; Gog et al., 2012). Mittelbach. (1990) reported that the addition of organic solvent in the reaction medium increases the reaction rates. Kaieda et al. (2001) and Antczak et al. (2009) reported that the solvents which are used in the large scale industry are volatile and dangerous to handle. They also suggested that the use of solvent-free system is more efficient than solvent system and reduce the cost of the recovery process and also the cost of distillation of solvent.

6.1.5. Effect of Alcohol Type

Methanol is the most polar alcohol widely used for the production of biodiesel (Rodrigues et al., 2008; Salis et al., 2005; Deng et al., 2005). In this study, methanol and 2-butanol were used in the transesterification process for the production of biodiesel. The biodiesel conversion yield obtained using NS88001 lipase with methanol at the reaction temperature of 35ºC and the reaction time of 4 h was 20.78, 24.85, 28.22, 30.68 and 27.09% for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, under the same reaction conditions the biodiesel conversion yield using NS88001 lipase with 2-butanol

was 17.22, 19.41, 23.13, 24.98 and 23.68% for the molar oil: alcohol ratio 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. The biodiesel conversion yield obtained using Novozyme 435 lipase with 2-butanol at the reaction time of 4 h was 18.32, 33.24, 34.99, 40.29 and 40.16% for the oil: alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, under the same reaction conditions the biodiesel conversion yield using Novozyme 435 lipase with methanol was 16.44, 31.08, 35.58, 36.92 and 34.68% for the molar oil: alcohol ratio 1:1, 1:2, 1:3, 1:4 and 1:5, respectively.

The biodiesel conversion yield was higher with methanol than 2-butanol. This is due to slow diffusion rate of the long carbon chain alcohol (Ghamguia et al., 2004). The enzyme activity and the biodiesel conversion yield using the secondary alcohol (2-butanol) were lower than the primary alcohol (methanol) when NS88001 lipase was used in the reaction system. This is similar to the results reported by Deng et al. (2005). Rodrigues et al. (2008) obtained high biodiesel conversion yield when using Novozyme 435 with methanol but the biodiesel conversion yield was reduced with an increase in carbon chain length of the alcohols (ethanol, propanol and butanol). Kose et al. (2002) reported that the methyl ester conversion decreased when increasing the carbon numbers of the primary alcohols. Nelson et al. (1996) reported a biodiesel conversion yield of 60-84% using Novozyme 435 with secondary alcohols. The biodiesel conversion yield decreased when feedstock grease reacted with methanol due to the high FFA content (> 9%) but it was effective with ethanol when FFA content was below < 22.4%. They stated that secondary alcohols are very effective in converting high concentrations of fatty acids to their corresponding esters. The conversion rate is slower when primary alcohol is used in the presence of water. Water appears to retard the conversion reaction when methanol is used but promotes formation of esters when *Candida antarctica* was used with secondary alcohols.

Salis et al. (2005) reported that the addition of branched chain alcohols into the reaction system improves the low temperature properties of the biodiesel fuel which is mainly used in the winter season. Iso et al. (2001) reported a high biodiesel conversion yield of 90% when *Pseudomonas cepacia* with 1-butanol used. Salis et al. (2005) reported that when *Pseudomonas cepacia* (PS-D) was used with 2-butanol, the initial reaction rate was slow due to the enzyme regio-specificity. Generally, the secondary alcohols react much slower than the

primary alcohols (Pinna et al., 2004; Salis et al., 2005). Several authors reported that during the bio-catalytic process, the slower reaction rate occurs when the alcohols react with acyl enzyme intermediates because of the steric hindrance (Xu et al., 2004; Salis et al., 2005). Nelson et al. (1996) reported that the *Rhizomucor meihei* and *Candida antarctica* were the best lipases to catalyze the transesterification process when using linear and branched chain alcohols. Salis et al. (2005) reported that butanol is completely miscible with the oil but methanol and ethanol are not fully miscible.

Novozyme 435 lipase is more active and has the ability to catalyze in the presence of low molecular weight alcohols (Martin and Otero, 2008; Rodrigues et al., 2008). Rodrigues et al. (2008) reported that the highest biodiesel conversion yields were achieved using Lipozyme TL-IM and Lipozyme RM-IM with the higher molecular weight alcohols (butanol) which indicated that the low molecular weight alcohols (methanol and ethanol) easily deactivate the enzyme by the substrates. Almost all triglycerides can be used as substrate in the enzymatic biodiesel production. The difference in the biodiesel conversion yield may be due to low viscosity of the substrates (oil).

The result obtained from this study further suggest a deactivating effect of low molecular weight alcohols on Novozyme 435, the effect being higher for methanolysis than for butanolysis. The differences in their behaviours could be due to partial deactivation of the biocatalyst by contact with a polar organic phase containing the alcohol and glycerol (formed as a byproduct of the reaction). This organic phase is only partially miscible with the substrate (oil) and could exist as droplets suspended in the reaction system. Shimada et al. (2002) suggested that methanol which is completely dissolved in the oil substrate mixture does not inactivate the biocatalyst. Also, as the reaction proceeds, the concentration of the methanol decreases as a consequence of the free glycerol produced in the reaction medium that serves to facilitate methanol extraction from the oil phase. At the beginning of the reaction, methanol is dissolved in the oil which results in the concentration of the substrate being almost equal to the oil. As the methanolysis reaction proceeds, glycerol is generated and forms a liquid phase which is not completely miscible with the oil. This second polar phase serves to extract methanol from the oil phase which results in a decreased concentration of the substrate leading to a decrease in the biodiesel conversion yield. Martin

and Otero (2008) and Watanabe et al. (1999) suggested that as the reaction proceeds, removal of glycerol from the substrates would increase the biodiesel conversion yield.

Gog et al. (2012) reported that methanol and ethanol are widely used to catalyze oil into biodiesel because they are easily available and are low in cost. However, these alcohols have denaturing ability of the enzyme in comparison with long chain aliphatic alcohols (Nelson et al., 1996; Shimada et al., 1999; Chen and Wu, 2003; Salis et al., 2005; Akoh et al., 2007). The degree of inactivation is inversely proportional to the carbon chain length of the alcohols (Chen and Wu, 2003). Therefore, stepwise addition of alcohol has been carried out to prevent lipase inactivation (Watanabe et al., 1999; Soumanou and Bornscheuer, 2003; Lu et al., 2007). Addition of organic solvents to the reaction system reduces the alcohol inhibition (Royon et al., 2007; Iso et al., 2001).

6.2. Enzymatic Transesterification by Combination Enzyme Catalysts

The biodiesel conversion yield using the combination of two enzymes (Novozyme 435 and NS88001) followed similar trend to those observed with individual enzymes. In this study, the maximum biodiesel conversion yield obtained from the combination of Novozyme 435 and NS88001 at 16 h reaction time with solvent system was 71.18% with methanol and 82.37% with 2-butanol. Similar trends were obtained in the solvent-free system and the maximum biodiesel conversion yields of 74.99 and 57.39% were obtained with methanol and 2-butanol, respectively.

The formation of esters from the oil increases with increasing reaction time (Freedman et al., 1984). Also, the rate of conversion of biodiesel from the fish oil was rapid during first 4 h followed by a steady increase in the conversion yield till the end of the experiment (16 h). Pinyaphong et al. (2011), Nelson et al. (1996), Watanabe et al. (1999), Iso et al. (2001), Chen et al. (2006), Martin and Otero (2008), Du et al. (2004), Xu et al. (2004), Salis et al. (2005) and Modi et al. (2006) observed similar trends from fish oil, tallow, cooking oil and vegetable oil.

Lee et al. (2006) reported that the biodiesel conversion from oil took place in a two steps using an enzyme mixture: (a) the *Candida rugosa* lipase hydrolyzed the oil to free fatty acids according to its non-specific site recognition which tends to hydrolyze the tri-glycerides, di-

glycerides and mono-glycerides without any acyl migration mechanism and (b) the *Rhizopus oryzae* lipase with 1,3 site specific esterifies the free fatty acids to methyl esters due to the combination of non regiospecific and regiospecific lipase with increasing the reaction time to 18 h. Then trends were observed in this study when using a combination of Novozyme 435 and NS88001 enzyme catalysts in the reaction. However, the maximum biodiesel conversion yield was obtained when using the combination of the two lipases at 16 h reaction time with solvent system due to their non-regiospecific nature.

6.3. Optimum Conditions

Table 6.1 shows the optimum conditions for both *Candida antarctica* (Novozyme 435) and experimental catalyst (NS88001) lipase individually and in combination. The optimum conditions for Novozyme 435 lipase, NS88001 lipase and the combination of lipases (Novozyme 435 and NS88001) with methanol and 2-butanol were 1:4 oil: alcohol molar ratio, 40ºC reaction temperature and 16 h reaction time.

The highest biodiesel conversion yield of 80.24% was obtained using Novozyme 435 and 2-butanol without solvent at 1:4 oil : alcohol molar ratio and the reaction temperature of 40ºC. Similar results were reported by Nelson et al. (1996). They stated that secondary alcohols are very effective in converting high concentrations of fatty acids to their corresponding esters. The conversion rate is slower when primary alcohol is used in the presence of water. Water appears to retard the conversion reaction when methanol is used but promotes formation of esters when *Candida antarctica* was used with secondary alcohols (Nelson et al., 1996).

The highest biodiesel conversion yield of 74.34% was obtained using NS88001 and methanol without solvent at 1:4 oil : alcohol molar ratio and the reaction temperature of 40ºC. The biodiesel conversion yield was higher with methanol than 2-butanol. This is due to slow diffusion rate of the long carbon chain alcohol (Ghamguia et al., 2004). The enzyme activity and the biodiesel conversion yield using the secondary alcohol (2-butanol) were lower than the primary alcohol (methanol) when NS88001 lipase was used in the reaction system.

Table 6.1. Optimum conditions for both enzymes individually and in combination.

Enzymes	Solvent System	Alcohol	Optimum conditions	Yield (%)
Novozyme 435	With	Methanol	1:4 oil : alcohol molar ratio, 40ºC, 16 h	65.86
		2-Butanol	1:4 oil : alcohol molar ratio, 40ºC, 16 h	76.66
	Without	Methanol	1:4 oil : alcohol molar ratio, 40ºC, 16 h	71.39
		2-Butanol	1:4 oil : alcohol molar ratio, 40ºC, 16 h	80.24
NS88001	With	Methanol	1:4 oil : alcohol molar ratio, 40ºC, 16 h	70.38
		2-Butanol	1:4 oil : alcohol molar ratio, 40ºC, 16 h	54.35
	Without	Methanol	1:4 oil : alcohol molar ratio, 40ºC, 16 h	74.34
		2-Butanol	1:4 oil : alcohol molar ratio, 40ºC, 16 h	49.29
Combination	With	Methanol	1:4 oil : alcohol molar ratio, 40ºC, 16 h	71.18
		2-Butanol	1:4 oil : alcohol molar ratio, 40ºC, 16 h	82.37
	Without	Methanol	1:4 oil : alcohol molar ratio, 40ºC, 16 h	74.99
		2-Butanol	1:4 oil : alcohol molar ratio, 40ºC, 16 h	57.39

The highest biodiesel conversion yield of 82.37% was obtained using the combination of enzymes (Novozyme 435 and NS88001) and methanol with hexane as solvent at 1:4 oil: alcohol molar ratio and the reaction temperature of 40ºC. The biodiesel conversion from oil took place in a two steps using the enzyme mixture: (a) the lipase enzyme hydrolyzed the oil to free fatty acids according to its non-specific site recognition which tends to hydrolyze the tri-glycerides, di-glycerides and mono-glycerides without any acyl migration mechanism and (b) the lipase with 1,3 site specific esterifies the free fatty acids to methyl esters due to the combination of non regiospecific and regiospecific lipase with increasing the reaction time to 16 h. Similar results were reported by Lee et al. (2006) and Xu et al. (2012). Therefore, the higher biodiesel conversion yield obtained using the combination of the two lipases was due to their non-regiospecific nature.

6.4. Glycerol

In this study, free glycerol was not obtained using Novozyme 435 and NS88001 individually and in combination using both methanol and 2-butanol with solvent and solvent system at all the oil: alcohol molar ratio, reaction temperature and reaction time. These could be due to the low alcohol concentration present in the reaction system. Theoretically, when 3 mole of alcohol reacts with 1 mole of triglycerides to give 3 mole of FAME and 1 mole of glycerol (byproduct). In the present study, 2.18 ml of oil and 8 ml n-hexane (total system = 10.18 ml) with 326 µl of alcohol (stoichiometric level with the ratio of 1:4) was used. The biodiesel conversion yield was 82.37% and no free glycerol was obtained in the solvent system. The remaining balance of 17.63% observed in this study was made of intermediates and/or bound glycerols such as monoacylglycerol (monoglycerides), diacylglycerol (diglycerides) and triacylglycerol (triglycerides). The glycerol is immiscible with oil and biodiesel and has a higher density than any other component in the liquid phase of the reaction system. The separation of glycerol is required in order to push the equilibrium towards product formation and also to reduces the catalytic activity of the enzyme by clogging the catalyst (Xu et al., 2011). However, it is difficult to identify and separate the glycerol phase on a laboratory scale using immobilized enzyme because the glycerol phase is relatively very small and colorless (Xu et al., 2011; Shimda et al., 1999).

6.5. Enzymatic Transesterification Model

Gog et al. (2012) reported that a two step mechanism (Ping-Pong Bi-Bi mechanism) is involved in the alcoholysis reaction of TAG molecule which is catalyzed by the enzyme (lipase) to produce a single ester bond in each step. In first step, the ester bond is hydrolyzed by the enzyme and releases the alcohol moiety which is followed by an esterification with the second substrate. This is the most commonly used mechanism to describe the alcoholysis of TAG molecule catalyzed by lipases (Kaieda et al., 1999; Xu, 2000; Paiva et al., 2000; Dossat et al., 2002; Gog et al., 2012).

$$E + ESs \leftrightarrow E.ESs \leftrightarrow F.Bp \leftrightarrow F + Bp \quad (6.1)$$

$$F + As \leftrightarrow F.As \leftrightarrow E.ESp \leftrightarrow E + ESp \quad (6.2)$$

Where:

E =Free enzymes

ESs= Ester substrate

F = Fatty acid

Bp = Alcohol moiety

As = Alcohol substrate

ESp= FAAE

s = Substrate

p = Product

Bommarius and Riebel- Bommarius (2000) reported that the mechanism of lipase involves the catalytic triad (Asp-His-Ser) as a charge relay system shown in Figure 6.1. The carboxylate group from aspartic acid is connected to histidine and the nitrogen from histidine is connected to alcohol group of serine. This catalytic triad is connected by hydrogen bonds. In the transesterification reaction, the first step is to make the serine alcohol a better nucleophile which is performed by histidine by pulling the proton off from the serine alcohol and forming an oxyanion. This serine oxyanion then attacks the carbonyl carbon of the substrates by forming a tetrahedral intermediate 1. The created oxyanion is stabilized by aspartate and histidine (amino acids) which is hydrogen bonded to the serine oxyanion. Then,

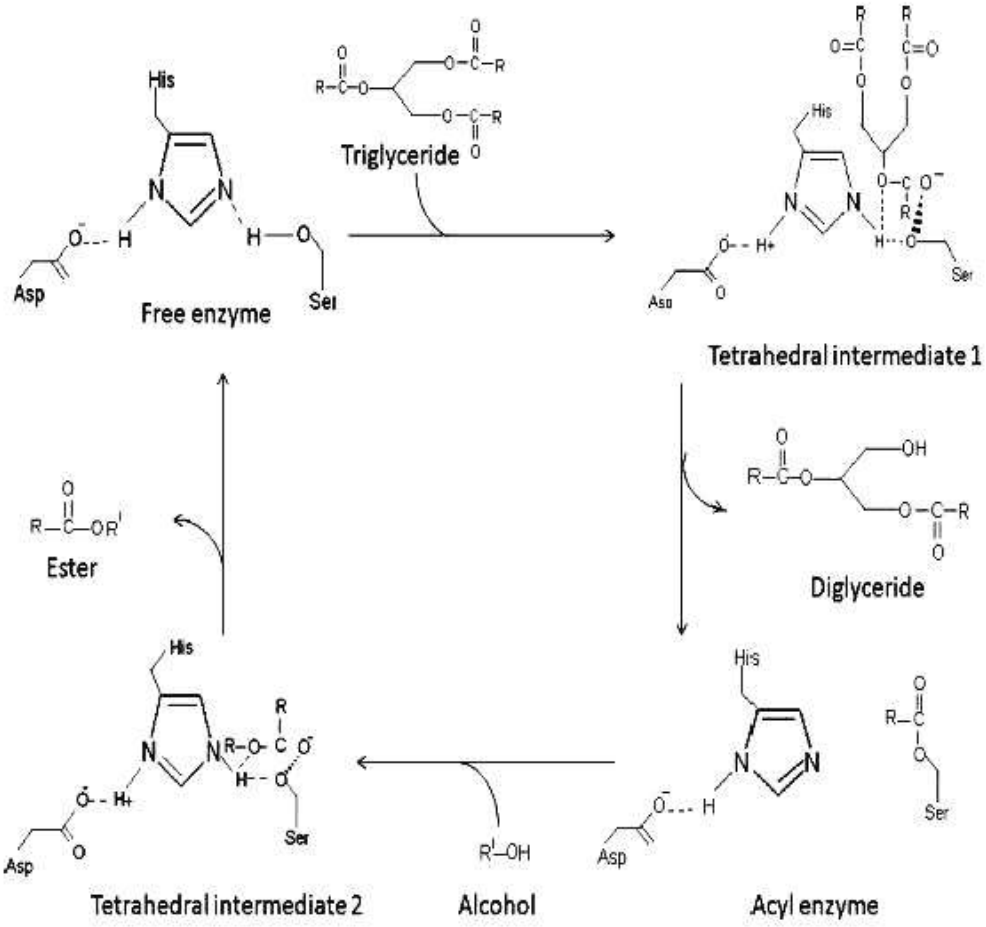


Figure 6.1. Mechanism of lipase in transesterification (Jegannathan et al., 2008).

the electrons from the oxyanion are pushed back to the carbonyl carbon, and the proton from histidine is transferred to diglyceride and then released subsequently (Al-Zuhair et al., 2007). Then, the serine ester reacts with alcohol to complete the transesterification process. The hydrogen from the alcohol molecule is removed by nitrogen from histidine and forming the alkyl oxide anion. Then the hydroxide attacks carbonyl carbon, the oxyanion intermediate is stabilized by a hydrogen bond (tetrahedral intermediate 2), the electrons are pushed back to the carbonyl carbon by forming free fatty acid. Then, oxygen from serine reclaims the hydrogen from the histidine to re-form the hydrogen bonding network. The aspartic acid serves to pull positive charge from the histidine when it is completely protonated (Jegannathan et al., 2008).

Pilarek et al. (2007) reported that the kinetic model of ping pong bi-bi mechanism has been considered to be an alcohol inhibition due to the irreversible bond cleavage in glycerides, a reversible isomerisation of monoglyceride and an irreversible deactivation of enzyme. The enzymatic alcoholysis kinetics of TAG molecule can be studied with respect to the parameters such as the enzyme type, reactants amount, and presence of solvents, mass transfer limitations, formation and conversion of intermediates and the temperature influencing the enzyme deactivation or the equilibrium limitation (Fjerbaek et al., 2009; Gog et al., 2012).

In this study, the biodiesel production took place in two steps. Initially, the substrate (fish oil) reacts with immobilized enzyme (E) which produces the fatty acids (DG and MG) and the alcohol moiety. Then, the fatty acids (DG and MG) reacted with alcohol which produces the fatty acid alkyl esters (FAAE) and Enzyme (E). In this study, the reactants and enzymes were not completely soluble in the solvent system. The system complexity increases when immobilized lipases are used with or without solvent because the reaction mixture results in a multi phase system and changes its nature during the reaction due to inhibition of operating parameters which makes the use of the model in the case inapplicable. Fjerbaek et al. (2009) reported that these equations are only applicable with homogenous phase where all reactants and enzymes are completely soluble in the solvent system.

6.6. Enzyme Reusability

The stability of enzyme Novozyme 435 with methanol in the solvent system slightly decreased after 10 cycles (from 65.86 to 63.48%) and then decreased rapidly reached zero after 30 cycles. In the solvent –free system with methanol, there was also initial slow decrease after 10 cycles (from 71.39 to 69.91%) followed by a rapid decrease in the enzyme stability after 10 cycles and the activity completely stopped after 20 cycles. Similar trend was observed by Lu et al. (2007) using stepwise addition of alcohol in the reaction mixtures.The short chain alcohols (methanol) rapidly deactivate the enzyme during repeated cycles which lead to increased cost of the catalyst in the production of biodiesel (Du et al., 2004).

On the other hand, there was small loss the stability of enzyme (Novozyme 435) in the solvent system with 2-butanol after 50 cycles. However, when it was used in the solvent – free system there was an initial slight decrease (from 80.24 to 79.52%) in the activity after 10 cycles followed a rapid decrease in the enzyme stability and the activity completely stopped after 20 cycles. Pinyaphong et al. (2011) reported similar trends for CPL lipase enzyme when used with methanol and t-butanol as solvent for 30 cycles.

The stability of enzyme NS88001 with methanol in the solvent system slightly decreased after 10 cycles (from 70.38 to 68.74%) and then decreased rapidly reached zero after 30 cycles. In the solvent –free system with methanol, there was also initial slow decrease after 10 cycles (from 74.38 to 71.81%) followed by a rapid decrease in the enzyme stability and the activity completely stopped after 20 cycles. Similar trend was observed by Lu et al. (2007) using stepwise addition of alcohol in the reaction mixtures. The short chain alcohols (methanol) rapidly deactivate the enzyme during repeated cycles which lead to increased cost of the catalyst in the production of biodiesel (Du et al., 2004).

On the other hand, the stability of enzyme (NS88001) with 2-butanol in the solvent system slightly decreased after 10 cycles (from 54.35 to 52.72%) and then decreased rapidly reached zero after 40 cycles. However, when it was used in the solvent –free system there was an initial slight decrease (from 49.29 to 48.36%) in the activity after 10 cycles followed a rapid decrease in the enzyme stability and the activity completely stopped after 20 cycles.

The stability of the combination of enzyme (Novozyme 435 and NS88001) with methanol in the solvent system slightly decreased after 10 cycles (from 71.18 to 70.23%) and then decreased rapidly reached zero after 30 cycles. In the solvent –free system with methanol, there was also initial slow decrease after 10 cycles (from 74.99 to 72.85%) followed by a rapid decrease in the enzyme stability after 10 cycles and the activity completely stopped after 20 cycles.

On the other hand, the stability of the combination enzyme (Novozyme 435 and NS88001) with 2-butanol in the solvent system slightly decreased after 20 cycles (from 82.37 to 77.23%) and then decreased rapidly after 50 cycles. However, when it was used in the solvent –free system there was an initial slight decrease (from 57.39 to 55.74%) in the activity after 10 cycles followed a rapid decrease in the enzyme stability and the activity completely stopped after 20 cycles.

Dossat et al. (1999) observed that during repeated use of lipase, the glycerol layer was formed on the surface of the enzymatic support that could cause the loss of activity by limiting substrate and product diffusion. Xu et al. (2004) reported that the inhibitory effect of glycerol could be eliminated by washing the immobilized lipase with iso-propanol to maintain its activity for 15 cycles. Xu et al. (2003) reported that the byproduct glycerol was not produced when methyl acetate was used in the reaction medium and observed that there was no loss in the lipase activity after it was reused for 10 cycles.

Rodrigues et al. (2008) stated that the decrease in the lipase activity could be due to factors such as desorption, substrate deactivation and product inhibition. They also reported that washing the lipase with non-polar solvents resulted in a greater retention of its activity by removing the substrate or product layer which is formed on the surface of the enzyme during the process.

The activity of the lipase was steady for 10 cycles because of less desorption or leaching of the bound enzyme which might be due to very tight binding of the lipase on the hydrophobic supports. The decrease in the activity of lipase after 10 cycles was due to the enzyme loss from the system because of desorption, severing of chemical bonds or erosion of the support material (Nawani et al., 2008).

CHAPTER 7. RECOMMENDATIONS

The following recommendations are made for future work

1. The effect of stirring speed on the rate of transesterification process should be studied.
2. The effect of enzyme concentration on the biodiesel conversion yield should be evaluated in solvent and solvent- free systems.
3. The recovery of alcohols and solvents should be evaluated and an economic analysis should be performed.
4. The kinetics for enzymatic transesterification with immobilized enzyme with and without solvent system should be evaluated.
5. The enzymatic transesterification process should be studied using packed bed reactors in order to evaluate the scale up parameters and to commercialize the product.

CHAPTER 8. CONCLUSIONS

The effectiveness of enzymatic transesterification of fish oil using *Candida antarctica* (Novozyme 435) and the experimental enzyme (NS88001) individually and in combination was studied. The effects of the reaction time (4, 8, 12 and 16 h), oil: alcohol molar ratio (1:1, 1:2, 1:3, 1:4 and 1:5), reaction temperature (35, 40, 45 and 50ºC), alcohol type (methanol and 2-butanol) and the solvent system (with and without hexane) on the biodiesel yield were evaluated. The stability of the enzymes (*Candida antarctica* (Novozyme 435) and the experimental enzyme (NS88001)) individually and in combination was determined. The following are the conclusions obtained from the study.

1. The effect of reaction time on the biodiesel conversion yield using *Candida antarctica* (Novozyme 435) and the experimental enzyme (NS88001) individually and in combination was highly significant at the 0.001 level.
    (a) The rate of the biodiesel conversion yield increased with increases in reaction time. Initially, the conversion of biodiesel was slow due to the initial mixing and dispersion of alcohol into the substrate. As the reaction proceeded further, the alcohol and substrate interacted with the enzyme the reaction rate increased very rapidly in the first 4 h and a maximum conversion yield of biodiesel was given after 16 h.
    (b) In the solvent system, increasing the reaction time from 4 to 16 h increased the biodiesel conversion yield by 53.59% using Novozyme 435 with methanol and by 56.19% using Novozyme 435 with 2-butanol and by 69.95% using NS88001 with methanol and by 58.04% using NS88001 with 2-butanol and by 60.06% using a combination of enzymes (Novozyme 435 and NS88001) with methanol and by 78.40% using a combination of enzymes (Novozyme 435 and NS88001) with 2-butanol, respectively.
    (c) In the solvent- free system, increasing the reaction time from 4 to 16 h increased the biodiesel conversion yield by 51.05% using Novozyme 435 with methanol and by 53.92% using Novozyme 435 with 2-butanol and by 77.16% using NS88001 with methanol and by 59.05% using NS88001 with 2-butanol and by 47.24% using a combination of enzymes (Novozyme 435 and

NS88001) with methanol and by 66.92% using a combination of enzymes (Novozyme 435 and NS88001) with 2-butanol, respectively.

2. The effect of oil: alcohol molar ratio on the biodiesel conversion yield using *Candida antarctica* (Novozyme 435) and the experimental enzyme (NS88001) individually and in combination was highly significant at the 0.001 level.
    (a) No reaction was observed at the oil: alcohol molar ratio of 1:1 for Novozyme 435 enzyme and at the oil: alcohol molar ratios of 1:1 and 1:2 for NS88001 enzyme in a solvent- free system.
    (b) The highest biodiesel conversion yield using *Candida antarctica* (Novozyme 435) and the experimental enzyme (NS88001) individually was obtained at the oil: alcohol molar ratio of 1:4.
    (c) In solvent system, increasing the oil: alcohol molar ratio from 1:1 to 1:4 increased the biodiesel conversion yield by 110.88% using Novozyme 435 with methanol and by 103.77% using Novozyme 435 with 2-butanol and by 94.74% using NS88001 with methanol and by 74.70% using NS88001 with 2-butanol.
    (d) In solvent- free system, increasing the oil: alcohol molar ratio from 1:1 to 1:4 increased the biodiesel conversion yield by 29.23% using Novozyme 435 with methanol and by 46.63% using Novozyme 435 with 2-butanol and by 17.94% using NS88001 with methanol and by 19.40% using NS88001 with 2-butanol.
    (e) In solvent system, increasing the oil: alcohol molar ratio from 1:4 to 1:5 decreased the biodiesel conversion yield by 2.94% using Novozyme 435 with methanol and by 9.36% using Novozyme 435 with 2-butanol and by 16.31% using NS88001 with methanol and by 8.51% using NS88001 with 2-butanol.
    (f) In solvent- free system, increasing the oil: alcohol molar ratio from 1:4 to 1:5 decreased the biodiesel conversion yield by 5.11% using Novozyme 435 with methanol and by 10.29% using Novozyme 435 with 2-butanol and by 2.95% using NS88001 with methanol and by 4.46% using NS88001 with 2-butanol.
    (g) At 1:4 oil: alcohol molar ratio, the highest biodiesel conversion yield was 71.18% with methanol and 82.37% with 2-butanol with solvent and 75.85%

with methanol and 57.39% with 2-butanol without solvent for the combination of enzymes (Novozyme 435 and NS88001).

3. The effect of alcohol type on the biodiesel conversion yield using *Candida antarctica* (Novozyme 435) and the experimental enzyme (NS88001) individually and in combination was highly significant at the 0.001 level.
    (a) The *Candida antarctica* (Novozyme 435) lipase showed the highest biodiesel conversion yield when using 2-butanol as alcohol.
    (b) The experimental enzyme (NS88001) lipase showed the highest biodiesel conversion yield when using methanol as alcohol.
    (c) The combination of enzymes (Novozyme 435 and NS88001) lipase showed the highest biodiesel conversion yield when using 2-butanol as alcohol.
4. The effect of reaction temperature on the biodiesel conversion yield using *Candida antarctica* (Novozyme 435) and the experimental enzyme (NS88001) individually and in combination was highly significant at the 0.001 level.
    a) The interaction between the substrate and polymer surface of the enzymes depends on the reaction temperature due to the hydrogen bonding and ionic interactions which plays a vital role in maintaining the thermostability of lipase in the system.
    b) The optimum reaction temperature was 40ºC for both the enzymes.
    c) In solvent system, increasing the reaction temperature from 35 to 40ºC increased the biodiesel conversion yield by 36.46% using Novozyme 435 with methanol and by 36.16% using Novozyme 435 with 2-butanol and by 36.05% using NS88001 with methanol and by 26.68% using NS88001 with 2-butanol.
    d) In solvent-free system, increasing the reaction temperature from 35 to 40ºC increased the biodiesel conversion yield by 40.69% using Novozyme 435 with methanol and by 35.47% using Novozyme 435 with 2-butanol and by 37.76% using NS88001 with methanol and by 32.21% using NS88001 with 2-butanol
    e) In solvent system, increasing the reaction temperature from 40 to 50ºC decreased the biodiesel conversion yield by 30.20% using Novozyme 435 with methanol and by 28.84% using Novozyme 435 with 2-butanol and by

25.49% using NS88001 with methanol and by 22.52% using NS88001 with 2-butanol.

f) In solvent-free system, increasing the reaction temperature from 40 to 50ºC decreased the biodiesel conversion yield by 39.89% using Novozyme 435 with methanol and by 30.32% using Novozyme 435 with 2-butanol and by 37.04% using NS88001 with methanol and by 27.32% using NS88001 with 2-butanol.

g) At 40ºC, the highest biodiesel conversion yield was 71.18% with methanol and 82.37% with 2-butanol with solvent and 75.85% with methanol and 57.39% with 2-butanol without solvent for the combination of enzymes (Novozyme 435 and NS88001).

5. The effect of solvent system on the of biodiesel conversion yield using *Candida antarctica* (Novozyme 435) and the experimental enzyme (NS88001) individually and in combination was highly significant at the 0.001 level.

   (a) The highest biodiesel conversion yield was obtained from Novozyme 435 lipase using solvent-free system.

   (b) The highest biodiesel conversion yield was obtained from NS88001 lipase using solvent system.

6. The activity of the enzymes was affected by the number of cycles.

   a) The stability of enzyme *Candida antarctica* (Novozyme 435) with methanol in the solvent system slightly decreased after 10 cycles and then decreased rapidly and stopped after 30 cycles. In the solvent –free system with methanol, the stability of the enzyme decreased slightly after 10 cycles which was followed by a rapid decrease and stopped after 20 cycles.

   b) The stability of enzyme *Candida antarctica* (Novozyme 435) with 2-butanol in the solvent system gradually decreased and reached 65.77% after 50 cycles. However, in the solvent –free system with 2-butanol, the stability of the enzyme decreased slightly after 10 cycles which was followed by a rapid decrease and stopped after 20 cycles.

   c) The stability of enzyme NS88001 with methanol in the solvent system slightly decreased after 10 cycles and then decreased rapidly reached zero after 30

cycles. In the solvent –free system with methanol, the stability of the enzyme decreased slightly after 10 cycles which was followed by a rapid decrease and stopped after 20 cycles.

d) The stability of enzyme NS88001 with 2-butanol in the solvent system slightly decreased after 10 cycles and then decreased rapidly reached zero after 40 cycles. However, in the solvent –free system with 2-butanol, the stability of the enzyme decreased slightly after 10 cycles which was followed by a rapid decrease and stopped after 20 cycles.

e) The stability of the combination of enzyme (Novozyme 435 and NS88001) with methanol in the solvent system slightly decreased after 10 cycles and then decreased rapidly reached zero after 30 cycles. In the solvent –free system with methanol, the stability of the enzyme decreased slightly after 10 cycles which was followed by a rapid decrease and stopped after 20 cycles.

f) The stability of the combination enzyme (Novozyme 435 and NS88001) with 2-butanol in the solvent system slightly decreased after 20 cycles and then decreased rapidly after 50 cycles. However, in the solvent –free system with 2-butanol, the stability of the enzyme decreased slightly after 10 cycles which was followed by a rapid decrease and stopped after 20 cycles.

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APPENDICES

APPENDIX A: Sample Calculation

1. Oil : Alcohol Molar Ratio (Stoichiometric calculation):

   Mol wt of fish oil= 990 g/mol

   Weight of oil = 2 grams

   Molar mass of methanol = 32 g/mol

   Molar mass of 2-butanol = 74 g/mol

   Density of oil= 0.917 g/ml

   Density of methanol = 0.790 g/ml

   Oil: alcohol molar ratio = 2 (g) /990 (g/mol) = 0.0020 (mol)

   = 0.0020 (mol)* 32 (g/mol) = 0.0646 (g)

   Volume of Methanol for 1:1= Molar mass of methanol/Density of Methanol

   = 0.0646 (g) / 0.790 (g/ml) = 0.081mL

   Therefore, volume of methanol for 1:1 oil: alcohol molar ratio is = 0.081mL

2. $\text{Conversion yield (wt \%)} = \frac{Peakarea Ax\ 100}{\sum(\text{Peak area A+Peak area B+}\cdots\text{+Peak area N})}$

   Peak area of Methyl Oleate: 3.59 e^5

   Total area: 1.19 e^6

Methyl Oleate (wt%) = $\frac{3.59\ e5}{1.19\ e6}$ x 100 = 30.16%

APPENDIX B: Data Obtained with Novozyme 435 Lipase

Table B1. Biodiesel conversion yield from fish oil using 0.5 grams of *Candida antarctica* (Novozyme 435) and methanol with hexane at different reaction temperatures and reaction times.

Time (h)	Oil: alcohol molar ratio	Reaction Temperature (ºC)															
		35				40				45				50			
		Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev
4	1:1	16.99	15.89	16.44	0.78	19.79	20.98	20.39	0.84	17.76	16.92	17.34	0.59	12.17	13.78	12.98	1.14
	1:2	30.24	31.92	31.08	1.19	37.44	38.81	38.13	0.97	35.8	37.04	36.42	0.88	29.56	30.91	30.24	0.95
	1:3	34.55	36.6	35.58	1.45	40.98	39.14	40.06	1.30	37.68	36.6	37.14	0.76	34.01	35.45	34.73	1.02
	1:4	36.19	37.64	36.92	1.03	42.28	43.47	42.88	0.84	39.17	40.67	39.92	1.06	36.21	37.92	37.07	1.21
	1:5	33.98	35.38	34.68	0.99	40.86	41.95	41.41	0.77	38.54	39.69	39.12	0.81	32.11	33.66	32.89	1.10
8	1:1	18.92	19.97	19.45	0.74	24.14	25.73	24.94	1.12	22.07	23.34	22.71	0.90	19.81	21.05	20.43	0.88
	1:2	32.41	34.04	33.23	1.15	39.04	41.12	40.08	1.47	37.7	36.23	36.97	1.04	34.14	35.66	34.90	1.07
	1:3	35.75	37.58	36.67	1.29	47.31	48.65	47.98	0.95	45.55	46.41	45.98	0.61	36.52	37.9	37.21	0.98
	1:4	39.47	40.89	40.18	1.00	50.67	52.17	51.42	1.06	49.61	50.64	50.13	0.73	38.72	39.94	39.33	0.86
	1:5	37.51	39.16	38.34	1.17	46.43	47.64	47.04	0.86	45.97	47.51	46.74	1.09	35.45	37.13	36.29	1.19
12	1:1	20.48	22.56	21.52	1.47	28.04	30.12	29.08	1.47	27.4	26.42	26.91	0.69	21.07	22.44	21.76	0.97
	1:2	36.82	38.49	37.66	1.18	44.75	46.41	45.58	1.17	41.66	40.93	41.30	0.52	35.45	36.59	36.02	0.81
	1:3	39.61	40.88	40.25	0.90	58.45	59.91	59.18	1.03	57.4	58.53	57.97	0.80	41.05	42.28	41.67	0.87
	1:4	42.27	44.11	43.19	1.30	61.84	63.02	62.43	0.83	61.64	60.39	61.02	0.88	43.4	44.72	44.06	0.93
	1:5	40.38	41.77	41.08	0.98	60.11	61.87	60.99	1.24	59.89	61.19	60.54	0.92	40.15	41.23	40.69	0.76
16	1:1	24.88	26.42	25.65	1.09	30.43	32.02	31.23	1.12	29.17	27.68	28.43	1.05	23.32	25.12	24.22	1.27
	1:2	40.43	42.21	41.32	1.26	49.98	51.42	50.70	1.02	49.16	48.02	48.59	0.81	38.84	40.35	39.60	1.07
	1:3	45.29	46.68	45.99	0.98	61.84	62.96	62.40	0.79	62.45	61.46	61.96	0.70	41.42	42.7	42.06	0.91
	1:4	47.34	49.17	48.26	1.29	65.01	66.7	65.86	1.20	63.29	61.76	62.53	1.08	45.14	46.79	45.97	1.17
	1:5	42.81	44.21	43.51	0.99	63.16	64.68	63.92	1.07	61.17	62.32	61.75	0.81	40.95	42.16	41.56	0.86

Table B2. Biodiesel conversion yield from fish oil using 0.5 grams of *Candida antarctica* (Novozyme 435) and methanol without hexane at different reaction temperatures and reaction times.

Time (h)	Oil: alcohol molar ratio	Reaction Temperature (ºC)															
		35				40								50			
		Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev
4	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	34.08	35.42	34.75	0.95	40.54	41.85	41.20	0.93	37.45	38.98	38.22	1.08	26.52	27.85	27.19	0.94
	1:3	37.45	38.62	38.04	0.83	43.86	44.95	44.41	0.77	40.86	41.72	41.29	0.61	27.94	29.97	28.96	1.44
	1:4	39.56	41.29	40.43	1.22	46.64	47.87	47.26	0.87	43.37	44.54	43.96	0.83	30.27	31.84	31.06	1.11
	1:5	35.88	36.97	36.43	0.77	44.08	45.22	44.65	0.81	41.21	42.11	41.66	0.64	23.66	24.91	24.29	0.88
8	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	36.14	37.92	37.03	1.26	43.98	45.41	44.70	1.01	41.87	43.04	42.46	0.83	28.45	29.83	29.14	0.98
	1:3	38.77	40.74	39.76	1.39	49.13	50.88	50.01	1.24	47.43	48.76	48.10	0.94	30.38	31.67	31.03	0.91
	1:4	41.58	42.86	42.22	0.91	53.45	54.75	54.10	0.92	50.91	51.94	51.43	0.73	33.51	34.88	34.20	0.97
	1:5	39.15	40.05	39.60	0.64	47.28	49.22	48.25	1.37	46.32	47.58	46.95	0.89	27.7	28.94	28.32	0.88
12	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	40.29	41.74	41.02	1.03	47.85	48.91	48.38	0.75	45.28	46.84	46.06	1.10	32.52	33.97	33.25	1.03
	1:3	43.16	44.94	44.05	1.26	62.27	63.76	63.02	1.05	59.58	60.44	60.01	0.61	34.28	35.91	35.10	1.15
	1:4	46.7	47.65	47.18	0.67	66.76	67.84	67.30	0.76	62.91	63.92	63.42	0.71	39.48	40.64	40.06	0.82
	1:5	44.44	45.54	44.99	0.78	63.09	64.49	63.79	0.99	58.11	59.08	58.60	0.69	30.66	31.89	31.28	0.87
16	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	43.28	44.43	43.86	0.81	54.66	55.82	55.24	0.82	51.87	52.9	52.39	0.73	34.77	36.83	35.80	1.46
	1:3	47.52	48.91	48.22	0.98	65.75	66.8	66.28	0.74	62.53	63.74	63.14	0.86	35.94	37.14	36.54	0.85
	1:4	50.19	51.28	50.74	0.77	70.82	71.95	71.39	0.80	65.82	67.14	66.48	0.93	42.08	43.74	42.91	1.17
	1:5	46.94	48.77	47.86	1.29	66.77	68.71	67.74	1.37	63.05	64.58	63.82	1.08	36.22	37.34	36.78	0.79

Table B3. Biodiesel conversion yield from fish oil using 0.5 grams of *Candida antarctica* (Novozyme 435) and 2-butanol with hexane at different reaction temperatures and reaction times.

Time (h)	Oil: alcohol molar ratio	Reaction Temperature (ºC)															
		35				40				45				50			
		Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev
4	1:1	17.69	18.95	18.32	0.89	22.92	24.6	23.76	1.19	20.75	19.52	20.14	0.87	17.13	18.62	17.88	1.05
	1:2	32.34	34.14	33.24	1.27	38.52	39.89	39.21	0.97	35.56	36.86	36.21	0.92	33.98	35.4	34.69	1.00
	1:3	34.19	35.78	34.99	1.12	39.64	41.19	40.42	1.10	38.51	37.29	37.90	0.86	35.63	37.18	36.41	1.10
	1:4	39.6	40.98	40.29	0.98	48.38	49.78	49.08	0.99	48.94	47.46	48.20	1.05	43.67	44.98	44.33	0.93
	1:5	40.76	39.55	40.16	0.86	41.05	42.21	41.63	0.82	41.47	42.86	42.17	0.98	39.02	40.4	39.71	0.98
8	1:1	19.42	21.21	20.32	1.27	26.12	27.62	26.87	1.06	21.23	22.66	21.95	1.01	18.12	19.58	18.85	1.03
	1:2	34.43	36.45	35.44	1.43	41.88	42.91	42.40	0.73	39.4	38.17	38.79	0.87	35.55	36.85	36.20	0.92
	1:3	40.55	41.84	41.20	0.91	48.53	50.1	49.32	1.11	45.12	46.24	45.68	0.79	39.62	41.47	40.55	1.31
	1:4	46.33	47.59	46.96	0.89	59.67	60.76	60.22	0.77	58.62	57.4	58.01	0.86	46.71	47.9	47.31	0.84
	1:5	41.62	42.48	42.05	0.61	48.22	49.4	48.81	0.83	48.03	46.98	47.51	0.74	43.6	44.88	44.24	0.91
12	1:1	22.92	24.64	23.78	1.22	30.68	32.19	31.44	1.07	27.3	25.91	26.61	0.98	20.42	21.64	21.03	0.86
	1:2	38.63	39.78	39.21	0.81	47.31	48.33	47.82	0.72	42.71	41.35	42.03	0.96	38.75	40.13	39.44	0.98
	1:3	43.44	44.86	44.15	1.00	55.95	57.14	56.55	0.84	47.4	48.6	48.00	0.85	45.2	46.72	45.96	1.07
	1:4	50.28	51.48	50.88	0.85	73.88	75.34	74.61	1.03	72.71	71.42	72.07	0.91	50.42	51.85	51.14	1.01
	1:5	44.9	46.51	45.71	1.14	62.11	63.25	62.68	0.81	58.01	59.14	58.58	0.80	46.11	47.41	46.76	0.92
16	1:1	28.01	29.37	28.69	0.96	36.71	38.52	37.62	1.28	35.6	34.19	34.90	1.00	23.83	25.65	24.74	1.29
	1:2	41.18	42.65	41.92	1.04	50.58	51.63	51.11	0.74	45.48	44.38	44.93	0.78	41.34	42.68	42.01	0.95
	1:3	49.78	51.16	50.47	0.98	60.22	61.53	60.88	0.93	56.35	55.13	55.74	0.86	52.46	54.12	53.29	1.17
	1:4	55.72	56.87	56.30	0.81	76.04	77.28	76.66	0.88	74.92	73.91	74.42	0.71	53.77	55.33	54.55	1.10
	1:5	48.61	49.82	49.22	0.86	68.83	70.12	69.48	0.91	67.86	68.8	68.33	0.66	50.14	51.42	50.78	0.91

Table B4. Biodiesel conversion yield from fish oil using 0.5 grams of *Candida antarctica* (Novozyme 435) and 2-butanol without hexane at different reaction temperatures and reaction times.

Time (h)	Oil: alcohol molar ratio	Reaction Temperature (ºC)															
		35				40				45				50			
		Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev
4	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	35.44	36.88	36.16	1.02	40.59	41.82	41.21	0.87	37.59	38.77	38.18	0.83	30.92	32.88	31.90	1.39
	1:3	37.29	38.78	38.04	1.05	43.27	44.85	44.06	1.12	42.62	43.91	43.27	0.91	32.55	33.71	33.13	0.82
	1:4	42.9	44.12	43.51	0.86	51.45	52.81	52.13	0.96	50.21	51.42	50.82	0.86	36.42	37.93	37.18	1.07
	1:5	35.05	36.65	35.85	1.13	47.33	48.51	47.92	0.83	44.8	45.91	45.36	0.78	33.76	35.65	34.71	1.34
8	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	38.55	39.64	39.10	0.77	44.7	45.82	45.26	0.79	42.65	43.89	43.27	0.88	32.58	33.84	33.21	0.89
	1:3	42.29	43.78	43.04	1.05	52.66	53.63	53.15	0.69	49.24	50.78	50.01	1.09	36.49	37.95	37.22	1.03
	1:4	48.82	49.92	49.37	0.78	61.94	63.82	62.88	1.33	60.48	61.53	61.01	0.74	42.73	43.79	43.26	0.75
	1:5	39.95	40.88	40.42	0.66	56.22	57.76	56.99	1.09	55.08	56.38	55.73	0.92	35.21	36.48	35.85	0.90
12	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	43.29	44.81	44.05	1.07	49.22	50.89	50.06	1.18	46.81	47.92	47.37	0.78	33.93	35.95	34.94	1.43
	1:3	47.37	48.99	48.18	1.15	57.92	59.83	58.88	1.35	52.54	53.77	53.16	0.87	41.76	42.84	42.30	0.76
	1:4	52.8	53.88	53.34	0.76	74.81	75.78	75.30	0.69	71.4	72.81	72.11	1.00	48.39	49.71	49.05	0.93
	1:5	46.54	47.73	47.14	0.84	68.44	69.65	69.05	0.86	63.23	64.49	63.86	0.89	36.64	37.92	37.28	0.91
16	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	46.95	47.86	47.41	0.64	53.92	55.52	54.72	1.13	52.21	53.74	52.98	1.08	35.59	36.97	36.28	0.98
	1:3	53.28	54.71	54.00	1.01	60.26	61.74	61.00	1.05	57.46	58.79	58.13	0.94	44.68	45.91	45.30	0.87
	1:4	58.57	59.88	59.23	0.93	79.55	80.92	80.24	0.97	76.85	77.88	77.37	0.73	55.18	56.64	55.91	1.03
	1:5	52.66	53.9	53.28	0.88	71.09	72.87	71.98	1.26	69.56	70.79	70.18	0.87	45.73	46.94	46.34	0.86

APPENDIX C: Data Obtained with NS88001 Lipase

Table C1. Biodiesel conversion yield from fish oil using 0.5 grams of NS88001 enzyme and methanol with hexane at different reaction temperatures and reaction times.

Time (h)	Oil: alcohol molar ratio	Reaction Temperature (ºC)															
		35				40				45				50			
		Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev
4	1:1	19.73	21.82	20.78	1.48	22.32	24.26	23.29	1.37	20.76	19.49	20.13	0.90	18.11	19.79	18.95	1.19
	1:2	24.13	25.56	24.85	1.01	30.56	31.96	31.26	0.99	30.14	28.59	29.37	1.10	25.42	27.52	26.47	1.48
	1:3	27.66	28.78	28.22	0.79	32.14	33.81	32.98	1.18	30.75	31.79	31.27	0.74	27.16	29.12	28.14	1.39
	1:4	30.24	31.12	30.68	0.62	40.75	42.06	41.41	0.93	32.68	31.36	32.02	0.93	28.23	29.64	28.94	1.00
	1:5	26.41	27.76	27.09	0.95	37.14	38.1	37.62	0.68	28.29	30.03	29.16	1.23	25.84	27.59	26.72	1.24
8	1:1	22.95	24.11	23.53	0.82	30.54	32.28	31.41	1.23	28.16	29.57	28.87	1.00	26.21	27.84	27.03	1.15
	1:2	29.62	31.02	30.32	0.99	38.46	39.64	39.05	0.83	36.44	37.89	37.17	1.03	28.95	30.77	29.86	1.29
	1:3	33.88	35.19	34.54	0.93	45.04	46.52	45.78	1.05	44.12	42.79	43.46	0.94	39.12	40.31	39.72	0.84
	1:4	35.12	36.95	36.04	1.29	54.48	55.83	55.16	0.95	53.09	51.86	52.48	0.87	43.26	45.02	44.14	1.24
	1:5	32.15	33.69	32.92	1.09	46.58	48.25	47.42	1.18	43.04	44.39	43.72	0.95	29.21	31.18	30.20	1.39
12	1:1	24.32	25.42	24.87	0.78	32.16	34.11	33.14	1.38	30.62	29.5	30.06	0.79	27.86	29.76	28.81	1.34
	1:2	32.56	34.6	33.58	1.44	40.74	41.96	41.35	0.86	40.35	38.93	39.64	1.00	35.64	36.89	36.27	0.88
	1:3	35.82	37.1	36.46	0.91	55.09	56.74	55.92	1.17	47.91	49.35	48.63	1.02	41.81	43.34	42.58	1.08
	1:4	48.27	49.42	48.85	0.81	65.31	66.56	65.94	0.88	59.35	58.27	58.81	0.76	46.59	48.28	47.44	1.20
	1:5	33.02	34.78	33.90	1.24	54.24	55.26	54.75	0.72	45.07	46.02	45.55	0.67	37.11	38.54	37.83	1.01
16	1:1	27.81	29.19	28.50	0.98	35.15	37.12	36.14	1.39	35.39	34.31	34.85	0.76	30.73	32.41	31.57	1.19
	1:2	35.11	36.68	35.90	1.11	46.89	48.64	47.77	1.24	45.78	44.26	45.02	1.07	36.68	38.14	37.41	1.03
	1:3	39.08	40.54	39.81	1.03	58.18	59.45	58.82	0.90	52.8	51.83	52.32	0.69	43.97	45.58	44.78	1.14
	1:4	51.13	52.32	51.73	0.84	69.44	71.32	70.38	1.33	59.88	58.52	59.20	0.96	51.76	53.12	52.44	0.96
	1:5	37.32	38.76	38.04	1.02	58.09	59.71	58.90	1.15	48.16	49.2	48.68	0.74	39.15	40.08	39.62	0.66

Table C2. Biodiesel conversion yield from fish oil using 0.5 grams of NS88001 enzyme and methanol without hexane at different reaction temperatures and reaction times.

Time (h)	Oil: alcohol molar ratio	Reaction Temperature (ºC)															
		35				40				45				50			
		Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev
4	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:3	28.48	29.86	29.86	0.98	36.14	37.92	37.03	1.26	33.28	34.86	34.07	1.12	26.13	27.72	26.93	1.12
	1:4	31.08	32.94	32.01	1.32	41.2	42.72	41.96	1.07	36.77	37.92	37.35	0.81	31.44	32.82	32.13	0.98
	1:5	26.11	27.24	26.68	0.80	37.98	39.84	38.91	1.32	32.81	34.53	33.67	1.22	28.22	29.92	29.07	1.20
8	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:3	36.71	37.99	37.35	0.91	47.46	48.73	48.10	0.90	45.02	46.25	45.64	0.87	31.38	32.88	32.13	1.06
	1:4	42.28	43.87	43.08	1.12	59.29	60.97	60.13	1.19	55.29	56.84	56.07	1.10	35.11	36.79	35.95	1.19
	1:5	35.45	36.51	35.98	0.75	55.8	56.94	56.37	0.81	49.53	50.92	50.23	0.98	32.05	33.98	33.02	1.36
12	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:3	38.24	39.55	38.90	0.93	59.77	61.41	60.59	1.16	52.44	53.57	53.01	0.80	37.85	39.42	38.64	1.11
	1:4	50.83	51.78	51.31	0.67	70.28	71.76	71.02	1.05	61.07	62.55	61.81	1.05	42.51	43.83	43.17	0.93
	1:5	35.74	36.98	36.36	0.88	68.31	69.89	69.10	1.12	53.31	54.72	54.02	1.00	35.14	36.78	35.96	1.16
16	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:3	43.66	44.92	44.29	0.89	62.14	63.91	63.03	1.25	55.63	56.98	56.31	0.95	39.23	40.95	40.09	1.22
	1:4	53.2	54.71	53.96	1.07	73.89	74.79	74.34	0.64	64.41	65.64	65.03	0.87	45.88	47.71	46.80	1.29
	1:5	40.58	42.69	41.64	1.49	71.38	72.89	72.14	1.07	61.77	62.9	62.34	0.80	44.94	46.83	45.89	1.34

Table C3. Biodiesel conversion yield from fish oil using 0.5 grams of NS88001 enzyme and 2-butanol with hexane at different reaction temperatures and reaction times.

Time (h)	Oil: alcohol molar ratio	Reaction Temperature (ºC)															
		35				40				45				50			
		Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev
4	1:1	16.26	18.18	17.22	1.36	23.45	24.58	24.02	0.80	23.13	21.78	22.46	0.95	18.31	20.11	19.21	1.27
	1:2	18.68	20.14	19.41	1.03	28.24	29.98	29.11	1.23	27.33	28.22	27.78	0.63	23.44	25.36	24.40	1.36
	1:3	22.54	23.71	23.13	0.83	31.66	33.23	32.45	1.11	29.78	30.81	30.30	0.73	27.55	29.38	28.47	1.29
	1:4	24.28	25.68	24.98	0.99	33.8	34.97	34.39	0.83	32	32.92	32.46	0.65	27.87	29.94	28.91	1.46
	1:5	23.05	24.31	23.68	0.89	26.13	27.34	26.74	0.86	24.66	25.52	25.09	0.61	22.72	23.89	23.31	0.83
8	1:1	19.1	20.97	20.04	1.32	25.94	28.02	26.98	1.47	24.01	25.5	24.76	1.05	21.58	23.24	22.41	1.17
	1:2	22.08	23.16	22.62	0.76	33.78	35.67	34.73	1.34	31.64	32.54	32.09	0.64	27.89	29.79	28.84	1.34
	1:3	26.91	28.19	27.55	0.91	35.87	37.51	36.69	1.16	35.13	33.78	34.46	0.95	31.51	32.91	32.21	0.99
	1:4	30.89	32.53	31.71	1.16	41.98	43.97	42.98	1.41	39.69	38.44	39.07	0.88	34.12	35.38	34.75	0.89
	1:5	26.14	27.71	26.93	1.11	40.21	42.11	41.16	1.34	33.82	34.68	34.25	0.61	32.03	33.06	32.55	0.73
12	1:1	23.78	25.89	24.84	1.49	29.96	31.78	30.87	1.29	27.76	29	28.38	0.88	25.31	26.9	26.11	1.12
	1:2	28.86	30.34	29.60	1.05	36.45	38.32	37.39	1.32	35.23	36.11	35.67	0.62	31.14	32.52	31.83	0.98
	1:3	33.97	35.78	34.88	1.28	45.36	47.11	46.24	1.24	39.43	40.47	39.95	0.74	37.31	39.08	38.20	1.25
	1:4	35.16	37.31	36.24	1.52	46.87	48.48	47.68	1.14	42.34	43.85	43.10	1.07	40.58	41.72	41.15	0.81
	1:5	33.22	34.87	34.05	1.17	42.95	44.53	43.74	1.12	37.16	38.58	37.87	1.00	34.01	35.31	34.66	0.92
16	1:1	25.35	26.52	25.94	0.83	30.28	31.94	31.11	1.17	29.93	28.77	29.35	0.82	25.37	27.22	26.30	1.31
	1:2	32.2	34.06	33.13	1.32	41.51	43.42	42.47	1.35	38.45	37.4	37.93	0.74	31.66	32.85	32.26	0.84
	1:3	37.64	38.85	38.25	0.86	49.15	50.21	49.68	0.75	44.73	43.38	44.06	0.95	39.98	41.72	40.85	1.23
	1:4	41.99	43.81	42.90	1.29	53.76	54.93	54.35	0.83	49.16	47.91	48.54	0.88	41.28	42.94	42.11	1.17
	1:5	38.12	39.29	38.71	0.83	48.8	50.64	49.72	1.30	40.5	41.8	41.15	0.92	34.98	36.77	35.88	1.27

Table C4. Biodiesel conversion yield from fish oil using 0.5 grams of NS88001 enzyme and 2-butanol without hexane at different reaction temperatures and reaction times.

Time (h)	Oil: alcohol molar ratio	Reaction Temperature (ºC)															
		35				40				45				50			
		Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev
4	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:3	20.84	21.95	21.40	0.78	28.57	29.81	29.19	0.88	26.77	27.89	27.33	0.79	18.94	20.71	19.83	1.25
	1:4	24.43	25.67	25.05	0.88	30.23	31.75	30.99	1.07	28.64	29.91	29.28	0.90	21.45	22.88	22.17	1.01
	1:5	21.76	22.91	22.34	0.81	25.05	26.43	25.74	0.98	25.41	27.07	26.24	1.17	15.89	17.54	16.72	1.17
8	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:3	23.96	25.44	24.70	1.05	32.33	33.78	33.06	1.03	30.19	31.43	30.81	0.88	23.56	24.75	24.16	0.84
	1:4	27.88	29.61	28.75	1.22	36.71	37.94	37.33	0.87	33.46	34.78	34.12	0.93	25.22	27.1	26.16	1.33
	1:5	24.22	25.77	25.00	1.10	33.16	34.65	33.91	1.05	29.3	30.94	30.12	1.16	18.48	19.91	19.20	1.01
12	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:3	27.53	28.97	28.25	1.02	37.75	38.88	38.32	0.80	35.85	37.31	36.58	1.03	27.43	29.28	28.36	1.31
	1:4	31.49	33.11	32.30	1.15	41.8	43.12	42.46	0.93	38.26	39.47	38.87	0.86	32.71	33.92	33.32	0.86
	1:5	26.75	27.82	27.29	0.76	36.23	37.67	36.95	1.02	34.6	35.98	35.29	0.98	23.87	25.52	24.70	1.17
16	1:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1:3	32.49	33.83	33.16	0.95	40.51	42.05	41.28	1.09	38.91	40.21	39.56	0.92	29.94	31.24	30.59	0.92
	1:4	36.67	37.89	37.28	0.86	48.68	49.89	49.29	0.86	45.79	47.32	46.56	1.08	35.03	36.61	35.82	1.12
	1:5	30.45	31.7	31.08	0.88	46.41	47.76	47.09	0.95	40.14	41.52	40.83	0.98	28.77	30.43	29.60	1.17

APPENDIX D: Data Obtained with the Combination of (Novozyme 435 and NS88001) Lipase

Table D1. Biodiesel conversion yield from fish oil using the combination of Novozyme 435 and NS88001 enzyme with methanol and 2-butanol in solvent and solvent free- system at different reaction times.

Time (h)	Biodiesel Conversion Yield (wt %)															
	Solvent System								Solvent-free System							
	Methanol				2-Butanol				Methanol				2-Butanol			
	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev	Trial 1	Trial 2	Avg	St. Dev
4	43.78	45.15	44.47	0.97	45.42	46.91	46.17	1.05	50.31	51.55	50.93	0.88	32.83	35.92	34.38	1.09
8	53.81	55.60	54.71	1.27	64.90	65.94	65.42	0.74	61.74	63.2	62.47	1.03	41.52	44.8	43.16	1.16
12	67.85	68.92	68.39	0.76	72.52	73.89	73.21	0.97	69.48	70.85	70.17	0.97	49.85	53.08	51.47	1.14
16	70.54	71.82	71.18	0.91	81.77	82.97	82.37	0.85	74.12	75.85	74.99	1.22	56.05	58.72	57.39	0.94