CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 BIODIESEL

The raw materials for the production of biodiesel, fatty acid alkyl ester, include biological sources such as vegetable oil, animal fat and recycle cooking grease or oil. Biodiesel can be produced by several processes and the property is similar to petrodiesel as shown in Table 2-1.

Table 2-1. Comparative properties of diesel and biodiesel

Fuel properties	**Diesel**	**Biodiesel**
Fuel Standard	ASTM D975	ASTM D6751
Lower Heating Value, Btu/gal	~129,050	~118,176
Kinematic Viscosity, at 40°C	1.3 - 4.1	4.0 – 6.0
Specific Gravity Kg/l, at 60°F	0.85	0.88
Density, lb/gal, at 15°C	7.079	7.328
Water and Sediment, vol%	0.05 max	0.05 max
Carbon, wt%	87	77
Hydrogen, wt%	13	12
Oxygen, by dif. wt%	0	11
Sulfur, wt%	0.05 max	0.0 to 0.0024
Boiling Point, °C	180 to 340	315 to 350
Flash Point, °C	60 to 80	100 to 170
Cloud Point, °C	-15 to 5	-3 to 12
Pour Point, °C	-35 to -15	-15 to 10
Cetane Number	40 - 55	48 – 65
Lubricity SLBOCLE, grams	2000-5000	>7,000
Lubricity HFRR, microns	300 – 600	<300

From Dwivedi *et al.*, 2006

2.1.1 Sources of raw materials

The feedstocks employed in biodiesel production are generally classified into vegetable oils, animal fats, and waste oils (Jeong *et al.*, 2009). Only oils from plants which are renewable, potentially inexhaustible source of energy and abundant or can be collected in a large amount with an appreciable quantity of oil are feasible to use for biodiesel preparation (Winayanuwattikun *et al.*, 2008).

Commonly accepted biodiesel raw materials include the oils from soybean (Watanabe *et al.*, 2002, Noureddini *et al.*, 2005 and Rosa *et al.*, 2008), canola (Dizge and Keskinler, 2008), rapeseed (Kamini and Lefuji, 2001), sunflower (Antolín *et al.*, 2002, Rashid *et al.*, 2008 and Dizge *et al.*, 2009) and palm oil. Moreover, there are several non- edible and waste plant oil seed species, such as physic nut (Tamalampudi *et al.*, 2008, Tiwari *et al.*, 2007 and Oliveira *et al.*, 2009), cotton seed (Köse *et al.*, 2002 and Qian *et al.*, 2008), pumpkin (Winayanuwattikun *et al.*, 2008 and Schinas *et al.*, 2009) etc., which could be utilized as sources for production of oil.

2.1.2 The production of biodiesel

Previous studies have shown that triglycerides hold promise as alternative diesel engine fuels. Some natural glycerides contain higher levels of unsaturated and saturated fatty acids. They can not be used as fuel in a diesel engine in their original from. The high viscosity, acid composition, and free fatty acid content of such oil, as well as gum formation due to oxidation and polymerization during storage and combustion, carbon deposits, and lubricating oil thickening are some of the more obvious problems (Darnoko *et al.*, 2000, Komers *et al.*, 2001 and Demirbas A., 2003). Consequently, considerable effort has gone into developing vegetable oil derivatives that approximate the properties and performance of hydrocarbon-based diesel fuels. Problems encountered in substituting triglycerides for diesel fuels are mostly associated with their high viscosity, low volatility and polyunsaturated character. There are various ways to produce biodiesel (Demirbas A., 2009) as follows:

2.1.2.1 Direct use and blending

Vegetable oil can be directly mixed with diesel fuel and may be used for running an engine. The blending of vegetable oil with diesel fuel were experimented successfully by various researchers. A diesel fleet was powered with a blend of 95% filtered used cooking oil and 5% diesel in 1982. A blend of 20% oil and 80% diesel was found to be successful (Narayan *et al.*, 1996). It has been proved that the use of 100% vegetable oil was also possible with some minor modifications in the fuel system. The high fuel caused the major problems associated with the use of pure vegetable oils as fuel viscosity in compression ignition engines. Micro-emulsification, pyrolysis and transesterification are the remedies used to solve the problems encountered due to high fuel viscosity (Ramadhas *et al.*, 2004).

2.1.2.2 Pyrolysis

Pyrolysis refers to chemical change caused by application of heat to get simpler compounds from a complex compound. The process is also known as cracking. Vegetable oils can be cracked to reduce viscosity and improve cetane number. The products of cracking include alkanes, alkenes, and carboxylic acids. Soyabean oil, cottonseed oil, rapeseed oil and other oils are successfully cracked with appropriate catalysts to get biodiesel (Ma and Hanna, 1999). By using this technique good flow characteristics were achieved due to reduction in viscosity. Disadvantages of this process include high equipment cost and need for separate distillation equipment for separation of various fractions. Also the product obtained was similar to gasoline containing sulfur which makes it less eco-friendly (Ma and Hanna, 1999)

2.1.2.3 Microemulsion

To solve the problem of high viscosity of vegetable oil, micro emulsions with solvents such as methanol, ethanol (Demirbas A., 2009), propanol (Ranganathan *et al.*, 2008) and butanol have been used. A micro emulsion is defined as the colloidal equilibrium dispersion of optically isotropic fluid microstructures with dimensions generally in the range of 1–150 nm formed spontaneously from two normally immiscible liquids and one or more ionic or non-ionic amphiphiles. These can improve spray characteristics by explosive vaporization of the low boiling constituents in the micelles. All micro emulsions with butanol, hexanol and octanol will meet the maximum viscosity limitation for diesel engines.

2.1.2.4 Transesterification

The most popular method of producing biodiesel is the transesterification of vegetable oils. Biodiesel obtained by transesterification process is a mixture of mono-alkyl esters of higher fatty acids. Transesterification is the alcoholysis of triglyceric esters resulting in a mixture of mono-alkyl esters and glycerol and the sequence of processes. The high viscosity component, glycerol, is removed and hence the product has low viscosity like the fossil fuels. The mixture of these mono-alkyl esters can therefore be used as a substitute for fossil fuels. The transesterification process can be done in a number of ways such as using an alkali catalyst, acid catalyst, biocatalyst, heterogeneous catalyst or using alcohols in their supercritical state. In the alkali process, sodium hydroxide (NaOH) or potassium hydroxide (KOH) (Leung and Guo, 2006, Sharma and Singh, 2008 and Tiwari *et al.*, 2007) is used as a catalyst along with methanol or ethanol. Initially, during the process, alcoxy is formed by reaction of the catalyst with alcohol and the alcoxy is then reacted with any vegetable oil to form biodiesel and glycerol. Glycerol, being denser settles at the bottom and biodiesel can be decanted. This process is the most efficient and least corrosive of all the processes and the reaction rate is reasonably high even at a low temperature of 60 °C. There may be risk of free acid or water contamination and soap formation is likely to take place which makes the separation process difficult (Ma and Hanna, 1999; Fukuda *et al.*, 2001; Barnwal and Sharma, 2005). The second conventional way of producing biodiesel is the application of an acid catalyst instead of a base. Any mineral acid can be used to catalyze the process. The most commonly used acids are sulfuric acid (Ramadhas *et al.*, 2004, Ghadge and Raheman, 2005, Tiwari *et al.*, 2007 and Guan *et al.*, 2009), sulfonic acid (Guerreiro *et al.*, 2006) and ferric sulfate (Wang *et al.*, 2007). Although yield is high, the acids, being corrosive, may cause damage to the equipment and the reaction rate was also observed to be low.

It has been recently found that enzymes such as lipase can be used to catalyze transesterification process by immobilizing them in a suitable support. The advantage of immobilization is that the enzyme can be reused without separation. Also, the operating temperature of the process is low (approximately 50 °C) compared to other techniques as shown in Table 2-2.

Table 2-2. Comparison between alkali-catalyzed and lipase-catalyzed methods for biodiesel production

	alkali-catalyzed process	lipase-catalyzed process
Reaction temperature	60-70 °C	30-40 °C
Free fatty acids in raw material	Saponified products	Methyl esters
Water in raw materials	Interference with the reaction	No influence
Yield of methyl esters	Normal	Higher
Recovery of glycerol	Difficult	Easy
Purification of methyl esters	Repeated washing	None
Production cost of catalyst	Cheap	Relatively expensive

From Fukuda *et al.,* 2001

2.2 Lipase

Lipase, triacylglycerol acylhydrolases (EC 3.1.1.3), constitutes a group of enzyme whose natural catalytic function is to hydrolyze ester bonds in tri-, di-, and monoglycerides (Villeneuve *et al.*, 2000, Cihangir and Sarikaya, 2004 and Châabouni *et al.*, 2008) and variety of compounds containing carboxylic ester moieties that are not acylglycerols. Lipase and phospholipase natural substrates are insoluble in water and their activity use maximum only at the lipid-water interface (Villeneuve *et al.*, 2000). This unique property known as interfacial activation may differ for each specific lipase. From the EC number of lipase, 3.1.1.3, whose components indicate the following groups of enzymes:

E.C.3.	Hydrolases
E.C.3.1.	Acting on ester bonds
E.C.3.1.1.	Carboxylic ester hydrolases
E.C.3.1.1.3	Triacylglycerol lipases

2.2.1 Source of lipase

Lipases from a large number of plant, animal and microorganism (Villeneuve *et al.*, 2000, Deng *et al.*, 2005 and Ibrahim, C.O., 2008) sources have been purified to homogeneity. Lipases isolated from different sources have a wide range of properties depending on their sources with respect to positional specificity, fatty acid specificity, thermostability, pH optimum, etc (Hasan *et al.*, 2006). Microbial enzymes are often more useful than enzymes derived from plants or animals (Ibrahim, C.O., 2008) because of their production is more convenient and safer, the great variety of catalytic activities available, the high yields possible, tend to have neutral or alkaline pH optima, ease of genetic and environmental manipulation to increase the yield of cells, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media (Hasan *et al.*, 2006).

2.2.2 Lipase produced by microorganisms

Both intracellular and extracellular lipases are produced by microorganism. Lipases are produced by a widespread number of microorganisms, bacteria, fungi and yeast. In particular, lipases produced by bacteria such as *Pseudomonas sp* (Karadzic et al., 2006). *Burkholderia cepacia* and fungi belonging to the genera *Penicillium, Rhizopus* and *Rhizomucor* and yeast such as *Candida sp.* are well-known industrial lipase producers.

The yeast *Candida rugosa* (previously named *Candida cylindracea)* is a major source of commercial lipase (CRL) broadly employed by various laboratories and industrial applications and classified as a non-specific lipase with respect to the position of the fatty acid chain released from the glycerol molecule. However, recently it has been recognised that CRL is indeed a heterogeneous mixture of different proteins since *C. rugosa* synthesises and secretes multiple lipase isoenzymes differing in biochemical properties and in substrate specificity (Maria and Gago, 1999).

2.2.3 Lipase property

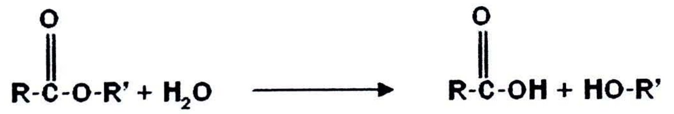
Lipolytic reactions occur at the lipid–water interface, implying that the kinetics cannot be described by Michaelis–Menten equations, as these are valid only if the catalytic reaction takes place in one homogenous phase. Lipolytic substrates usually form equilibrium between monomeric, micellar and emulsified states, resulting in the need for a suitable model system to study lipase kinetics. The monolayer technique has been used extensively and, more recently, an oil-drop technology has been put forward in which lipase kinetics are monitored by automatic analysis of the profile of an oil drop hanging in water; the decrease in the interfacial tension between the oil and water caused by lipase hydrolysis is measured as a function of time. The best-known phenomenon emerging from early kinetic studies of lipolytic reactions became known as 'interfacial activation', describing the fact that the activity of lipases is enhanced towards insoluble substrates that form an emulsion. Lipases, in contrast to esterases, were therefore defined as carboxylesterases acting on emulsified substrates.

2.2.4 Enzymatic reaction of lipase

Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface and do not hydrolyze dissolved substrates in the bulk fluid (Vakhlu *et al.*, 2005). A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. Lipases are serine hydrolases. Lipases display little activity in aqueous solutions containing soluble substrates (Sharma *et al.*, 2001).

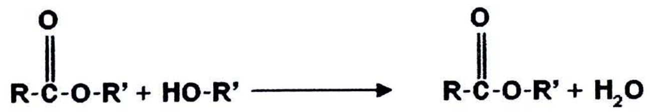
Lipases have been used in numerous applications of which the most important one is in the production of fine chemicals and oleochemicals. The products include fatty acids from hydrolysis of lipids, esters or glycerides via esterification reactions and modified lipids and structural triglycerides via the transesterification reactions, namely acidolysis, alcoholysis and interesterification (Ibrahim C.O., 2008) (Figure 2-1).

2.2.4.1 Hydrolysis



$$R\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R' + H_2O \longrightarrow R\text{-}\overset{\overset{O}{\|}}{C}\text{-}OH + HO\text{-}R'$$

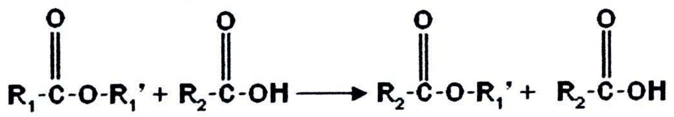
2.2.4.2 Esterification



$$R\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R' + HO\text{-}R' \longrightarrow R\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R' + H_2O$$

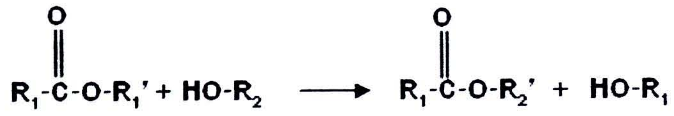
2.2.4.3 Transesterification

2.2.4.3.1 Acidolysis



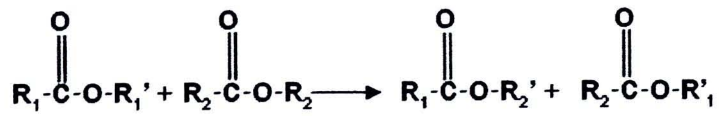
$$R_1\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R_1' + R_2\text{-}\overset{\overset{O}{\|}}{C}\text{-}OH \longrightarrow R_2\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R_1' + R_2\text{-}\overset{\overset{O}{\|}}{C}\text{-}OH$$

2.2.4.3.2 Alcoholysis



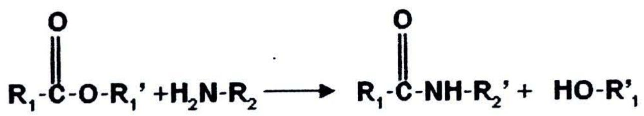
$$R_1\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R_1' + HO\text{-}R_2 \longrightarrow R_1\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R_2' + HO\text{-}R_1$$

2.2.4.3.3 Interesterification



$$R_1\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R_1' + R_2\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R_2 \longrightarrow R_1\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R_2' + R_2\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R'_1$$

2.2.4.3.4 Aminolysis



$$R_1\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}R_1' + H_2N\text{-}R_2 \longrightarrow R_1\text{-}\overset{\overset{O}{\|}}{C}\text{-}NH\text{-}R_2' + HO\text{-}R'_1$$

Figure 2-1 Different reactions catalyzed by lipase in aqueous and non-aqueous solutions.

Due the wide variety of environmental condition, lipases are often easily inactivated and difficult to be separated from the reaction system for reuse. Consequently, the further industrial applications of lipase are limited. By an appropriate choice of the immobilization process, operational costs of industrial processes involving lipase can be significantly reduced (Hung *et al.*, 2003 and Chang *et al.*, 2008)

2.2.5 Applications of lipases

Lipases are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals (Cihangir and Sarikaya, 2004, Dandavate and Madamwar, 2007 and Rajendran *et al.*, 2008). Lipase can be used to accelerate the degradation of fatty waste (Masse *et al.*, 2001) and polyurethane.

2.3 Immobilization

Research and development work provided a bewildering array of support materials and methods for immobilization. Much of the expansion may be attributed to developments provide specific improvements for a given application. Surprisingly, there have been few detailed and comprehensive comparative studies on immobilization methods and supports. Therefore, no ideal support material or method of immobilization has emerged to provide a standard for each type of immobilization. Selection of support material and method of immobilization is made by weighing the various characteristics and required features of the enzyme and cell application against the properties, limitations, characteristics of the combined immobilization support. A number of practical aspects should be considered before embarking on experimental work to ensure that the final immobilized enzyme or cell preparation is fit for the planned purpose or application and will operate at optimum effectiveness. This part provides some background to assist in choice evaluation for support and method of immobilization.

2.3.1 Choice of support

The characteristics of the matrix are of paramount importance in determining the performance of the immobilized enzyme system. Ideal support properties include physical resistance to compression, hydrophilicity, inertness toward enzymes ease of derivatization, biocompatibility, resistance to microbial attack, and availability at low cost.

Supports can be classified as inorganic and organic according to their chemical composition (Table 2-3). The organic supports can be subdivided into natural and synthetic polymer.

Table 2-3. Classification of supports

Organic

Natural polymers

- Polysaccharides: cellulose, dextrans, agar, agarose, chitin, alginate
- Protein: collagen, albumin
- Carbon

Synthetic polymers

- Polystyrene
- Other polymer: polyacrylate polymethacrylates, polyacrylamide, polyamides, vinyl and allyl-polymers

Inorganic

Natural minerals: bentonite, silica

Processed materials: glass (nonporous and controlled pore), metals, controlled pore metal oxides

The physical characteristics of the matrices (such as mean particle diameter, swelling behavior, mechanical strength, and compression behavior) will be of major importance for the performance of the immobilized. In particular, pore parameters and particle size determine the total surface area and thus critically affect the capacity for binding of enzymes. Nonporous supports show few diffusional limitations but have a low loading capacity (Öztürk *et al.*, 2007). Therefore, porous supports are generally preferred because of the greater protection from the environment. Porous supports should have a controlled pore distribution in order to optimize capacity and flow properties. In spite of the many advantages of inorganic carriers (e.g., high stability against physical, chemical, and microbial degradation), most of the industrial applications are performed with organic matrices.

2.3.2 Methods of enzyme immobilization

Enzyme immobilization technology may be an effective means to perform enzyme reuse and to improve its activity and stability (Chang *et al.*, 2008). A number of method for immobilization of enzymes have been reported in the literatures review such as adsorption onto an insoluble material, covalent linking to an insoluble carrier, entrapping enzymes within the matrix and encapsulation in gel bead. The relative merits each are discussed briefly below.

2.3.2.1 Adsorption

Immobilization by adsorption is the simplest method and involves reversible surface interactions between enzyme/cell and support material. The forces involved are mostly electrostatic, such as Van der Waals interactions, hydrophobic interactions, hydrogen bonds, ionic bonds (Villeneuve *et al.*, 2000), although hydrophobic bonding can be significant. These forces are very weak, but sufficiently large in number to enable reasonable binding. For example, it is known that yeast cells have a surface chemistry that is substantially negatively charged so that use of a positively charged support will enable immobilization. Existing surface chemistry between the enzyme/cell and support is utilized so no chemical activation/modification is required and little damage is normally done to enzyme or cells in this method of immobilization. The procedure consists of mixing together the biological components and a support with adsorption properties, under suitable conditions of pH, ionic strength and others, for the period of incubation, followed by collection of the immobilized material and extensive washing to remove nonbound biological components.

2.3.2.2 Covalent linkage

This method of immobilization involves the formation of a covalent bond between the enzyme and support material. The bond is normally formed between functional groups present on the surface of the support and functional groups belonging to amino acid residues on the surface of the enzyme. A number of amino acid functional groups are suitable for participation in covalent bond formation. Those that are most often involved are the amino group (NH_2) of lysine or arginine, the carboxyl group (CO_2H) of aspartic acid or glutamic acid, the hydroxyl group (OH) of serine or threonine, and the sulfydryl group (SH) of cysteine.

It is important to choose a method that will not inactivate the enzyme by reacting with amino acids at the active site. So, if an enzyme employs a carboxyl group at the active site for participation in catalysis, it is wise to choose a reaction that involves amino groups for the covalent bond with the support. Chemical methods of crosslinking normally involve covalent bond formation between the enzyme by means of a bi- or multifunctional reagent, such as glutaraldehyde toluene and diisocyanate. However, the toxicity of such reagents is a limiting factor in applying this method to living cell and many enzymes.

2.3.2.3 Entrapment

Immobilization by entrapment differs from adsorption and covalent binding in that enzyme molecules are free in solution, but restricted in movement by the lattice structure of a gel. The porosity of the gel lattice is controlled to ensure that the structure is tight enough to prevent leakage of enzyme or cell, yet at the same time allow free movement of substrate and product. Inevitably, the support will act as a barrier to mass transfer, and although this can have serious implications for reaction kinetics, it can have useful advantages since harmful cells, protein and enzyme are prevented from interaction with the immobilization biocatalyst. Entrapment can be achieved by mixing an enzyme with a polyionic polymer material and then cross linking the polymer with multivalent cations in an ion-exchange reaction to from a lattice structure that traps the enzyme or cell (ionotropic gelation). Alternatively, it is possible to mix the enzyme with chemical monomers that are then polymerized to from a cross linked polymeric network, trapping the enzyme in the interstitial spaces of the lattice. The latter method is more widely used, and a number of acrylic monomers are available for the formation of hydrophilic copolymers.

2.3.2.4 Encapsulation

Encapsulation of enzyme and or cell can be achieved by enveloping the biological components within various forms of semi permeable membranes. It is similar to entrapment in that the enzymes and cell are free in solution, but restricted in space. Large proteins or enzymes cannot pass out of or into the capsule, but small substrates and products can pass freely across the semipermeable membrane. Many materials have been used to construct microcapsules varying from 10-100 μm in diameter; for example, nylon and cellulose nitrate have proven popular. The problems associated with diffusion are more acute and many result in rupture of the membrane if products from a reaction accumulate rapidly. A further problem is that the immobilized cell or enzyme particle may have a density fairly similar to that of the bulk solution with consequent problems in reactor configuration, flow dynamics, and so on.

Since most enzymes applied for industrial uses need to be immobilized in order to be reusable and therefore reduce the cost of the operation, there are many methods to immobilize enzyme. Each method provides different advantages and disadvantages depending upon its nature as shown in Table 2-4.

Table 2-4. Comparative methods for the immobilization of enzyme

Characteristics	Adsorption	Covalent binding	Entrapment	Membrane confinement
Preparation	Simple	Difficult	Difficult	Simple
Cost	Low	High	Moderate	High
Binding force	Variable	Strong	Weak	Strong
Enzyme leakage	Yes	No	Yes	No
Applicability	Wide	Selective	Wide	Vary wide
Running problems	High	Low	High	High
Matrix effects	Yes	Yes	Yes	No
Large diffusional barriers	No	No	Yes	Yes
Microbial protection	No	No	Yes	Yes

Reference: http://www.org /ippagele/ipdate/2004/05/file/e200405-1101.pdf

After immobilization of lipase, changes were observed in enzyme activity, optimum pH, affinity to substrate and stability.

2.4 Literature reviews

The extent of immobilization changes depended on the source of enzyme, the type of support and the method of immobilization. (Bayramoğlu and Arica, 2008)

2.4.1 Review of lipase immobilization

From previous literature, studies on different support materials and protocols for lipase immobilization and the effects of operational conditions on the enzyme activity and enantioselectivity are also explored.

Takaç and Bakkal immobilized *Candida rugosa* lipase on Amberlite XAD 7 and immobilization under the best reaction conditions in achieving high activity. The authors found that pH 6 and 45°C were obtained for immobilization *C.rugosa* lipase adsorbed on Amberlite XAD 7 was used in the hydrolysis of racemic Naproxen methyl ester (Takaç and Bakkal, 2007).

Huang and Cheng immobilized alkali lipase from *Penicillium expansum* on biomodal ceramic foam and determined on the preferable immobilization conditions. The optimal conditions for immobilization of lipase were found at pH 8, 12 g lipase/g support, 4 hr immobilizing time and 20 °C immobilizing temperature (Huang and Cheng, 2008).

Chang *et al.* immobilized *Candida rugosa* lipase by adsorption on Celite. The immobilization conditions and characterization of the immobilized enzyme were investigated. The optimum immobilization conditions were as follows: immobilization time 59.1 min, immobilization temperature 10.7 °C and enzyme support ratio 0.5 (w/w): the highest specific activity obtained was 18.16 U/mg-protein with activity yield of 34.1% (Chang *et al.*, 2007).

Yeşiloğlu immobilized *Candida rugosa* lipase by non-covalently on bentonite. The authors found that the optimum pH of bentonite-immobilized lipase was 7.5, which is slightly higher than the free enzyme, the value of the pH 7.0 (Yeşiloğlu Y., 2005).

Gitlesen *et al.* immobilized different lipases by adsorption on polypropylene powder. The adsorption of lipase from *Candida rugosa* lipase was better than other sources of lipase. Adsorption of a crude lipase from *Candida rugosa* was fast and equilibrium was reached in 30 and 100 min for protein and lipase activity adsorption respectively (Gitlesen *et al.*, 1997).

Lei *et al.* immobilized porcine pancreas lipase by covalent on magnetic microspheres. The authors found that the activity yield was up to 63% (±2.3%) and enzyme loading of 39 (±0.5) mg/g support. The resulting immobilized lipase had higher optimum temperature compared with those of free lipase and exhibited better thermal, broader pH stability and excellent reusability (Lei *et al.*, 2009).

Yang *et al.* immobilized lipase from *Candida* sp. 99-125 by physical adsorption onto macroporous resins (such as NKA-9, AB-8, H103 and D4020). They reported that NKA-9 was an appropriate support because 98.98% degree of immobilization was obtained. As lipase was adsorbed on NKA in heptane to produce biodiesel, the batch conversion rate reached 97.3% when the three step methanolysis protocol was used (Yang *et al.*, 2006).

Ye *et al.* immobilized lipase from *Candida rugosa* by adsorption and chemical bonding onto chitosan-modified poly (acrylonitrile-co-maleic acid) membrane surface. The activity retention of the immobilized lipase on the chitosan-tethered membrane by adsorption (54.1%) is higher than that by chemical bonding (44.5%) (Ye *et al.*, 2006).

Feresti and Ferreira studied the immobilized lipase from *Candida rugosa, Pseudomonas fluorescens* and *Candida antarctica* B onto chitosan and glutaraldehyde pretreated chitosan powder. The prepared biocatalysts were assayed in the direct esterification of oleic acid and ethanol to produce the ethyl oleate. The immobilization of lipase from *Candida antarctica* B onto untreated chitosan powder led to 75% conversion of the fatty acid in 24 hr of reaction.They concluded that *Candida antarctica* B was the most active (Feresti and Ferreira, 2007).

2.4.2 Review of transesterification catalyzed by lipase

Authors/year	Oil/enzyme	Acyl acceptor	Conversion (%)	Technique employed
Watanabe *et al.* (2000)	Vegetable oil, Novozyme 435	Methanol	90-93	Stepwise addition of methanol
Samukawa *et al.* (2000)	Soybean oil, Novozyme 435	Methanol	97	Stepwise addition methanol and preincubation of enzyme in methyl oleate and soyabean oil
Ban *et al.* (2001)	Vegetable oil, *Rhizopus oryzae*	Methanol	90	Stepwise addition of methanol and application of glutaraldehyde for stability of enzyme
Iso *et al.* (2001)	Triolein, *Pseudomonas flourescens*	Butanol	90	Butanol was used as an acyl acceptor and no solvent was used
Shimada *et al.* (2002)	Waste cooking oil, Novozyme 435	Methanol	90	Stepwise addition of methanol
Bako *et al.* (2002)	Sunflower oil, Novozyme 435	Methanol	97	Stepwise addition of methanol and removal of glycerol by dialysis
Du *et al.* (2004)	Soy bean oil, Novozyme 435	Methyl acetate	92	A novel acyl acceptor, methyl acetate which had no inhibitory effects was used

Authors/year	Oil/enzyme	Acyl acceptor	Conversion (%)	Technique employed
Xu *et al*. (2004)	Soy bean oil, Novozyme 435	Methanol	98	Stepwise addition of methanol and removal of glycerol using the solvent, iso-propanol
Li *et al*. (2006)	Rapeseed oil, Novozyme 435 & Lipozyme TL IM	Methanol	95	Combined use of Lipozyme TL IM and Novozyme 435 along with tert-butanol as solvent
Royon *et al*. (2007)	Cotton seed oil, Novozyme 435	Methanol	97	tert-Butanol was used as a solvent
Modi *et al*. (2007)	Jatropha oil, Novozyme 435	Ethyl acetate	91.3	Ethyl acetate having no inhibitory effects was used
Hama *et al*. (2007)	Soy bean oil, *Rhizopus oryzae*	Methanol	90	Stepwise addition of methanol in a packed bed reactor
Dizge and Keskinler (2009)	Canola oil, *Thermomyces lanuginosus*	Methanol	90	Stepwise addition of methanol
Lu et al. (2008)	Glycerol trioleate, *Candida* sp. 99-125	Methanol & CCl_4	90.58	Three step methanolysis in different solvents systems
Dizge *et al*. (2009)	Sunflower oil, *Thermomyces lanuginosus*	Methanol	97	Stepwise addition of methanol and flow rate of substrate

Authors/year	Oil/enzyme	Acyl acceptor	Conversion (%)	Technique employed
Halim, S.F.A. and Kamaruddin, A.H. (2008)	Waste cooking palm oil, Novozyme 435	Methanol & *tert*-butanol	88	Optimal condition in batch system
Watanabe *et al.* (2002)	Soybean oil, *Candida antarctica*	Methanol	93.8	Stepwise addition of methanol
Lu *et al.* (2007)	Lard, *Candida sp.* 99-125	Methanol	87.4	n-hexane was used as a solvent
Kösa *et al.* (2002)	Cotton seed, *Candida antarctica*	Isoamyl alcohol	94	New alcohol type on the alcoholysis
Noureddini *et al.* (2005)	Soybean oil, *Pseudomonas cepacia*	Methanol & Ethanol	67 & 65	-
Kamini, N.R. and Iefuji,H. (2001)	Vegetable oil, *Cryptococcus spp.* S-2	Methanol	80.2	Amount of water in reaction
Nie *et al.* (2006)	Salad oil, *Candida sp.* 99-125	Methanol	96	Optimal conditions in a fixed bed reaction
Gao *et al.* (2009)	Chinese tallow kernel oil, lipase	Methanol	93.86	-