ENZYMATIC TRANSESTERIFICATION OF WASTE ANIMAL FATS FOR PRODUCTION OF BIODIESEL

by

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ABSTRACT

The process of transesterification is the exchange of the organic group R" of an ester with the organic group R'of an alcohol, often catalyzed by acid, base or enzyme. Biodiesel, a mixture of monoalkyl esters of long chain fatty acids, is produced from vegetable oils, animal fats and fish oils by transesterification in presence of alcohol. Biodiesel is a fuel which can be used in a mixture of other fuels or alone. The base catalyzed transesterification method of biodiesel production is not suitable for waste animal fat as it contains 10–15% free fatty acids which result in higher soap formation and cause extensive downstream processing. Enzyme catalyzed transesterification can overcome the problem of soap formation and multi-step purification of end products and results in a higher purity biodiesel. Lipase is the enzyme widely used in the process of enzymatic transesterification. Various lipases have been used to transesterify triglycerides with short chain alcohols to alkyl esters. The objectives of this study were to screen lipase enzymes for the transesterification process and to use the best lipase for biodiesel production from waste animal fat. Enzymatic transesterification by individual and combined enzyme catalysts (Novozyme 435 and NS88001) was first carried out to investigate the effects of reaction time (4, 8, 12 and 16 hour), oil : alcohol molar ratios (1:1, 1:2, 1:3, 1:4 and 1:5), the effects of alcohol type (methanol and 2-butanol) and reaction temperature (35, 40, 45 and 50°C) on biodiesel yield in solvent and solvent-free systems. The highest conversion yield of biodiesel (96.67%) was obtained from a combination of Novozyme and NS88001 lipase with the optimal reaction condition of oil : 2-butanol molar ratio of 1:4, enzyme concentration of 25% (12.5% w/w of each enzyme), hexane as solvent, a 45°C reaction temperature, a reaction time of 16 h and a mixing speed of 200 rpm. The reusability of lipase enzymes by individual and combination of enzyme catalysts (Novozyme 435 and NS88001) with solvent and solvent-free systems was also investigated in order to reduce the cost of the process. The lipase enzymes lost their activity after being reused for 30 cycles in solvent-free systems and after 10 cycles in solvent system.

LIST OF ABBREVIATIONS AND SYMBOLS USED

°C: Degree Celsius

h : Hours

nm : Nanometer

ml : Milliliter

µl : Microliter

BSTFA : N, O - Bis (Trimethylsilyl)-Trifluroacetamide

DPA : Methyl all-cis-7,10,13,16,19-docosapentaenoate

EPA : Methyl all-cis-5,8,11,14,17- eicosapentaenoate

FAAE : Fatty Acid Alkyl Esters

FAME : Fatty Acid Methyl Esters

FFA : Free Fatty Acids

FLF : Free Lipase form

GC : Gas Chromatography

Imm : Immobilized lipase form

PCMC : Protein-coated microcrystals

RMIM : Immobilized *Rhizomucor miehei*

RPM : Rotation per minute

TLIM : Immobilized *Thermomyces lanuginose*

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CHAPTER 1. INTRODUCTION

The high demand for fossil fuels and their limited supply has prompted the search for alternative renewable fuel sources such as biodiesel, bioethanol and biogas from biomass materials (Saka and Kusidana, 2001; Xie and Li, 2006). Biodiesel is a renewable energy source that can be used as an alternative fuel source in compression-ignition engines instead of fossil fuels (Demirbas, 2003a, Knothe *et al*., 2005). It has certain qualities over diesel such as being sulfur free, non-toxic, biodegradable and has non-carcinogenic compounds (Venkataraman, 2002). These characteristics make it more greener and eco-friendly than diesel (Bondioli *et al*., 1995; Akoh *et al*., 2007; Basha *et al*., 2009; Shafiee and Topal, 2008; Robles *et al*., 2009).

There are many raw materials that can be used as a source for the production of biodiesel including plant oils, animal fats, microbial mass and other waste materials (Akoh *et al*., 2007). The most popular plants used as a feedstock are jatropha, canola, coconut, cottonseed, groundnut, karanj, olive, palm, peanut, rapeseed, safflower, soybean and sunflower (Demirbas, 2003a; Akoh *et al*., 2007; Robles *et al*., 2009). The most popular animal sources used as a feedstock are beef tallow, chicken fat, lamb fat, lard, yellow grease, hemp oil, waste cooking oil and the greasy by-product from omega-3 fatty acid production (Demirbas, 2003a; Marchetti *et al*., 2008; Ranganathan *et al*., 2008; Antczak *et al*., 2009). The main component of fats and oils are triacylglycerols (triglycerides) which are made of different types of fatty acids with one glycerol (glycerine) being the backbone. The types of fatty acids present in the triglycerides determine the fatty acids profile. Fatty acid profiles from plants and animal sources are different and each fatty acid has its own chemical and physical properties which can be a major factor influencing the properties of biodiesel.

Transesterification is a classic chemical process used to convert the vegetable oils and animal fats to biodiesel. Usually, a short chain alcohol is used with the feedstock to convert methyl esters and glycerin. The objective is to reduce the viscosity of oil by turning it into biodiesel. Transesterification can proceed with three catalysts: acid, alkali and enzyme. With an acid catalyst, the proton is donated to the carbonyl group which makes it more reactive. A base catalyst is used to remove the proton from alcohol which makes the reactants more

reactive (Schuchardt *et al*., 1998). Both acid and alkali methods require more energy and a downstream processing step for removing the by-product glycerin. Base catalysts are widely in use by the industries to produce the biodiesel. An enzymatic catalyst cleaves the backbone of the glycerol which makes the reactants more reactive, giving the product without the need for a downstream processing step. The glycerol can be extracted easily and the energy required for the process is minimal. The aim of this study was to investigate the potential of producing biodiesel from animal rendering waste using enzyme catalysts.

CHAPTER 2. OBJECTIVES

The aim of present study was to optimize the enzymatic transesterification process for the production of biodiesel from animal rendering waste. The specific objectives were:

1. To study the effectiveness of enzymatic transesterification using the experimental lipase catalyst (NS88001) and *Candida antarctica* (Novozyme 435) individually and in combination.
2. To study the effects of four operating parameters on the biodiesel yield:
    a) Types of alcohol (Methanol and 2-Butanol).
    b) Alcohol feedstock ratio (1:1, 1:2, 1:3, 1:4 and 1:5).
    c) Reaction temperature (35, 40, 45 and 50°C).
    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 of the lipase enzyme catalysts (NS88001) and *Candida antarctica* (Novozyme 435) in the transesterification process.

CHAPTER 3. LITERATURE REVIEW

3.1. Feedstock for Biodiesel Production

Marchetti *et al*. (2008) suggests waste fats and oils, crude or refined, can be used as feedstocks for the production of biodiesel. Often fats and oils are obtained from plant derivatives, animal fats, microbial substances and waste materials (Akoh *et al*., 2007). More than 300 plant derivatives have been used for the production of biodiesel (Subramanian *et al*., 2005). Plant oils from jatropha, karanj, canola, coconut, cottonseed, groundnut, olive, palm, peanut, rapeseed, safflower, sunflower, and soybean oils are the most popular derivatives used in production of biodiesel (Demirbas, 2003b; Akoh *et al*., 2007; Robles *et al*., 2009). Animal fats like beef tallow, lard, yellow grease and fish oils are the most used sources in the production of biodiesel. Even autotrophic organisms like algae are used in the production of biodiesel (Vasudevan and Briggs, 2008). Waste materials used in production of biodiesel include hemp waste, restaurant grease and waste cooking oils (Demirbas, 2003a; Marchetti *et al*., 2008; Ranganathan *et al*., 2008; Antczak *et al*., 2009). Table 3.1. shows the physical properties of various bio-oils (Barnwal and Sharma. (2005); Karmakar *et al*., 2010). Table 3.2. describes the fatty acids composition of oils obtained from some feedstocks (Akoh *et al*., 2007; Marchetti *et al*., 2007).

The composition of oil or fat is the most important factor affecting biodiesel production. The feedstock for biodiesel production should have higher levels of unsaturated fatty acids, long chain fatty acids and oxidation stability (Robles *et al*., 2009). Higher level of oleic acid (unsaturated fatty acids with 18 carbons and a single double bond) may produce less viscous with high quality biodiesel (Knothe, 2005; Robles *et al*., 2009).

The usage of plant derived oils in the production of biodiesel has significantly impacted food, feed and oleo-chemical industries (Li *et al*., 2007; Jegannathan *et al*., 2008). Production of biodiesel has to compete with other industries like food, chemical,

Table 3.1. Physical properties of various feedstocks (Barnwal and Sharma, 2005; Karmakar *et al*., 2010).

Oils and fats	MIU (wt %)	Density (Kg/ m^3)	Kinematic viscosity at 40°C (mm^2/s)	Cetane No. (°C)	High Heating Value (MJ/Kg)	Flash point (°C)	Saponification Value	Iodine Value
Canola	0.85	911.5	34.72	37.6	39.7	246	189.80	-
Soybean	0.77	913.8	28.87	37.9	39.6	254	195.30	128-143
Sunflower	0.65	916.1	35.84	37.1	39.6	274	19.14	125-140
Palm	0.03	918	44.79	42.0	-	267	208.63	48-58
Peanut	-	902.6	39.60	41.8	39.8	271	191.50	84-100
Corn	1.67	909.5	30.75	37.6	39.5	277	183.06	103-128
Rice bran	2.73	918.5	36.68	-	-	-	201.27	90-108
Sesame	-	913.3	36.0	41.8	39.4	260	196.50	103-116
Cottonseed	-	914.8	33.50	-	39.4	234	198.50	103-115
Jatropha	0.16	940.0	33.90	-	38.65	225	200.80	82-98
Neem	2.16	918.5	50.30	-	-	-	209.66	65-80
Karanja	0.72	936.5	43.61	-	-	-	188.50	81-90
Mahua	-	960.0	24.50	-	36.0	232	190.5	58-70
Linseed	0.64	923.6	25.75	34.6	39.3	241	187.63	-
Coconut	2.74	918.0	27.26	-	-	-	267.56	7.5-10.5
Castor	0.41	955.0	251.20	37.4	37.4	-	191.08	83-86
Tobacco	-	917.5	27.70	-	-	-	191.50	125-154
Beef Tallow	0.84	-	45.34	-	-	-	198.0	-
Yellow grease	0.68	-	132.10	-	-	-	198.36	-

Table 3.2. Fatty acid profile of oils and fats used for production of biodiesel (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

livestock feed and cosmetic for the feedstock (McNeff *et al*., 2008). The demand for vegetable oils for biodiesel production may increase the demand for fertilizer which will significantly increase greenhouse emission to the atmosphere (Jegannathan *et al*., 2008).

3.2. Animal Fats and Oils

Animal fats include rendered animal fats, fish oils and milk fat (cow's milk). Cow milk fat can be processed into butter and oil is produced from butter (De Greyt and Huyghebaert, 1993). The milk from sheep and other animals has not been used to produce fats. Rendered animal fats (also called waste animal fats) are produced in high quantities by the slaughter houses around the world as a byproduct from meat production (Sonntag, 1979a). Rendered fats are used for oil production, unlike plant oils which can be used for human consumption. Fish oils are byproducts from fish processing. There are also some fish which are caught mainly for oil production (Osman *et al*., 2001). Fish such as shark, whale, salmon, haddock and mackerel are used as oil sources.

The composition of fatty acids in oils from milk fats, rendered animal fats and fish oil are different. The short chain fatty acids from milk are larger than other fats, ranging from C_4 to C_{10} (Balcao and Malcata, 1998). Animal fats have more saturated and monounsaturated fatty acids whereas fish oils consist of polyunsaturated fatty acids (Ma *et al*., 1998). The composition of fatty acids in animal fats is important for fuel generation. Fatty acids can be altered by influencing animal diet (Canakci *et al*., 1999). In poultry, the fatty acids (especially linoleic acid) can be altered by increasing the grains in their diet. Increases in peanut and corn in pig diet can produce softer lard (Yang *et al*., 2003).

3.2.1. ***Butter Fats***

Butter fats are mainly from cow's milk and can be further processed to cheese, butter or kept in milk (De Greyt and Huyghebaert, 1993). Milk has both short and medium chain fatty acids with some other low levels *trans* unsaturation level fatty acids (Poisson *et al*., 1999). The fat in milk comprises of triacylglycerols (97 - 98%), cholestrol (0.2 - 0.4%), phospholipids (0.2 - 1.0%) and a few monoacylglycerols and diacylglycerols (Balcao and Malcata, 1998).

The composition of milk fats can be varied by changing the diet of the cattle. There are more than 500 distinct fatty acids in the milk, many are relatively low in concentration and may not affect the composition (Balcao and Malcata, 1998). The saturated fatty acids C_4 - C_{18} comprise 28 - 31% and include oleic, vaccenic acid (11*t*-18:1) (O'Donnell, 1993). One of the major components in C_{16} and C_{18} make about 4 - 8% in the composition (Balcao and Malcata, 1998). The milk also contains odd - chain members, low level of iso-, anteiso-, and very low level of linoleic and linoleic acids which are not all-*cis* isomers. Evidence of oxo (keto) and lactones can also be found (O'Donell, 1993; Balcao and Malcata, 1998).

Dairy fat consumption was about 6.3 million tonnes in 2002. The major countries consuming dairy fats are European Union-15 countries (1.52 million tonnes), India (1.57 million tonnes) and the least consuming countries are Pakistan (490000 tonnes) and Central Europe (200000 tonnes). The major exports of dairy fats are from New Zealand supplying almost 90% of the butter (FAO, 2011).

3.2.2. ***Lard***

Lard can be found in the body of pigs. The fatty acids composition of lard mainly comprises of oleic acids (41.6 %), palmitic acid (23.9%) and some linoleic acid (13.2%) (Yang *et al*., 2003). Lard contains (67.3%) of palmitic acids in the *sn*-2 position of triacylglycerols (Yang *et al*., 2003). Due to its composition, the physical properties of lard allow it to easily melt when randomized. Lard contains high levels of cholesterol and has no natural antioxidants. Hence, the level of oxidation should be maintained by adding natural or synthetic antioxidants to the oil (Akoh and Moussata, 1998; Gunstone, 1999; Yang *et al*., 2003).

3.2.3. ***Tallow***

Tallow consists of fats from lamb and cattle but mainly produced from cattle. A picture of Tallow is shown in Figure 3.1. The properties and composition of crude beef tallow are shown in Table 3.3 (Sonntag, 1979a). The fatty acids composition of tallow mainly comprises of oleic acid (26 - 50%), stearic acid (6 - 40%), palmitic acid (17 -37%), myristic

Figure 3.1. Beef Tallow.

Table 3.3.Properties and composition of crude beef tallow (Sonntag, 1979a).

Characteristics	Value
Fatty acids (wt%)	
Myristic acid	2-8
Palmitic acid	24-37
Stearic acid	14-29
Oleic acid	40-50
Linoleic acid	1-5
Iodine number	35-48
Saponification number	193-202
Titre (°C)	40-46
Melting point (°C)	47-50
GS_3	15-28
GS_2U	46-52
GSU_2	20-37
GU_3	0-2

GS_3 : Glycerol from Trisaturates
GS_2U : Glycerol from Disaturates
GSU_2 : Glycerol from Diunsaturates
GU_3 : Glycerol from Triunsaturates

acid (1 - 8%), linoleic acid (0 - 5%) and other branched-chain acids (Ma *et al*., 1998). The fatty acids are mostly saturated and monounsaturated fatty acids (Canakci *et al*., 1999) and only a minimum amount of essential fatty acids can be found in the tallow. Presence of *trans* unsaturation is about (~ 5%) (Canakci *et al*., 1999). Tallow has high cholesterol level and lacks in natural antioxidants (Nelson *et al*., 1996). The world production of tallow is about 8 million tonnes (FAO, 2011).

3.2.4. ***Fish Oil***

Fish oil can be obtained as a byproduct from the fish processing industries. Fish oils contain more saturated fatty acids (myristic and palmitic acids) than other oils and fats (Haagsma *et al*., 1982). The fatty acids also consist of polyunsaturated fatty acids present in *n*-3 C_{20} and C_{22}. Osman *et al*. (2001) reported on the fatty acid composition of different fish oils and indicated that fish oil which contains *n-3* acids can be a source of energy in feed. The fish processing industry used to subject the fish oil to a partial hydrogenation process to produce margarine and spreads for commercial use. However, the consumption of these materials are now limited (Guil-Guerrero and Belarbi, 2001). Currently, the pharmaceutical industry uses the oil (without the process of partial hydrogenation) for production of more unsaturated fatty acids, vitamins and other valuable products. Cod liver oil has been used for the production of EPA and DHA which has *n-3* fatty acids (Guil-Guerrero and Belarbi, 2001). The annual production of cod liver oil is approximately 10000 tonnes (FAO, 2011). Shark liver oil is being used for the production of vitamin A and alkoxyglycerols for treatment of cancer. Table 3.4 shows the fatty acids composition of various fish oils (Haagsma *et al*., 1982; Osman *et al*., 2001; Guil-Guerrero and Belarbi, 2001).

3.3. Feedstock Properties

Clogging and solidification can occur in the biofuels at lower temperatures, due to the higher level of saturation of fatty acids (Pinto *et al*., 2005; Akoh *et al*., 2007; Demirbas, 2008). Higher levels of unsaturated fatty acids in biofuels can reduce clogging,

Table 3.4. Fatty acids composition of various oils (Haagsma *et al*., 1982; Osman *et al*., 2001; Guil-Guerrero and Belarbi, 2001).

Fatty acids	Fish oils							Vegetable oils (%)	Tallow	Lard
	Spanish mackerel (%)	Menhaden (%)	Black pomfret (%)	Hardtail Scad (%)	Indian mackerel (%)	Yellow striped scad (%)	Cod liver oil (%)			
C 14:0	1.16	5.97	2.07	1.35	1.04	1.82	4.7	-	-	-
C 16:0	2.32	6.98	4.66	3.15	3.26	2.38	11.4	11.4	29.0	15.5
C 16:1	1.16	9.64	0.52	0.90	0.16	2.34	9.1	-	-	17.3
C 17:0	0.58	0.35	0.52	0.45	0.98	0.53	-	-	-	-
C18:0	0.88	3.51	1.56	1.80	0.98	1.59	2.2	4.4	24.5	0.3
C 18:1	2.05	7.05	1.56	1.35	1.79	2.57	24.9	20.8	44.5	1.3
C 20:0	0.29	2.94	0.52	0.45	0.81	0.26	-	0.3	-	-
C 20:1	0.58	2.34	1.04	0.95	0.32	0.26	12	-	-	-
C 22:0	0.58	0.38	0.52	0.90	0.16	0.26	-	-	-	-
C 22:1	0.29	0.40	0.52	0.45	0.32	0.53	4.8	-	-	-
C 23:0	0.29	0.42	0.52	0.90	0.16	0.26	-	-	-	-
C 24:0	6.39	0.26	1.04	0.45	0.32	0.79	-	-	-	-

solidification, viscosity and results in high levels of pour and cloud point properties, meaning that biodiesel can be used in both warm and cold weather condition. Hence, the saturation and unsaturation levels of fatty acids can have a remarkable impact on biodiesel quality. If saturated fatty acids are at high levels, the cetane index and combustion temperature are high, then the pour and cloud points are low, which may lead to higher viscosity in the biodiesel. If unsaturated fatty acids levels are high, the cetane index and combustion temperature are low and may affect the quality of biodiesel (Robles *et al*., 2009). The level of oxidation stability is one of the factors in the feedstock which may affect biodiesel use in extreme conditions. Biodiesel derived from soybeans (plant source) cannot be suited for North America's climate because of oxidation instability (Vasudevan and Briggs, 2008; Marchetti *et al*., 2008). Table 3.5 shows the physical properties of methyl esters from various feedstocks (Karmakar *et al*., 2010).

Vasudevan and Briggs (2008) stated that blending with diesel (2, 20, 80% of biodiesel) can improvise the stability of biodiesel and output energy level. Fukuda *et al*. (2001) and Harding *et al*. (2007) reported that the emission of carbon dioxide and methane can be reduced by increasing the percentage of blending ratio. Fukuda *et al*. (2001) reported that by using B80 the emission of carbon dioxide has been reduced by 15.66%. Vasudevan and Briggs (2008) stated that biofuels without blending with diesel can reduce the emission of both carbon dioxide and methane to zero emission.

3.4. Structure and Nomenclature of Fatty Acids

Normally fatty acids are monocarbonic in nature and derived from aliphatic hydrocarbons. Most of the fats fall under the families of alkane- and alkene- fatty acids. The numbering of the carbon chain follows the carbonyl-end to trace functional groups. To number the double bonds beginning from the methyl-group at the tail. In addition, the prefix

Table 3.5. Physical properties of methyl esters from various feedstocks (Karmakar *et al*., 2010).

Oils and fats	Cloud point (°C)	Density (Kg/ m^3)	Kinematic viscosity at 40°C (mm^2/s)	Cetane No. (°C)	Heating Value (MJ/Kg)	Flash point (°C)	Cold filter plugging point	Oxidation stability at 110°C (h)
Linseed	-3.8	892.5	3.75	-	-	-	-8	0.2
Rapeseed	-3.3	882.0	4.43	54.40	-	-	-13	7.6
Sunflower	3.4	880.0	4.43	49.0	33.5	183	-3	0.9
Soybean	1.0	884.0	4.03	45.0	33.5	178	-4	2.1
Peanut	5.0	883.0	4.9	54.0	33.6	176	17	2.0
Palm	13.0	876.0	5.7	62.0	33.5	164	12	4.0
Rice bran	0.3	885.5	4.95	-	-	-	-3	0.4
Coconut	0.0	807.3	2.72	-	-	110	-4	35.5
Olive	-	-	4.5	57.0	-	178	-6	3.3
Castor	-13.4	899.0	15.25	-	-	-	7	1.1
Corn	-2.8	885.0	4.4	53.0	-	170	-12	2.2
Jatropha	2.7	879.5	4.8	-	39.2	135	0	2.3
Mahua	-	850.0	3.98	-	37.0	208	6	-
Neem	14.4	884.5	5.21	-	-	-	11	7.1
Sesame	-6.0	867.3	4.2	50.48	40.4	170	-10	-
Moringa oleifera	13.3	877.2	4.83	67.07	-	-	13	2.3
Tobacco	-	882.0	5.2	-	44.6	-	-	-
Tung	10.0	903.0	7.53	-	-	-	-11	0.4
Beef tallow	16.0	874.0	4.82	-	8.0	-	14	1.6
Yellow grease	6.0	882.5	4.55	-	-	-	2	5.2

"n" or "ω" is used with the number of carbon atoms. The physical properties are highly dependent on the structure of fatty acids. The structure of fatty acids can be altered by rearranging the double bond or the configuration (*cis*- and *trans*-) which can give different physical properties. Figure 3.2 shows the structure of saturated, unsaturated, monounsaturated and polyunsaturated fatty acids (Gunstone *et al*., 1999).

3.4.1. ***Saturated Unbranched Fatty Acids***

Most of the natural lipids from animal and plant sources contain 10-40% saturated fatty acids (Gurr and Harwood, 1991; Rustan and Drevon, 2005). Saturated fatty acids have straight hydrocarbon chains with an even number of carbon atoms and no double bonds. The most common saturated fatty acids from animal and plant sources have 14, 16 and 18 carbon numbers (Gurr and Harwood, 1991; Rustan and Drevon, 2005). Saturated fatty acids are filled by hydrogen on each carbon atoms. They have only one single bond attached onto one carbon atom with two hydrogen atoms on either side (Rustan and Drevon, 2005).

Unlike longer chains fatty acids, acetic acid (C_2) does not occur in natural triglycerides. The minimum level of fatty acids that belongs to triglycerides is (C_4) butyric acid which is found in butter. Other familiar fatty acids include lauric, myristic, capric, palmitic and stearic acids. The physical states of fatty acids are liquid up to C_8 (Mattson and Grundy, 1985).

3.4.2. ***Unsaturated Unbranched Fatty Acids***

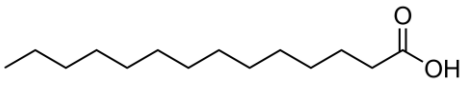
Unsaturated fatty acids have at least one double bond between carbons atoms. They can be classified into two distinct groups by geometric configurations, *cis* and *trans* configurations (Roche, 1999). *Cis* configuration double bond have two hydrogen atom attached on the same side of the carbon chain molecule. *Trans* configuration double bond

have two hydrogen atoms on either side of the carbon chain molecule, similar to saturated fatty acids (Roche, 1999; Rustan and Drevon, 2005).

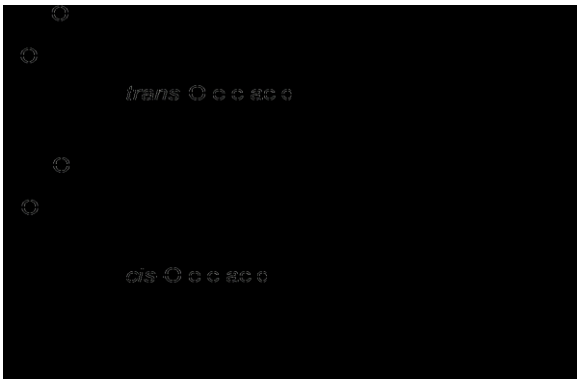
Some of the fatty acids are essential for humans as the human system cannot generate these specific unsaturated unbranched fatty acids (Watkins *et al*., 2002). The availability of such fatty acids depends on outside supplies from plants and fish (Roche, 1999). Unsaturated fatty acids are divided into two groups: (a) monounsaturated fatty acids (b) polyunsaturated fatty acids.

3.4.2.1. Monounsaturated Fatty Acids: The main characteristics of monounsaturated fatty acids are that they have one double bond with two hydrogen atoms attached on either side of the carbon atoms in the fatty acids chain and can occur on different positions of carbon atoms (Roche, 1999; Rustan and Drevon, 2005). The common monounsaturated fatty acids have a chain length of 16-22 carbon atoms with a single double bond and cis configuration (i.e. they orient in same direction). By hydrogenation, the orientation can be changed to cis and then to trans configuration (Roche, 1999). Their mobility is restricted due to the double bond in the fatty acid chain. Cis configurations are thermodynamically stable and have lower melting points than trans configurations (Roche, 1999).

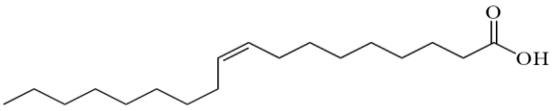
The lowest carbon number of edible fatty acids found in this classification is caproleic acid $C_{10:1}$ which is found in milk fat. Oleic acid is the only monounsaturated fatty acid in this group and is a major fatty acid in animal fats (Khosla and Hayes, 1996). However, some fatty acids have lower spectrum of monounsaturated fatty acids (Grundy, 1987). However, in most cases, the range of monoenoic acids varies from $C_{10:1}$ to $C_{18:1}$ in milk fats, from $C_{16:1}$ to $C_{24:1}$ in fish oil and up to $C_{30:1}$ in seed oils (Mattson and Grundy, 1985).



(a) Saturated unbranched fatty acids



(b) Unsaturated unbranched fatty acids



(c) Monosaturated fatty acids

(d) Polyunsaturated fatty acids

Figure 3.2. Structure of fatty acids (Gunstone *et al*., 2007).

3.4.2.2. Polyunsaturated Fatty Acids: Polyenic acids are derived from adding cis- and trans-isomers to monoenic acids. Polyunsaturated fatty acids with double bonds are isolated from edible oils and fats (SanGiovanni et al., 2000). There are some polyunsaturated fatty acids that are available in vegetable oil (Raes et al., 2004). These polyenic acids are all cis- isomers and mammals must acquire from vegetable sources as they cannot be synthesized by mammals (Ruxton et al., 2004). There are some organs (especially where fats are inside the organs) and fish oil where highly unsaturated fatty acids can be found (Mattson and Grundy, 1985). Non edible oils and fats are composed of conjugated polyunsaturated fatty acids. Linoleic and linolenic acid are polyenic fatty acids and considered to be essential fatty acids in mammal diet. The alkene fatty acid families are found to be rare in this group.

3.5. Enzyme Catalyst

Lipases are enzymes that can be extracted from animals, plants and microorganisms. Lipases can hydrolyze fats and oils (Maruyama *et al*., 2000; Sellapan and Akoh, 2005; Akoh *et al*., 2007). Most of the animal lipases are extracted from the pancreas whereas lipases from plants are extracted from oat seed, castor seed and papaya latex (Akoh *et al*., 2007). Higher yields of lipase are obtained from microbial sources than from animals and plants. The production and commercialization of microbial lipases is much easier than animals and plants (Hasan *et al*., 2006; Akoh *et al*., 2007; Antczak *et al*., 2009). Most of the lipases in the biotechnological applications and industries are from microbes.

Lipases have different physicochemical properties and specific activity (Aires-Barros *et al*., 1994; Abramic *et al*., 1999). They have been used as novel catalyst in applications such as the production of biodiesel, flavour compounds, agrochemicals and enantiospore pharmaceuticals (Jaegar and Eggert, 2002). Lipases can hydrolyze 15 triacylglycerols (Jaegar and Reetz, 1998; Salis *et al*., 2005; Joseph *et al*., 2008). Because of their stability they hydrolyze the remaining triacylglycerols in the reactions (Jegannathan *et al*., 2008). Lipases react with ester bonds of carboxylic acids to hydrolyze the fats or oil (Joseph *et al*., 2008). However, some restriction on lipase reactions occurs due to the length of the fatty acids chains. Many of the natural lipase catalysts can catalyse the fats from triacylglycerol to fatty acid alkyl esters (FAAE's) (Akoh *et al*., 2007; Joseph *et al*., 2008). They can be both

regiospecific (only act on specific bonds in the triglycerides) or non- regiospecific (act on entire bonds in the triglycerides) (Robles *et al*., 2009).

Lipases are widely used in the production of biodiesel. They can be used in differential media systems like monophasic and biphasic systems. Enzymes can be produced in bulk quantities due to their extracellular, vital and versatile nature. The advantages of using lipases for biodiesel production are: (a) they can simplify the downstream separation of biodiesel from glycerol as by product, (b) reusability and recovery of immobilized lipases is possible (c) they show more tolerance towards short chain alcohols and (d) they have high thermostability (Bacovsky *et al*., 2007; Kato *et al*., 2007; Robles *et al*., 2009). The disadvantage of using lipases as catalysts in the production of biodiesel is that the cost of enzymes is higher than the chemical catalysts (acid or alkali) and inhibition of glycerol in the reaction mixture can promote reduction in biodiesel yield due to loss of catalyst activity in the reaction (Marchetti *et al*., 2008; Robles *et al*., 2009).

3.6. Microbial Lipases

Microbial lipases are extracted from either bacteria or fungi. Due to their high yield and bulk quantities, they are used in the transesterification process. There are 38 distinguishable lipases commonly derived from bacteria sources and are used in biodiesel production (Gupta *et al*., 2004). The common lipases recommended for the production of biodiesel are *Aspergillus niger*, *Bacillus thermoleovorans*, *Burkholderia cepacia*, *Candida antarctica* (Novozyme 435), *Candida* sp 99-125, *Candida cylindracea*, *Candida rugosa*, *Chromobacterium viscosum*, *Fusarium heterosporum*, *Fusarium oxysporum*, *Getrichum candidum*, *Humicola lanuginose*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Rhizomucor miehei*, *Rhizopus oryzae*, *Thermomyces lanuginose*, *Mucor miehei*, *Oospora lactis*, *Penicillium cyclopium*, *Penicillium roqueforti*, *Pseudomonas aeruginosa, Pseudomonas putida*, *Rhizopus arrhizus*, *Rhizopus chinensis Rhizopus circinans*, *Rhizopus delemr*, *Rhizopus fusiformis*, *Rhizopus japonicus* (NR400), *Rhizopus stolonifer* (NRRL1478), *Rhodotorula rubra*, *Saccharomyces cerevisiae* and *Staphylococcus hyicus*. Tables 3.6-3.12 show some lipases used for biodiesel production. The most productive lipases used for the

Table 3.6. Enzymatic production of biodiesel using lipase *Candida* sp. 99-125.

Lipase form	Feedstock	Acyl-acceptor	Alcohol to substrate ratio	Solvent	Temperature (°C)	Other conditions	Yield (%)	Authors
Imm	Rapeseed oil	Methanol	3:1 molar ratio added in 3 days	Petroleum ether	40	36h, 180 rpm, batch stirred reactor	83	Deng *et al.* (2005); Nie *et al.* (2006); Tan *et al.* (2006)
Imm	Salad oil	Methanol	-	n-hexane	40	36h, 180 rpm, batch stirred reactor	95	Deng *et al.* (2005) Nie *et al.* (2006); Tan *et al.* (2006)
Imm	Waste oil	Methanol	-	Petroleum ether	40	36h, 180 rpm, batch stirred reactor	92	Dung *et al.* (2005) Nie *et al.* (2005); Tan *et al.* (2006)
Imm	Vegetable oil	Methanol	-	Petroleum ether	40	36h, 180 rpm, batch stirred reactor	96	Dung *et al.* (2005) Nie *et al.* (2006)

Imm: Immobilized Lipase
Rpm: Rotation per minute

Table 3.7. Enzymatic production of biodiesel using lipase *Pseudomonas cepacia*.

Lipase form	Feedstock	Acyl-acceptor	Alcohol to substrate ratio	Solvent	Temperature (°C)	Other conditions	Yield (%)	Authors
Free	Soybean oil	Methanol	3:1 molar ratio added in 3 steps	-	35	90h, 150 rpm	> 80	Kaeida *et al.* (2001);
Imm	Sunflower oil	1-butanol	3:1 molar ratio added in 4 steps	-	40	24 h	88.4	Deng *et al.* (2005)
Imm	Soybean oil	Methanol	-	None	-	-	67	Noureddini *et al.* (2005)
Imm		Ethanol	-	None	-	-	65	Noureddini *et al.* (2005)
Imm	Palm kernel oil	Ethanol		None	-	-	72	Abigor *et al.* (2000)
Imm	Palm kernel oil	T-butanol	-	None	-	-	62	Abigor *et al.* (2000)
Imm	Palm kernel oil	N-propanol	-	None	-	-	42	Abigor *et al.* (2000)
Imm	Mahua oil	Ethanol	4:1 molar ratio	-	40	6 h, 200 rpm	96	Kumari *et al.* (2006)
Imm	Jatropha oil	Ethanol	4:1 molar ratio	-	50	8 h, 200 rpm	98	Shah and Gupta *et al.* (2005)
PCMC	Mahua oil	Ethanol	4:1 molar ratio	-	40	2.5 h, 200 rpm	99	Kumari *et al.* (2005);

Imm: Immobilized Lipase
Rpm: Rotation per minute
PCMC: Protein-coated microcrystals

Table 3.8. Enzymatic production of biodiesel using lipase *Pseudomonas fluoresces*.

Lipase form	Feedstock	Acyl-acceptor	Alcohol to substrate ratio	Solvent	Temperature (°C)	Other conditions	Yield (%)	Authors
Free	Soybean oil	Methanol	3:1 molar ratio added in 3 steps	None	35	90 h, 180 rpm	90	Kaieda *et al.* (2001)
Imm	Soybean oil	Methanol	-	N-heptane	-	Use of recombiant	92	Lou *et al.* (2006)
Imm	Sunflower oil	Methanol	4.5:1 molar ratio added in 3 steps	None	40	24 h, 180 rpm	> 95	Soumanou and Bornscheuer (2003)
Imm	Sunflower oil	Iso-butanol	3:1 molar ratio added in 4 steps	-	40	24 h	45.3	Deng *et al.* (2005)

Imm: Immobilized Lipase
Rpm: Rotation per minute

Table 3.9. Enzymatic production of biodiesel using lipase *Rhizomucor miehei*.

Lipase form	Feedstock	Acyl-acceptor	Alcohol to substrate ratio	Solvent	Temperature (°C)	Other conditions	Yield (%)	Authors
Imm	Sunflower oil	Methanol	3:1 molar ratio added in 3 steps	n-hexane	40	30 h, 200 rpm	> 80	Soumanou and Bornscheuer (2003)
Imm	Sunflower oil	Ethanol	3:1 molar ratio added in 4 steps	n-hexane	40	24 h	79.1	Soumanou and Bornscheuer (2003)
Imm	Soybean oil	Methanol	-	n-hexane	-	-	92.2	Shieh *et al.* (2006)

Imm: Immobilized Lipase
Rpm: Rotation per minute

Table 3.10. Enzymatic production of biodiesel using lipase *Rhizopus oryzae*.

Lipase form	Feedstock	Acyl-acceptor	Alcohol to substrate ratio	Solvent	Temperature (°C)	Other conditions	Yield (%)	Authors
Imm Whole cell	Jatropha oil	Methanol	3:1	-	30	36 h, glutaraldehyde	80	Tamalampudi *et al.* (2005)
Imm	Soybean oil	Methanol	-	-	37	165 h, 150 rpm	71	Matsumoto *et al.* (2006)
Imm	Soybean oil	Methanol	-	None	-	-	80-90	Kaieda *et al.* (2006)
Imm	Soybean oil	Methanol	-	-	-	Stepwise addition of methanol, glutaraldehyde treatment	90	Ban *et al.* (2005)

Imm: Immobilized Lipase
Rpm: Rotation per minute

Table 3.11. Enzymatic production of biodiesel using lipase *Thermomyces lanuginose*.

Lipase form	Feedstock	Acyl-acceptor	Alcohol to substrate ratio	Solvent	Temperature (°C)	Other conditions	Yield (%)	Authors
Imm	Sunflower oil	Methanol	3:1 molar ratio added in 3 steps	n-hexane	40	30h, 200 rpm	>60	Soumanou and Bornscheuer (2003)
	Sunflower oil	1 propanol	3:1 molar ratio added in 4 steps	-	40	24h	89.8	Nie *et al.* (2006);
	Sunflower oil	2 propanol	3:1 molar ratio added in 4 steps	-	40	24h	72.8	Tan *et al.* (2006)
Imm	Rapeseed oil	Methanol	4:1	Tert-butanol	35	12h, 130 rpm	95	Deng *et al.* (2005)

Imm: Immobilized Lipase
Rpm: Rotation per minute

Table 3.12. Enzymatic production of biodiesel using lipase *Mucor miehei*.

Lipase form	Feedstock	Acyl-acceptor	Alcohol to substrate ratio	Solvent	Temperature (°C)	Other conditions	Yield (%)	Authors
Imm	Sunflower oil	Ethanol	3:1	none	30	5h	83	Selmi and Thomas (1998)
Free	Sunflower oil	Ethanol	3:1	Petroleum ether	45	5h	82	Mittelbach (1990)
Free	Tallow	Methanol	3:1	Hexane	45	8h, 200 rpm	94.8	Nelson *et al.* (1996)
Free	Rapeseed oil	Methanol	3:1	Hexane	45	5h, 200 rpm	77.3	Nelson *et al.* (1996)
		Ethanol	3:1	Hexane	45	5h, 200 rpm	98.2	Nelson *et al.* (1996)
Free	Soybean oil	Methanol	3:1	Hexane	45	5h, 200 rpm	75.4	Nelson *et al.* (1996)
		Ethanol	3:1	Hexane	45	5h, 200 rpm	97.4	Nelson *et al.* (1996)

Imm: Immobilized Lipase
Free: Free Lipase
Rpm: Rotation per minute

production of biodiesel are *Candida antarctica* (Novozyme 435), *Candida rugosa, Pseudomonas fluorescens*, *Rhizopus oryzae* (Vasudevan and Briggs, 2008)

For both methanolysis and ethanolysis, *Candida antarctica* (Novozyme 435) showed high activity in plant derived oil and animal tallow with 2-butanol as secondary alcohol. It showed lower yield with other long and short chained alcohol (Nelson *et al*., 1996). Mittelbach (1990) reported a 90% conversion yield using *Candida antarctica* (Novozyme 435) with methanol in solvent free medium and 82% conversion yield using *Candida antarctica* (Novozyme 435) as catalyst in solvent free medium. The increase in carbon length of alcohol potentially reduces the conversion yield in the reaction (Rodriguez *et al*., 2008). Salis *et al*. (2005) and Li *et al*. (2006) reported 45% conversion yield with solvent free medium but 90% conversion yield was reported when methanol and *tert*-butanol was involved as solvent in the system. Noureddini *et al*. (2005) reported 67% and 65% for methanolysis and ethanolysis in a solvent free medium using *Pseudomonas cepacia* as enzyme catalyst, respectively. Salis *et al*. (2005) reported 100% conversion yield in solvent free butanolysis. Rodriguez *et al*. (2008) reported that *Rhizomucor miehei* in butanolysis with solvent gave highest yield than short chain alcohol and butanolysis gave 99% conversion yield in solvent free system with the same enzyme catalyst. Li *et al*. (2006) reported 85% conversion yield using methanolysis in a *tert*-butanol system in the reaction and also stated that more than one lipase (combination of *Rhizopus oryzae* and *Candida rugosa*) can be used as catalyst in the medium to reduce the cost and to optimize the conversion. Li *et al*. (2006) obtained 95% conversion yield using a combination of *Candida antarctica* and *Thermomyces lanuginose*.

Lipases can be classified based on their applications into extracellular and intracellular lipases. Extracellular lipases are obtained from organisms and have been extracted and purified. Intracellular lipases are enzymes that are present within the organisms (Robles *et al*., 2009). Jegannathan *et al*. (2008) states that both extracellular and intracellular lipases (microorganisms) can be immobilized.

3.6.1. *Extracellular Lipase*

Extracellular lipases are extracted from microbes and further purified. These enzymes can be produced by solid state fermentation and liquid fermentation methods. Immediately after the fermentation step, the harvesting and purification steps follow, yielding pure lipase as biocatalysts for various applications (Balaji and Ebenezer, 2008; Barberis *et al*., 2008). The complexity of producing the enzyme is mainly due to the purification step, which determines the structure and function of the lipase (Palekar *et al*., 2000; Saxena *et al*., 2003). The cost of the purification step is relatively high (Bandman *et al*., 2000; Joseph *et al*., 2008). Major known commercialized enzymes are extracellular lipases (Robles *et al*., 2009). The Novozyme 435 is the most popular enzyme produced from *Candida antarctica*. The lipases RM IM and TL IM are produced from *Rhizomucor miehei* and *Thermomyces lanuginose*, respectively (Robles *et al*., 2009).

3.6.2. *Intracellular Lipase*

The term intracellular lipase means that the activity of the lipase can be utilized within the cell (Robles *et al*., 2009). Microbial enzymes are mainly intracellular lipases due to their cost and viability which has led to the use of whole cell. The major step to effectively reduce cost is by removing the purification process in the production of extracellular lipase. Iftikhar *et al*. (2008) stated that for production of biodiesel in bulk quantities, the intracellular enzyme can be introduced directly in the whole cell as support biomass. The immobilization step can be effective and fast for some intracellular lipases in comparison to extracellular lipases as a complex purification step is not required (Fukuda *et al*., 2001). Some intracellular lipases have been produced from the following microorganisms and used as biocatalysts: *Candida antarctica*, *Rhizopus oryzae*, and *Saccharomyces cerevisae*. (Fukuda *et al*.,2008; Robles *et al*.,2009). A comparison of the steps involved in the immobilization of extracellular and intracellular lipases is shown in Figure 3.3.

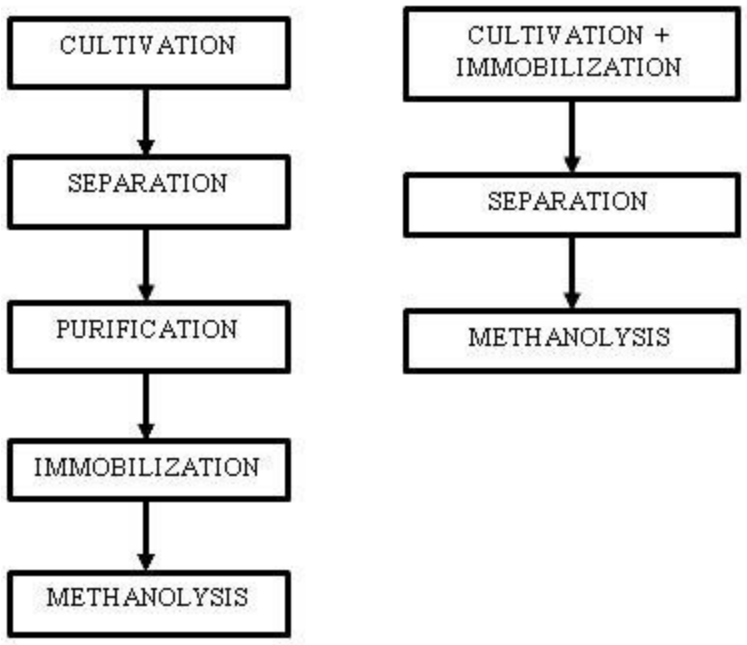


Figure 3.3. Comparison of steps involved in the immobilization (Jegannathan *et al*., 2008).

3.7. Lipase Immobilization Techniques

Immobilization of lipase means that the lipase is trapped and its movement is restricted within the space (Jegannathan *et al*., 2008). The purpose of using immobilized enzymes over mobile enzymes is to provide longer activation period and lower enantioselectivity (Klibanov, 1983; Kamori *et al*., 2000). The major benefits for the immobilization of lipases include: (a) the reusability of lipase in the reaction, (b) the separation process is minimized between enzyme and products and (c) the continuous process can be established through packed bed reactors (Peilow and Misbah, 2001). Recovering the lipase from the products gives a higher conversion yield than free lipase. Salah *et al*. (2007) reported that 25% conversion yield was obtained from immobilized lipase and 3% was obtained from free lipase. Low level of inhibition of acyl acceptor was seen with immobilized enzyme compared free lipase. The immobilized enzymes are more stable in thermal and chemical reactions and have improved mechanical property (Awang *et al*., 2007; Bhushan *et al*., 2008).

Dizge *et al*. (2009a) stated that the major cost in the production of biodiesel was the immobilized enzyme due to its carrier and support matrix material which covers about 85-90% of cost. For an efficient and cost effective process, the support matrix material and its carrier should be of low cost. Robles *et al*. (2009), Malcata *et al*. (1990) and Jegannathan *et al*. (2008) suggested that there are several factors to be considered when selecting the support materials: (a) it should be stable under both thermal and chemical reactions, (b) has good mechanical properties, (c) loading factor and (d) high resistance, all of which will depend on both hydrophobic or hydrophilic properties.

Immobilization techniques use either chemical or containment methods to immobilize the lipase within the cells. Figure 3.4 illustrates both chemical and containment techniques. Immobilization methods can be further categorized into four groups namely: adsorption, cross linking, entrapment, and encapsulation (Klibanov; 1983). Adsorption and cross linking are subjected to chemical methods while entrapment and encapsulation are subjected to physical methods (Malcata *et al*., 1990; Illanes *et al*., 2008; Jegannathan *et al*., 2008; Vaidya *et al*., 2008; Nasratun *et al*., 2009). Figure 3.5 shows various types of enzyme binding. Klibanov (1983) suggests that each method has its own complexity, activity and properties

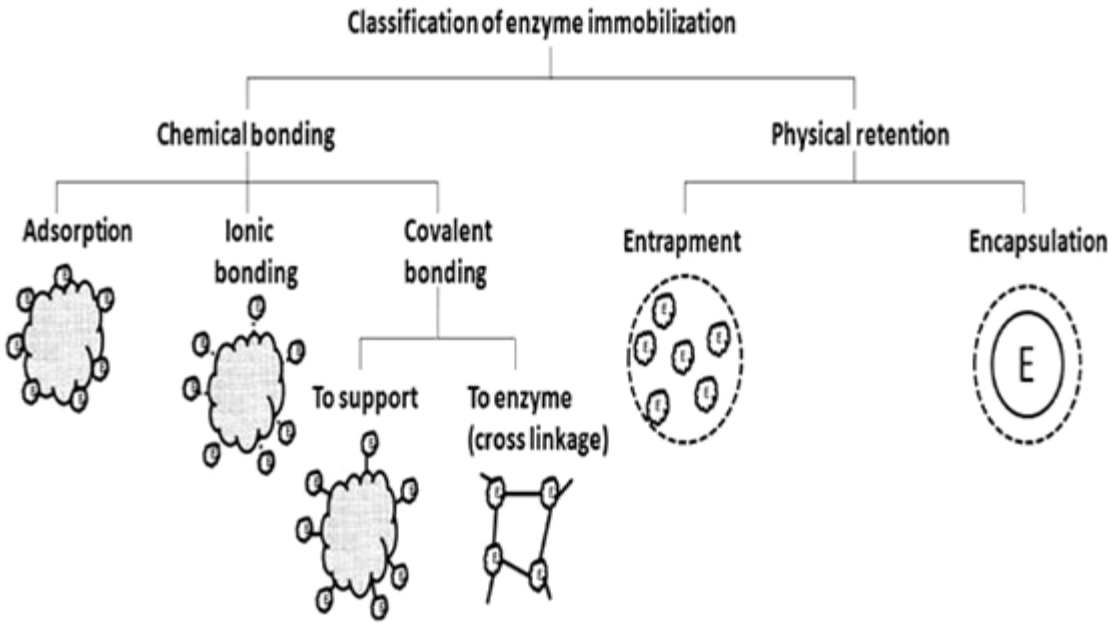


Figure 3.4. Types of Immobilization techniques (Jegannathan *et al*., 2008).

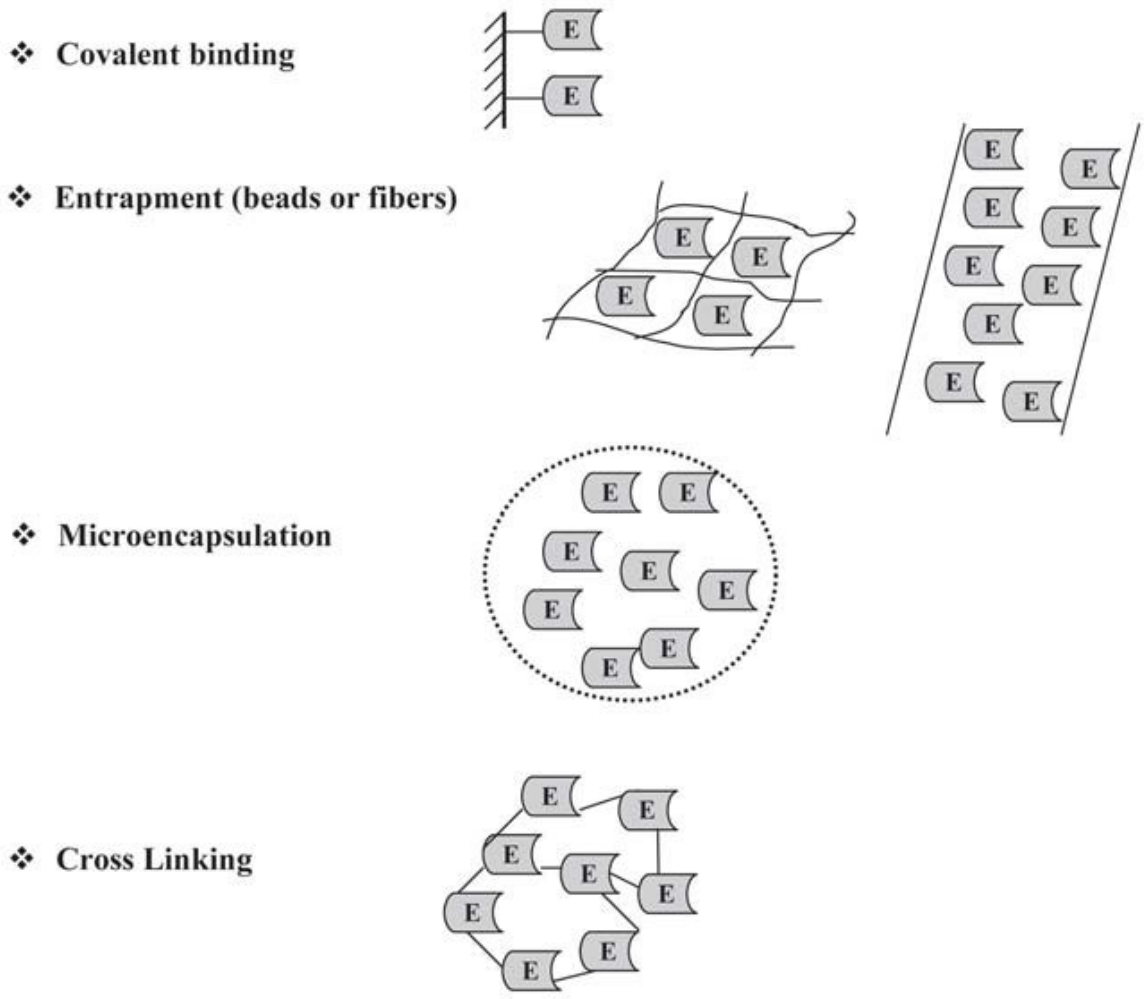


Figure 3.5. Various types of enzyme binding (a) Covalent binding (b) Entrapment (c) Microencapsulation (d) Cross-linking (Jegannathan *et al*., 2008).

(Nasratun *et al*., 2009; Malcata *et al*., 1990). However, these techniques can be used for both extracellular and intracellular lipases (Klibanov, 1983).

3.7.1. ***Adsorption***

Adsorption is a basic technique that involves electrostatic forces. The enzymes are atrracted to the surface of the support matrix material by means of the weak forces (Yong and Al-Duri, 1996; Fernandez-Lafuente *et al*., 1998). Porous support matrix materials are commonly used for large scale continuous packed bed reactors (Gao *et al*., 2006). Carriers which employ covalent bonds are preferred due to their cost effectiveness, high mass transfer when interacting with substrates, high activity and absence of chemical additives (Fukuda *et al*., 2001; Gao *et al*., 2006). The major carriers that uses covalent bonds are ceramic and porous glass, sand, cellulose and metallic oxides (Klibanov, 1983). Other major carriers include celite, silica gel, sepharose, sephadex (Malcata *et al*., 1990; Jegannathan *et al*., 2008). Salis *et al*. (2005) achieved 100% conversion yield of biodiesel using diatomaceous earth as carrier and *Pseudomonas cepacia* as enzyme catalyst. Talukder *et al*. (2006) reported that using acrylic resin as the carrier with *Candida antarctica* as enzyme catalyst gave 97% conversion yield. Table 3.13 shows biodiesel production using the immobilized lipases enzyme by various immobilization techniques (Jegannathan *et al*., 2008)

In the production of biodiesel these techniques have their limitations. Loss of interaction between support matrix material and enzyme can occur during the transesterification process when the concentration of glycerol is high (Malcata *et al*., 1990; Jegannathan *et al*., 2008). The disadvantage of this technique is that when the enzyme is absorbed in the reaction it loses its stability and the recovery and reusability of the enzymes is difficult (Jegannathan *et al*., 2008).

3.7.2. ***Cross-linking***

Cross-linking occurs when one lipase is linked with another lipase to form a strong structure using reagents such as gluteraldehyde, bisdiazobenxidine and hexamethylene diisocyanate (Malcata *et al*., 1990; Lopez-serano *et al*., 2002). The most commonly used

Table 3.13. Biodiesel production using immobilized lipase enzyme by various immobilization techniques (Jegannathan *et al*., 2008).

Immobilization method	Carrier used	Enzyme	Oil	Alcohol	Solvents	Conversion yield (%)	References
Adsorption	Toyonite-200M	*Pseudomonas fluorescens*	Sunflower	1-propanol	-	91	Iso *et al*. (2001)
Adsorption	Celite	*Pseudomonas cepacia*	Jatropha	Ethanol	Petroleum ether	98	Shah *et al*. (2006)
Adsorption	Macroporous anion exchange resin	*Mucor miehei*	Sunflower	Ethanol	t-Butanol	82	Mittlebach *et al*. (1990)
Adsorption	-	*Candida antarctica*	Cottonseed	Methanol	t-Butanol	95	Royan *et al*. (2007)
Adsorption	-	*Candida antarctica*	Rapeseed	Methanol	-	95	Li *et al*. (2006)
Adsorption	Macroporous acrylic resin	*Candida antarctica*	Jatropha	Ethyl acetate	Hexane	91.3	Mukesh *et al*. (2007)
Adsorption	Polypropylene EP 100	*Pseudomonas fluorescens*	Sunflower	Methanol	THF	91	Soumanou *et al*. (2003)
Adsorption	Acrylic resin	*Candida antarctica*	Palm	Methanol	t-Butanol	97	Talukder *et al*. (2006)
Adsorption	Silica gel	*Candida antarctica*	Soybean oil	Methanol	Ionic liquids	94	Wang *et al*. (2006)
Adsorption	Acrylic resin	*Candida antarctica*	Soybean	Methanol	-	80	Sung *et al*. (2007)
Adsorption	Celite-545	*Chromobacterium viscosum*	Jatropha	Ethanol	-	92	Shah *et al*. (2004)
Adsorption	Anion resin	*Porcine pancreatic*	Sunflower	Ethanol	-	80	Yesiloglu (2004)
Adsorption	-	*Candida antarctica*	Soybean	Methyl acetate		92	Xu *et al*. (2003)
Adsorption	Non-polar resin	*Candida* sp. 99-125	Soybean	Methanol	Hexane	98.8	Yang *et al*. (2006)

Table 3.13. Continued.

Immobilization method	Carrier used	Enzyme	Oil	Alcohol	Solvents	Conversion yield (%)	References
Adsorption	Diatomaceous earth	*Pseudomonas cepacia*	Sunflower	2-Butanol		100	Salis *et al*. (2005)
Adsorption	Acrylic resin	*Candida antarctica*	Soybean	Methyl acetate		92	Du *et al*. (2004)
Adsorption	Acrylic resin	*Candida antarctica*	Crude Jatropha	Methanol	2-Propanol	92.8	Mukesh *et al*. (2006)
Adsorption	Textile membrane	*Candida* sp. 99-125	Salad	Methanol	Hexane	96	Lu *et al*. (2006)
Adsorption	Macroporous anion resin	*Candida antarctica*	Palm kern oil	Ethanol	Supercritical CO_2	63	Oliveira *et al*. (2001)
Adsorption	Hydrotalcite	*Thermomyces langinosus*	Waste cooking oil	Methanol		92.8	Yagiz *et al*. (2007)
Crosslinking	Glutaraldehyde	*Pseudomonas cepacia*	Madhuca	Ethanol		92	Kumari *et al*. (2007)
Entrapment	Hydrophobic sol-gel	*Pseudomonas cepacia*	Soybean	Methanol		56	Noureddine *et al*. (2005)
Entrapment	Phyllosilicate sol-gel	*Pseudomonas cepacia*	Tallow and grease	Ethanol		94	Hsu *et al*. (2001)
Encapsulation	Silica aerogel	*Burkholderiacepacia*	Sunflower	Methyl acetate	Isooctane	64	Orcaire et al. (2006)

reagent for cross-linking is gluteraldehyde (Jegannathan *et al*., 2008). Cross-linking can occur both inter-molecularly or intra-molecularly which are more stable support matrix in biodiesel production (Klibanov, 1983). The disadvantage of this technique is that separation of cross-linked lipase from the product is difficult because of their small size (Jegannathan *et al*., 2008). The conversion yield of biodiesel using the cross-linking technique is about 90%. Kumari *et al*. (2007) reported a 92% conversion yield using glutaraldehyde as the carrier for *Pseudomonas cepacia* when producing biodiesel from madhuca oil.

3.7.3. ***Entrapment***

Entrapment is a technique where the lipase is entrapped inside the matrix of a polymer material such as alginate (Cheetam *et al*., 1979; Malcata *et al*., 1990; Shtelzer *et al*., 1992; Illanes *et al*., 2008). Immobilization through entrapment gives more activity and stability than the adsorption technique (Malcata *et al*., 1990). The polymers used in entrapment are either non-covalent or covalent. The major gels used in these techniques are calcium alginate, kappacarrageenan and methylenebisacylamide (Klibanov, 1983). The technique is not complex, has strong structure and the recovery and reusability are better compared to adsorption (Meter *et al*., 2007) but mass transfer has some limitation (Malcata *et al*., 1990). Jegannathan *et al*. (2008) reported 65% of conversion yield with entrapped lipase which is comparatively lower than both adsorption and crosslinking because of the mass transfer limitation. Hsu *et al*. (2004) achieved 94% conversion yield using phyllosilicate sol-gel as the carrier and *Pseudomonas cepacia* as the enzyme catalyst. Noureddini *et al*. (2005) reported that using hydrophobic sol-gel as the carrier with *Pseudomonas cepacia* as the enzyme catalyst gave 56% conversion yield.

3.7.4. ***Encapsulation***

Encapsulation is similar to the entrapment technique. However, the difference is the confinement of the lipase within porous materials like capsules or beads (Malcata *et al*., 1990). This technique can be used favorably for microencapsulating the enzyme (Serralheiro *et al*., 1990; Vicente *et al*., 1994). The separation process of enzyme from the biodiesel is simple because the structure of encapsulation is strong and the lipase cannot flow out of the

capsule material which also enhances mass transfer (Khan and Vulfson, 2001). Malcata *et al*. (1990) reported a low conversion yield due to the membrane permeability limitation and found lipase activity to be limited when reacting with larger molecules like triglycerides. The formation of a film layer can be seen while using this type of immobilized enzyme which can reduce mass transfer and the yield of the biodiesel (Antczak *et al*., 2009; Fjerbaek *et al*., 2009). Orcaire *et al*. (2006) reported that the most common carrier used for biodiesel production is silica aerogel and observed a 64% conversion yield using *Burkholderiacepacia* as the enzyme catalyst (Jegannathan *et al*., 2008).

3.8. Properties of Lipase

3.8.1. ***Specificity***

The regioselectivity for specific positions on the triglyceride molecule is used to determine the lipase specificity. Classification of lipases can also be based on selectivity of regioselectivity (acyl position) on the glycerol backbone (Chandler, 2001). Three types of lipase have been identified non-specific, 1, 3 specific and 2 specific (Koskinen and Klibanov, 1996; Rahman *et al*., 2005). Non-specific regioselective lipase does not have any specificity towards the ester bonds of the triglyceride molecules, 1, 3 specific regio-selective lipase reacts with the highest position of ester bonds in the triglyceride molecule but neglects all middle ester bonds and 2 specific regio-selective lipase reacts only with middle ester bonds but neglects all higher position ester bonds on the triglyceride molecules (Macrae, 1983). The most common non-specific lipases are from *Candida antarctica*, *Candida cylindracea*, *Candida rugosa*, and *Pseudomonas cepacia*. There are only a few 2 specific lipases mentioned in literature such as *Geotricum candidum* which is not used in transesterification. Major lipases which have 1, 3 specificity are from *Rhizopus oryzae*, *Thermomyces lanuginosus*, *Aspergillus niger*, and *Rhizomucor miehei* (Shimada *et al*., 1997; Fukuda *et al*., 2001; Lanser *et al*., 2002; Robles *et al*., 2009).

The specificity was initially believed not to have such an influence on the conversion yield of biodiesel because these lipases do not react with all the ester bonds of the triglycerides. Antczak *et al* (2009) showed that the specificity is vital for the reaction and the yield can increase from 66% to more than 90 %. Fukuda *et al* (2001) stated that because acyl

migration was spontaneous, the conversion yield was higher than expected. Studies in thin layer chromatography showed that the 2 specific lipases migrate either to 1 or 3 specific positions on partial triglycerides in aqueous phase (Fukuda *et al*., 2009). Addition of silica gel in the reaction mixture and the use of immobilization support (which is polar) can increase the acyl migration and the productivity of the reaction (Akoh *et al*., 2007; Robles *et al*., 2009).

Ergan *et al*. (2006) reported that *Candida rugosa* (which is a non-specific lipase catalyst) gave 70% conversion yield from high-erucic acid rapeseed oil. Lipase catalyst that are 1,3 specific like those from *Rhizopus arrhizus* and *Mucor miehei* gave 100% conversion yield from high-erucic acid rapeseed oil in the absence of organic solvents in the system (Ergan *et al.,*2006). Linfield (1984) reported that a non-specific *Candida cylindracea* lipase catalyst transesterifed fats completely within 12-16 hours. Lyberg and Adlercreutz (2008) reported that *Pseudomonas fluorescens* and *Pseudomonas cepacia* catalyzed eicosapentaenoic acid (EPA) completely and *Candida rugosa* catalyzed docosahexaenoic acid (DHA) completely due to regiospecificity.

3.8.2.***Stability***

In the production of biodiesel, stability of the enzymes is vital as the enzyme should maintain its activity throughout the transesterification process (Moreira *et al*., 2007; Zheng *et al*., 2009). Lipases cannot sustain their activity at the same level due to factors such as high temperature, impurities and surface properties of the reactors which may inhibit or deactivate the enzyme. However, enzymes can better sustain their activity *in-vivo*, when they are in their natural environment (Klibanov, 1983). Enzymes can be deactivated or destabilized due to mechanical forces, short chain alcohols, glycerol and water content (Malcata *et al*., 1990; Marchetti *et al*., 2007; Robles *et al*., 2009). Inhibition due to alcohols and thermal degradation may also cause destabilization of the enzymes in long term (Torres *et al* 2008). To increase the stability of an enzyme, the enzyme can be modified with respect to its genetic and chemical and physical properties (Malcata *et al*., 1990; Reetz, 2002; Mateo *et al*., 2007; Illanes *et al*., 2008).

Li *et al*. (2006) reported that the conversion yield of methyl esters had no loss of activity after 200 cycles with no interference of glycerol in the reaction for Lipozyme TL IM and Novozyme 435 catalyst in the solvent system. In a solvent-free system, the stability of lipase was drastically reduced due to glycerol adsorption on the surface of the immobilized lipase catalyst. Shimada *et al*. (2002) reported that stepwise addition of alcohol can reduce the inhibition effect of alcohol and maintain the stability of lipase for a longer time. Du *et al*. (2004) reported that the stability of lipase catalyst could be reduced due to the inhibition effect of methanol and high production of glycerol in the reaction. Noureddini *et al*. (2005) reported that the stability of lipase catalyst *Pseudomonas cepacia* on sol-gel support as immobilized carriers with soybean oil was stable for 72 hours at 35°C. Reetz *et al*. (2006) reported that both hydrogen bonding and ionic interactions were responsible for the thermal stability of immobilized lipase catalyst. Shah *et al*. (2007) reported that at a pH of 7, *Pseudomonas cepacia* on celite as the immobilized carrier had more stability in both aqueous and non-aqueous media in the reaction. Yagiz *et* al. (2007) reported that loss of lipase stability was observed at a pH of 8.5 after 7 cycles using immobilized lipase TL IM on hydrotalcite as the immobilized carrier. Shah *et al*. (2007) stated that inactivation of the enzyme catalyst can occur due to higher pH than 7.

3.8.3. ***Recovery and Reuse***

Recovery and reusability are the main considerations when using immobilized lipases in the transesterification process because their cost is relatively high (90% of the overall cost of the process). To be cost effective, the lipase should be reused many times without losing its stability and catalytic activity. Immobilization of the enzyme gives it the ability to maintain its activity and stability under adverse environmental conditions. Fernandez-Lafuente *et al*. (1998), Bhushan *et al*. (2009) and Gao *et al*. (2006) suggested that the separation of immobilized lipase could occur in packed bed reactors without a filtration process which would be more economical. However, the strength, activity and reusability of immobilized lipase depend on the type of immobilization technique and support matrix material (Fukuda *et al*., 2009; Robles *et al*., 2009). Lee *et al*. (2002) reported that the addition of methanol in a stepwise manner reduced the inhibition of lipase and achieved 85% conversion yield after 8 cycles. The stability of the enzyme could be maintained using solvents to reduce the

inhibiting factors of short chain alcohols. Fukuda *et al*. (2008) reported that pretreatment of gluteraldehyde gave more stability and longevity for several recycles and about 70% yield was obtained compared to the expected conversion yield of about 50%.

The washing of enzymes between reactions can maintain higher conversion yield for many cycles. Li *et al*. (2007) displayed no loss of fatty acid methyl esters (FAME) yield after 200 cycles by washing immobilized lipase with *tert*-butanol between each reaction. Huang *et al*. (2010) also showed that the immobilized lipase can be used many times without losing its activity and stability. Lee *et al*. (2008) stated that the use of isopropanol as the regenerating solvent for the immobilized lipase, gave more than 80% conversion yield after 5 cycles. Salah *et al*. (2007) reported that the use of hexane as the regenerating agent maintains the activity of lipase for three cycles.

3.9. Biodiesel Production

3.9.1. *Direct use and Blending*

Bartholomew (1981) suggested that vegetable oil could be used as an alternative fuel source to provide energy. In 1980, Caterpillar, a company that manufactures heavy machinery, investigated the use of vegetable oil in their plant in Brazil. They used 100% vegetable oil without any petroleum in the pre-combustion chamber. Using 100% vegetable oil as a source for energy was not realistic and vegetable oil was blended with diesel fuel in the ratio of 1:4 (Ma and Hanna, 1999). Ziejewski *et al*. (1986) reported that sunflower oil was blended with diesel fuel in the ratio of 1:4. Schlautman *et al*. (1986) reported that soybean oil was blended with diesel fuel in the ratio of 1:4 which was used for a longer run in diesel engine. The advantages of using vegetable oil as a fuel include: portability, availability and renewability. The disadvantages include: high viscosity, low volatility, reactive unsaturated hydrocarbons chains, deterioration of vegetable oil and insufficient combustion (Pryde, 1983; Ma and Hanna, 1999). Vegetable oil can cause a problem for direct injection engines after prolonged use in a longer run. Ma and Hanna (1999) stated that using vegetable oil (direct or blends with petroleum) in diesel engines would not be practical because of the gumming of oil during storage, high FFA's, thickening of oil, deposits of carbon and acid composition.

3.9.2. *Microemulsions*

A microemulsion is defined as optically isotropic fluid microstructure dispersion in a colloidal equilibrium state, commonly with dimension of 1-150 nm, formed instantaneously between two immiscible liquids such as oil and water (Schwab *et al*., 1987). Microemulsions can develop spray characteristics by vaporization in the micelles which have low boiling point (Pryde, 1984). Both ionic and non-ionic microemulsions of ethanol in soybean oil were considered good grade 2 diesels with low cetane number and high energy content (Goering *et al*., 1982b). Using butanol, hexanol, and octanol in a microemulsion resulted in a maximum visocity grade 2 diesels. In methanol micellar solubilization, 2-octanol was an effective amphiphile for both triolein and soybean oil. The use of methanol was preferred over ethanol for economical purposes (Schwab *et al*., 1987; Ma and Hanna, 1999).

Ziejewski *et al*. (1984) reported that a winterized emulsion volume of 53% alkali-refined sunflower oil and 33.4% 1-butanol with 190 proof ethanol gave lower viscosity and less ash content (< 0.01%). Increasing the 1-Butanol concentration showed better spray patterns in the lubricating oil. Goering, (1984) reported that an emulsion of methanol, 2-Octanol, cetane improver and soybean oil in the ratio 13.3:33.3:1:52.7 reduced the viscosity, ash content and cetane number in the lubricating oil. Ma and Hanna (1999) reported that increasing the viscosity of lubricating oil by using emulsions can cause the irregular injection of fuel, deposits of heavy carbon and incomplete combustion in combustion engines.

3.9.3. *Thermal cracking (Pyrolysis)*

Thermal cracking is defined as the conversion of one substance to another with heat alone or with heat and catalyst (Sonntag, 1979b). To yield small particles, chemical bonds are cleaved (Weisz *et al*., 1979). Characterization and optimization of the thermal cracking process is difficult because products and paths cannot be easily determined. Many fats or oils (vegetable oils, animal fats, and fatty acid methyl esters) can be pyrolyzed by thermal cracking (Sonntag, 1979b). Pyrolysis of vegetable oils to synthesize petroleum products have been investigated using catalytic cracking (Pioch *et al*., 1993). Billaud *et al*. (1995) pyrolyzed rapeseed oil to give methyl esters using tubular reactor with 500-850°C and in

nitrogen. Demirbas (2003b) reported that 78.3% conversion yield of gasoline was obtained using $ZnCl_2$ as catalyst with sunflower oil at 660K. Pioch *et al*. (1993) reported that using silica- alumina and zeolite as catalysts with palm oil stearin and copra oil gave conversion yields of 84% and 74%, respectively. Katikaneni *et al*. (1995) reported that a 81- 99% conversion yield was achieved using silica- alumina as the catalyst. Leng *et al*. (1999) reported that when using selective zeolite as the catalyst, palm oil was converted to hydrocarbons. Sang *et al*. (2003) reported a maximum conversion yield of palm oil to hydrocarbons and gasoline, using micromesoporous zeolite as catalyst, of 90 and 48%, respectively. Zhenyi *et al*. (2004) reported a maximum yield of diesel can be obtained with temperatures higher than 675K. The major disadvantages of this process are that the equipment is expensive and the process is not cost effective. Although it has the same chemical quality of petroleum products (diesel and gasoline), removal of oxygen from the fuel during the cracking process eliminates the beneficial aspects of petroleum products.

3.9.4. ***Transesterification***

Unlike microemulsion and thermal cracking which are problematic, transesterification has become the preferable method for the production of biodiesel (Ma and Hanna, 1999; Akoh *et al*., 2007; Robles *et al*., 2009; Ranganathan *et al*., 2008). The transesterification reaction occurs when alcohol reacts with triglycerides to give esters and glycerol as a by-product. Short chain alcohol like methanol, ethanol, octanol and other branched alcohols are widely used in the transesterification process (Fukuda *et al*., 2001). Alcohols and esters are likely to produce FAME's (Robles *et al*., 2009).

Figure 3.6 shows the transesterification process which consists of three continuous steps: (a) the conversion of triglycerides to diglycerides, (b) the conversion of diglycerides to monoglycerides and (c) the conversion of monoglycerides to methyl esters and glycerin (Freedman *et al*., 1984; Noureddini and Zhu, 1997; Marchetti *et al*., 2008). One fatty acid alkyl ester (FAAE) molecule is produced from each conversion of fats/oils by alcohol as shown in Figure 3.7 (Leung *et al*., 2010). Several catalysts (acids, alkali and enzymes) were

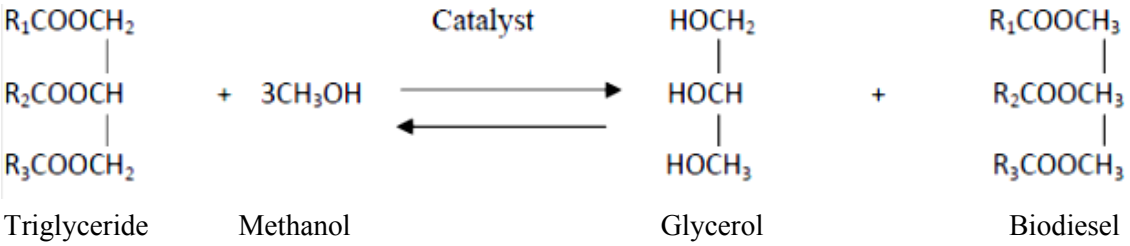


Figure 3.6. Overall reaction of the transesterification process (Leung *et al*., 2010).

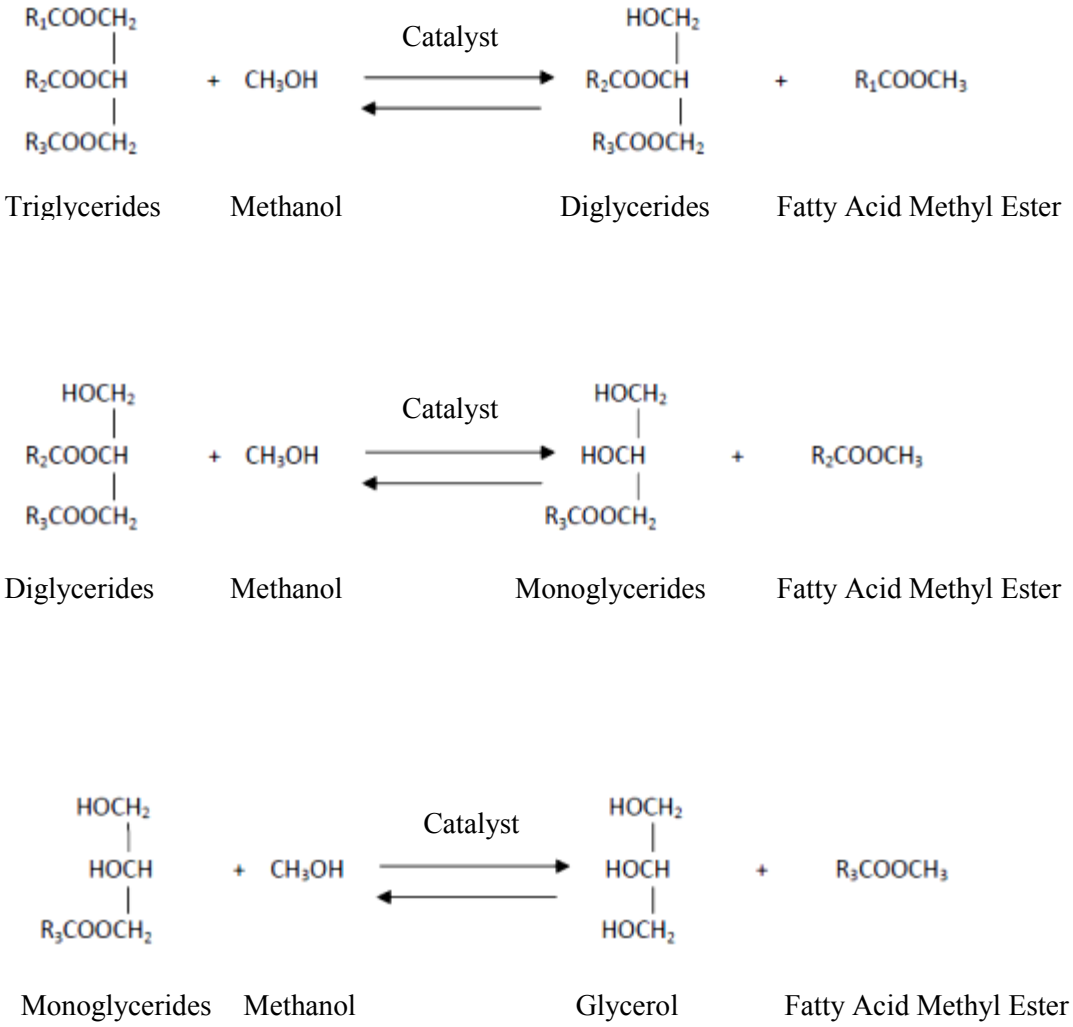


Figure 3.7. Stepwise reaction of transesterification (Murugesan *et al*., 2009).

used to increase the rate of transesterification reaction for the production of biodiesel (Bacovsky *et al*., 2007; Murugesan *et al*., 2009; Leung *et al*., 2010). McNeff *et al*. (2008) suggested that using the catalyst may affect the rate of reaction, purity of feedstock, purification process of the product. Factors such as mixing intensity, alcohol to oil ratio, concentration of catalyst, and temperature can also affect the reaction rate considerably (Marchetti *et al*.,2007).

3.9.4.1. Chemicals: Both acids and alkalis are used as catalysts in chemical transesterification. Alkali catalysts are commercially used because of their cost effectiveness, minimum reaction time and low temperature and pressure environment (Bacovsky et al., 2007; Leung et al., 2010). Acid catalysts are not widely used as alkali catalysts. The major acids used for the transesterification process are sulfuric acid, hydrochloric acid and sulfonic acid. Acid catalysts can achieve a high yield without the formation of soap. The disadvantages of using acids as catalysts are that corrosion can occur in the reaction and the rate of reaction is slow compared to alkali catalysts (Freedman et al., 1984; Bacovsky et al., 2007).

Alkali catalysts have higher conversion yields but the major disadvantage of these catalysts is their effect on the purification process of biodiesel as saponified products are produced. Figure 3.8 shows the alkali process of transesterification for biodiesel production (Ranganathan *et* al., 2008). Many free fatty acids and water in the reaction mixture reduce the efficiency of the transesterification process (Leung and Guo, 2006; Marchetti et al., 2008). The purification step removes water from the transesterification process (0.2 ton of waste water per ton of biodiesel is produced) which makes the process expensive and not environmentally friendly (Fjerbaek et al., 2009). The major alkali catalysts used commercially are sodium hydroxide (NaOH) and potassium hydroxide (KOH) (Schuchardt et al., 1998; Marchetti et al., 2008; Robles et al., 2009).

Both acid and alkali catalysts consume energy due to the complexity of the purification process (Xu and Wu, 2003). A separation process is required after completion of the transesterification process to separate biodiesel from impurities, monoglycerides, diglycerides, triglycerides, catalyst, glycerol, monoacylglycerols and diacylglycerols. The

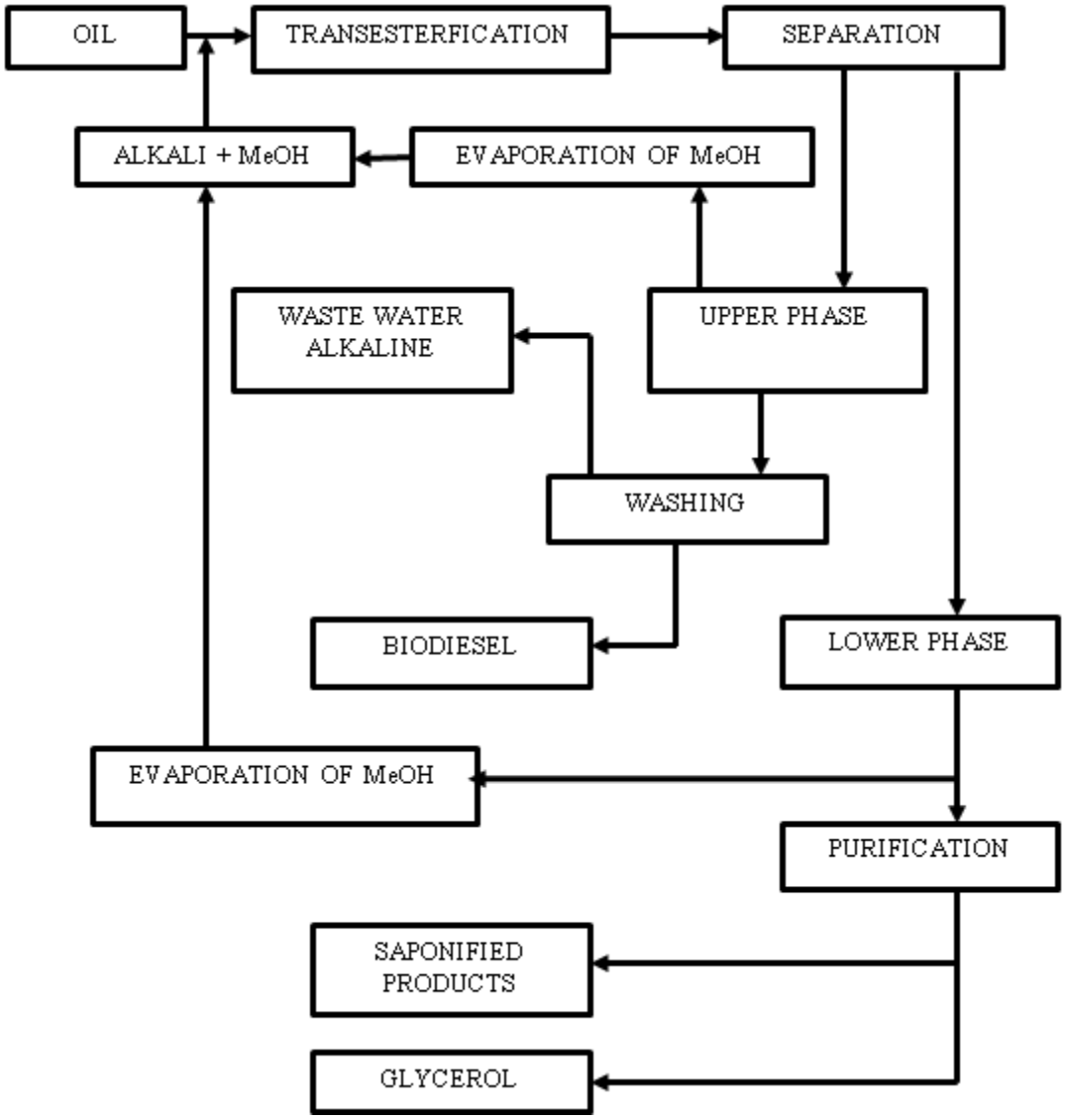


Figure 3.8. Alkali process of transesterification of biodiesel production (Ranganathan *et al*., 2008).

separation process involves few steps including gravitational settling or centrifugation (to separate glycerol from the end product), deodorization and pigment removal (Antczak *et al*., 2009; Banerjee and Chakraborty, 2009).

3.9.4.2. Enzymes: The use of enzyme catalysts reduces the complications associated with acid and alkali catalysts. Enzyme transesterification has proved to be more effective in reducing feedstock limitations, removal of downstream processing step and easy separation of glycerol from biodiesel (Jegannathan et al., 2008). In contrary to alkali catalysts, enzyme catalysts do not allow formation of soap in the reaction and hence the presence of free fatty acids in the reaction is not a problem (Harding et al., 2007; Fjerbaek et al., 2009). The waste water produced with enzyme catalysts are lower than that produced with acid catalysts (Dizge and Keskinler, 2008). Unlike chemical catalysts (which do not convert insoluble feedstock in the reaction), enzyme catalysts converts entire free fatty acids in the reaction to product allowing waste oil and fats from all sources to be used as the feedstock (Fukuda et al., 2001). Enzymes can be used in immobilized forms so that the separation process of enzyme catalysts from the FAAE's is simplified and the enzyme can be reused (Akoh et al., 2007; Robles et al., 2009). Table 3.14 illustrates the differences between the alkali and enzyme catalysts.

3.10. Enzymatic Transesterification

Enzymes are used widely in various applications like detergents, genetic engineering, leather, baking, starch hydrolysis, production of fructose, drug intermediates, bio-surfactants and biodiesel (Kudli-Shrinivas, 2007; Shah *et al*., 2003). Enzymes are biocatalyst which eliminates the requirement of excess energy by reducing the downstream processing and the problems associated with both alkali and acid catalyst (Roberts, 1989; Arnold, 1998). China is the first and major producer of biodiesel using lipase as the catalyst, producing 20,000 tons of biodiesel per annum (Du *et al*., 2008). The schematic diagram of producing biodiesel from enzyme is showed in Figure 3.9. There are advantages of using lipase as biocatalyst compared to acid or alkali catalyst such as the absence of soap formation, separation of high quality glycerol as by-product, no washing step is required to esterify both free fatty acids

Table 3.14. Comparison of alkali catalyst and biocatalyst transesterification (Shah *et al*., 2003, Fukuda *et al*., 2001).

Major factors	Alkali catalyst transesterification	Biocatalyst transesterification
Temperature	60-80ºC	20-60ºC
Presence of FFA's in feed stock	Soap formation	Complete conversion into the methyl ester
Presence of water	Soap formation is more likely as hydrolysis of the oil may take place	No effect on final product
Yield of biodiesel production	High, nearly 99%	Comparatively lower than alkali catalyst, around 90%
Downstream processing	Multi-step purification of end products	None
Biodiesel production cost	Cheap as catalysts comparatively cost less	Very expensive as biocatalyst are expensive
Commerialization	100% commercialized	China and Brazil
Waste water generation	Saline and alkaline effluent needs treatment before discharge	No waste water generation

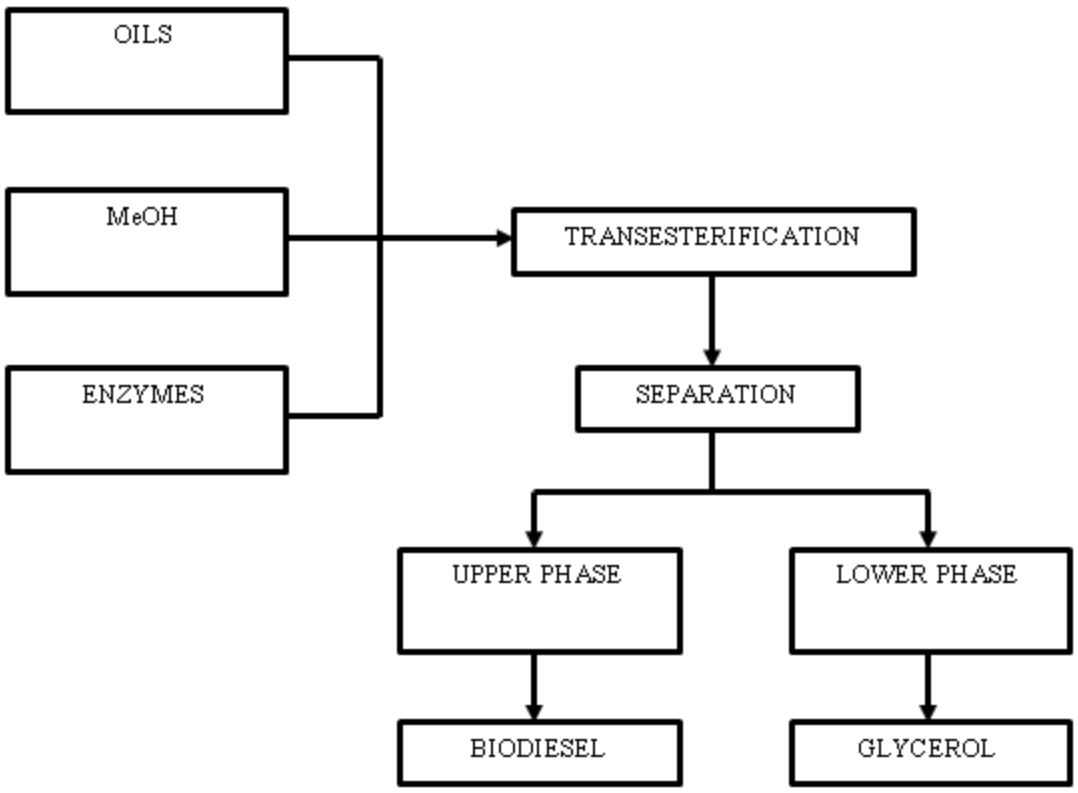


Figure 3.9. Enzymatic production of biodiesel (Ranganathan *et al*., 2008).

and triglycerides, no limitation in raw material, require less energy for conversion of free fatty acids to FAAE's and lower molar ratios are required than chemical transesterifcation (Narasimharao *et al*., 2007; Tamalampudi *et al*., 2008; Fjerbaek *et al*., 2009). The disadvantages of enzymatic transesterification process are slower rate of reaction, lengthy reaction time, high dosage of catalyst is required and high production cost (Bacovsky *et al*., 2007; Jeong and Park., 2008; Fjerbaek *et al*., 2009).

3.11. Factors Affecting Enzymatic Transesterification

In the transesterification process, the factors affecting the rate of conversion of biodiesel include the selection of alcohol, use of solvents, alcohol to oil molar ratio, water activity and reaction temperature.

3.11.1. *Selection of Alcohol*

Alcohols can be divided in two types namely long chain alcohols and short chain alcohols. Long chain alcohols can be used in the transesterification reaction but the conversion yield is lower than that obtained with the short chain alcohols because they inhibit the lipase activity (Coggon *et al*., 2007). Short chain alcohols like methanol and ethanol are widely used in the transesterification process for the enzymatic production of biodiesel. Other short chain alcohols can be used in the process including propanol, iso-propanol, 2-propanol, n-butanol and iso-butanol (Iso *et al*., 2001; Antczak *et al*., 2009; Varma and Madras, 2010). Salis *et al*. (2005) used different types of short chain alcohol with *Pseudomonas cepacia* without a solvent system and obtained a conversion yield of 40% with methanol, 93% with ethanol, 99% with propanol, 99% with 1-butanol, 83% with 2-butanol, 99% with 2-methyl-1-propanol and 99% with pentanol.

Short chain alcohols like methanol and ethanol are cost effective and but are responsible for deactivation and inhibition of immobilized lipase (Chen and Wu, 2003; Samukawa *et al*., 2000). The deactivation of enzyme was reported by insoluble methanol present in the oil or fats (Salis *et al*., 2005; Al-zuhair *et al*., 2007). Glycerol also inhibits the immobilized lipase. It deactivates and destabilizes the lipase because it has the tendency to get absorbed by the surface support matrix (Kumari *et al*., 2009).

Deactivation of the enzyme is determined by the decrease in carbon atoms in the alcohol (Chen and Wu, 2003; Ranganathan *et al*., 2008). Antczak *et al*. (2009) states that the rate of transesterification process is directly proportional to the length of alcohol carbon chain and indicated that ethanol is more favorable than methanol in some reactions.

Some researchers have suggested ways to avoid the inhibition of the enzyme by short chain alcohol including stepwise addition of short chain alcohols or adding it in sequence (Shimada *et al*., 1997; Watanabe *et al*., 2002; Soumanou and Bornscheuer, 2003; Matassoli *et al*., 2009) and using a solvent system (Nelson *et al*., 1996; Mittelbach, 1990; Modi *et al*., 2007). Stepwise addition of short chain alcohol is applicable only for methanol because ethanol has less of an inhibition effect towards immobilized lipase. To prevent the methanol inhibition effect, the ratios of oil: fat should be maintained below 3 and for ethanol it should maintain below 11 (Robles *et al*., 2009). Lee *et al*. (2008) obtained a 98.92% conversion yield using stepwise addition of methanol and 65% conversion yield when methanol was added in a batch process. Every lipase has different inhibition level and lipases that are extracted from *Pseudomonas* are more resistant towards alcohol inhibition than lipases extracted from *Thermomyces lanuginosa* and *Rhizomucor miehei* (Fjerbaek *et al*., 2009).

3.11.2. ***Use of Solvents***

Solvents are used to lower the inhibition effect of alcohol by increasing its solubility (Kumari *et al*., 2009). Solvents can also solubilize the by-product glycerol which can prevent the surface coating of the immobilized enzyme and the inhibition effect (Royon *et al*., 2007). Solvent systems provide a homogenous mixture between reactants and products which reduces the inhibition of enzymes and stabilizes the immobilized lipase in the reaction (Ranganathan *et al*., 2008; Fjerbaek *et al*., 2009). The homogenous mixture readily reduces the problems associated with multiple phase reactions and mass transfer reduction due to the high viscosity of the oil/fat substance (Fjerbaek *et al*., 2009). Vasudevan and Briggs (2008) stated that the rate of the transesterification reaction increases in the solvent system when compared to a solvent free system.

The solvents commonly used in the transesterification process are hydrophobic in nature and include hexane, n-heptane, petroleum ether and cyclohexane (Holmberg and Hult, 1990; Nelson *et al*., 1996; Soumanou and Bornscheuer, 2003; Ghamgui *et al*., 2004; Lara and park, 2004; Coggon *et al*., 2007). The most stable solvent commonly used is hexane which has moderate polarity towards enzymes (Li *et al* 2006; Fjerbaek *et al*., 2009). Tert-butanol and 2-butanol are alcohols which can also be used as solvent that can be used for regeneration of lipase (Robles *et al*., 2009). Royon *et al*. (2007) showed that the *Candida antarctica* (Novozyme 435) conversion yield was higher when *tert*-butanol was introduced to the solvent system. In a methanolysis reaction, the enzyme catalyst *Thermomyces lanuginosa* showed a conversion of 10% in solvent free system but when *tert*-butanol was added, a conversion yield of about 75% was obtained (Li *et al*., 2006). Qin *et al*. (2008) investigated the methanolysis of soybean oil using an enzyme from *Rhizopus chinensis* as a catalyst with different solvents and found n-heptane to be the optimum solvent with respect to efficiency. The conversion yields for the solvents were 84.2, 73.5, 73.4, 71.1 and 65.8% when n-octane, iso-octane, petroleum ether, acetone and cyclohexane were used as solvents with *tert*-butanol as alcohol in the reaction, respectively.

The solvents are used in the reaction to reduce the inhibitory effect of short chain alcohols but there are some disadvantages of using solvents in the reaction mixture including (a) addition processing is required to separate biodiesel product from the solvents, (b) organic solvents are unstable and hazardous, (c) the volume of reactors must be increased and (d) using solvents increases the overall cost for the producing the biodiesel (Ranganathan *et al*., 2008; Fjerbaek *et al*., 2009).

3.11.3. ***Alcohol : Substrate Molar Ratio***

In the transesterification process, the alcohol : oil ratio is a vital part of the reaction, where the alcohol : oil should be more than one molar ratio to enable the process to proceed at specific rate. The rate of the reaction is directly proportional to the alcohol : oil ratio (Antczak *et al*., 2009). Deactivation of the enzyme occurs when alcohol is insoluble in the reaction. Alcohol must be dissolved completely in the reaction mixture to prevent the deactivation of lipase and to increase the reaction rate (Jeong and Park, 2008). In solvent free

methanolysis, the concentration of methanol is inversely proportional to the activity of lipase in the reaction (Iso *et al*., 2001; Kose *et al*., 2002; Chen *et al*., 2006).

When selecting an alcohol it must have more than three carbons, If the carbons are less than three, the alcohol has a tendency to inhibit lipase in the reaction. The stoichiometric ratio of both methanol and ethanol are 1:3 and 2:3, respectively. The inhibition of lipase can be restricted by dissolving the alcohol completely in the reaction mixture within their stoichiometric ratios (Shimada *et al*., 2002; Robles *et al*., 2009). Matassoli *et al*. (2009) suggests that the ratios of methanol and ethanol to oil in solvent system must be 1:3 and 1:6, respectively.

In a solvent-free reaction, the inhibitory effect of lipase can be lowered when the addition of alcohol occurs in a stepwise manner (Selmi and Thomas, 1998; Kose *et al*., 2002; Vasudevan and Briggs, 2008). The molar ratios of short chain alcohols like methanol to oil must be around 3:1 (Antczak *et al*., 2009). In ethanol, the molar ratio of ethanol: oil reachs 11:1 (Robles *et al*., 2009; Munio *et al*., 2008).

In the butanolysis of triolein with the enzyme catalyst *Pseudomonas cepacia*, the molar ratios 3:1, 6:1, 9:1, and 12:1 were used and the optimum ratio was found to be in the range of 3:1 - 6:1. The conversion yield in that range was 100% after 4 hours of reaction but the ratios 9:1 and 12:1 showed 100% conversion yield after 5 and 6 hours, respectively (Salis *et al*., 2005). In the methanolysis of rapeseed oil using *Candida antartctica,* the optimum ratio was between 2:1 and 5:1, which gave a high conversion yield. The 6:1 ratio gave low yield due to inhibition effect of lipase in the reaction (Jeong and Park, 2008). However, the optimum level of molar ratio depends on the alcohol, lipase and feedstock used (Shimada *et al*., 2002; Robles *et al*., 2009; Matassoli *et al*., 2009).

3.11.4. ***Water Activity***

Water activity is one of the vital factors in enzymatic transesterification which sustains the three dimensional structure of the enzyme and determines the FAME yield and rate of reaction (Jegannathan *et al*., 2008; Lu *et al*., 2009). It can be expressed as water activity or percentage concentration (Antczak *et al*., 2009). The optimum water activity increases the

activity of lipase and reduces the hydrolysis in the enzymatic transesterification process even with short chain alcohols (Noureddini *et al*., 2005; Akoh *et al*., 2007; Jegannathan *et al*., 2008). Optimization of water activity depends on different factors such as the reaction system, alcohols, lipase source, immobilization technique, and stability of enzyme (jegannathan *et al*., 2008; Antczak *et al*., 2009). Few lipases such as those from *Candida rugosa, Pseudomonas cepacia,* and *Pseudomonas fluorescens* do not react with alcohols if there is no water activity but they show high conversion yield with water activity between 1% and 20% (Akoh *et al*., 2007; Fjerbaek *et al*., 2009). The conversion yield of *Rhizopus oryzae* was high with water activity between 4% and 30%. The water activity for some lipase can lead to no reaction. For example, the lipase from *Candida antarctica* does not like water in transesterification process (Deng *et al*., 2005; Fjerbaek *et al*., 2009). Robles *et al*. (2009) suggests that the water activity leads to flooding the pores which tends to lower the reaction rate. Li *et al*. (2006) stated that the optimum water activity must be 2% or less for transesterification process to give high conversion yield. He found that when *Thermomyces lanuginosa* and *Candida antarctica* were used in combination with tert-butanol as solvent, the water activity was maintained above 2% which gave low methyl ester yield.

3.11.5. ***Reaction Temperature***

According to Marchetti *et al*. (2008), lipases are thermally stable within the temperature range of 20°C - 70°C. However, the rate of conversion is highly dependent on temperatures outside this range. Antczak *et al*. (2009) states that the optimum temperature of immobilized lipase depends upon stability of lipase, type of solvent and type of alcohol. Jeong and Park (2008) performed a transesterification process with reaction temperature between 25°C - 55°C and found the optimum reaction temperature to be 40°C. Lee *et al*. (2008) showed an optimum reaction temperature of 45°C using combination of *Rhizopus oryzae* and *Candida rugosa* with methanol as the alcohol.

3.12. Glycerol

Production of glycerol can be both by microbial fermentation or chemical synthesis from petro-chemical feedstocks. Tradionally, glycerol is produced as a by-product of hydrolyzing the fats from the feedstock (Wang *et al*., 2001). Glycerol is also known as glycerin or

glycerine. It is a simple alcohol with many applications in various industries such as cosmetics, paint, automotive, food, tobacco, pharmaceuticals, pulp and paper, leather and textile industries (Biebl et al. 1998; Wang *et al*., 2001). Various chemicals can be obtained from glycerol as feedstock. Biebl et al. (1998) reported that glycerol can be used as a feedstock in the chemical synthesis of poly trilmethylene or polyterephthalate which can enhance certain physical properties (good resilience, stain resistance and low static generation) of fiber used in the textile industries. The conversion of (5 -15%) glycerol to (75 - 90%) dihydroxyacetone using *Acetobacter suboxidans* bacterium as the medium in submerged fermentation is an example of using glycerol as a feedstock for industrial fermentations (Wang *et al*., 2001). The dihydroxyacetone can be further converted from dihydroxyacetone kinase to dihydroxyacetone phosphate which is a substrate molecule for aldolases to produce optically active sugar derivatives (Itoh *et al*., 1999). Table 3.15 shows the usage of glycerol in various applications.

The annual production of glycerol was 600,000 tons in 2001. The production of glycerol from hydrolysis of fats has decreased, due to soap being replaced by detergents in the developing countries and industrial nations (Agarwal, 1990; Rehm, 1998). Also, the production of glycerol can be obtained from the oxidation or chlorination of propylene. However, the cost of propylene is high and there are associated environmental concerns (Wang *et al*., 2001), thus the production of glycerol from propylene has been in decline. Glycerol can also be produced as a byproduct during the microbial fermentation of sugar to ethanol using *Saccharomyces cerevisiae* in a redox-neutral process (Agarwal, 1990; Wang *et al*., 2001). This method became more attractive and cost effective than the chemical synthesis from petrochemical feedstocks or the recovery as a byproduct of the soap manufacture process from fats (Wang *et al*., 2001).

Table 3.15. Usage of glycerol in various applications (Wang *et al*., 2001)

Field of Use	Percent Use (%)			
	USA	Europe	Japan	China
Drugs	39.5	23.1	34.0	5.0
Tobacco	15.8	2.5	5.3	7.0
Glycerintriacetate	ND	14.4	ND	ND
Food	14.5	5.6	ND	ND
Polyether alcohol	10.5	13.1	11.6	5.2
Paints	9.2	13.1	19.5	49.0
Cellophane	2.0	4.4	3.8	1.5
Dynamite	0.6	3.1	1.9	3.1
Toothpaste	ND	ND	ND	16.0
Cosmetics	ND	ND	ND	6.0
Miscellaneous	7.9	20.7	23.9	7.2

*ND = No Data
USA Production = 160,000 tons/yr
Europe Production = 190,000 tons/yr
Japan Production = 50,000 tons/yr
China Production = 80,000 tons/yr

CHAPTER 4. MATERIALS AND METHODS

4.1. Experimental Materials

4.1.1. *Glassware*

The glassware used in the experiment included test tubes, 50 ml conical flasks, gas chromatography vials, micro pipettes and funnels. All glassware were washed with soap, and tap water and rinsed with distilled water.

4.1.2. *Chemicals*

Methanol, ethanol, 2-butanol, n-hexane, tertrahydrofuran, N, O - Bis (Trimethylsilyl)-Trifluroacetamide (BSTFA), Sodium methoxide, 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), 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) and methyl-stearidonate was purchased from Cayman Chemical (Ann Arbor, Michigan, USA).

4.1.3. *Equipment*

The equipment used in the experiment included: gas chromatography (Hewlett Packard 5890 series II, Agilent, Mississauga, Ontario, Canada) with AT-FAME 30 m x 0.32 mm x 0.25 μm HELIFLEX capillary columns, (Alltech Associates, Inc., Deerfield, Illinois, USA), Isotemp oven (655 F, Fisher Scientific Toronto, Ontario, Canada), microprocessor controlled water bath (280 Series, Fisher Scientific, Toronto, Ontario, Canada) at 90°C and reciprocal shaking bath (2850 Series, Fisher Scientific, Toronto, Ontario, Canada) at 45°C and balance (Mettler AE 200 Scale -Mettler Toledo Canada, Mississauga, Ontario, Canada).

4.1.4. ***Animal Tallow***

The animal wastes were obtained from S.F Rendering, Centreville Nova Scotia. Samples (10 Kg) were collected and stored at - 20°C. The waste was obtained as beef tallow that had been rendered by the company. The sample material was yellowish in colour.

4.1.5. ***Enzymes***

Immobilized Lipase from *Candida antartica* (Novozyme 435) was purchased from Sigma Aldrich (St. Louis, Missouri, USA) and experimental immobilized lipase NS-88001 was obtained from Novozymes North America Inc, (Franklinton, North Carolina, USA).

4.2. Experimental Design

The experimental work was divided into four parts. The first part of the experiments was carried out to convert animal fats to oil and characterize the produced oil.

The second part of the experiments was carried out to investigate the effects of alcohol (methanol and 2-butanol), reaction temperature (35, 40, 45, 50°C), oil : alcohol molar ratio (1:1, 1:2, 1:3, 1:4, 1:5), reaction time (4, 8, 12, 16 hour) and system (solvent and solvent-free system) on the effectiveness of the lipase *Candida antarctica* (Novozyme 435) and the experimental lipase (NS88001) individually. Table 4.1 shows the number of experimental runs.

The third part of the experiments was carried out to investigate the effects of alcohol (methanol and 2-butanol), oil : alcohol molar ratio (1:1, 1:2, 1:3, 1:4, 1:5), reaction time (4, 8, 12, 16 hour) and system (solvent and solvent-free system) on the combined lipases (Novozyme 435 and NS88001) at constant reaction temperature of 45°C. Table 4.2 shows the number of experimental runs.

The fourth part of the experiments was carried out to investigate the reusability of the lipase for both lipases (individually and combination). Reusability of individual and

Table 4.1. Enzymatic transesterification using individual enzyme

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

No. of replicates = 3
Total no. of samples = 960

Table 4.2. Enzymatic transesterification using a combination of enzymes

Factors	Parameters
Enzymes	*Candida antarctica* (Novozyme 435) and experimental enzyme (NS88001)
Alcohol	Methanol and 2-butanol
Oil: alcohol ratio	1:1, 1:2, 1:3, 1:4 and 1:5
Reaction temperature	45°C
Reaction time	4, 8, 12 and 16 hours
System	Solvent and solvent-free

No. of replicates = 3
Total no. of samples = 240

Table 4.3. Reusability of lipase

Factors	Parameters
Enzymes	*Candida antarctica* (Novozyme 435), experimental lipase (NS88001) and Combination of both enzymes
Alcohol	Methanol and 2-butanol
Oil: alcohol ratio	1:3
Reaction temperature	45°C
Reaction time	8 hours
System	Solvent and solvent-free

No. of cycles = 50

a combinations of enzyme catalysts were determined based on the number of cycles. Table 4.3 shows the reusability conditions for Novozyme 435, NS88001 and combination of both (Novozyme 435 and NS88001) enzyme catalyst.

4.3. Experimental Procedure

4.3.1. *Purification of Crude Animal Tallow*

The animal tallow was first heated to 105-110°C with constant stirring at 50 rpm in a round bottom flask for one hour. During the process of melting the fats, the top layer consisting of bubbles and impurities was discarded regularly. Then, the extracted crude animal tallow oil was filtered four times using vacuum filtration with ultra filter paper (Whatman No.40, Fisher Scientific, Toronto, Ontario, Canada). The oil percentage was calculated as follows

$$\% \text{ oil} = \left(\frac{Mass\ of\ oil}{Total\ fat}\right) * 100 \qquad (4.1)$$

4.3.2. *Enzymatic Transesterification Process*

Biodiesel production from animal fats was carried out using enzymatic transesterification method. In this procedure, both individual and a combination of enzyme catalysts were optimized.

4.3.2.1. Optimization of Individual Enzyme Catalyst: The enzymatic transesterification was done in order to extract fatty acid methyl esters from the animal fats by individual enzyme catalyst as shown in Figure 4.1. The two enzymes were evaluated individually and five oil : alcohol molar ratios (1:1, 1:2, 1:3, 1:4 or 1:5), four reaction temperatures (35, 40, 45 or 50°C) and four reaction times (4, 8, 12 or 16 hours) were investigated. The homogenized oil (2.3 ml corresponding to 2 g of fat) was placed into a 50 ml conical flask and heated on a hot plate (PC-620, Corning, New York, New York, USA). The immobilized enzyme (Novozyme

435 or NS88001) of 25% of the oil weight (0.5 g) was added to the flask. The appropriate amount of alcohol (methanol or 2-butanol) was added based on stoichiometric oil : alcohol molar ratio (1:1, 1:2, 1:3, 1:4 or 1:5). The solution was mixed using a reciprocal shaking bath (2850 Series, Fisher Scientific, Toronto, Ontario, Canada) at 200 rpm. The desired temperature (35, 40, 45 or 50°C) was selected. After the desired reaction time was completed (4, 8, 12 or 16 hours), the enzyme was filtered by vacuum filtration as recommended by Nelson et al. (1998). Samples (100µl) were taken from the mixture and analyzed using a gas chromatography system (Hewlett Packard 5890 series II, Agilent, Mississauga, Ontario, Canada). The same procedure was repeated till the results from all the investigated parameters (oil: alcohol molar ratios, reaction temperatures and reaction times) were evaluated.

4.3.2.2. Optimization of Combination Enzyme Catalysts: Figure 4.2 shows the enzymatic transesterification process with a combination of enzyme catalysts. The two enzymes were evaluated in a combination (12.5% each on weight basis) and five oil : alcohol molar ratios (1:1, 1:2, 1:3, 1:4 or 1:5), and four reaction time (4, 8, 12 or 16 hours) were investigated at the reaction temperature of 45°C. The optimal condition of individual enzymes was determined from the previous step. The homogenized oil (2.3 ml corresponding to 2g of fat) was placed into a 50 ml conical flask, heated on a hot plate (PC-620, Corning, New York, USA) and 12.5% (based on oil weight) of each immobilized enzyme (Novozyme 435 and NS88001) was added to the flask. The appropriate amount of alcohol (methanol or 2-butanol) was added based on the desired stoichiometric oil : alcohol molar ratio (1:1, 1:2, 1:3, 1:4 and 1:5). The solution was mixed using a reciprocal shaking bath (2850 Series, Fisher Scientific, Toronto, Ontario, Canada) at 200 rpm and the optimum reaction temperature (45°C). After the desired reaction time (4, 8, 12 or 16 hours), the enzyme was filtered by vacuum filtration as recommended by Nelson et al. (1998). Samples (100 µl) were taken from the mixture and analyzed using a gas chromatography system (Hewlett Packard 5890 series II, Agilent, Mississauga, Ontario, Canada). The same procedure was repeated till all the parameters (oil: alcohol molar ratio and reaction times) were evaluated.

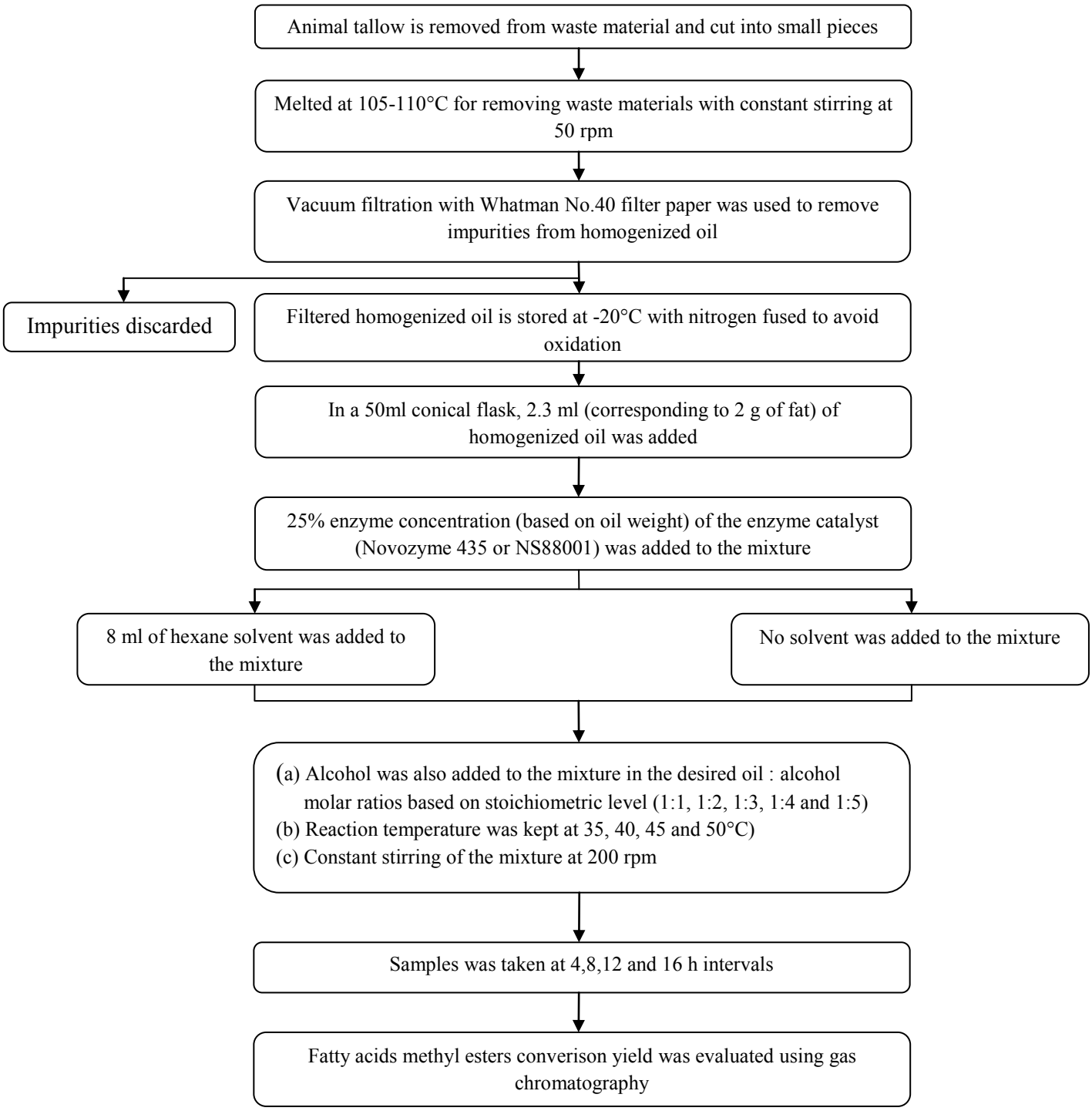


Figure 4.1. Enzymatic transesterification of individual enzyme catalyst by solvent and solvent-free systems.

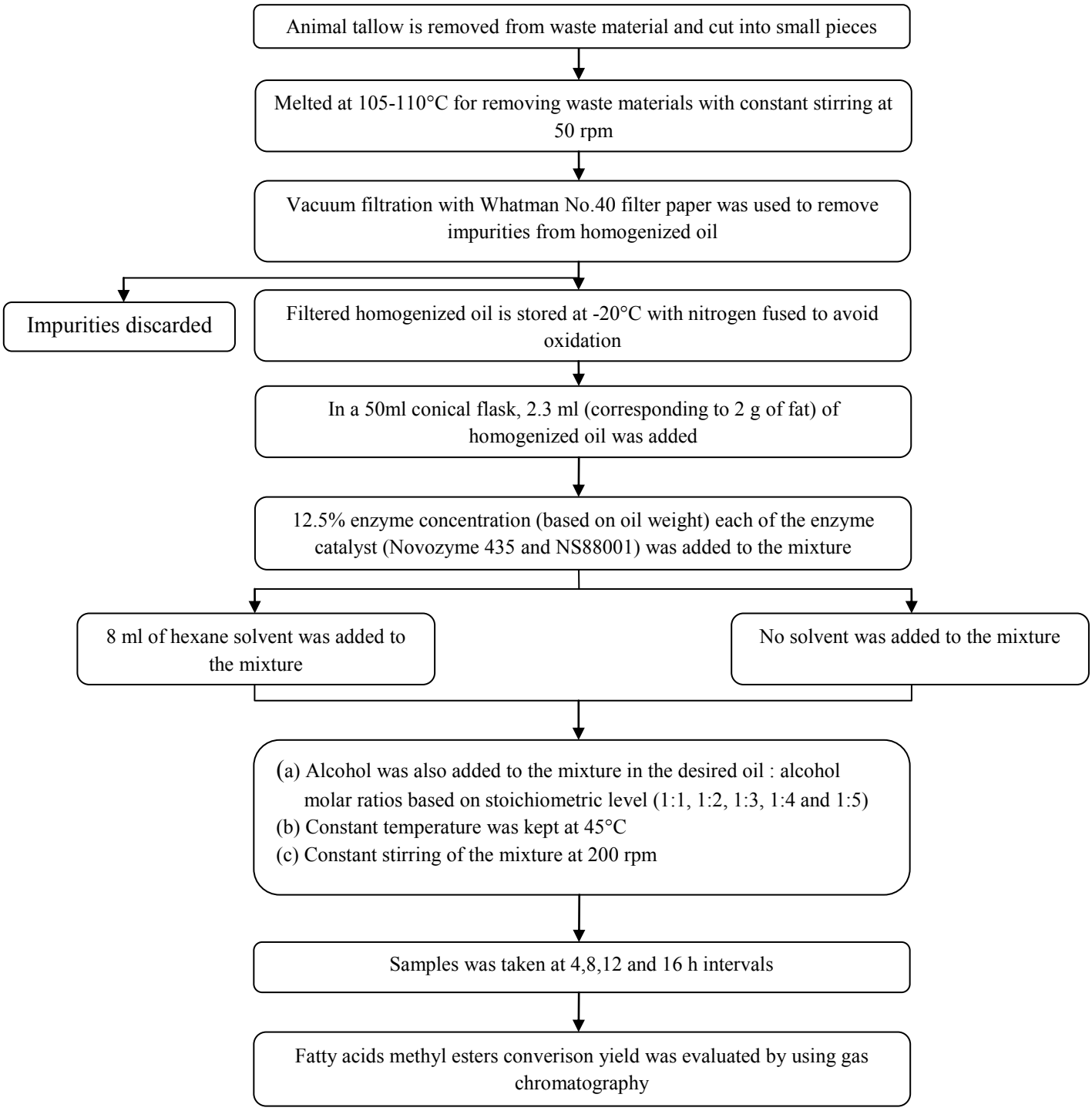


Figure 4.2. Enzymatic transesterification of combination enzyme catalyst by solvent and solvent-free systems.

4.3.3. *Reusability of Lipase*

The homogenized oil was placed into a 50 ml conical flask and heated on a hot plate (PC-620, Corning, New York, USA). The appropriate amount of enzymes (1:3 oil : alcohol molar ratio) was added and the solution was mixed using a reciprocal shaking bath, (2850 Series, Fisher Scientific, Toronto, Ontario, Canada) at 200 rpm and 45°C for 8 hours. The reaction was stopped by removing the enzyme from the system with vacuum filtration. The enzyme catalysts were washed with distilled water three times and then washed once with tert-butanol. The washed enzyme catalysts were dried at 45°C for an hour in an oven Isotemp oven (655 F, Fisher Scientific, Toronto, Ontario, Canada). The dried enzymes were reintroduced in the system and the sample procedure was repeated 50 times.

4.4. Experimental Analysis

4.4.1. *Determination of yield*

A 100 µL aliquot was taken from the transesterification process at selected time intervals (4, 8, 12 and 16 hours) and flushed with nitrogen in the waterbath (280 series, Fisher Scientific, Toronto, Ontario, Canada) at 45°C in order to evaporate the hexane. A 10 mg portion 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 15 minutes. The sample was then cooled to room temperature for few minutes after which 5 mL of hexane was added. An aliquot of 1.5 mL 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 a gas chromatography system, coupled with flame ionization detector (FID) (HP5890 Series II, Agilent Technologies, Mississauga, Ontario, Canada). An AT-FAME capillary column 30 m in length, 0.32 mm of internal

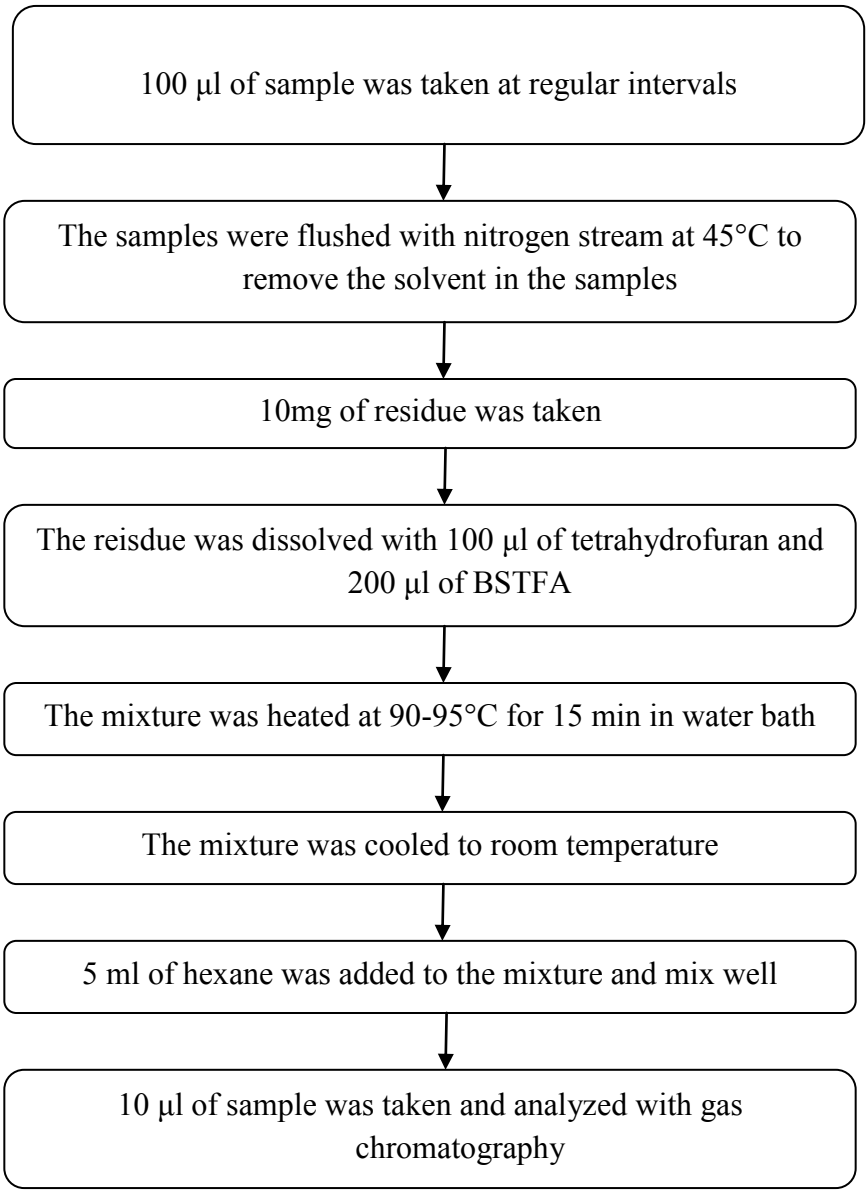


Figure 4.3. Sample preparation procedure for Gas chromatography.

diameter and 0.25 μm film thicknesses (Alltech Associates, Inc., Deerfield, Illinois, USA) was used for analyses. The column is a highly polar and stable bonded polyethylene glycol phase. The separated samples were injected directly into the column with the initial oven temperature of 60°C, followed by a flow rate of (20°C/min). A final temperature of 280°C 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.

$$\text{Conversion yield of Peak area A (wt \%)} = \frac{Peak\ area\ A\ x\ 100}{\sum(\text{Peak area A+Peak area B+}\cdots\text{+Peak area N})} \quad (4.2)$$

Where:

Peak area A = Methyl Oleate

Peak area B = Methyl Sterate

Peak area N = No. of Impurities

4.4.2. *Statistical analysis*

All the statistical analyses were conducted using Minitab statistics software (Version 16.2.2, Minitab Inc, Pennsylvania, USA) the data conversion yield and standard errors were calculated. Analysis of variance (ANOVA) was performed on the data to test the effects of the parameters and their interactions. Tukey's multiple tests were performed to determine the differences among the levels of each parameter. The α-level chosen was 0.05.

CHAPTER 5. RESULTS

5.1. Characterization of Animal Tallow

Table 5.1 shows the composition of the animal tallow used in this study. The filtration process removed about 7.5 % of the total weight of tallow as impurities present in the animal fats. The homogenized oil was characterized by gas chromatography to identify and quantify the fatty acid composition of the tallow. Five fatty acids were identified in the animal tallow: oleic acids (44%), palmitic acids (28%), stearic acids (26%), linoleic acids (1%), and myristic acids (1%).

5.2. Enzymatic Transesterification by Individual Enzyme Catalyst

Enzymatic transesterification by individual enzyme catalysts (Novozyme 435 or NS88001) was first carried out to investigate the effects of reaction time (4, 8, 12 and 16 hour), oil : alcohol molar ratios (1:1, 1:2, 1:3, 1:4 and 1:5) and reaction temperature (35, 40, 45 and 50°C) on biodiesel yield in solvent and solvent-free systems. All trials showed that no conversion yield of biodiesel was obtained with solvent and without solvent system when methanol was used with the enzyme Novozyme 435 and when 2-butanol was used with the enzyme NS88001. Therefore, 2-butanol was used with the enzyme Novozyme 435 whereas methanol was used with the enzyme NS88001 in this study. The results are shown in Tables 5.2-5.5.

Tables 5.6 shows the analysis of variance performed on the yield data. The effect of enzyme type, molar ratio, reaction time, reaction temperature and solvent system were highly significant at the 0.001 level. All interactions between the parameters were also highly significant at the 0.001 level.

The results obtained from Tukey's Grouping (Table 5.7) indicated that the two enzymes were significantly different from each other at the 0.05 level. The highest mean yield of 63.33% was obtained with *Candida antarctica* (Novozyme 435). The four levels of oil: alcohol molar ratio (1:1, 1:2, 1:3 and 1:5) were significantly different from one another at the

Table 5.1. Composition of animal tallow.

Parameters	Value
Impurities (Kg)	0.375
Oil (%)	92.5
Impurities (%)	7.5
Fatty acids (wt%)	
Oleic acid	44
Palmitic acid	28
Stearic acid	26
Linoleic acid	1
Myristic acid	1

Tallow =5 kg

Table 5.2.Biodiesel yield (wt%) from animal tallow 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 Ratios	Reaction Temperature (°C)		
		40	45	50
4	1:1	45.26 ± 0.91	59.29 ± 1.19	52.70 ± 1.05
	1:2	49.85 ± 1.00	60.31 ± 1.21	54.46 ± 1.09
	1:3	49.93 ± 1.00	72.81 ± 1.46	62.08 ± 1.24
	1:4	57.62 ± 1.15	68.65 ± 1.37	60.68 ± 1.21
	1:5	30.25 ± 0.61	62.51 ± 1.25	54.52 ± 1.09
8	1:1	48.62 ± 0.97	62.94 ± 1.26	58.52 ± 1.17
	1:2	58.78 ± 1.18	64.09 ± 1.28	62.13 ± 1.24
	1:3	59.06 ± 1.18	73.41 ± 1.47	64.79 ± 1.30
	1:4	62.69 ± 1.25	69.09 ± 1.38	67.96 ± 1.36
	1:5	42.09 ± 0.84	66.05 ± 1.32	63.15 ± 1.26
12	1:1	52.30 ± 1.05	67.97 ± 1.36	64.57 ± 1.29
	1:2	61.03 ± 1.22	69.27 ± 1.39	67.36 ± 1.35
	1:3	65.98 ± 1.32	75.73 ± 1.51	69.91 ± 1.40
	1:4	68.35 ± 1.37	72.68 ± 1.45	68.89 ± 1.38
	1:5	47.63 ± 0.95	66.83 ± 1.34	65.10 ± 1.30
16	1:1	52.94 ± 1.06	73.40 ± 1.47	69.21 ± 1.38
	1:2	62.06 ± 1.24	74.59 ± 1.49	69.48 ± 1.39
	1:3	69.39 ± 1.39	77.23 ± 1.74	72.48 ± 1.55
	1:4	69.85 ± 1.40	75.68 ± 1.51	74.09 ± 1.48
	1:5	62.31 ± 1.25	73.32 ± 1.47	71.86 ± 1.44

Table 5.3. Biodiesel yield (wt%) from animal tallow using 0.5 grams of *Candida antarctica* (Novozyme 435) with 2-butanol as alcohol and without hexane at different reaction times, oil : alcohol molar ratios and reaction temperatures.

Time (h)	Oil: Alcohol Molar Ratios	Reaction Temperature (°C)		
		40	45	50
4	1:1	Not extractable	Not extractable	Not extractable
	1:2	68.57 ± 1.37	84.49 ± 1.69	50 ± 1.00
	1:3	73.98 ± 1.48	85.67 ± 1.71	54.70 ± 1.09
	1:4	76.85 ± 1.54	87.47 ± 1.75	79.89 ± 1.60
	1:5	49.45 ± 0.99	84.62 ± 1.69	37.67 ± 0.75
8	1:1	Not extractable	Not extractable	Not extractable
	1:2	71.23 ± 1.42	88.23 ± 1.76	65.42 ± 1.31
	1:3	79.50 ± 1.59	91.81 ± 1.84	67.58 ± 1.58
	1:4	80.36 ± 1.61	89.29 ± 1.79	85.49 ± 1.71
	1:5	50.26 ± 1.01	86.85 ± 1.74	48.93 ± 0.98
12	1:1	Not extractable	Not extractable	Not extractable
	1:2	76.54 ± 1.53	89.97 ± 1.80	79.98 ± 1.60
	1:3	81.65 ± 1.63	92.54 ± 1.85	92.17 ± 1.84
	1:4	85.64 ± 1.71	93.70 ± 1.87	93.53 ± 1.87
	1:5	53.24 ± 1.06	90.28 ± 1.81	58.99 ± 1.18
16	1:1	Not extractable	Not extractable	Not extractable
	1:2	82.53 ± 1.65	92.86 ± 1.86	91.3 ± 1.83
	1:3	86.35 ± 1.73	95.13 ± 1.90	93.76 ± 1.88
	1:4	86.79 ± 1.74	95.21 ± 1.90	93.80 ± 1.88
	1:5	69.56 ± 1.39	91.33 ± 1.83	64.91 ± 1.70

Table 5.4. Biodiesel yield (wt%)from animal tallow using 0.5 grams of Experimental catalyst (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 Ratios	Reaction Temperature (°C)		
		40	45	50
4	1:1	42.87 ± 0.86	58.90 ± 1.18	55.20 ± 1.10
	1:2	46.51 ± 0.93	73.85 ± 1.48	63.10 ± 1.26
	1:3	58.39 ± 1.17	77.89 ± 1.56	69.28 ± 1.39
	1:4	78.07 ± 1.56	84.35 ± 1.69	73.24 ± 1.46
	1:5	51.31 ± 1.03	69.20 ± 1.38	46.73 ± 0.93
8	1:1	47.29 ± 0.95	63.67 ± 1.27	60.90 ± 1.22
	1:2	74.71 ± 1.49	79.85 ± 1.60	64.90 ± 1.30
	1:3	85.81 ± 1.72	88.94 ± 1.78	77.63 ± 1.55
	1:4	81.02 ± 1.62	85.62 ± 1.71	78.42 ± 1.57
	1:5	70.41 ± 1.41	79.56 ± 1.59	54.98 ± 1.10
12	1:1	60.09 ± 1.20	78.72 ± 1.57	65.91 ± 1.32
	1:2	74.98 ± 1.50	84.69 ± 1.69	71.92 ± 1.44
	1:3	85.93 ± 1.72	92.11 ± 1.84	82.25 ± 1.64
	1:4	82.82 ± 1.66	92.86 ± 1.86	79.87 ± 1.60
	1:5	72.94 ± 1.46	87.81 ± 1.76	69.13 ± 1.38
16	1:1	66.82 ± 1.34	78.97 ± 1.58	70.03 ± 1.40
	1:2	79.97 ± 1.60	92.56 ± 1.85	72.85 ± 1.46
	1:3	88.23 ± 1.76	94.43 ± 1.89	92.97 ± 1.86
	1:4	88.26 ± 1.77	95.75 ± 1.92	91.24 ± 1.82
	1:5	75.10 ± 1.50	88.04 ± 1.76	77.95 ± 1.56

Table 5.5. Biodiesel yield (wt%) from animal tallow using 0.5 grams of Experimental catalyst (NS88001) with Methanol as alcohol and without hexane at different reaction times, oil : alcohol molar ratios and reaction temperatures.

Time (h)	Oil: Alcohol Molar Ratios	Reaction Temperature (°C)		
		40	45	50
4	1:1	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable
	1:3	72.80 ± 1.46	74.16 ± 1.48	38.40 ± 1.77
	1:4	77.86 ± 1.56	80.10 ± 1.60	58.40 ± 1.17
	1:5	47.69 ± 0.95	59.74 ± 1.19	32.60 ± 0.65
8	1:1	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable
	1:3	77.58 ± 1.55	80.22 ± 1.60	46.60 ± 0.93
	1:4	82.46 ± 1.65	84.85 ± 1.70	67.90 ± 1.36
	1:5	75.05 ± 1.50	76.23 ± 1.52	40.10 ± 0.80
12	1:1	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable
	1:3	77.92 ± 1.56	82.10 ± 1.64	57.90 ± 1.16
	1:4	83.40 ± 1.67	87.67 ± 1.75	77.08 ± 1.54
	1:5	78.61 ± 1.57	78.97 ± 1.58	53.51 ± 1.07
16	1:1	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable
	1:3	83.05 ± 1.66	93.16 ± 1.86	62.81 ± 1.26
	1:4	87.09 ± 1.74	94.04 ± 1.88	84.19 ± 1.68
	1:5	78.97 ± 1.58	80.76 ± 1.62	70.98 ± 1.42

Table 5.6. ANOVA of biodiesel yield using the enzymes Novozyme 435 and NS88001 (individually) at different oil: alcohol molar ratios, reaction times and reaction temperatures with and without solvent.

Source	DF	SS	MS	F	P
Total	719	599331			
Model					
EN	1	3170	3170.1	1479.2	0.001
MR	4	229478	57369.5	26769.5	0.001
RTI	3	23049	7682.9	3584.9	0.001
RTE	2	18225	9112.6	4252.1	0.001
SY	1	46063	46063.4	21493.9	0.001
EN*MR	4	40222	10055.4	4692.0	0.001
EN*RTI	3	222	73.8	34.5	0.001
EN*RTE	2	2900	1449.8	676.5	0.001
EN*SY	1	43648	43648.0	20366.8	0.001
MR*RTI	12	2560	213.3	99.5	0.001
MR*RTE	8	5317	664.7	310.1	0.001
MR*SY	4	118906	29726.4	13870.8	0.001
RTI*RTE	6	1330	221.7	103.4	0.001
RTI*SY	3	323	107.5	50.2	0.001
RTE*SY	2	3521	1760.4	821.4	0.001
EN*MR*RTI	12	1096	91.4	42.6	0.001
EN*MR*RTE	8	2322	290.2	135.4	0.001
EN*MR*SY	4	40740	10185.0	4752.5	0.001
EN*RTI*RTE	6	557	92.8	43.3	0.001
EN*RTI*SY	3	451	150.4	70.2	0.001
EN*RTE*SY	2	688	344.1	160.6	0.001
MR*RTI*RTE	24	1034	43.1	20.1	0.001
MR*RTI*SY	12	1537	128.1	59.8	0.001
MR*RTE*SY	8	3322	415.2	193.7	0.001
RTI*RTE*SY	6	1138	189.6	88.5	0.001
EN*MR*RTI*RTE	24	1354	56.4	26.3	0.001
EN*MR*RTI*SY	12	1065	88.8	41.4	0.001
EN*MR*RTE*SY	8	2521	315.1	147.0	0.001
MR*RTI*RTE*SY	24	870	36.3	16.9	0.001
EN*MR*RTI*RTE*SY	24	663	27.6	12.9	0.001
Error	486	1042	2.1		

DF: Degree of freedom
SS: Sum of square
MS: Mean of square
R^2 : 99.67%
EN = Enzymes
MR = Molar ratios
RTI = Reaction time
RTE = Reaction temperature
SY= Solvent System

Table 5.7. Tukey's Grouping of the various parameters (using individual enzymes).

Factors	Level	N	Mean (%)	Tukey Grouping
Enzyme Type	Novozyme 435	360	63.05	A
	NS88001	360	58.85	B
Oil : Alcohol Molar Ratios	1:1	144	30.37	A
	1:2	144	53.63	B
	1:3	144	76.01	C
	1:4	144	80.02	C
	1:5	144	64.74	D
Reaction Time (hours)	4	180	52.76	A
	8	180	59.22	B
	12	180	63.83	BC
	16	180	67.99	C
Reaction Temperature (°C)	40	240	57.79	A
	45	240	68.05	B
	50	240	57.02	A
Solvent System	Hexane	360	68.95	A
	Without Hexane	360	52.95	B

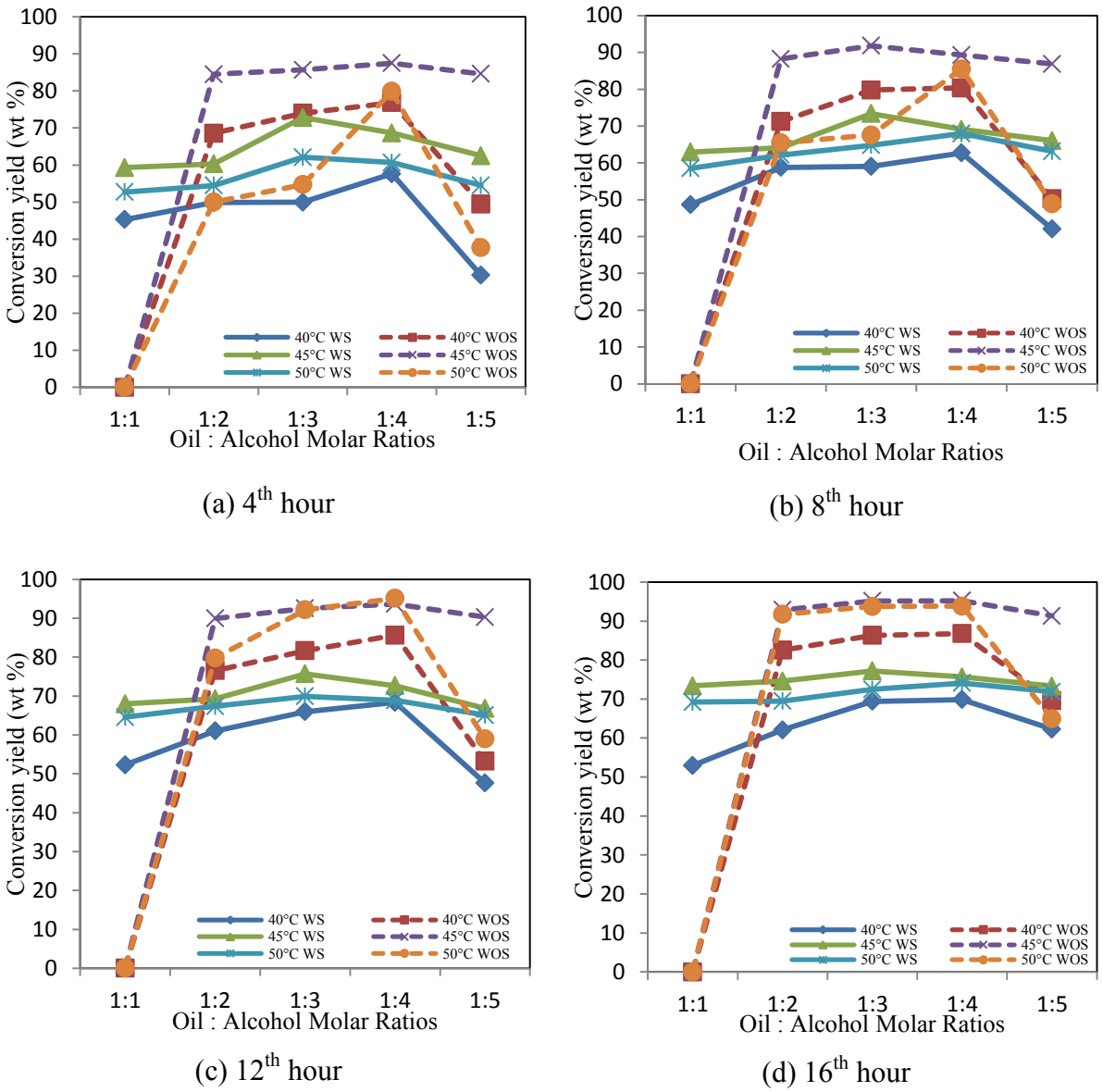
Groups with the same letter are not significantly different at the 0.05 level.

0.05 level. However, the oil: alcohol molar ratios of 1:3 and 1:4 were not significantly different from each other but were significantly different from other oil: alcohol molar ratios at the 0.05 level. The highest mean yield of 80.02% was obtained with the 1:4 oil : alcohol molar ratio. The reaction times 4, 8 and 12 hour were significantly different from one another at the 0.05 level but the reaction time 12 was not significantly different from the reaction times 8 and 16 hour at the 0.05 level. The highest mean yield of 67.99% was achieved with 16 hour reaction time. The reaction temperatures 40 and 50°C were not significantly different from each other but were significantly different from the reaction temperature 45°C at the 0.05 level. The highest mean yield of 68.05% was obtained at the reaction temperature 45°C. The difference between hexane and without-hexane was significantly different at the 0.05 level. The highest mean yield of 68.95% was obtained with hexane as a solvent system.

5.2.1. *Effect of Oil : Alcohol Molar Ratio*

Figures 5.1 and 5.2 show the effect of oil : alcohol molar ratio on the conversion yield using two enzymes at different reaction temperatures, reaction times and solvent systems. Generally, there was an increase in the biodiesel conversion yield by the enzymes catalyst (Novozyme 435 and NS88001) with increases in oil : alcohol molar ratios from 1:1 to 1:4 followed by decrease in conversion yield with a further increase in the oil : alcohol ratios from 1:4 to 1:5 for all reaction time (4, 8, 12 and 16 h) and reaction temperatures (40, 45 and 50°C) with and without solvent.

Figure 5.1 shows the effect of oil : alcohol molar ratios on the biodiesel conversion yield by Novozyme 435. The conversion yield of biodiesel in the solvent system at the 4 h increased from 45.26 to 57.62% (27.30%), from 59.29 to 68.65% (15.78%) and from 52.70 to 60.68% (15.14%) with increases in oil : alcohol molar ratios from 1:1 to 1:4 for the reaction temperatures of 40, 45 and 50°C, respectively. A further increase in the oil : alcohol molar ratio from 1:4 to 1:5 decreased the biodiesel conversion yield from 57.62 to 30.25% (47.50%), from 68.65 to 62.51% (8.94%) and from 60.68 to 54.52% (11.29%) for the reaction temperatures of 40, 45 and 50°C, respectively. However, the conversion yield of biodiesel at the 4 h in solvent-free system increased from 68.57 to 76.85% (12.07%), from 84.49 to 87.47% (3.40%) and from 50 to 79.89% (37.41%) with increases in oil : alcohol



(a) 4th hour

(b) 8th hour

(c) 12th hour

(d) 16th hour

Figure 5.1. Effect of oil : alcohol (2-butanol) molar ratio on biodiesel conversion yield by *Candida antarctica* (Novozyme 435) at different reaction temperatures and reaction times with and without solvent (WS= with solvent, WOS= without solvent).

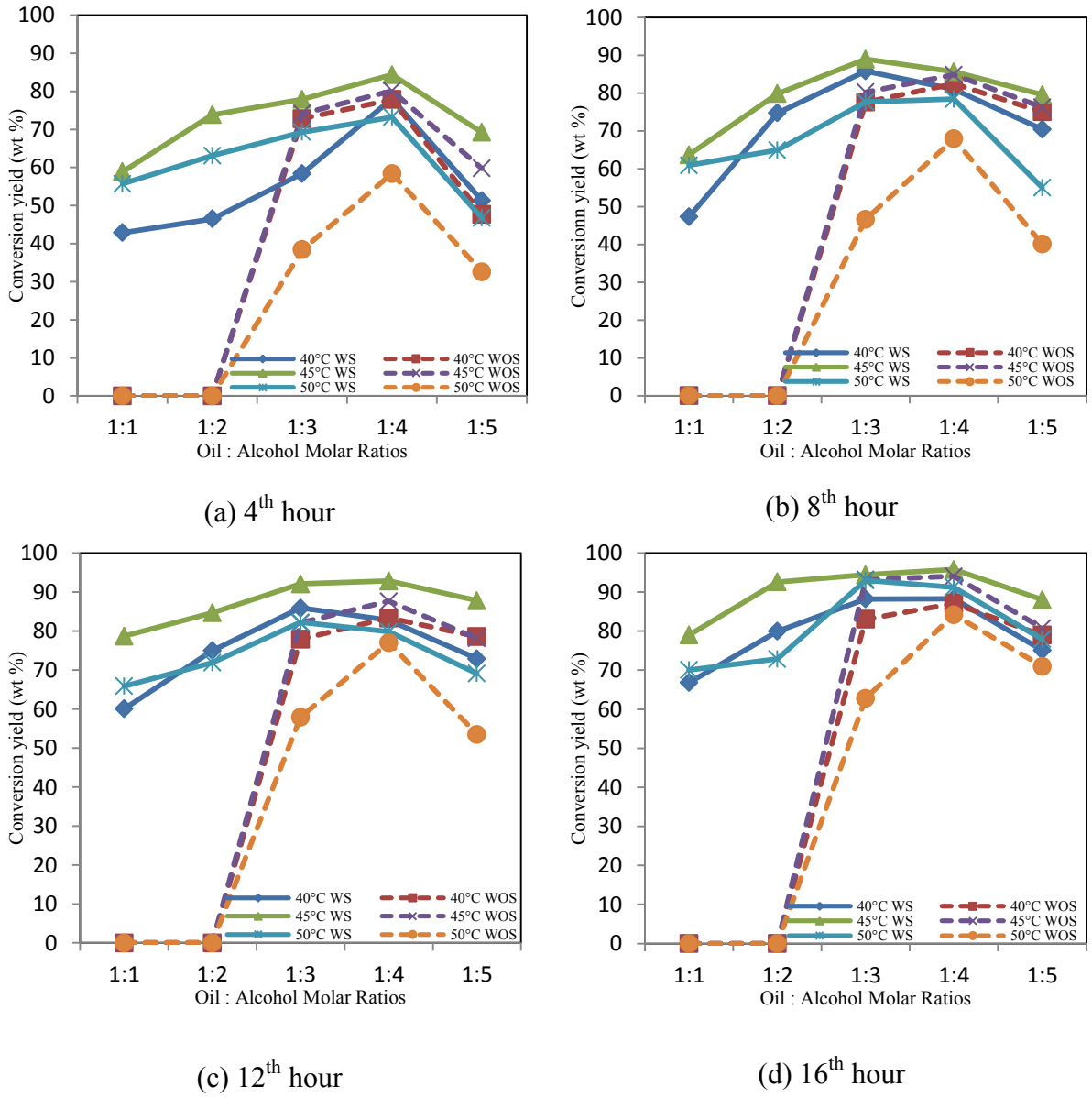


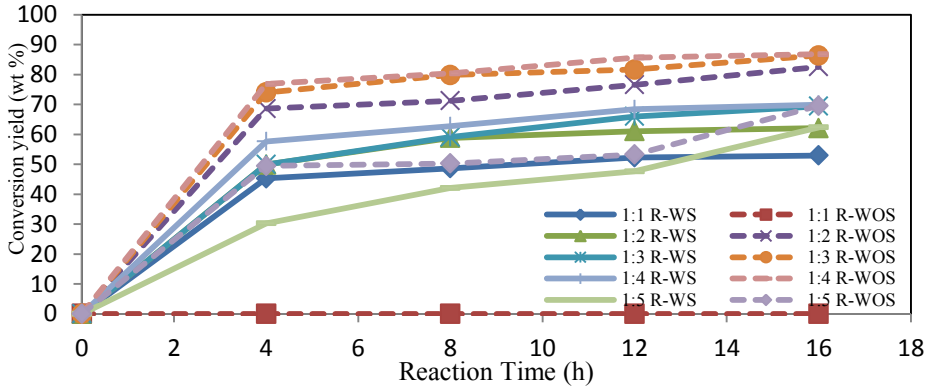
Figure 5.2. Effect of oil : alcohol (methanol) molar ratio on biodiesel conversion yield by experimental enzyme (NS88001) at different reaction temperatures and reaction times with and without solvent (WS= with solvent, WOS= without solvent).

molar ratios from 1:2 to 1:4 for the reaction temperatures of 40, 45 and 50°C, respectively. No conversion yield was observed in the solvent -free system at the oil : alcohol molar ratio of 1:1. A further increase in oil : alcohol molar ratios from 1:4 to 1:5, decreased the biodiesel conversion yield from 76.85 to 49.45% (35.65%), from 87.47 to 84.62% (3.25%) and from 79.89 to 37.67% (52.55%) for the reaction temperatures of 40, 45 and 50°C respectively. Similar trends were observed at the 8, 12 and 16 h at all reaction temperatures (40, 45 and 50°C) with and without solvent.

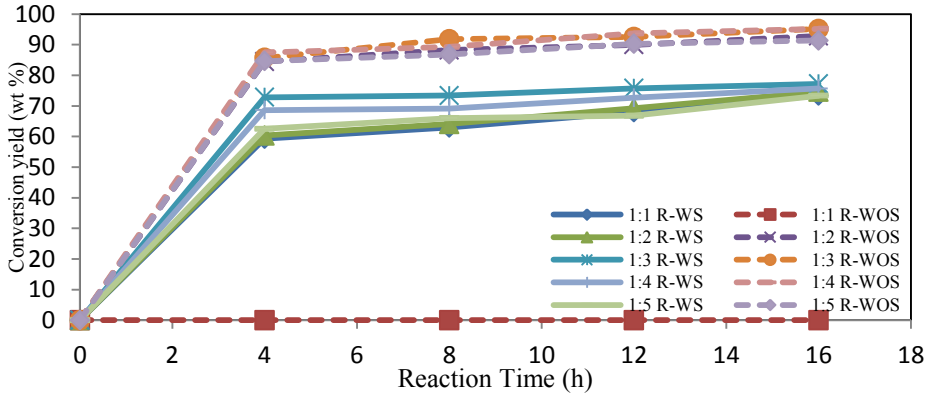
Figure 5.2 shows the effect of oil : alcohol molar ratios on the biodiesel conversion yield by NS88001. The conversion yield of biodiesel in the solvent system at the 4 h increased from 42.87 to 78.07% (82.13%), from 58.9 to 84.35% (43.20%) and from 55.20 to 73.24% (31.49%) with increases in the oil : alcohol molar ratios from 1:1 to 1:4 for the reaction temperatures of 40, 45 and 50°C, respectively. A further increase in the oil : alcohol molar ratio from 1:4 to 1:5 decreased the biodiesel conversion yield from 78.07 to 51.31% (34.27%), from 84.35 to 69.2% (17.96%) and from 73.24 to 46.73% (36.19%) for the reaction temperatures of 40, 45 and 50°C, respectively. However, in the solvent-free system, the conversion yield of biodiesel at the 4 h increased from 72.80 to 77.86% (6.95%), from 74.16 to 80.1% (8%) and from 38.4 to 58.4% (52.08%) with increases in the oil : alcohol molar ratios from 1:2 to 1:4 for the reaction temperatures of 40, 45 and 50°C, respectively. A further increase in the oil : alcohol from 1:4 to 1:5 decreased the biodiesel conversion yield from 77.86 to 47.69% (38.74%), from 80.1 to 59.74% (25.41%) and from 58.4 to 32.6% (44.17%) for the reaction temperatures of 40, 45 and 50°C, respectively. Similar trends were observed at the 8, 12 and 16 h at all reaction temperature (40, 45 and 50°C) with and without solvent.

5.2.2. *Effect of Reaction Time*

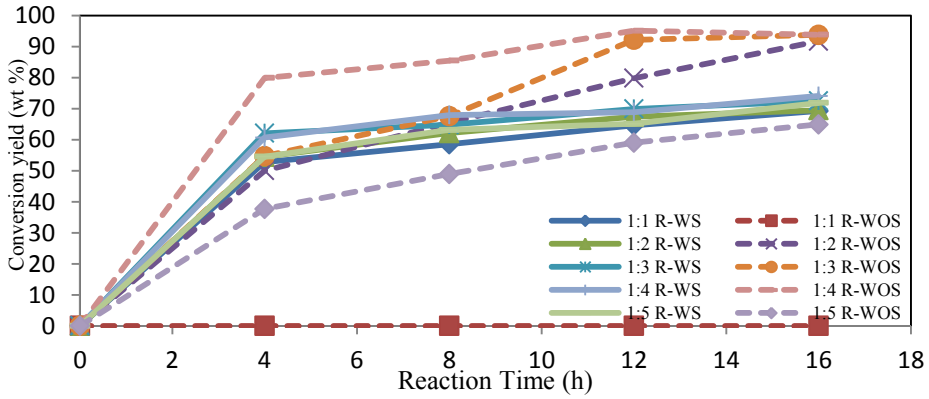
Figures 5.3 and 5.4 show the effect of reaction time on the conversion yield using the two enzymes at different reaction temperatures, oil: alcohol molar ratios, reaction times and solvent systems. Generally, there was an initial rapid increase in the biodiesel conversion yield by the enzymes catalysts (Novozyme 435 and NS88001) with increases in reaction time



(a) 40°C

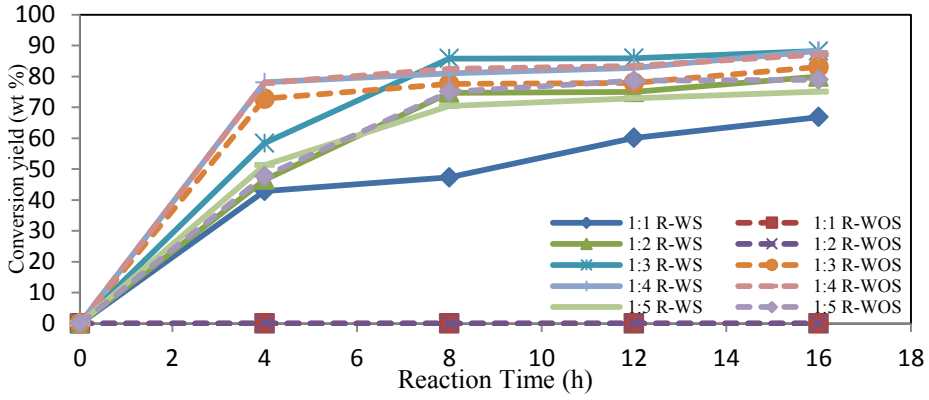


(b) 45°C

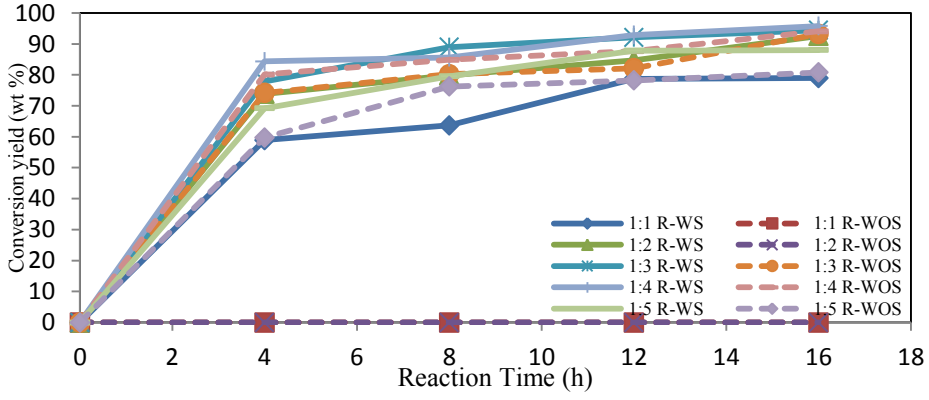


(c) 50°C

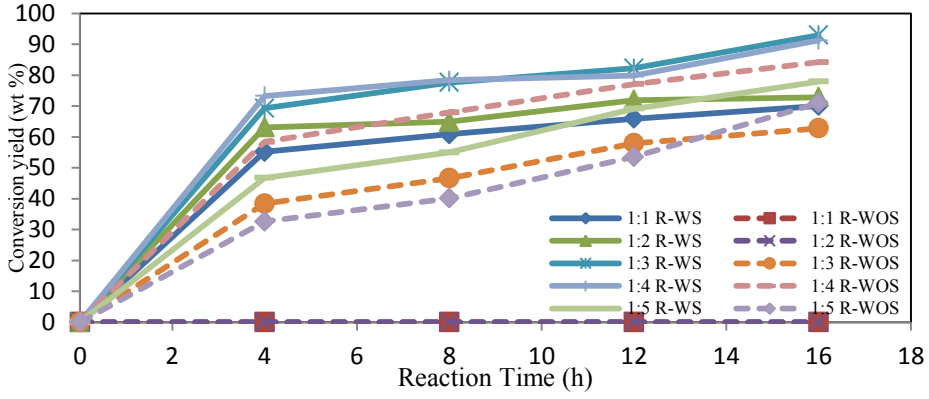
Figure 5.3. Effect of reaction time on biodiesel conversion yield by *Candida antarctica* (Novozyme 435) at different reaction temperatures and oil : alcohol (2-butanol) molar ratios with and without solvent (R= oil : alcohol molar ratios, WS= with solvent and WOS= without solvent).



(a) 40°C



(b) 45°C



(c) 50°C

Figure 5.4. Effect of reaction time on biodiesel conversion yield by experimental enzyme (NS88001) at different reaction temperatures and oil : alcohol (methanol) molar ratios with and without solvent (R= oil : alcohol molar ratios, WS= with solvent and WOS= without solvent).

during the first 4 hours followed by a gradual increase thereafter till the end of the experiment (16 h) for all reaction temperatures (40, 45 and 50°C) and oil : alcohol molar ratios (1:1, 1:2, 1:3, 1:4 and 1:5) with and without solvent.

Figure 5.3 shows the effect of reaction time on the biodiesel conversion yield by Novozyme 435. The conversion yield of biodiesel in a solvent system at the 40°C reached 45.26%, 49.85%, 49.93%, 57.62% and 30.25% for the oil : alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. Further increases in the reaction time from 4 h to 16 h, increased the biodiesel conversion yield from 45.26 to 52.94% (16.9%), from 49.85 to 62.06% (24.49%), from 49.93 to 69.39% (38.97%), from 57.62 to 69.85% (21.22%) and from 30.25 to 62.31% (105.98%) for the oil : alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, in solvent-free system, the biodiesel conversion yield of biodiesel at 40°C reached 68.57%, 73.98%, 76.85% and 49.45% after 4 h for the oil : alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. No reaction was observed at the 1:1 oil : alcohol molar ratio at the 40°C in the solvent-free system. Further increases in reaction time from 4 h to 16 h, increased the biodiesel conversion yield from 68.57 to 82.53% (20.35%), from 73.98 to 86.35% (16.72%), from 76.85 to 86.79% (12.93%) and from 49.45 to 69.56% (40.66%) for the oil : alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. Similar trends were observed at the 45 and 50°C at the oil : alcohol molar ratios of of 1:1, 1:2, 1:3, 1:4 and 1:5 with and without solvent and no reaction was also observed at the 1:1 oil : alcohol molar ratio for the 45 and 50°C in solvent-free system.

Figure 5.4 shows the effect of reaction time on the biodiesel conversion yield by NS88001. The conversion yield of biodiesel in a solvent system at the 40°C reached 42.87%, 46.51%, 58.3%, 78.07% and 51.31% for the oil : alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. Further increases in the reaction time from 4 h to 16 h, increased the biodiesel conversion yield from 42.87 to 66.82% (55.86%), from 46.51 to 79.97% (71.94%), from 58.39 to 88.23% (51.10%), from 78.07 to 88.26% (13.05%) and from 51.31 to 75.10% (46.36%) for the oil : alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, in solvent-free system, the conversion yield of biodiesel at the 40°C reached 72.80%, 77.86% and 47.69% after 4 h for the oil : alcohol molar ratios of 1:3, 1:4 and 1:5,

respectively. No reaction was observed at 1:1 and 1:2 oil : alcohol molar ratio at the 40°C in solvent-free system. Further increases in reaction time from 4 h to 16 h increased the biodiesel conversion yield from 72.80 to 83.05% (14.95%), from 77.86 to 87.09% (11.85%) and from 47.69 to 78.97% (65.59%) the oil : alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. Similar trends were observed at the 45 and 50°C for the oil : alcohol molar ratios of 1:3, 1:4 and 1:5 with and without solvent. However, no reactions were observed with the 1:1 and 1:2 oil : alcohol molar ratios at the 45 and 50°C in solvent-free system.

5.2.3. *Effect of Reaction Temperature*

Figures 5.5 and 5.6 show the effect of reaction temperature on the biodiesel conversion using the two enzymes at different reaction times, reaction temperatures, oil : alcohol molar ratios and solvent systems. There was an increase in biodiesel conversion yield by the enzymes Novozyme 435 and NS88001 when the reaction temperature was increased from 40 to 45°C followed by a decrease in conversion yield of biodiesel when the reaction temperature was further increase from 45 to 50°C for all reaction times (4, 8, 12 and 16 hours) and oil : alcohol molar ratios (1:1, 1:2, 1:3, 1:4 and 1:5) with and without solvent systems. No reactions were observed at 35°C reaction temperature at all reaction time (4, 8, 12 and 16 h) and all oil : alcohol molar ratios (1:1, 1:2, 1:3, 1:4 and 1:5) with and without solvent.

Figure 5.5 shows the effect of reaction temperature on the biodiesel conversion yield by Novozyme 435. The conversion yield of biodiesel at the 4 h increased from 45.26 to 59.29% (30.99%), from 49.85 to 60.31% (20.98%), from 49.93 to 72.81% (45.82%), from 57.62 to 68.65% (19.14%), from 30.25 to 62.51% (106.64%) when the reaction temperature was increased from 40 to 45°C for the oil : alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. A further increase in reaction temperature from 45 to 50°C, decreased the conversion yield from 59.29 to 52.70% (11.11%), from 60.31 to 54.46% (9.69%), from 72.81 to 62.08% (14.73%), from 68.65 to 60.68% (11.60%), from 62.51 to 54.52% (12.78%) for the oil : alcohol molar ratios 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. However, in solvent-free system, the conversion yield of biodiesel at the 4 h increased from 68.57 to 84.49% (23.21%), from 73.98 to 85.67% (15.80%), from 76.85 to 87.47% (13.81%), from 49.45 to

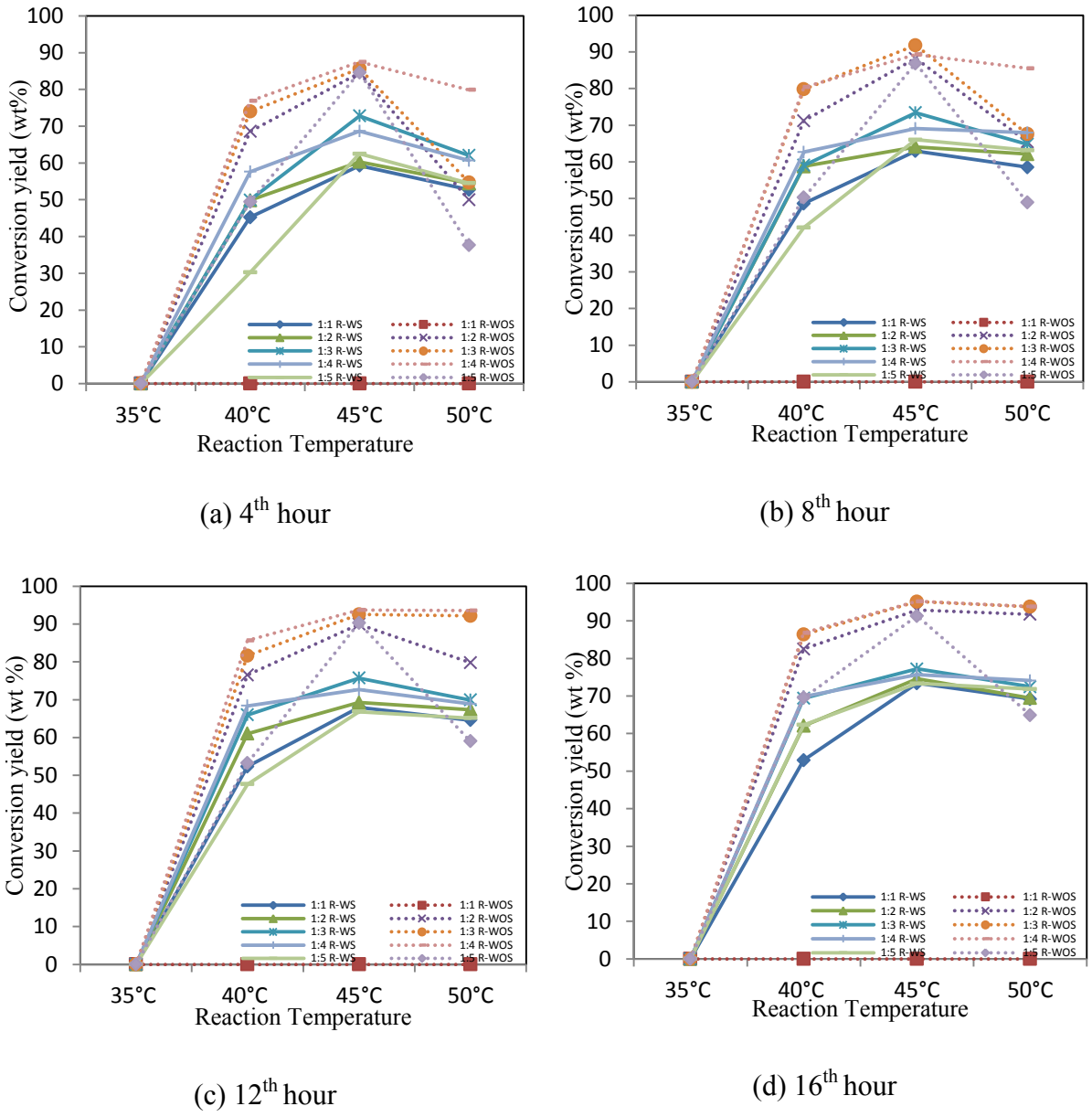
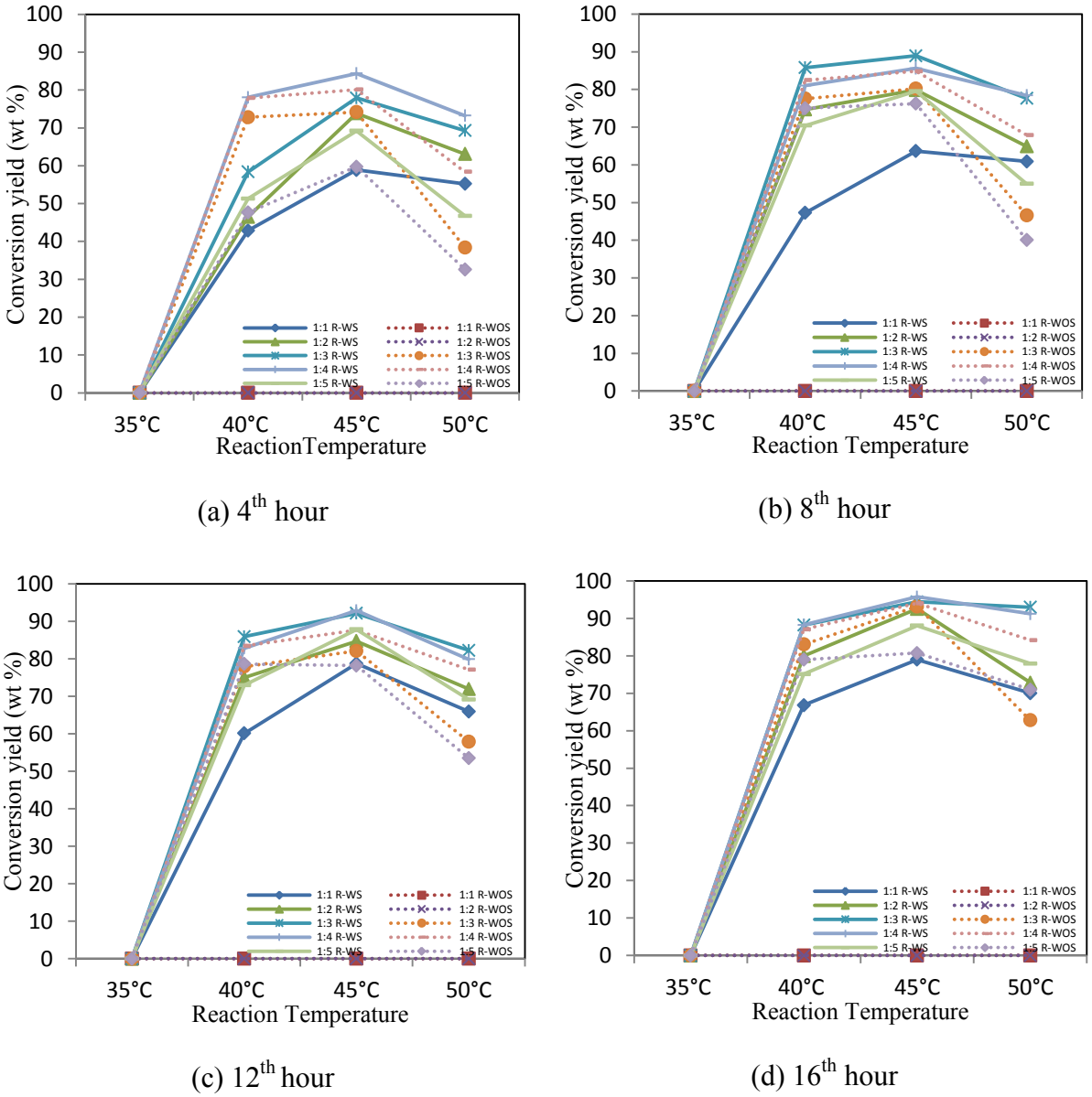


Figure 5.5. Effect of reaction temperature on the biodiesel conversion yield by *Candida antarctica* (Novozyme 435) at different oil : alcohol (2-butanol) molar ratios and reaction times with and without solvent (R= oil : alcohol molar ratios, WS= with solvent and WOS= without solvent).



(a) 4th hour

(b) 8th hour

(c) 12th hour

(d) 16th hour

Figure 5.6. Effect of reaction temperature on the biodiesel conversion yield by experimental enzyme (NS88001) at different oil : alcohol (methanol) molar ratios and reaction times with and without solvent (R= oil : alcohol molar ratios, WS= with solvent and WOS= without solvent).

84.62% (71.12%) when the reaction temperature was increased from 40 to 45°C for the oil : alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. No reaction was observed at 1:1 oil : alcohol molar ratio at 4 h in solvent-free system. A further increases in reaction temperature from 45 to 50°C decreased the conversion yield from 84.49 to 50% (40.82%), from 85.67 to 54.70% (36.15%), from 87.47 to 79.89% (8.66%), from 84.62 to 37.67% (55.48%) for the oil : alcohol molar ratios 1:2, 1:3, 1:4 and 1:5, respectively. Similar trends were observed with the 8, 12 and 16 h at the oil : alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5 with and without solvent and no reactions were observed at the 1:1 oil : alcohol molar ratio for the 8, 12 and 16 h in solvent-free system.

Figure 5.6 shows the effect of reaction temperature on the biodiesel conversion yield by NS88001. The conversion yield of biodiesel at the 4 h increased from 42.87 to 58.90% (37.39%), from 46.51 to 73.85% (58.78%), from 58.39 to 77.89% (33.39%), from 78.07 to 84.35% (8.04%), from 51.31 to 69.20% (34.86%) when the reaction temperature was increased from 40 to 45°C for the oil : alcohol molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. A further increase in reaction temperature from 45 to 50°C decreased the biodiesel conversion yield from 58.90 to 55.20% (5.43%), from 73.85 to 63.10% (14.55%), from 77.89 to 69.28% (11.05%), from 84.35 to 73.24% (13.17%), from 69.20 to 46.73% (32.47%) 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 conversion yield of biodiesel at the 4 h increased from 72.80 to 74.16% (1.86%), from 77.86 to 80.10% (2.87%), from 47.69 to 59.74% (25.26%) for the oil : alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. No reactions were observed at the 1:1 and 1:2 oil : alcohol molar ratios at the 4 h in solvent-free system. A further increase in reaction temperature from 45 to 50°C decreased the conversion yield of biodiesel from 74.16 to 38.40% (48.22%), from 80.10 to 58.40% (27.09%), from 59.74 to 32.60% (45.76%) 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 at the oil : alcohol molar ratios of 1:3, 1:4 and 1:5 with and without solvent. No reactions were observed at the 1:1 oil : alcohol molar ratio for the 8, 12 and 16 h in solvent-free system.

5.2.4. ***Effect of Solvent***

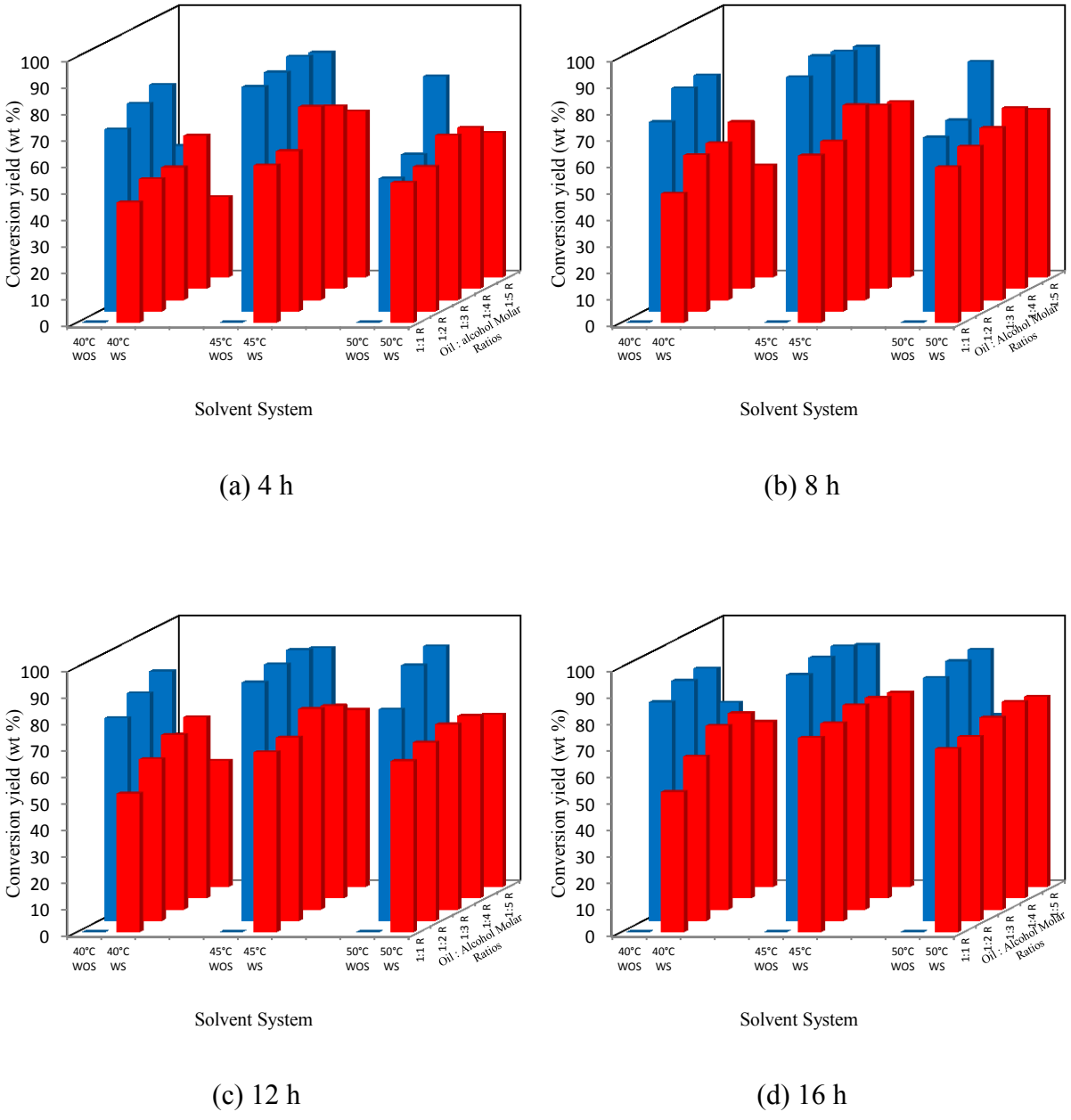
Figure 5.7 shows the effect of solvent on the biodiesel conversion using Novozyme 435 at different reaction times, reaction temperatures and oil : alcohol molar ratios. No reaction was observed at the 1:1 oil : alcohol molar ratio without solvent. The solvent-free system achieved higher biodiesel conversion yield than the solvent system at all other oil : alcohol molar ratios, reaction temperatures and reaction times.

Figure 5.8 shows the effect of solvent system on the biodiesel conversion using NS88001 at different reaction times, reaction temperatures and oil : alcohol molar ratios. No reaction was observed at the 1:1 and 1:2 oil : alcohol molar ratios without solvent. The solvent-free system achieved high biodiesel conversion yield at all the oil : alcohol molar ratios at the 40°C and 4 h. However, the solvent system achieved higher conversion yield of biodiesel than the solvent-free system at all other oil : alcohol molar ratios, reaction temperatures and reaction times.

5.3. Enzymatic Transesterification by a Combination of Enzyme Catalysts

The transesterification results of the individual enzymes showed that the reaction temperature of 45°C was the optimum. Therefore, the enzymatic transesterification by a combination of the two enzymes was carried out at the optimum reaction temperature of 45°C to investigate the effects of alcohol type (methanol and 2-butanol), reaction time (4, 8, 12 and 16 hour) and oil : alcohol molar ratio (1:1, 1:2, 1:3, 1:4 and 1:5) on biodiesel conversion yield in solvent and solvent-free systems. The results are shown in Tables 5.8 and 5.9.

Tables 5.10 and 5.11 show the analysis of variance and Tukey's grouping performed on the biodiesel conversion yield by the combination of *Candida antarctica* Novozyme 435 and experimental catalyst NS88001. The effects of alcohol, molar ratios, reaction temperature and solvent system 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



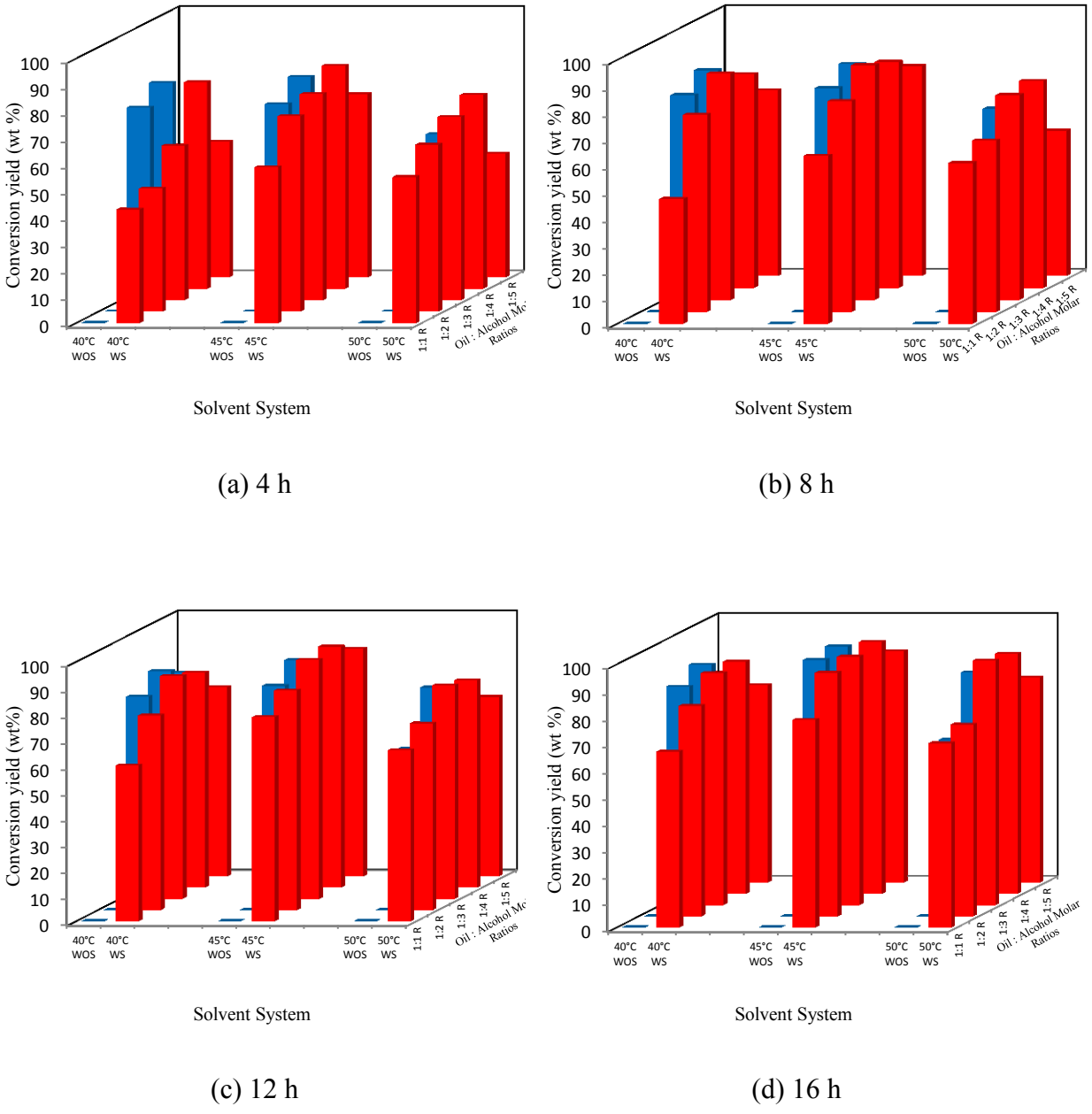
(a) 4 h

(b) 8 h

(c) 12 h

(d) 16 h

Figure 5.7. Effect of solvent system on the biodiesel conversion yield by *Candida antarctica* (Novozyme 435) at different oil : alcohol (2-butanol) molar ratios and reaction times with and without solvent (R= oil : alcohol molar ratios, WS= with solvent and WOS= without solvent).



(a) 4 h

(b) 8 h

(c) 12 h

(d) 16 h

Figure 5.8. Effect of solvent system on the biodiesel conversion yield by experimental catalyst (NS88001) at different oil : alcohol (methanol) molar ratios and reaction times with and without solvent (R= oil : alcohol molar ratios, WS= with solvent and WOS= without solvent).

Table 5.8. Biodiesel yield (wt%) from animal tallow using combined Novozyme 435 and NS88001 with different alcohols at a reaction temperature of 45 °C and different reaction times using hexane as solvent.

Reaction Time (h)	Oil: Alcohol Molar Ratio	Alcohol Type	
		Methanol	2-Butanol
4	1:1	33.65 ±0.67	42.60 ± 0.85
	1:2	36.90 ±0.74	59.40 ± 1.19
	1:3	39.70 ±0.79	72.40 ± 1.45
	1:4	42.30 ±0.85	75.30 ± 1.51
	1:5	30.80 ±0.62	64.20 ± 1.28
8	1:1	45.09 ± 0.90	53.10 ± 1.06
	1:2	46.40 ± 0.93	65.90 ± 1.32
	1:3	59.80 ± 1.20	80.30 ± 1.61
	1:4	64.50 ± 1.29	85.40 ± 1.71
	1:5	46.80 ± 0.94	73.60 ± 1.47
12	1:1	56.09 ± 1.12	65.71 ± 1.31
	1:2	57.06 ± 1.14	82.92 ± 1.66
	1:3	72.61 ± 1.45	83.05 ± 1.66
	1:4	79.30 ± 1.59	95.85 ± 1.92
	1:5	61.12 ± 1.22	75.90 ± 1.52
16	1:1	70.30 ± 1.41	79.14 ± 1.58
	1:2	86.23 ± 1.72	86.52 ± 1.73
	1:3	93.86 ± 1.88	86.76 ± 1.74
	1:4	91.29 ± 1.83	96.67 ± 1.93
	1:5	73.76 ± 1.48	85.90 ± 1.72

Table 5.9. Biodiesel yield (wt%) from animal tallow using combined Novozyme 435 and NS88001 with different alcohols at a reaction temperature of 45 °C and different reaction times without solvent.

Reaction Time (h)	Oil: Alcohol Molar Ratio	Alcohol Type	
		Methanol	2-Butanol
4	1:1	Not extractable	Not extractable
	1:2	58.96 ± 1.18	55.60 ± 1.11
	1:3	67.24 ± 1.34	65.91 ± 1.32
	1:4	67.20 ± 1.34	66 ± 1.32
	1:5	52.99 ± 1.06	63.16 ± 1.26
8	1:1	Not extractable	Not extractable
	1:2	73.80 ± 1.48	66.33 ± 1.33
	1:3	80.55 ± 1.61	70.98 ± 1.42
	1:4	74.91 ± 1.50	84.97 ± 1.70
	1:5	62.23 ± 1.24	73.64 ± 1.47
12	1:1	Not extractable	Not extractable
	1:2	67.47 ± 1.35	76.16 ± 1.52
	1:3	95.87 ± 1.92	76.5 ± 1.53
	1:4	89.51 ± 1.79	95.51 ± 1.91
	1:5	63.28 ± 1.27	63.70 ± 1.27
16	1:1	Not extractable	Not extractable
	1:2	52.86 ± 1.06	68.81 ± 1.38
	1:3	85.40 ± 1.71	74.93 ± 1.50
	1:4	79.54 ± 1.59	87.03 ± 1.74
	1:5	35.94 ± 0.72	60.65 ± 1.21

Table 5.10. ANOVA of biodiesel conversion yield using the Enzymes Novozyme 435 and NS88001 in combination at different oil : alcohol molar ratios, reaction times and alcohols.

Source	DF	SS	MS	F	P
Total	239	158738			
Model					
MR	4	79332	19832.9	1332.71	0.001
RTI	3	14911	4970.2	333.98	0.001
SY	1	7278	7278.2	489.07	0.001
AL	1	5001	5001.1	336.06	0.001
MR*RTI	12	1997	166.4	11.18	0.001
MR*SY	4	30389	7597.2	510.50	0.001
MR*AL	4	1787	446.7	30.02	0.001
RTI*SY	3	8417	2805.7	188.53	0.001
RTI*AL	3	520	173.5	11.66	0.001
SY*AL	1	2960	2959.6	198.87	0.001
MR*RTI*SY	12	1523	126.9	8.53	0.001
MR*RTI*AL	12	1487	123.9	8.33	0.001
MR*RTI*SY*AL	12	651	54.3	3.65	0.001
Error	167	2485	14.9		

DF: Degree of freedom
SS: Sum of square
MS: Mean of square
R^2 : 99.28%
MR: Molar ratios
RTI: Reaction time
SY: Solvent
AL: Alcohol type

Table 5.11. Tukey's Grouping of the biodiesel conversion yield for the various parameters using a combination of the two enzymes.

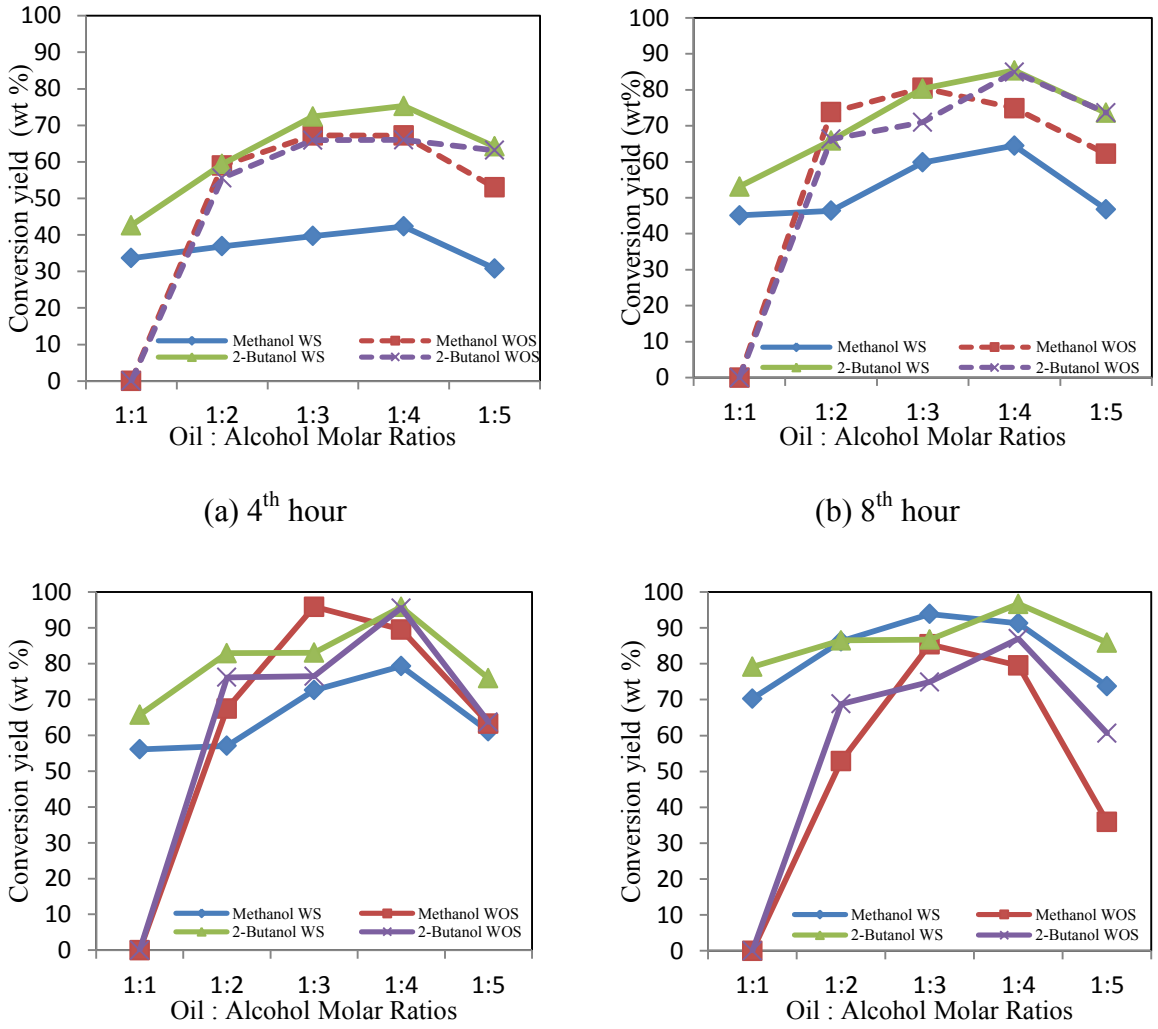
Factors	Level	N	Mean (%)	Tukey Grouping
Oil : Alcohol Molar ratios	1:1	48	27.86	A
	1:2	48	65.61	B
	1:3	48	75.35	C
	1:4	48	79.19	C
	1:5	48	61.73	B
Reaction time (hours)	4	60	49.72	A
	8	60	60.41	B
	12	60	67.88	C
	16	60	69.78	C
Alcohol type	Methanol	120	57.38	B
	2- butanol	120	66.51	A
Solvent	Hexane	120	67.45	A
	Without Hexane	120	56.44	B

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

Grouping indicated that the oil : alcohol molar ratios 1:2 and 1:5 were not significantly different from one another at the 0.05 level. Also, the oil : alcohol molar ratios 1:3 and 1:4 were not significantly different from one another at the 0.05 level. The highest mean yield of 79.19% was obtained with the 1:4 molar ratio. The reaction times 4, 8 and 12 h were significantly different from each other at the 0.05 level but the reaction times 12 and 16 h were not significantly different from one another at the 0.05 level. The highest mean yield of 69.78% was obtained with 16 hour. The two alcohols were significantly different from each other at the 0.05 level. The highest mean yield of 66.51% was obtained with 2-butanol. The two solvent systems were significantly different from each other at the 0.05 level. The highest mean yield of 67.45% was obtained with hexane as solvent system.

5.3.1. *Effect of Oil : Alcohol Molar Ratio*

Figure 5.9 shows the effects of oil : alcohol molar ratio on the biodiesel conversion yield using a combination of Novozyme 435 and NS88001 at different reaction times, oil : alcohol molar ratios and solvent systems. Generally, there was an increase in the biodiesel conversion yield by the combination of enzyme catalysts (Novozyme 435 and NS88001) with increases in the oil : alcohol molar ratio from 1:1 to 1:4 followed by a decrease in conversion yield when the oil : alcohol molar ratios was further increased from 1:4 to 1:5 for all reaction times (4, 8, 12 and 16 h) with both alcohols (methanol and 2-butanol) with and without solvent. The conversion yield of biodiesel in the solvent system at the 4 h increased from 33.65 to 42.30% (25.75%) and from 42.60 to 75.30% (76.76%) with increases in the oil : alcohol molar ratio from 1:1 to 1:4 for methanol and 2-butanol, respectively. A further increase in the oil : alcohol molar ratio from 1:4 to 1:5 decreased the biodiesel conversion yield from 42.30 to 30.80% (27.18%) and from 75.30 to 64.20% (14.74%) for methanol and 2-butanol, respectively. However, in the solvent-free system, the conversion yield of biodiesel at the 4 h increased from 58.96 to 67.20% (13.97%) and from 55.60 to 66%(18.70%) with increases in the oil : alcohol molar ratio from 1:2 to 1:4 for methanol and 2-butanol, respectively. No reaction was observed at the 1:1 oil : alcohol molar ratio at the 4 h in the solvent-free system. A further increase in the oil : alcohol molar ratios from 1:4 to 1:5 decreased the biodiesel conversion yield from 67.20 to 52.99% (21.14%) and from 66 to



(a) 4th hour

(b) 8th hour

(c) 12th hour

(d) 16th hour

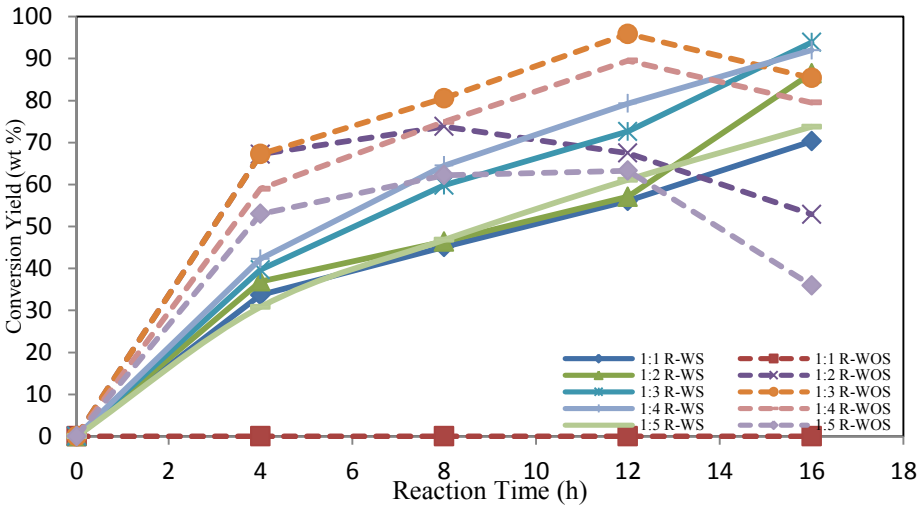
Figure 5.9. Effect of molar ratio on the biodiesel conversion yield using a combination of Novozyme 435 and NS88001 at different reaction times and alcohols with and without solvent (WS= With solvent, WOS= Without solvent).

63.16% (4.30%) for methanol and 2-butanol, respectively. Similar trends were observed at the reaction times of 8, 12 and 16 h for the two alcohols (methanol and 2-butanol) with and without solvent but no reactions were observed at the 1:1 oil : alcohol molar ratio for the 8, 12 and 16 h in the solvent-free system.

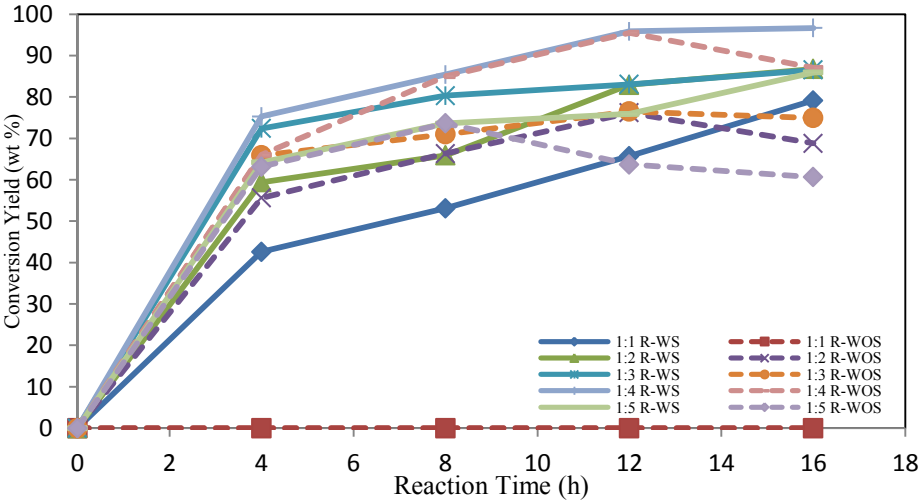
5.3.2. *Effect of Reaction Time*

Figure 5.10 shows the effect of reaction time on the biodiesel conversion yield using a combination of the enzymes Novozyme 435 and NS88001. In the solvent system, there was a rapid increase in the biodiesel conversion yield by the combination of Novozyme 435 and NS88001 with increases in the reaction time during the first 4 hours followed by a gradual increase till the end of the experiment (16 h) for both alcohols (methanol and 2-butanol) at all oil : alcohol molar ratios (1:1, 1:2, 1:3, 1:4 and 1:5). However, in the solvent-free system, the biodiesel conversion yield rapidly increased during the first 4 hours followed by gradual increase till the12 h for both alcohols (methanol and 2-butanol) at the oil : alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5. A further increases in reaction time from 12 h to 16 h decreased the conversion yield for both alcohols (methanol and 2-butanol) at the oil : alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5.

The conversion yield of biodiesel in a solvent system reached 33.65%, 36.90%, 39.70%, 42.30% and 30.80% for methanol and 42.60%, 59.40%, 72.40%, 75.30% and 64.20% for 2-butanol after 4 h for the molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. Further increases in the reaction time from 4 h to 16 h increased the biodiesel conversion yield from 33.65 to 70.30 (108.91%), from 36.90 to 86.23% (133.68%), from 39.70 to 93.8% (136.27%), from 42.30 to 91.29% (115.81%) and from 30.80 to 73.76% (139.48%) for methanol and from 42.60 to 79.14% (85.77%), from 59.40 to 86.76% (46.06%), from 72.40 to 86.52% (19.50%), from 75.30 to 96.67% (28.37%) and from 64.20 to 85.90% (33.80%) for 2-butanol for the 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 reached 58.96%, 67.24%, 67.20% and 52.99% for methanol and 55.60%, 65.91%, 66% and 63.16% for 2-butanol after 4 h for the oil : alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. No reactions were observed at the 1:1 oil : alcohol molar ratio in the solvent-free system. Further increases in reaction time



(a) Methanol



(b) 2-Butanol

Figure 5.10. Effect of reaction time on the biodiesel conversion yield by a combination of Novozyme 435 and NS88001 using different alcohols and oil : alcohol molar ratios with and without solvent (R= oil : alcohol molar ratios, WS= with solvent and WOS= without solvent).

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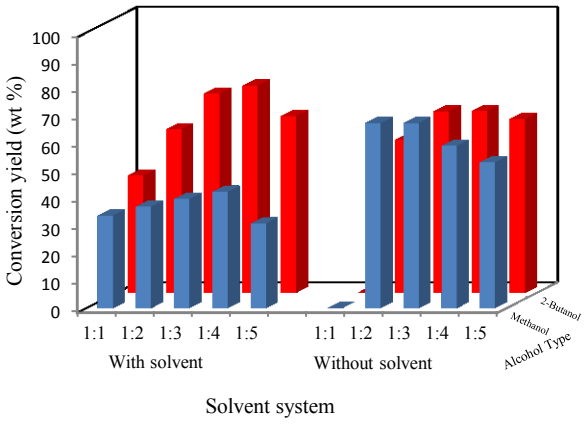
from 4 h to 12 h increased the biodiesel conversion yield from 58.96 to 67.47% (14.43%), from 67.24 to 95.87% (42.66%), from 67.20 to 89.51% (33.19%) and from 52.99 to 63.28% (19.41%) for methanol and from 55.60 to 76.16% (36.97%), from 65.91 to 76.5% (16.06%), from 66 to 95.51% (44.71%), and from 63.16 to 63.70% (0.85%) for 2-butanol for the oil : alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively. A further increase in reaction time from 12 h to 16 h decreased the biodiesel conversion yield from 67.47 to 52.86% (21.65%), from 95.87 to 85.40% (10.92%), from 89.51 to 79.54% (11.13%) and from 63.28 to 35.94% (43.20%) for methanol and from 76.16 to 68.81% (9.54%), from 76.5 to 74.93% (2.05%), from 95.51 to 87.03% (8.87%) and from 63.70 to 60.65% (4.78%) for the oil : alcohol molar ratios of 1:2, 1:3, 1:4 and 1:5, respectively.

5.3.3. *Effect of Alcohol Type*

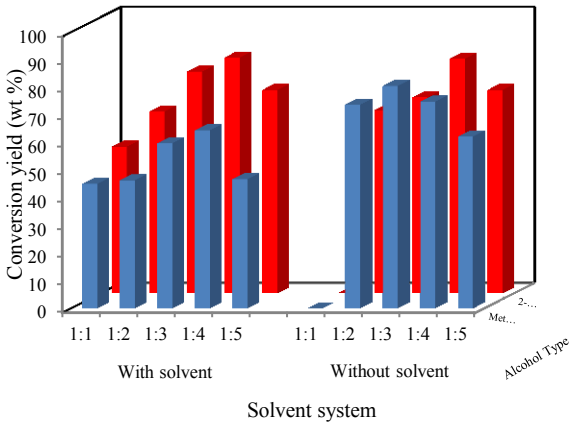
The effect of alcohol on the biodiesel conversion yield at different oil : alcohol molar ratios, reaction time and solvent systems are shown in Figure 5.11. No reaction was observed at the 1:1 oil : alcohol molar ratio without solvent for both alcohols (methanol and 2-butanol). Generally, a higher biodiesel conversion yield was achieved using 2-butanol with most reaction times and oil : alcohol molar ratios without solvent. However, at 8 h reaction time, the 1:2 and 1:3 oil :alcohol molar ratios achieved high conversion yield in solvent-free system for methanol. Also, at 12 h reaction time , the 1:3 oil : alcohol molar ratio achieved high biodiesel conversion yield in solvent-free system with methanol

5.3.4. *Effect of Solvent*

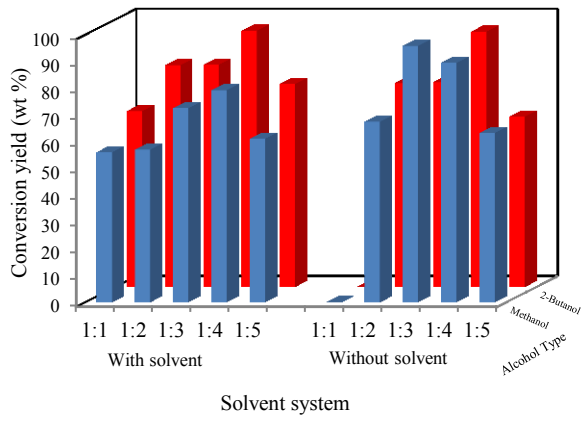
Figure 5.12 shows the effect of solvent system on the biodiesel conversion yield using a combination of Novozyme 435 and NS88001 at different alcohol types, reaction times and oil : alcohol molar ratio. No reaction was observed at the 1:1 oil : alcohol molar ratio without solvent for both alcohols (methanol and 2-butanol). The solvent system achieved the high biodiesel conversion yield at the 4, 8, 12 h reaction times for methanol at all oil : alcohol molar ratios. However, the solvent-free system achieved the higher biodiesel conversion yield at the 16 h reaction time for all oil : alcohol molar ratios. Higher biodiesel conversion



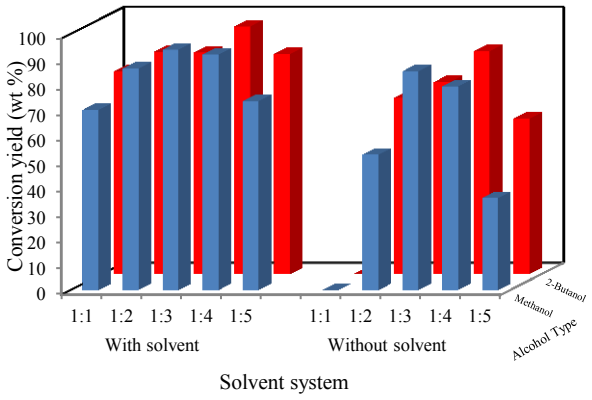
(a) 4th hour



(b) 8th hour

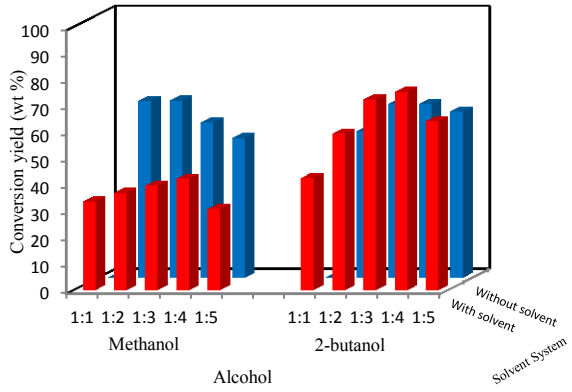


(c) 12th hour

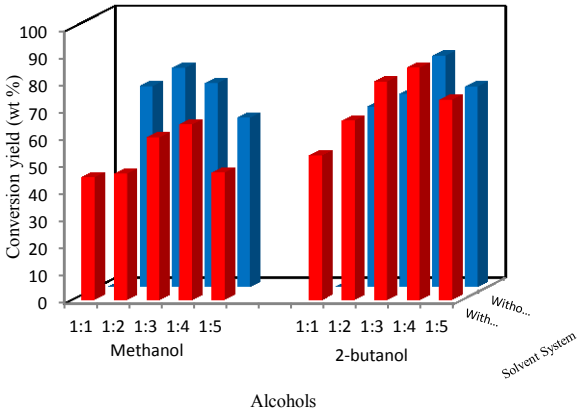


(d) 16th hour

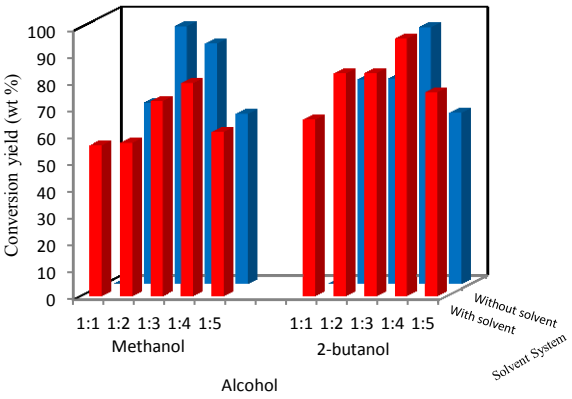
Figure 5.11. Effect of alcohol type on the biodiesel conversion yield using a combination of Novozyme 435 and NS88001 at different molar ratios and alcohols with and without solvent (R= molar ratios, WS= with solvent and WOS= without solvent).



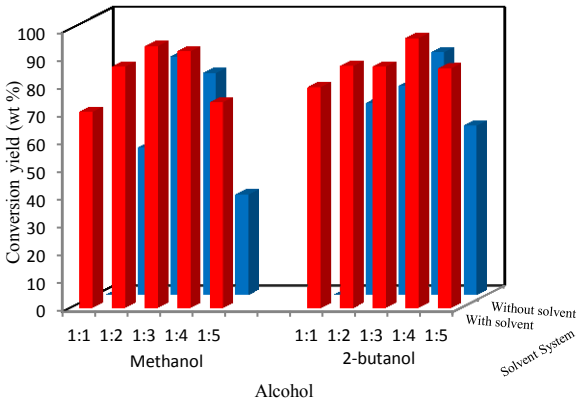
(a) 4 h



(b) 8 h



(c) 12 h



(d) 16 h

Figure 5.12. Effect of solvent system on biodiesel conversion yield using a combination of Novozyme 435 and NS88001 at different molar ratios and alcohols with and without solvent (R= molar ratios, WS= with solvent and WOS= without solvent).

yields were achieved with solvent using 2-butanol at all reaction times and oil : alcohol molar ratios.

5.4. Enzyme Reusability

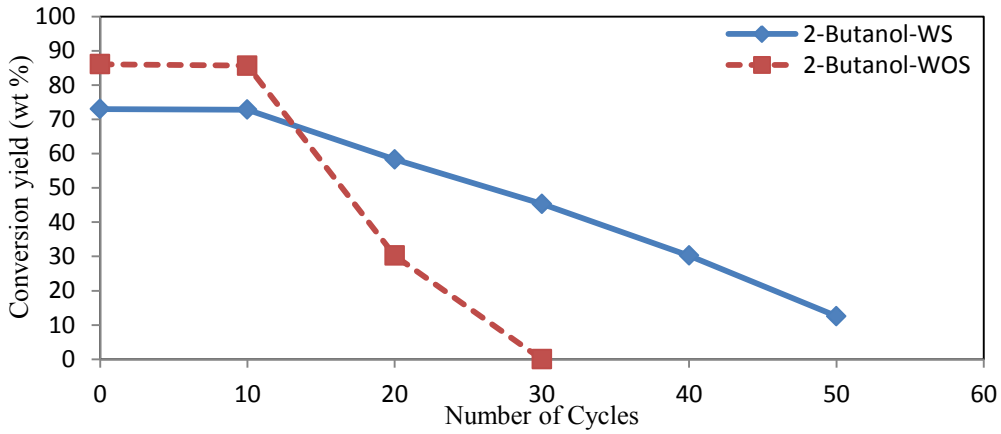
The effect of the number of enzyme cycles on the conversion yield using different enzymes at the optimum conditions (45°C, 1:3 oil : alcohol molar ratio, 8 h and solvent system) is shown in Table 5.12 and Figure 5.13. In the solvent system, there was a gradual decrease in conversion yield by the enzymes Novozyme 435 and NS88001 individually and in a combination with increases in the number of cycles. However, in the solvent-free system, there was a rapid decrease in the biodiesel conversion yield by the enzymes Novozyme 435 and NS88001 individually and in combination with increases in the number of cycles.

In a solvent system, when the number of cycles was increased to 10 cycles, the conversion yield of biodiesel by Novozyme 435 with 2-butanol decreased slightly from 73.02 to 72.81% (0.28%). A further increase in the number of cycles from 10 to 50 decreased the biodiesel conversion yield from 72.81 to 12.56% (82.84%). However, in solvent-free system the conversion yield of biodiesel with 2-butanol decreased from 86.09 to 85.67% (0.48%) with increases in the number of cycles to 10. A further increase in the number of cycles from 10 to 30 decreased the conversion yield from 85.67 to 0.00% (100%). No reaction was observed after 30 cycles. No reaction was observed with methanol as alcohol in both the solvent and solvent-free system.

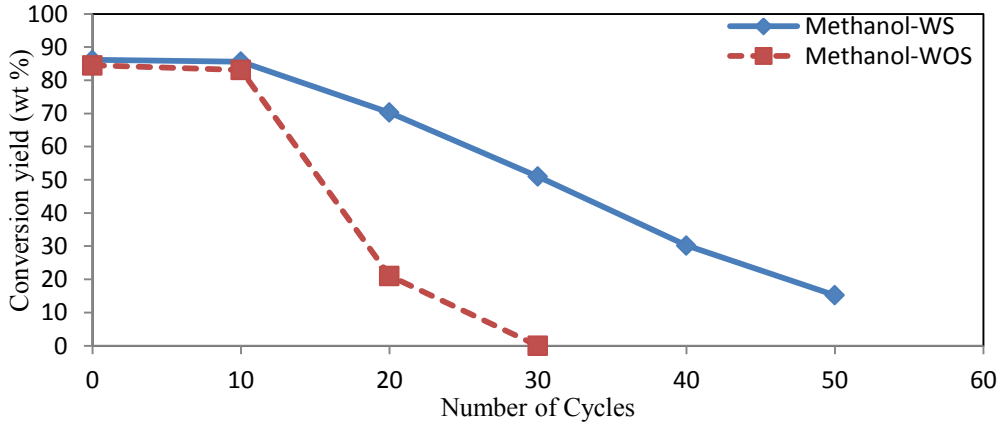
In a solvent system, when the number of cycles was increased to 10, the conversion yield of biodiesel by NS88001 with methanol decreased slightly from 86.12 to 85.6% (0.6%). A further increase in the number of cycles from 10 to 50 decreased the conversion yield from 85.6 to 15.2% (82.24%), respectively. However, in the solvent-free system, the biodiesel conversion yield of biodiesel with methanol decreased gradually from 84.5 to 83.12% (1.63%) with increases in number of cycles to 10 cycles. A further increase in the number of cycles from 10 to 30 decreased the biodiesel conversion yield rapidly from 83.12 to 0.00%

Table 5.12. Reusability of Novozyme 435, NS88001 and Combination of Novozyme 435 and NS88001 with solvent at 45°C for 8 hours.

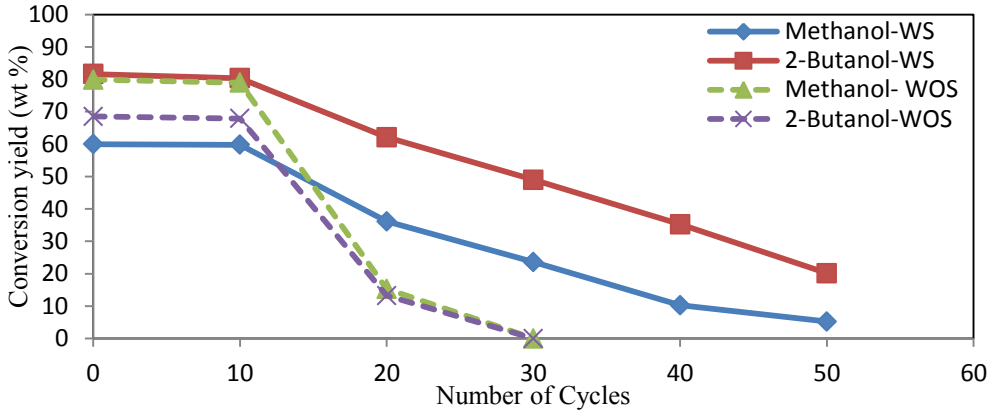
Enzymes	Solvent System	Alcohol	Number of Cycles (yield %)					
			0	10	20	30	40	50
Novozyme 435	With	Methanol	-	-	-	-	-	-
		2-butanol	73.02	72.81	58.29	45.32	30.24	12.56
	Without	Methanol	-	-	-	-	-	-
		2-butanol	86.09	85.67	30.25	-	-	-
NS88001	With	Methanol	86.12	85.6	70.29	50.98	30.21	15.2
		2-butanol	-	-	-	-	-	-
	Without	Methanol	84.5	83.12	21.06	-	-	-
		2-butanol	-	-	-	-	-	-
Combination	With	Methanol	60	59.8	36.13	23.58	10.23	5.20
		2-butanol	81.6	80.3	62.1	48.96	35.26	20.12
	Without	Methanol	79.87	78.95	15.29	-	-	-
		2-butanol	68.52	67.89	13.21	-	-	-



(a) Novozyme 435



(b) NS88001



(c) Combination

Figure 5.13. Reusability of Novozyme 435, NS88001 and Combination of Novozyme 435 and NS88001 with and without solvent at 45°C for 8 hours (WS= With solvent and WOS= Without solvent).

(100%). No reaction was observed after 30 cycles. No reaction was observed with 2-butanol as alcohol in both the solvent and solvent-free system.

In a solvent system, when the number of cycles was increased to 10, the conversion yield of biodiesel by a combination of Novozyme 435 and NS88001 decreased slightly from 60 to 59.8% (0.33%) for methanol and 81.6 to 80.3% (1.59%) for 2-butanol. A further increase in the number of cycles from 10 to 50 decreased the conversion yield from 59.8 to 5.20% (91.3%) for methanol and 80.3 to 20.12% (74.94%) for 2-butanol, respectively. However, in the solvent-free system the conversion yield of biodiesel decreased gradually from 79.87 to 78.95% (1.15%) for methanol and 68.52 to 67.89% (0.91%) for 2-butanol with an increase in the number of cycles to 10 cycles. An further increase in the number of cycles from 10 to 30 cycles decreased the conversion yield from 78.95 to 0.00% (100%) for methanol and 67.89 to 0.00% (100%) for 2-butanol, respectively. No reaction was observed after 30 cycles.

CHAPTER 6. DISCUSSION

6.1. Extraction Profiles of the Raw Material

After melting and homogenizing the animal tallow, the impurities (7.5%) were removed by filtration. The fatty acids analysis by Hilditch procedure (Budge *et al*., 2006) indicated that the homogenized oil contained high percentages of oleic acid (44%), palmitic acid (28%) and stearic acid (26%) as well as lower percentages of myristic acid (1%) and linoleic acid (1%). A high concentration of oleic acid improves the characteristics of biodiesel resulting in a high cetane index and combustion temperature (Robles *et al*., 2009). Biodiesel produced from feedstocks containing a high level of oleic acid showed similar characteristics to these of conventional diesel (Knothe, 2005; Robles *et al*., 2009).Therefore, the biodiesel produced for oil extracted from animal tallow is expected to have good characteristics as a biofuel.

The extracted oil can be transformed to biodiesel by chemical or enzymatic transesterification. Watanabe *et al*. (2002), Dorado *et al*. (2004) and Kulkarni and Dalai (2006) reported that oxidized oil can inhibit the chemical transesterification process and increase the oxidation of methyl esters. Kulkarni and Dalai (2006) stated that an increase in the oxidation of methyl esters might increase the cetane number which tends to delay the ignition time in the engine. However, oxidized vegetable oil did not inhibit the formation of methyl esters from the methanolysis process by *Candida antarctica* lipase (Kulkarni and Dalai, 2006). Nelson *et al*. (1996) and Watanabe *et al*. (2002) reported that oxidation in crude tallow or oil containing high free fatty acids is a common problem and no negative effects of the oxidized oil substrate on the transesterification process by enzyme was observed. Watanabe *et al*. (2002) stated that in the enzymatic process, the oxidized substrate becomes a non recognition site for the enzyme to bind and the process continues with the substrates which are not oxidized. However, the authors stated that using oxidized oil might reduce the biodiesel stability. Nelson *et al*. (1996) reported that the stability of biodiesel can be increased by blending the biodiesel with conventional diesel especially in cold environment. In this study, enzymatic transesterification was carried out and no oxidation stability test was performed on crude tallow oil nor was antioxidants used.

6.2. Enzymatic Transesterification

6.2.1. *Effect of Oil : Alcohol Molar Ratios*

Increasing in the oil : alcohol molar ratio from 1:1 to 1:4 at 4 h with solvent increased the conversion yield of biodiesel for Novozyme 435 by 27.30, 15.78 and 15.14% and when the oil : alcohol molar ratio was further increased from 1:4 to 1:5 at 4 h, the biodiesel conversion yield was decreased by 47.50, 8.94 and 10.15% at the reaction temperatures of 40, 45 and 50°C, respectively. Similar trends were seen with both enzymes for all reaction times with and without solvent.

Chen *et al*. (2006) and Kumari *et al*. (2009) obtained similar results from waste cooking oil and jatropha oil. Chen *et al*. (2006) reported that increasing the oil : alcohol molar ratio from 1:1 to 1:4 promoted the methanolysis reaction with waste cooking oil, but the formation of methyl esters decreased when the oil : alcohol molar ratios was increased from 1:4 to 1:5 due to an excess of methanol in the system. They suggested that the excess methanol distorted the essential water layer needed to stabilize the structure of the enzyme. It is likely that this could explain, the similar results obtained by the enzyme catalyst with waste animal fats in the present study. Kumari *et al*. (2009) reported that the biodiesel conversion yield increased when the oil : alcohol molar ratio was increased up to 1:4 and then decreased when the oil : alcohol molar ratio was further increased to 1:5. The decrease in the formation of methyl esters was similar to that observed in the present study.

In this study, the highest biodiesel conversion yields of 77.23% and 95.75% were achieved using *Candida antarctica* Novozyme 435 and NS88001 at 25% enzyme concentration, respectively. Nelson *et al*. (1996) reported a similar biodiesel conversion yield of (83.8%) using *Candida antarctica* (SP 435) with 25% of enzyme concentration. The decrease in conversion yield of methyl esters from oil substrate at higher oil : alcohol molar ratios might be due to the presence of insoluble methanol in the reaction system. Tamalampudi *et al*. (2008) suggested that this would cause the active site on the surface of the lipase to be locked resulting in less access of Novozyme 435 to the surface of oil substrate. Dizge and Keskinler (2008) also reported that the use of excessive amount of

methanol (short-chain alcohol) might deactivate the lipase in the reaction. In this study, the increase of the oil : alcohol molar ratio from 1:4 to 1:5 deactivated the lipase catalyst and resulted in low conversion yield. It is likely that once the maximum level of esters is formed, a further increase in number of moles of alcohol decreases the formation of methyl esters in the reaction due to enzyme inactivation (Nelson *et al*. 1996; Chen *et* al. 2006; Bernardes *et* al. 2007; Dizge and Keskinler. 2008; Tamalampudi *et al*. 2008; Andre *et al*. 2008).

Reports have suggested that the theoretical 1:3 stoichiometric oil : alcohol molar ratio is needed to complete the reaction due to the following continous steps (a) the conversion of triglycerides to diglycerides, (b) the conversion of diglycerides to monoglycerides and (c) the conversion of monoglycerides to methyl esters and glycerol (Freedman *et al*., 1984; Noureddini and Zhu, 1997; Marchetti *et al*., 2008). From the results obtained in this study, increases in the oil : alcohol molar ratio from 1:1 to 1:4 at 4 h with solvent increased the conversion yield of biodiesel for Novozyme 435 by 27.30, 15.78 and 15.14% at the reaction temperatures of 40, 45 and 50°C, respectively. Similar trends were seen with both enzymes for all reaction times with and without solvent under same conditions. The lipase catalyst (Novozyme 435 and NS88001) showed different activity due to their mass transfer, formation of esters, use of alcohol and solvent system. 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 of the reaction to their corresponding esters. After the alcohol had dispersed and reached a maximum conversion biodiesel yield at 1:4 oil : alcohol molar ratio, no significance increase in the conversion yield of biodiesel was observed by using both enzymes. The conversion yield was decreased when the oil : alcohol molar ratio was increased from 1:4 to 1:5 due to alcohol inhibition. Short chain alcohols such as methanol are responsible for deactivation and inhibition of immobilized lipase (Chen and Wu, 2003; Samukawa *et al*., 2000). Deactivation of enzyme likely occurred by the insoluble alcohol present in the reaction due to its tendency to be absorbed by the surface support matrix (Salis *et al*., 2005; Al-zuhair *et al*., 2007). The excess of stoichiometric 1 : 3 oil : alcohol molar ratios ensures the rate of reaction and leads to higher biodiesel conversion yield. However, the excess amount of the alcohol might decrease the activity and distort the spatial confirmation of lipase structure and cause the lipase to deactivate. The optimum oil : alcohol

molar ratio used by several researchers was 1:3 using Novozyme 435. However, in this study the optimum level was achieved at 1:3 and 1:4 oil : alcohol molar ratio in a solvent system using Novozyme 4.5 and NS88001, respectively. In a solvent-free system, a 1:4 oil : alcohol molar ratio was the optimum level for both Novozyme435 and NS88001 due to the presence of free fatty acids in the substrate.

6.2.2. *Effect of Reaction Time*

When the reaction time was increased from 4 to 16 h at 40°C, the increases in biodiesel conversion yield for Novozyme 435 were 16.96, 24.49, 38.97, 21.22 and 105.98% with the solvent system and 20.35, 16.72, 12.93 and 40.66% with the solvent-free system 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 for all reaction temperatures and oil : alcohol molar ratios with and with solvent.

Nelson *et al*. (1996) , Chen *et al*.(2006) and Modi *et al*. (2006) observed similar trends from crude tallow, waste cooking oil and vegetable oil. Nelson *et al*. (1996) reported a maximum biodiesel conversion yield of 83.8 % at 16 h with 1:3 molar ratio using 25% concentration of the enzyme *Candida antarctica* (SP 435) with hexane and 2-butanol alcohol in the system. Chen (2006) achieved a maximum biodiesel conversion yield of 85.12% at 30 h with 1:4 oil : alcohol molar ratio using 30% concentration of the immobilized enzyme *Rhizopus oryzae* and waste cooking oil as substrate. Modi *et al*. (2006) reported that a maximum biodiesel conversion yield of 93.4% was achieved at 8 h with 1:4 oil :alcohol molar ratio using the enzyme *Candida antarctica* (Novozyme 435) with vegetable oil. The biodiesel conversion yields obtained in this study with both enzymes were slightly higher than those reported in the literature and were achieved in a shorter time due to the non-regiospecific characteristics of the enzyme catalyst of Novozyme 435. However, it was not known whether NS88001 was regiospecific or non-regiospecific. Higher conversion yield of biodiesel was obtained at 16 h of reaction time in this study, which may indicate that NS88001 is non-regiospecific.

In the present study, the maximum conversion yield of 77.23 % was obtained by Novozyme 435 with solvent and 2-butanol at the 16 h whereas the conversion yield of 95.75% was obtained by NS88001 with methanol in the solvent system under the same condition. Similar patterns were obtained with solvent-free systems under the same conditions. At the initial phase of the reaction, the enzymes, oil and alcohol appeared to be static and the reaction started when the stirring speed reached 200 rpm which promoted the initial mixing and increased the mass transfer between substrate and enzyme catalyst. Formation of esters increased with increase in reaction time from 1h to 4h. Freedman *et al*. (1984), Ma *et al*. (1998), Leung and Guo, (2006), Meher *et al*. (2006), Alamu *et al*. (2007) and Eevera *et al*. (2009) reported that the rate of conversion of fatty acid esters increased with increases in reaction time as the reaction proceeds rapidly due to the initial mixing and dispersion of alcohol into the oil substrate and the activation of enzyme. After alcohol is dispersed, it rapidly interacts with fatty acids giving a maximum conversion yield. However, a further increase in the reaction time may decrease conversion yield due to the backward reaction of transesterification (Chen *et al*., 2006). Kose *et al*. (2001) and Li *et al*. (2006) reported that the initial reaction in a solvent-free system might take a longer period to activate the enzyme in the system.

6.2.3. *Effect of Reaction Temperature*

In this study, when the reaction temperature was increased from 40 to 45°C at 4 h for Novozyme 435 with the solvent system, the increases in conversion yield of biodiesel were 30.99, 20.98, 45.82, 19.14 and 106.64 % and when the reaction temperature was further increased from 45 to 50°C at 4 h, the biodiesel conversion yield decreased by 11.11, 9.69, 9.57, 16.65 and 12.78% for the 1:1, 1:2, 1:3, 1:4 and 1:5 oil : alcohol molar ratios, repectively. Similar trends were seen with both enzymes Novozyme 435 and NS88001 for all reaction times and oil : alcohol molar ratios with and without solvent.

Chen *et al* (2006), Dizge and Keskinler. (2008), Rodrigues *et al*. (2008) and Nie *et al*. (2006) observed similar trends from waste cooking oil, canola oil, vegetable oil and salad oil. Chen *et al* (2006) reported that the biodiesel conversion yield increased (reaching a maximum of 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 during conversion of waste cooking oil to methyl esters using Lipozyme RM IM. Dizge and Keskinler (2008) reported that the biodiesel conversion yield increased (reaching a maximum of 85.8%) 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 when converting canola oil to methyl esters using Lipozyme TL. Rodrigues *et al*. (2008) reported that a maximum biodiesel conversion yield of 53% was achieved at 35°C and then decreased with increases in reaction temperature above 35°C during conversion of soybean oil to methyl esters using Novozyme 435. Nie *et al*. (2006) reported that a maximum biodiesel conversion yield of 90% was obtained at 40°C and increasing the reaction temperature above 40°C decreased the biodiesel conversion yield. In this study, higher conversion yield were obtained at 45°C which was higher than those reported in the literature.

In this study, when the reaction temperature was increased from 40 to 45°C at 4 h for Novozyme 435 with the solvent system, the increases in conversion yield of biodiesel were 30.99, 20.98, 45.82, 19.14 and 106.64 % for the 1:1, 1:2, 1:3, 1:4 and 1:5 oil : alcohol molar ratios, repectively. Similar pattern was followed by NS88001 with and without solvent system under same condition. However, increasing the reaction temperature leads the substrate oil to reduce the viscosity and enhances the mass transfer between substrate and enzyme catalyst. Due to this effect, an increase in conversion yield of biodiesel can be obtained. Reetz *et al*. (1996), Kumari *et al*. (2009) and Antczak *et al*. (2009) reported that interactions between enzyme polymer surface and substrate appears to be dependent on reaction temperature due to hydrogen bonding and ionic interactions which play important roles in maintaining the thermostability of lipase in the system. When the reaction temperature was further increased from 45 to 50°C at 4 h for Novozyme 435, the biodiesel conversion yield decreased by 11.11, 9.69, 9.57, 16.65 and 12.78% for the 1:1, 1:2, 1:3, 1:4 and 1:5 oil : alcohol molar ratios, respectively. A higher temperature may denature the specific structure of enzymes which results in a decrease in the methyl esters formation. Denaturation of enzyme support matrix may also promote 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 conversion yield of methyl

esters due to inhibition of enzyme activity by higher temperature. Nie *et al*. (2006) also reported that higher temperature can give faster reaction but exceeding the optimum temperature may lead the enzyme denaturing. However, the optimum reaction temperature is dependent on other parameters such as oil : alcohol molar ratio, enzyme activity, stability and type of system used.

6.2.4. ***Effect of Solvent***

In this study, a maximum biodiesel conversion yields of 95.21% and 95.75% were obtained by the Novozyme 435 and enzyme NS88001 in the solvent system at 45°C and 1:4 oil : alcohol molar ratio, respectively.

Mittelbach (1990), Soumanou and Bornscheuer (2003) and Kumari *et al*. (2007) reported similar biodiesel conversion yields with solvent and solvent-free systems. Mittelbach (1990) reported conversion yields of biodiesel of 80% and 76% using 10% of *Pseudomonas* lipase concentration with methanol and ethanol for 14 h at 50°C with and without solvent, respectively. Soumanou and Bornscheuer (2003) reported that immobilized lipase Lipozyme TL IM showed similar rates of transesterification (82 and 80% biodiesel conversion yield) with and without solvent at 25 and 30 h. Kumari *et al*. (2007) reported that a maximum biodiesel conversion yield of 94% was obtained with 1:4 oil : alcohol molar ratio at 55°C and 48 h using *Enterobacter aerogenes* with t-butanol as solvent. They indicated that using solvent significantly reduced the negative effects of methanol and glycerol in the reaction system. Xu *et al*. (2003) and Shimada *et al*. (2002) reported that the decreases in biodiesel conversion yield in solvent-free system using immobilized Lipozyme TL at oil : alcohol molar ratio of 1:1 were because of inactivation of lipase due to the presence of insoluble methanol in reaction.

In this study, n-hexane was used as a solvent. Fjerbaek *et al*. (2008) and Antczak *et* al. (2009) reported that the use of organic non polar solvents for transesterification process might help to reduce the viscosity of the oil substrate and increase the mass transfer between the enzyme and the substrate. The authors suggested that by using n-hexane in the reaction might help to stabilize the enzyme in the reaction inspite of toxicity of alcohol. Nie *et al*.

(2006) reported that a maximum conversion yield of biodiesel of 96% was observed when with using *Candida* sp 99-125 with salad oil and n-hexane (non-polar solvent) as solvent in the system and when acetone (polar solvent) was used the yield decreased to (40%). They indicated that the organic non-polar solvents with a log *P* value (value obtained by octanol/ water experiments to determine the non polarity of a solvent) greater than 2 are considered to be suitable in the transesterification reaction due to their hydrophobic property so that water cannot be stripped from the enzyme and the spatial conformation of the active site of the enzyme is maintained. The authors suggested that n-hexane (log $P = 3.5$) can preserve the catalytic reaction, thus increasing the biodiesel conversion yield. Lu *et al*. (2009) suggested that the n-hexane increased the biodiesel conversion yield with less water residue in the reaction and the non-polar solvent which promotes the usage of short chained alcohols like methanol (a polar alcohol). Antczak *et* al. (2009) and Kaieda *et* al. (2001) reported that the solvents used in large scale industries are volatile and potentially dangerous to handle. These authors also suggests that use of solvent-free system in order to reduce the cost of the recovery process of the solvent and cost of distillation of solvent.

6.2.5. ***Selection of Alcohol***

In this study, *Candida antarctica* Novozyme 435 showed reaction with 2-butanol but no reaction was observed when methanol was used as alcohol for all reaction time. This is due to the presence of traces amounts of water in the reaction which did not promote methyl esters formation when methanol was used as reported by Nelson *et al*. (1996). The alcohol 2-butanol was extremely effective in converting high fatty acids content in the oil to corresponding ester in the reaction. Nelson *et al*. (1996) reported that the conversion of oil to esters by *Candida antarctica* SP 435 as catalyst was retarded when methanol was used in the reaction due to the presence of water in the reaction while 2-butanol promoted the ester formation in the reaction. The author also reported that using 2-butanol did not affect the rate of conversion for both solvent and solvent-free systems. Haas *et al*. (2002) reported that methanol and water can increase the denaturation of lipase in the system. However, using 2-butanol needs some traces of water molecule to catalyze the reaction.

Using the experimental catalyst (NS88001) resulted in the formation of methyl esters when using methanol in the systems but no ester formation was observed when 2-butanol was used. Antczak *et al*. (2009) reported that using long chain alcohol might require more reaction time due to their length of hydrocarbon chain of alcohol. Ghamgui *et al*. (2004) reported that *Rhizomucor oryzae* showed conversion of esters in the reaction with and without n-hexane as solvent when using polar (short chain) alcohol but lower conversion yield was obtained using long chain alcohol as substrate due to the slower diffusion rate of the long-chain alcohol. Tamalampudi *et al*. (2008) reported that using methanol (short-chain alcohol) could easily diffuse due to their low molecular weight and high polarity resulting in higher reaction rate.

Nelson *et al*. (1996), Ghamgui *et al*. (2004) and Tamalampudi *et al*. (2008) obtained similar results from *Candida* sp 435, immobilized *Rhizomucor oryzae* and immobilized whole cell *Rhizopus oryzae.* Nelson *et al*. (1996) reported that the *Candida antarctica* Sp 435 showed a maximum biodiesel conversion yield of 83.8% from crude tallow using 2-butanol and a 25.7% conversion yield of biodiesel using methanol with solvent. Ghamgui *et al*. (2004) reported that using ethanol, propanol and butanol showed similar results (between 75-83% biodiesel conversion yield) while using pentanol and hexanol showed lower conversion yield of biodiesel (20.56-29.03%) when using immobilized *Rhizomucor oryzae* with oleic acid for synthesizing 1-butyl oleate. Tamalampudi *et al*. (2008) reported that higher conversion yield was achieved using methanol as alcohol while ethanol, n-propanol and n-butanol showed lower conversion yield of biodiesel when using immobilized whole cell *Rhizopus oryzae* with jatropha oil.

6.2.6. ***Enzymatic Transesterfication by Combination Enzyme Catalyst***

Generally, the biodiesel conversion yield by the two enzymes combined (Novozyme 435 and NS88001) followed similar pattern to those observed with individual enzymes. The biodiesel conversion yield increased when the oil :alcohol molar ratio was increased from 1:1 to 1:4 reaching 29.85% (with methanol) and 22.15% (with 2-butanol) and then decreased to 19.20% (with methanol) and 11.14% (with 2-butanol) when the oil : alcohol molar ratio was further increased from 1:4 to 1:5 with both alcohols in the solvent (with n-hexane). However,

in solvent-free system, a maximum biodiesel conversion yield of 95.87% and 95.51% were obtained at 1:3 oil : alcohol molar ratios (with methanol) and at 1:4 oil : alcohol molar ratios (with 2-butanol), respectively. Li *et al*. (2006) and Lee *et al*. (2006) obtained similar trends using 1:3 oil : alcohol molar ratio of Novozyme 435 and Lipozyme TL IM with methanol and *Candida rugosa* and *Rhizopus oryzae* with methanol. Li *et al*. (2006) suggested that the 1:4 oil : alcohol molar ratio was needed to complete the reaction, but an excess of methanol over the optimum ratio might induce toxicity in the reaction and distort the enzyme support which leads to inactivation of enzyme. Lee *et al*. (2006) and Talukder *et al*. (2006) reported that higher oil : alcohol molar ratios than 1:3 using primary alcohol might inhibit the transesterification process due to the presence of insoluble methanol in the system that can lead to enzyme deactivation.

In this study, the maximum conversion yield obtained from the combination of Novozyme 435 and NS88001 at the 16 h with the solvent system was 93.86% with methanol and 96.67% with 2-butanol. However, in the solvent-free system the maximum conversion yield of 95.87% (with methanol) and 95.51% (with 2-butanol) was obtained at the 12 h. Several researchers (Lee *et al.*, 2006; Leung and Guo, 2006; Alamu *et al*., 2007; Ma *et al*., 1998; Eevera *et al*., 2009) reported that the transesterification reaction proceeds rapidly at the beginning due to mixing and dispersion of alcohol into substrate and activation of enzyme. After dispersion of alcohol, the lipase starts to convert the oil to methyl esters which results in rapid production of fatty acids methyl esters. However, in solvent-free system the maximum conversion yield was obtained at the 12 h and decreased when the reaction time was increased to 16h. The longer reaction times may have decreased the conversion yield due to the backward reaction of transesterification and the enzyme deactivation. The system involving the combination of enzyme catalyst was not converted completely which could be due to the excess amount of alcohol absorbed in the support matrix causing the conversion yield of biodiesel to decrease considerably. Lee *et al*. (2006) reported that the conversion of oil to biodiesel took place in a two steps manner using an enzyme mixture: (a) the immobilized lipase *Candida rugosa* 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* which

is 1,3 site specific lipase then esterifies the free fatty acids to methyl esters due to its combination of non regiospecific and regiospecific of the lipase which increased the reaction time to 18 h. Similar reactions were observed in this study when using a combination of Novozyme 435 and NS88001 enzyme catalysts in the reaction. However, in this study the maximum conversion yield of biodiesel using the combination of the two lipases was obtained at 16 h with solvent system due to their non-regiospecific in nature.

6.3. Glycerol

In this study, the maximum yield of free glycerol (0.06%) was obtained using a combination of lipase catalyst (Novozyme 435 and NS88001), 2-butanol, a 1: 4 oil: alcohol molar ratio, a 45°C reaction temperature and a 16 h reaction time with the solvent system. No free glycerol was detected in the gas chromatography analysis for all samples. These could be due to the low alcohol concentration present in the system. Theoretically, 3 mol of alcohol react with 1 mol of triglycerides to give 3 mol of FAME and 1 mol of glycerol as the byproduct. In the present study, we have used 2.3 ml of oil and 8 ml n-hexane (total system = 10.3 ml) with 384 µl of alcohol (stoichiometric level with the ratio of 1:4). This gave 96.67% of FAME and only 0.06% of free glycerol as the byproduct in the solvent system. The small traces of the glycerol might be absorbed by the support matrix of immobilized lipase.

The transesterification process consists of three continuous steps: (a) the conversion of triglycerides to diglycerides, (b) the conversion of diglycerides to monoglycerides and (c) the conversion of monoglycerides to fatty acid methyl esters (FAME) and free glycerol (Freedman *et al*., 1984; Noureddini and Zhu, 1997; Marchetti *et al*., 2008). The remaining balance of 3.27% observed in this study was made of intermediates and/or bound glycerols such as monoacylglycerol (monoglycerides), diacylglycerol (diglycerides). Dizge *et al*. (2009a) reported maximum biodiesel conversion yield of 97% and free glycerol of 0.01% using 92 g of canola oil, 40.02 g of methanol, an oil: alcohol molar ratio of 1:6, a 50°C reaction temperature and a 24 h reaction time. The author also reported that 2.79% were intermediates and/or bound glycerol. Xu *et al*. (2011) reported that the low concentration of glycerol (as the byproduct of the reaction) was not detected by the gas chromatography. Their reaction conditions were 10 g of rapeseed oil, 5% concentration of Novozyme 435, 1.8

ml of ethanol a 1:1 oil : alcohol molar ratio, a 35°C raction temperature and a 24 h reaction time.

Dossat *et al*. (1999) and Xu *et al*. (2011) reported that the possible mechanisms of glycerol inhibition include: (a) the mass transfer between substrate and enzyme can be restricted by bound glycerol or free glycerol clogging in the active site of the enzyme which tends to decrease the conversion yield of biodiesel and (b) the hygroscopic nature of glycerol might reduce the avaibility of water content and affect the enzyme activity in the reaction. Dossat *et al*. (1999) reported that the decrease in the conversion yield of biodiesel was due to the limitation of mass transfer between substrate and enzyme and the bound glycerol which caused the clogging of the active site of the enzyme. The author also stated that the produced glycerol in the reaction can be absorbed by the support matrix of immobilized enzyme. Shimada *et al*. (1999) reported that at the beginning of the reaction the formation of glycerol was absent and as the reaction proceeds the formation of free and bound glycerol was high. The glycerol in the system tends to deposit on the surface of the immobilization support matrix and inhibits the enzyme activity. Similar, trends were observed with the combination of enzymes (Novozyme 435 and NS88001) for both alcohols at the reaction temperature of 45°C in the solvent-free system.

6.4. Enzymatic Transesterification Model

Al-zuhair *et al*. (2007) reported that the kinetics of biodiesel follows the Ping-Pong Bi Bi mechanism (Figure 6.1) with most of the proposed earlier models using long chain fatty acids with lipase as catalyst. The proposed mechanism of transesterification by enzymatic catalysis takes place in four steps: (a) enzyme-substrate complex formed due to addition of nucleophillic oxygen in the O-H group which is present on the enzyme, (b) the conjugated acid of the amine transfers the proton to the alkyl oxygen of the substrate and formation of glycerol moiety (if triacylglyceride was the substrate, diacylglyceride would form with glycerol moiety and so on), (c) the oxygen atom from a alcohol molecule is added to the carbon atom of the C=O of acyl enzyme intermediate, thus acylated enzyme-alcohol complex is formed and (d) oxygen from the enzyme complex is eliminated and the proton is transferred from the conjugated acids from the amine, resulting in fatty acids methyl esters

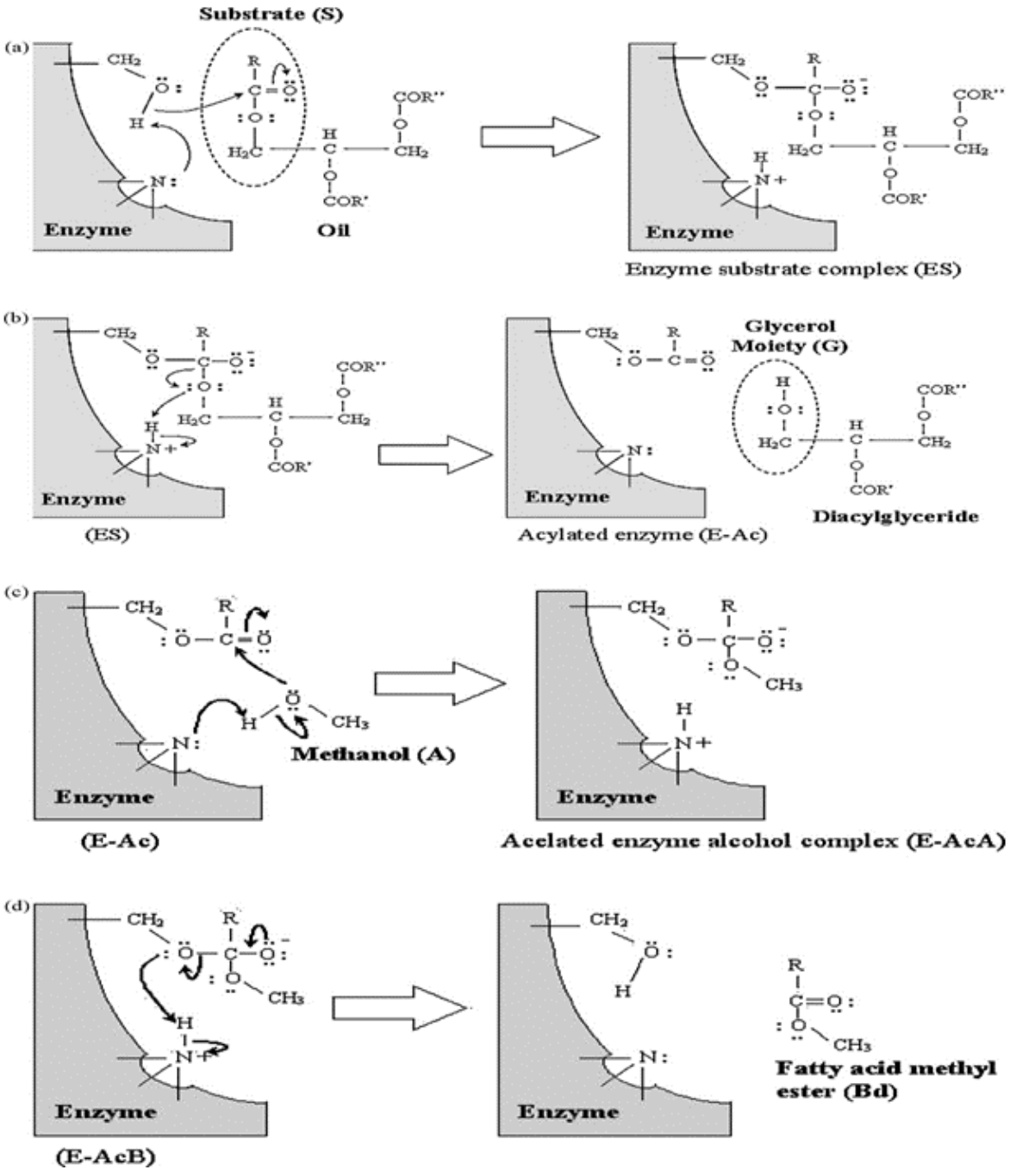


Figure 6.1. Mechanism of enzymatic production of FAME (Al-zuhair *et al*., 2007)

(FAME). The kinetics of fatty acid methyl esters (FAME) from triacylglycerides are described as follows (Al-zuhair *et al*., 2007):

$$E + S \underset{k_{-1}}{\overset{}{\longleftrightarrow}} E.S \quad (6.1)$$

$$E.S \longleftrightarrow E.Ac.G \quad (6.2)$$

$$E.Ac.G \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} E.Ac + G \quad (6.3)$$

$$E.Ac + A \underset{k_{-3}}{\overset{k_3}{\longleftrightarrow}} E.Ac.A \quad (6.4)$$

$$E.Ac.A \longleftrightarrow E.Bd \quad (6.5)$$

$$E.Bd \underset{k_{-4}}{\overset{k_4}{\longleftrightarrow}} E + Bd \quad (6.6)$$

where:

$k_1, k_{-1}, k_2, k_{-2}, k_3, k_{-3}, k_4, k_{-4}$ = rate constants

E.S = enzyme-substrate complex

E.Ac.G = acylated enzyme-glycerol moiety complex

E.Ac.A = acylated enzyme-alcohol complex

k_4, k_{-4} = rate constants for the product formation

BD = Biodiesel as product

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 which is not the case in the present study. The complexity increases when immobilized lipases are used in solvent system and/or solvent-free system because using the immobilized enzyme results in a multi phase system and changes its nature during the reaction due to inhibition

factors by operating parameters. Limitations of mass transfer of immobilized enzymes should be examined with molecular size of substrates and products. Many researchers (Fjerbaek *et al*., 2009; Al-zuhair *et al*., 2007; Kumari *et al*., 2009) have stated that the accuracy of the kinetic model cannot be predictable when using immobilized lipase in the reaction system and using a solvent-free system.

6.5. Enzyme Reusability

In this study, the activity of lipase enzyme catalyst with solvent system decreased gradually after 10 and reached zero after 50 cycles, but in solvent-free system there was a rapid decrease in conversion yield after 10 cycles and activity stopped after 30 cycles. Dossat *et* al. (1999), Xu *et al*. (2003), Soumanou and Bornscheuer (2003), Ghamgui *et al*. (2004) and Bernardes *et al*. (2007) obtained similar results from immobilized Lipozyme *Thermomyces lanuginosus*, immobilized Lipozyme *Rhizomucor miehei* and immobilized *Rhizopus oryzae*.

As reported by other researchers, the reduction in enzymatic activity may be due to the decreased interaction between lipase and substrate, while repeated use of enzyme in the reaction without removing glycerol from the system might inhibit the interaction between the substrate and lipase (Dossat *et* al., 1999 ; Soumanou and Bornscheuer., 2003 and Ghamgui *et al*., 2004). However, the immobilized lipase was rinsed with water and alcohol in between cycles which might have removed the glycerol from the surface of the support matrix. In a solvent system, the inhibition of glycerol has been found to have less influence towards lipase activity due to the solubility of glycerol in the system (Dossat *et* al., 1999).

Xu *et al*. (2003) reported that while using methyl acetate as acyl acceptor, no glycerol was produced in the reaction with no loss of enzyme activity for 10 cycles in the reaction. However, the byproduct from the reaction was triacetylglycerol instead of glycerol which did not affect the product quality. Bernardes *et al*. (2007) reported that in a solvent-free system, deposition of water on the surface of lipase might reduce the conversion yield of biodiesel. Xu *et al*. (2003) and Shimada *et al*. (2002) reported that the decreases in biodiesel conversion yield in a solvent-free system using immobilized Lipozyme TL at oil : alcohol molar ratio of

1:1 were because of the inactivation of lipase due to the presence of insoluble methanol in the reaction.

6.6. Other Considerations

In order for this study to be industrially relevant, there are other considerations that should be addressed. For example, the various unsaturated components in the raw material might result in different degradation levels of biodiesel product. The addition of antioxidants in the biodiesel might help to increase the stability of biodiesel and delay the oxidation process. These factors would be important to investigate for industrial biodiesel production. In this study as well as in several studies in the literature, biodiesel production has been carried out in a batch system with mixing at 200 rpm. However, scaling up the process might require a greater level of mixing that requires costly energy input and this should be investigated. The enzyme concentration was kept at 25% in this study (which also used by several researchers) due to many factors such as: mixing intensity, type of feedstock, volume of the system and reaction time. However, increasing the volume of the substrate might need the enzyme concentration to be varied based on the fatty acids composition and free fatty acids content. In this study, Novozyme 435 and NS88001 had restrictions on the alcohol type and reaction time due to their specificity and mass transfer limitations. However, different immobilization techniques or support matrices might help to increase the mass transfer and allow greater flexibility with the alcohol type.

As mentioned previously, kinetics has been difficult to study in multiphase systems due to their complexity. However, for a better understanding of the inhibition level of alcohol, glycerol and other impurities in the system, the study of the kinetics is required to increase the efficiency of the process. In addition, studying the enzymatic transesterification process in a continuous packed bed reactor might help to optimize the industrial process due to their large throughput capacity. In this study, a 2 ml system was used based on that 96 µl of methanol and 102 µl of 2-butanol at stoichiometric level. However, due to the low volumes, it was not possible to recover the alcohol. Increasing the volume of the system might also require that the solvents and alcohol be recovered from the system and recycled to reduce the cost of the process and aid in the commercialization of the biodiesel.

CHAPTER 7. CONCLUSIONS

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

1. The effect of alcohol type on the conversion yield of biodiesel using *Candida antarctica* (Novozyme 435) and experimental catalyst (NS88001) individually and in combination was significant at the 0.001 level.
   (a) The lipase *Candida antarctica* (Novozyme 435) showed highest conversion yield using 2-butanol as alcohol.
   (b) Experimental catalyst (NS88001) obtained highest conversion yield by using methanol as alcohol.
   (c) The combination of Novozyme 435 and NS88001 obtained highest conversion yield using 2-butanol as alcohol.
2. The effect of oil : alcohol molar ratios on the conversion yield of biodiesel using *Candida antarctica* (Novozyme 435) and experimental enzyme (NS88001) individually and in combination was highly significant at the 0.001 level.
   (a) No reaction was observed at 1:1 oil : alcohol molar ratio for *Candida antarctica* Novozyme 435 and 1:1 and 1:2 oil : alcohol molar ratio for experimental enzyme NS88001 and 1:1 oil : alcohol molar ratio for combination of *Candida antarctica* Novozyme 435 and experimental enzyme NS88001 with solvent-free system.
   (b) The highest conversion yield of biodiesel using *Candida antarctica* Novozyme 435 and experimental enzyme (individually and in combination) was obtained at the 1:4 molar ratio.
   (c) In the solvent system, increasing the oil : alcohol molar ratio from 1:1 to 1:4 increased the conversion yield of biodiesel by 3.10% for *Candida antarctica*

Novozyme 435 (with 2-butanol) and by 21.24% for experimental enzyme NS88001 (with methanol) and by 29.85% (with methanol) and 22.15% (with 2-butanol) for the combination of *Candida antarctica* (Novozyme 435) and experimental enzyme (NS88001).

(d) In solvent-free system, increasing the oil : alcohol molar ratio from 1:1 to 1:4 increased the biodiesel conversion yield by 2.53% for *Candida antarctica* Novozyme 435 (with 2-butanol) and by 0.94% for experimental enzyme NS88001 (with methanol) and by 50.47% (with methanol) and 26.47% (with 2-butanol) for the combination of *Candida antarctica* (Novozyme 435) and experimental enzyme (NS88001).

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

(a) The rate of conversion of fatty acid esters increases with increase in reaction time as the reaction proceeds slowly at the beginning due to the initial mixing and dispersion of alcohol into the oil substrate and activation of enzyme. After dispersion of alcohol, the enzyme rapidly interacted with fatty acids esters giving a maximum conversion yield.

(b) In the solvent system, increasing the reaction time from 4 to 16 h increased the conversion yield of biodiesel by 6.07% for *Candida antarctica* Novozyme 435 (with 2-butanol) and by 13.51% for experimental enzyme NS88001 (with methanol) and by 136.42% (with methanol) and 28.37% (with 2-butanol) for the combination of (*Candida antarctica* Novozyme 435 and experimental enzyme NS88001) with solvent system.

(c) In solvent free system, increasing the reaction time from 4 to 16 h increased the conversion yield of biodiesel by 8.84% for *Candida antarctica* Novozyme 435 (with 2-butanol) and by 17.40% for experimental enzyme NS88001 (with methanol).

(d) For the combination of (*Candida antarctica* Novozyme 435 and experimental enzyme NS88001) without solvent system, the increase in reaction time from 4

to 12 h increased the conversion yield of biodiesel by 42.57% with methanol and by 44.90% with 2-butanol.

4. The effect of reaction temperature on the conversion yield of biodiesel by *Candida antarctica* (Novozyme 435) and experimental enzyme (NS88001) individually and in combination was highly significant at the 0.001 level.
    (a) The optimum reaction temperature was 45°C for both enzymes.
    (b) The interactions between enzyme polymer surface and substrate appears to be dependent on reaction temperature due to hydrogen bonding and ionic interactions which play a important roles in maintaining the thermostability of lipase in the system. Higher temperature may denature the specific structure of enzymes which results in decrease in methyl esters formation.
    (c) In the solvent system, increasing the reaction temperature from 40 to 45°C increased the biodiesel conversion yield by 11.29% for *Candida antarctica* Novozyme 435 (with 2-butanol) and by 8.48% for experimental enzyme NS88001 (with methanol) with solvent system.
    (d) In solvent free system, increasing the reaction temperature from 40 to 45°C increased the biodiesel conversion yield by 9.70% for *Candida antarctica* Novozyme 435 (with 2-butanol) and by 7.98% for experimental enzyme NS88001 (with methanol) without solvent system.
    (e) At 45°C, the highest biodiesel conversion yield was 93.86% with methanol and 96.67% with 2-butanol with solvent and 95.87% with methanol and 95.51% with 2-butanol without solvent system for combination of (*Candida antarctica* Novozyme 435 and experimental enzyme NS88001).
5. The effect of solvent system on the conversion yield of biodiesel using *Candida antarctica* (Novozyme 435) and experimental enzyme (NS88001) individually and in combination was highly significant at the 0.001 level.
    (a) The lipase *Candida antarctica* (Novozyme 435) showed highest conversion yield of biodiesel when using solvent free system.
    (b) The experimental catalyst (NS88001) and the combination of (*Candida antarctica* Novozyme 435 and experimental enzyme NS88001) showed the highest conversion yield when using solvent system.

(c) n-hexane in the reaction helped to stabilize the enzyme in the reaction inspite of toxicity of alcohol.

6. In solvent system, the activity of experimental lipase (NS88001) and *Candida antarctica* (Novozyme 435) individually and in combination with solvent system was reduced after 10 cycles and stopped after 50 cycles.
7. In solvent-free system, the activity of the enzymes was deactivated after 10 cycles and stopped after 30 cycles.

CHAPTER 8. RECOMMENDATIONS

The following recommendations are made for future work

1. The effect of antioxidants on the quality of oil, transesterification efficiency and quality of biodiesel should be evaluated.

2. The effect of stirring speed on the rate of transesterification should be studied.

3. The effect of enzyme concentration of the lipase catalyst should be evaluated in both solvent and solvent-free systems.

4. An immobilized enzyme system should be tested and the structure and function of the enzymes should be evaluated in order to improve the stability of the enzyme and reduce the inhibition factors.

5. The kinetics for enzymatic transesterification with immobilized enzyme with and without solvent system should be evaluated.

6. The enzymatic transesterification process should be studied in the continuous process in packed bed reactors to evaluate the scale up parameters and in order to commericialize the product.

7. The recovery of solvents and alcohols should be evaluated and an economic analysis should be performed on the process.

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APPENDICES

APPENDIX A: Sample Calculations

Sample Calculation

1. Molarity Calculation:

M = moles of solute / litre of solvent = 0.00232 (mol) / 0.008 (L)= 0.29 (mol/L)

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

Mol wt of tallow= 858.5 g/mol

Weight of oil = 2 grams

Molar mass of methanol = 32.04 g/mol

Density of methanol = 0.7918 g cm^3

Oil : alcohol molar ratio = 2 (g) /858.5 (g/mol) = 0.00232 (mol)

= 0.00232 (mol)* 32.04 (g/mol) = 0.0746 (g)

= 0.0746 (g) / 0.7918 (g cm^3) = 0.0942 cm^3

Oil : alcohol molar ratio for 1:1 = 0.0942 cm^3

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

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 (wt %)

APPENDIX B: Data obtained with NOVOZYME 435

Table B1. Biodiesel yield from animal tallow using 0.5 grams of *Candida antarctica* (Novozyme 435) with 2-butanol as alcohol and with hexane at different reaction temperatures and reaction times.

Time (h)	oil: alcohol	Reaction Temperature														
		40°C					45°C					50°C				
		Trial 1	Trial 2	Trial 3	Avg	St. Dev	Trial 1	Trial 2	Trial 3	Avg	St. Dev	Trial 1	Trial 2	Trial 3	Avg	St. Dev
4	1:1	46.17	43.65	45.96	45.26	1.40	60.48	58.68	58.71	59.29	1.03	53.75	53.67	50.68	52.70	1.75
	1:2	50.85	49.23	49.47	49.85	0.87	61.52	59.49	59.92	60.31	1.07	55.55	55.09	52.74	54.46	1.51
	1:3	50.93	51.54	47.32	49.93	2.28	74.27	71.65	72.51	72.81	1.33	63.32	64.59	58.33	62.08	3.31
	1:4	58.77	57.21	56.88	57.62	1.01	70.02	69.73	66.20	68.65	2.13	61.89	59.87	60.28	60.68	1.07
	1:5	30.86	31.85	28.05	30.25	1.97	63.76	63.98	59.79	62.51	2.36	55.61	53.79	54.16	54.52	0.96
8	1:1	49.59	51.26	45.01	48.62	3.24	64.20	61.97	62.65	62.94	1.14	59.69	59.73	56.14	58.52	2.06
	1:2	59.96	57.98	58.40	58.78	1.04	65.37	63.45	63.45	64.09	1.11	63.37	63.91	59.11	62.13	2.63
	1:3	60.24	58.56	58.38	59.06	1.03	74.88	72.54	72.81	73.41	1.28	66.09	63.82	64.46	64.79	1.17
	1:4	63.94	61.32	62.81	62.69	1.32	70.47	70.99	65.81	69.09	2.85	69.32	66.22	68.34	67.96	1.58
	1:5	42.93	40.75	42.59	42.09	1.17	67.37	65.05	65.73	66.05	1.19	64.41	64.55	60.49	63.15	2.31
12	1:1	53.35	51.32	52.23	52.30	1.01	69.32	66.25	68.34	67.97	1.57	65.86	63.94	63.91	64.57	1.12
	1:2	62.25	63.45	57.39	61.03	3.21	70.66	68.30	68.85	69.27	1.23	68.71	68.45	64.92	67.36	2.11
	1:3	67.30	66.21	64.43	65.98	1.45	77.24	74.21	75.74	75.73	1.52	71.31	70.59	67.83	69.91	1.84
	1:4	69.72	67.89	67.44	68.35	1.20	74.13	73.54	70.37	72.68	2.03	70.27	69.84	66.56	68.89	2.03
	1:5	48.58	49.21	45.10	47.63	2.22	68.17	67.29	65.03	66.83	1.62	66.40	66.89	62.01	65.10	2.69
16	1:1	54.00	52.50	52.32	52.94	0.92	74.87	72.89	72.44	73.40	1.29	70.59	67.23	69.81	69.21	1.76
	1:2	63.30	60.20	62.68	62.06	1.64	76.08	73.98	73.71	74.59	1.30	70.87	69.89	67.68	69.48	1.63
	1:3	70.78	68.54	68.85	69.39	1.21	78.97	76.85	75.87	77.23	1.58	72.55	71.29	73.59	72.48	1.15
	1:4	71.25	70.25	68.05	69.85	1.63	77.19	74.76	75.09	75.68	1.32	75.57	73.15	73.55	74.09	1.30
	1:5	63.56	61.52	61.85	62.31	1.09	74.79	72.50	72.67	73.32	1.27	73.30	70.99	71.29	71.86	1.25

Table B2. Biodiesel yield from animal tallow using 0.5 grams of *Candida antarctica* (Novozyme 435) with 2-butanol as alcohol and without hexane at different reaction temperatures and reaction times.

Time (h)	oil: alcohol	Reaction Temperature														
		40°C					45°C					50°C				
		Trial 1	Trial 2	Trial 3	Avg	St. Dev	Trial 1	Trial 2	Trial 3	Avg	St. Dev	Trial 1	Trial 2	Trial 3	Avg	St. Dev
4	1:1	0.00	0.00	0.00			0.00	0.00	0.00			0.00	0.00	0.00		
	1:2	71.28	67.23	67.20	68.57	2.35	85.22	85.45	82.80	84.49	1.47	49.41	51.06	49.53	50.00	0.92
	1:3	74.57	74.87	72.50	73.98	1.29	86.10	86.95	83.96	85.67	1.54	54.78	54.98	54.34	54.70	0.33
	1:4	77.35	77.89	75.31	76.85	1.36	87.92	86.51	87.98	87.47	0.83	79.93	80.21	79.53	79.89	0.34
	1:5	48.90	50.99	48.46	49.45	1.35	84.63	85.00	84.23	84.62	0.39	38.20	36.42	38.39	37.67	1.09
8	1:1	0.00	0.00	0.00			0.00	0.00	0.00			0.00	0.00	0.00		
	1:2	73.01	70.87	70.01	71.30	1.55	88.62	89.60	86.47	88.23	1.60	65.07	66.00	65.19	65.42	0.51
	1:3	79.97	81.25	78.18	79.80	1.54	93.01	92.45	89.97	91.81	1.61	68.33	66.23	68.18	67.58	1.17
	1:4	79.77	82.56	78.75	80.36	1.97	91.52	88.85	87.50	89.29	2.04	86.76	85.93	83.78	85.49	1.54
	1:5	49.90	51.63	49.25	50.26	1.23	88.21	87.23	85.11	86.85	1.58	48.65	49.24	48.90	48.93	0.30
12	1:1	0.00	0.00	0.00			0.00	0.00	0.00			0.00	0.00	0.00		
	1:2	79.07	75.54	75.01	76.54	2.21	90.80	90.94	88.17	89.97	1.56	80.96	80.20	78.18	79.78	1.43
	1:3	82.39	82.54	80.02	81.65	1.42	94.80	92.13	90.69	92.54	2.09	92.34	91.02	93.15	92.17	1.08
	1:4	86.02	86.97	83.93	85.64	1.56	95.04	94.23	91.83	93.70	1.67	94.48	94.01	92.10	93.53	1.26
	1:5	52.52	55.02	52.18	53.24	1.55	92.50	89.87	88.47	90.28	2.04	59.97	59.00	58.00	58.99	0.99
16	1:1	0.00	0.00	0.00			0.00	0.00	0.00			0.00	0.00	0.00		
	1:2	82.47	83.56	81.56	82.53	1.00	93.93	93.65	91.00	92.86	1.61	91.95	92.54	89.50	91.33	1.61
	1:3	87.33	87.10	84.62	86.35	1.50	94.23	96.13	95.03	95.13	0.95	94.32	94.65	92.31	93.76	1.27
	1:4	88.20	87.12	85.05	86.79	1.60	95.53	96.79	93.31	95.21	1.76	95.04	94.33	92.04	93.80	1.57
	1:5	66.96	72.52	69.20	69.56	2.80	91.95	92.54	89.50	91.33	1.61	64.13	65.70	64.90	64.91	0.79

APPENDIX C: Data obtained with NS88001

Table C1. Biodiesel yield from animal tallow using 0.5 grams of experimental lipase (NS88001) with methanol as alcohol and with hexane at different reaction temperatures and reaction times.

Time (h)	oil: alcohol	Reaction Temperature														
		40°C					45°C					50°C				
		Trial 1	Trial 2	Trial 3	Avg	St. Dev	Trial 1	Trial 2	Trial 3	Avg	St. Dev	Trial 1	Trial 2	Trial 3	Avg	St. Dev
4	1:1	41.73	44.87	42.01	42.87	1.74	59.90	59.08	57.72	58.90	1.10	55.90	56.60	54.61	55.70	1.01
	1:2	45.91	48.04	45.58	46.51	1.34	74.85	74.33	72.37	73.85	1.30	63.46	64.00	61.84	63.10	1.13
	1:3	58.85	59.10	57.22	58.39	1.02	78.85	78.49	76.33	77.89	1.36	68.78	71.06	68.00	69.28	1.59
	1:4	77.07	79.63	77.51	78.07	1.37	84.52	84.87	83.66	84.35	0.62	71.85	75.31	72.56	73.24	1.83
	1:5	50.61	52.34	50.98	51.31	0.91	67.20	70.58	69.82	69.20	1.78	47.05	47.34	45.80	46.73	0.82
8	1:1	46.97	48.56	46.34	47.29	1.14	64.49	64.12	62.40	63.67	1.12	61.90	61.12	59.68	60.90	1.12
	1:2	75.68	75.23	73.22	74.71	1.31	80.76	80.54	78.25	79.85	1.39	66.10	65.00	63.60	64.90	1.25
	1:3	87.11	86.23	84.09	85.81	1.55	89.08	89.13	88.61	88.94	0.29	78.75	78.06	76.08	77.63	1.39
	1:4	82.35	80.51	80.20	81.02	1.16	85.60	86.35	84.91	85.62	0.72	78.36	80.05	76.85	78.42	1.60
	1:5	69.90	71.82	69.51	70.41	1.24	78.56	81.15	78.97	79.56	1.39	55.78	55.28	53.88	54.98	0.98
12	1:1	60.29	61.09	58.89	60.09	1.11	79.72	79.29	77.15	78.72	1.38	66.24	66.90	64.59	65.91	1.19
	1:2	75.98	75.48	73.48	74.98	1.32	82.69	86.38	85.00	84.69	1.87	72.92	72.36	70.48	71.92	1.28
	1:3	87.15	86.43	84.21	85.93	1.53	91.11	93.95	91.27	92.11	1.60	81.86	84.29	80.61	82.25	1.87
	1:4	83.71	83.59	81.16	82.82	1.44	94.33	93.25	91.00	92.86	1.70	79.69	81.65	78.27	79.87	1.70
	1:5	74.27	73.07	71.48	72.94	1.40	89.19	88.19	86.05	87.81	1.60	67.91	71.03	68.45	69.13	1.67
16	1:1	67.97	67.01	65.48	66.82	1.25	79.97	79.55	77.39	78.97	1.38	69.03	71.43	69.63	70.03	1.25
	1:2	81.07	80.47	78.37	79.97	1.42	93.94	93.03	90.71	92.56	1.67	73.90	73.26	71.39	72.85	1.30
	1:3	88.23	88.99	87.47	88.23	0.76	93.76	95.99	93.54	94.43	1.36	94.57	93.23	91.11	92.97	1.74
	1:4	88.03	89.26	87.49	88.26	0.91	96.21	96.20	94.84	95.75	0.79	92.29	92.01	89.42	91.24	1.59
	1:5	76.70	73.80	74.80	75.10	1.47	90.64	87.20	86.28	88.04	2.30	78.95	78.51	76.39	77.95	1.37

Table C2. Biodiesel yield from animal tallow using 0.5 grams of experimental lipase (NS88001) with methanol as alcohol and without hexane at different reaction temperatures and reaction times.

Time (h)	oil: alcohol	Reaction Temperature														
		40°C					45°C					50°C				
		Trial 1	Trial 2	Trial 3	Avg	St. Dev	Trial 1	Trial 2	Trial 3	Avg	St. Dev	Trial 1	Trial 2	Trial 3	Avg	St. Dev
4	1:1	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		
	1:3	73.80	73.26	71.34	72.80	1.29	73.36	75.64	73.48	74.16	1.29	38.10	39.89	37.21	38.40	1.36
	1:4	78.76	78.52	76.30	77.86	1.35	79.80	81.00	79.50	80.10	0.79	58.97	59.00	57.23	58.40	1.01
	1:5	47.55	48.78	46.74	47.69	1.03	59.13	61.54	58.55	59.74	1.59	33.51	33.25	31.04	32.60	1.36
8	1:1	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		
	1:3	77.61	78.15	76.98	77.58	0.59	79.38	81.82	79.46	80.22	1.39	47.07	47.06	45.67	46.60	0.81
	1:4	83.30	83.01	81.07	82.46	1.21	86.32	85.08	83.15	84.85	1.59	68.90	68.26	66.54	67.90	1.22
	1:5	74.02	76.55	74.58	75.05	1.33	75.23	77.75	75.71	76.23	1.34	40.95	40.26	39.09	40.10	0.94
12	1:1	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		
	1:3	79.18	78.22	76.36	77.92	1.43	81.10	83.74	81.46	82.10	1.43	58.90	58.06	56.74	57.90	1.09
	1:4	83.09	84.66	82.45	83.40	1.14	87.60	88.99	86.42	87.67	1.29	76.08	78.62	76.54	77.08	1.35
	1:5	78.92	79.04	77.87	78.61	0.64	79.02	78.98	76.52	78.17	1.43	54.53	55.00	51.00	53.51	2.19
16	1:1	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		
	1:3	82.24	84.71	82.20	83.05	1.44	93.15	94.03	92.30	93.16	0.87	63.81	63.07	61.55	62.81	1.15
	1:4	85.85	88.83	86.59	87.09	1.55	94.80	94.16	93.16	94.04	0.83	83.73	85.87	82.97	84.19	1.51
	1:5	80.02	79.50	77.39	78.97	1.39	81.80	81.34	79.14	80.76	1.42	72.28	71.10	69.56	70.98	1.36

APPENDIX D: Data obtained with Combination of (Novozyme 435 and NS88001)

Table D1. Biodiesel yield from animal tallow using 0.5 of combined Novozyme 435 and NS88001 with different alcohols at reaction temperatures and reaction times with solvent.

Time (h)	oil: alcohol	Alcohol									
		Methanol					2-butanol				
		Trial 1	Trial 2	Trial 3	Avg	St. Dev	Trial 1	Trial 2	Trial 3	Avg	St. Dev
4	1:1	31.54	35.21	34.20	33.65	1.90	44.12	43.45	40.23	42.60	2.08
	1:2	37.16	37.64	35.90	36.90	0.90	60.59	60.59	57.02	59.40	2.06
	1:3	38.67	41.52	38.91	39.70	1.58	71.81	73.85	71.54	72.40	1.26
	1:4	43.38	40.31	43.21	42.30	1.73	75.00	76.81	74.09	75.30	1.38
	1:5	31.44	31.42	29.54	30.80	1.09	63.61	65.48	63.51	64.20	1.11
8	1:1	44.10	46.98	44.19	45.09	1.64	52.24	55.02	52.04	53.10	1.67
	1:2	47.91	45.82	45.47	46.40	1.32	66.52	66.60	64.58	65.90	1.14
	1:3	57.58	60.59	61.23	59.80	1.95	82.33	79.88	78.69	80.30	1.85
	1:4	64.31	63.21	65.98	64.50	1.39	85.95	84.03	86.22	85.40	1.19
	1:5	46.95	47.59	45.86	46.80	0.87	73.19	75.07	72.54	73.60	1.32
12	1:1	55.45	57.21	55.61	56.09	0.97	66.71	66.02	64.40	65.71	1.19
	1:2	56.16	58.20	56.82	57.06	1.04	80.92	84.58	83.26	82.92	1.85
	1:3	73.09	71.25	73.49	72.61	1.19	85.07	82.69	81.39	83.05	1.87
	1:4	78.39	80.89	78.62	79.30	1.38	95.45	96.77	95.33	95.85	0.80
	1:5	60.54	60.95	61.87	61.12	0.68	76.34	76.98	74.38	75.90	1.35
16	1:1	69.89	71.71	69.30	70.30	1.25	78.25	80.72	78.45	79.14	1.37
	1:2	85.43	87.95	85.31	86.23	1.49	87.76	87.50	85.02	86.76	1.51
	1:3	91.47	95.74	94.37	93.86	2.18	85.92	87.85	85.79	86.52	1.15
	1:4	91.51	90.21	92.15	91.29	0.99	97.80	96.23	95.98	96.67	0.99
	1:5	74.26	72.43	74.59	73.76	1.16	86.90	86.62	84.18	85.90	1.49

Table D2. Biodiesel yield from animal tallow using 0.5 of combined Novozyme 435 and NS88001 with different alcohols at reaction temperatures and reaction times without solvent.

Time (h)	oil: alcohol	Alcohol									
		Methanol					2-butanol				
		Trial 1	Trial 2	Trial 3	Avg	St. Dev	Trial 1	Trial 2	Trial 3	Avg	St. Dev
4	1:1	0.00	0.00	0.00			0.00	0.00	0.00		
	1:2	66.54	68.54	66.52	67.20	1.16	56.12	56.71	53.97	55.60	1.44
	1:3	64.16	68.58	68.98	67.24	2.68	64.26	67.23	66.24	65.91	1.51
	1:4	57.23	60.14	59.51	58.96	1.53	65.47	67.32	65.21	66.00	1.15
	1:5	51.28	54.05	53.64	52.99	1.49	62.96	64.42	62.10	63.16	1.17
8	1:1	0.00	0.00	0.00			0.00	0.00	0.00		
	1:2	71.61	75.28	74.51	73.80	1.93	65.35	67.66	65.98	66.33	1.19
	1:3	79.48	82.16	80.01	80.55	1.42	69.51	72.40	71.03	70.98	1.45
	1:4	75.93	75.39	73.41	74.91	1.33	84.26	86.67	83.98	84.97	1.48
	1:5	61.35	63.47	61.87	62.23	1.11	74.58	74.17	72.17	73.64	1.29
12	1:1	0.00	0.00	0.00			0.00	0.00	0.00		
	1:2	67.05	68.82	66.54	67.47	1.20	75.16	77.68	75.64	76.16	1.34
	1:3	97.34	96.06	94.21	95.87	1.57	77.44	77.09	74.97	76.50	1.34
	1:4	89.60	90.85	88.08	89.51	1.39	95.92	96.01	94.60	95.51	0.79
	1:5	64.06	64.55	61.23	63.28	1.79	64.44	64.21	62.45	63.70	1.09
16	1:1	0.00	0.00	0.00			0.00	0.00	0.00		
	1:2	53.68	53.10	51.80	52.86	0.96	69.81	69.19	67.43	68.81	1.23
	1:3	85.25	86.08	84.87	85.40	0.62	75.93	75.43	73.43	74.93	1.32
	1:4	79.11	80.90	78.61	79.54	1.20	88.08	86.98	86.03	87.03	1.03
	1:5	36.41	36.66	34.75	35.94	1.04	61.06	61.86	59.03	60.65	1.46