Enhanced Lipase – Catalyzed Triglyceride Hydrolysis

Submitted by

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Abstract

The use of microbial lipases for the hydrolysis of natural oils such as triglyceride esters is a green alternative to conventional high temperature, high pressure steam-based technologies and other chemical synthesis. The hydrolytic splitting of the esters is necessary for the downstream production of high value chemical products such as coatings, adhesives, and high-performance personal care products. Deployment of enzymatic methods enables conversions to be achieved at close to ambient temperature and pressure with positive impacts on energy utilization and product purity. Enzymatic splitting of triglyceride esters is limited often by slow kinetics due to mass transfer limitations and by challenges of economic enzyme recycling.

Immobilization of lipases on a solid carrier or support has proven to be an effective alternative method and, in some cases, considered superior to the use of lipases in aqueous media. Enhanced thermal and chemical stability, activity, recoverability, and reusability of the biocatalyst (lipase) are all potential advantages of immobilization. Efficacy of immobilization depends significantly on the mechanical integrity of the support, availability, and adjustable characteristics such as porosity, surface area, particle size a functional groups present, and the type of lipase used. The performance of microbial lipase derived from *Candida rugosa* immobilized onto polymeric methacrylate-based resins with no surface functionalization (ECR1030M), epoxy & butyl functionalization (ECR8285) and octadecyl functionalization (ECR8806M) supplied by Purolite® Lifetech™ Resins Corporation was studied for triglyceride hydrolysis. The main mechanism involved in lipase immobilization was adsorption using hydrophobic interactions. Sizing of protein using the dynamic light scattering technique suggested immobilization was surface dominant. The resins were characterized using FT-IR spectrometry, N_2 adsorption, contact angle measurements and scanning electron microscopy. Continuously stirred batch reactors operated at 100 RPM were

used to compare the performance of the immobilized lipase by measuring the release of free fatty acids (FFA). Octadecyl functionalized methacrylate polymer resins showed superior performance. Comparing the octadecyl functionalized resins with un-functionalized methacrylate resins, a four-fold increase in activity retention was observed in multicycle experiments. Epoxy & butyl functionalized resins showed lower performance compared to octadecyl functionalized resin but higher than un-functionalized methacrylate resins. Performance in the presence of crosslinking agents such as, Glutaraldehyde, (3-Aminopropyl) triethoxysilane and itaconic acid applied during the immobilization protocol for methacrylate based polymeric resins and superior performance was observed in the case of itaconic acid on functionalized methacrylate resins.

Prior work has shown that mass transfer rates and reaction rates can be intensified by increasing interfacial area for lipase catalyzed triglyceride hydrolysis using electrostatic spray reactors. However, the possible effect of oriented external electrical field on the lipase catalytic activity has hitherto not been considered. The performance of microbial lipase derived from *Candida rugosa* in aqueous solutions was analyzed in the presence of a steady DC externally applied electrical voltage. The reaction was conducted in three different batch type reactors: (1) In a quiescent (fixed interface) reactor; (2) In a stirred tank batch reactor, and (3) In a recirculating tubular flow reactor. It was concluded that the oriented external electrical field has a positive effect on all three-reactor system studied, showing reaction rate enhancement, independent of interfacial area. Further studies conducted using reverse polarity, increased electrode distance and for immobilized lipase system has shown that lipase undergoing conformational changes due to an oriented external electrical field is the main driving mechanism for this noted enhanced performance.

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Chapter 1 : Introduction to oils and fats.

1.1. Importance of oils and fats and their market growth.

Every year, the human population uses nearly 320 million tons of fats and oils obtained from animal and vegetable sources. These renewable resources are used as raw materials for the manufacture of many edible products including butter, shortenings, margarine, salad oils, and cooking oils and other non-edible products including animal feeds, soaps, personal care products, paints, lubricants, and greases [1, 2]. The global production rate of vegetable oils has increased from 137.3 million tons per year in 2010 to 209.1 million tons per year in 2020. The most popular vegetable oil consumed in United States is soybean oil due to its high production rate of 10.9 million tons per year [1, 3]. **Figure 1.1** shows global consumption of various vegetable oils for the year 2020 [1]. Thus, the oils and fats industry are of global importance, providing economic stability in countries throughout Asia, Europe, South America, and North America.

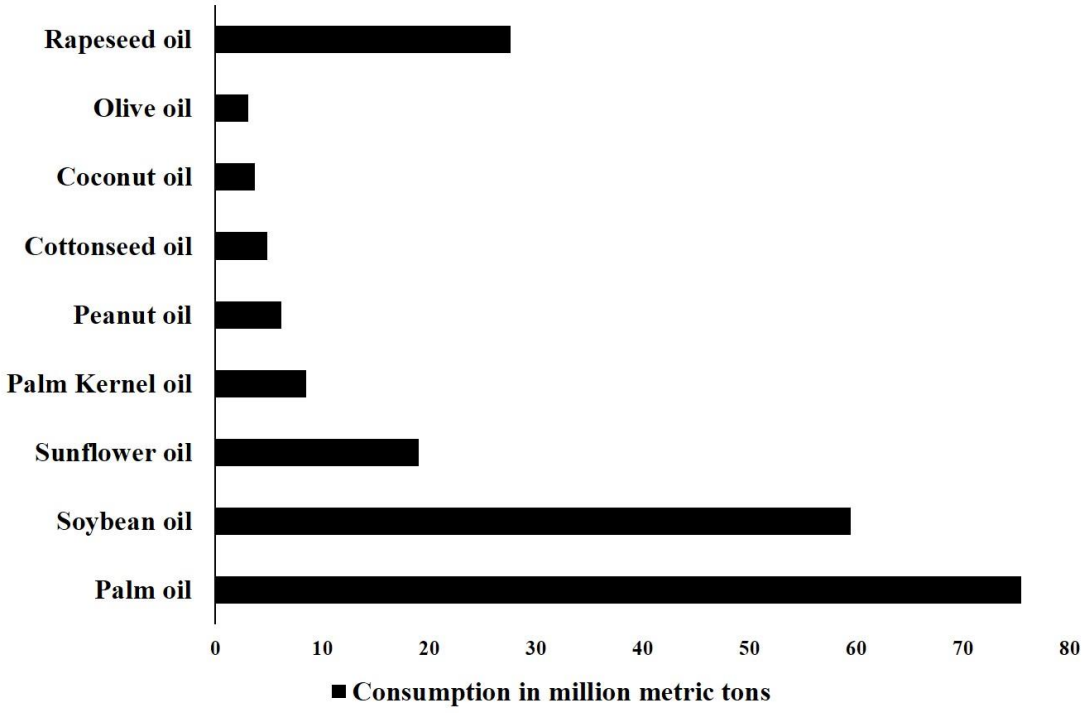


Figure 1.1. Global consumption of various vegetable oils.

1.2. General physical properties of oils and fats.

Fats and oils are often distinguished from one another based on their physical appearance. In general, fats are solid or semi-solid at room temperatures (22 ± 2°C) and pressures (1 atm) while oil remains to be a liquid in these conditions [4]. **Table 1.1** shows the physical properties of some commonly consumed natural oils and fats by human population [5-7]. It can be seen from the table that palm and coconut oils are exceptions since they would be solid in certain regions of the world yet are liquid in their respective country of origin and make and are still considered as oils. Apart from their difference in physical appearance, fats are insulators of heat and electricity while most oils are good conductors of heat but poor conductors of electricity [8]. However, irrespective of the above differences, both these classes of compounds are majorly made up of triacylglycerols (or more commonly known as triglycerides).

Table 1.1. Typical physical properties of some commonly consumed natural oils and fats by human population.

Name	Melting Point (°C)	Specific gravity (g/mL) At 15.5°C	Kinematic Viscosity (cP) At 37.8°C
Beef Tallow	40 – 50	0.90	-
Lard	30 – 35	0.96	35
Butter	32 – 35	0.86	42
Palm oil	35 – 37	0.92	30.9
Cocoa butter	34 – 38	0.86	-
Coconut oil	24 – 25	0.92	28.0
Olive oil	-6	0.91	46.6
Soybean oil	-16	0.92	31.8
Sunflower oil	-17	0.92	33.3
Rapeseed oil	-10	0.90	30.92

Triglycerides (or Triacylglycerol or TG) make up to more than 95% by weight for most of the oils and fats with other minor components including mono and diglycerides, free fatty acids, phosphatides, sterols, fatty alcohols, fat-soluble vitamins, and other impurities depending on the source [9]. A single glycerol molecule contains three alcohol (-OH) functional groups and all these groups are esterified to form a single triglyceride molecule. **Figure 1.2** represents the structure of a single triglyceride molecule [10]. In nature, triglycerides are formed from carboxylic acids. These are commonly known as fatty acids and are represented by R_a, R_b and R_c in the figure.

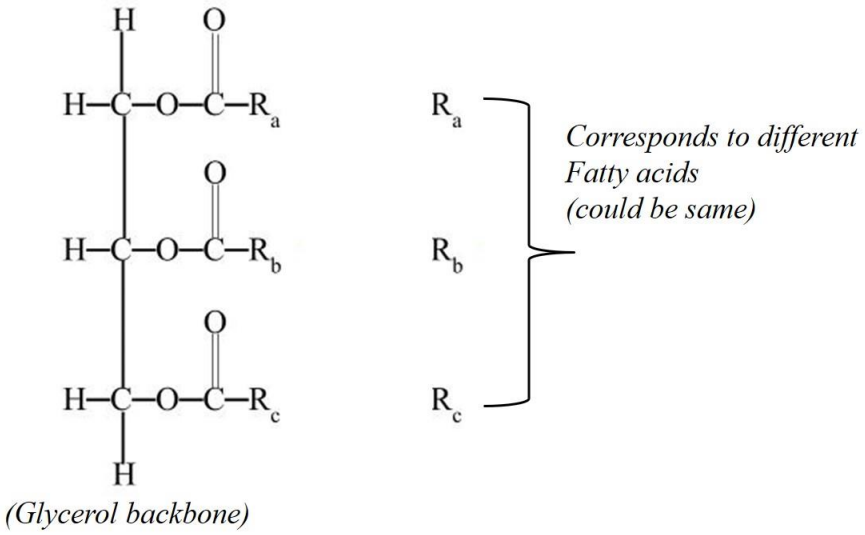


Figure 1.2. Structural representation of a single triglyceride molecule.

1.3. Biochemistry of fatty acids

Fatty acids (FA) are generally classified into three types: saturated (mostly found in animal fats), monounsaturated (includes canola, olive, and peanut oils) and polyunsaturated (includes soybean, sunflower, corn, and most nut oils) [11]. Irrespective of their type, all fatty acids are composed of long aliphatic chain with a monocarboxylic acid moiety at the terminal end [12]. In

saturated fatty acids, all the carbon atoms are attached to maximum possible number of hydrogen atoms. **Figure 1.3** shows the structure of chemical bonds associated with saturated and unsaturated fatty acid molecule [13]. The degree of unsaturation is given by the number of hydrogen atoms missing (unsaturated) [14]. Unsaturated fatty acids show cis-trans isomerism due to the presence of double bonds.

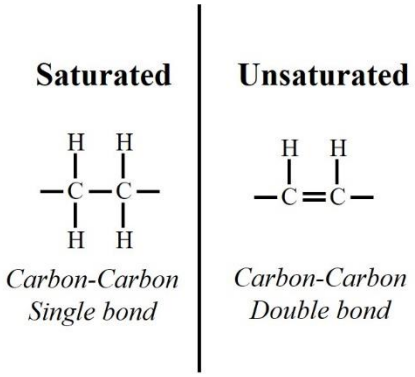


Figure 1.3. Structural representation of chemical bonds associated with saturated and unsaturated fatty acid molecule.

Typically, most oils are more unsaturated (fewer hydrogen atoms), and these bonds are associated with fatty acids section of the triglyceride molecule and hence, it is evident that the properties of oils and fats are profoundly dependent on the types of fatty acids (chain length, degree of unsaturation and stereo chemistry) involved in their makeup [15]. However, certain properties such as solubility and poor electrical conductivity remain the same for most of the fats, oils, and fatty acids. They exhibit high solubility in organic solvents such as acetone, benzene, toluene, and chloroform and minimal or no solubility in water [16].

There have been more than 300 different fatty acids identified from various sources of oils and fats from across the world with some unusual number of double bonds and oxidations or epoxidations [17]. However, more than 90% of fatty acid molecules attached to triglyceride

molecules in commonly sourced vegetable oils such as soybean oils, rapeseed oils or sunflower oils and animal fats such as lards, beef tallows or butters are primarily composed of an even number of carbon atoms ranging from 4 (butanoic acid) to 24 (lignoceric acid) carbons long. Fatty acids are described by the number of carbon atoms in the chain, followed by a colon and additional numbers which indicate the number of double bonds. Therefore, for those fatty acids in the 18-carbon series we have C18:0 which represents steric acid, C18:1 for oleic acid, C18:2 for linoleic acid and C18:3 for linolenic acid [18]. These double bonds can also exhibit in 'cis' and 'trans' form which is dependent on the arrangement of carbon chains across one or more double bonds [19]. Naturally occurring unsaturated fatty acids show cis type configuration. For example, C18:1 in cis configuration represents oleic acid and in trans configuration represents elaidic acid [20]. **Table 1.2** shows average fatty acid compositions for some of the commonly used fats and oils [21-26].

Table 1.2. Average fatty acid compositions of commonly used fats and oils.

Name	Fatty acid component												
	<14:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:0
Beef Tallow	0.9	3.7	24.9	4.2	18.9	36.0	3.1	0.6	-	0.3	-	-	-
Lard	0.5	1.3	23.8	2.7	13.5	41.2	10.2	1.0	-	1.0	-	-	-
Butter	23.8	8.2	21.3	1.8	9.8	20.4	1.8	1.2	-	-	-	-	-
Palm oil	0.1	1.0	43.5	0.3	4.3	36.6	9.1	0.2	-	0.1	-	-	-
Cocoa butter	-	0.1	25.4	0.2	33.2	32.6	2.8	0.1	-	0.0	-	-	-
Coconut oil	58.7	16.8	8.2	-	2.8	5.8	1.8	-	-	-	-	-	-
Olive oil	-	0.1	11.0	0.8	2.2	72.5	7.9	0.6	-	-	-	-	-
Soybean oil	-	0.1	10.3	0.2	3.8	22.3	51.0	6.8	-	-	-	-	-
Sunflower oil	-	0.1	5.9	0.1	4.5	19.5	65.7	-	-	-	-	-	-
Rapeseed oil	-	-	1.7	-	0.9	12.3	12.7	7.6	1.2	5.8	0.9	59.4	0.5

1.4. Modifications of oils and fats

The oils and fats industry is one in which many chemical engineering techniques are applied. Fats and oils obtained naturally do not always have the required physical and chemical properties for specialized purposes. On the contrary, many other renewable raw materials that do have the ideal properties required but are too expensive or not available in plenty. Modification of oils and fats offers an adequate solution for this purpose. Modified oils and fats offer changes to the physical and chemical properties which can be utilized for wider purposes, thus making them suitable for many uses or for making oils and fats with desirable properties available in sufficient quantities [27]. Some of the processes that offer the opportunity of modifying oils and fats with their respective notable applications are summarized below.

1.4.1. Hydrolysis

Oils and fats undergo hydrolysis reaction when treated with mineral acids, alkalis, super-heated steam (fat splitting) or by use of enzyme lipase to produce free fatty acids (FFA) and glycerol upon completion. When hydrolysis reaction is carried out in the presence of an alkali such as sodium hydroxide (NaOH) or potassium hydroxide (KOH) it leads to the formation of sodium or potassium salts of fatty acids which are commonly put together to form soaps and the associated reaction is called saponification and is one among the most important chemical processes that is carried out in abundance [28]. The hydrolysis of triglycerides into FFAs and glycerol as shown in **Figure 1.4**, is an important pre-requisite process required in the oleochemical industry to produce various fatty acids which are utilized as raw materials in the manufacture of various personal care products, cosmetics, and pharmaceutical drugs [29, 30]. For example, stearic acid is used as one of the raw materials in the manufacture of soaps, shampoos, lotions, and shaving creams [31].

1.4.2. Esterification

Esterification as shown in **Figure 1.4**, is the reverse process of hydrolysis and the reaction is carried out between polyhydric alcohols and fatty acids. Industrially, the esterification reaction is carried out between glycerol and fatty acids at 180 – 230°C between three and six hours under vacuum conditions. However, inorganic catalysts such as iron, magnesium or zinc oxides or organic catalysts such as naphthalene or sulphonic acid can also be used to speed up esterification reactions [32]. Esterification processes finds their applications in pharmaceutical and perfumery industries. For example, they are used in the manufacture of (S)-ibuprofen ester using (R,S)-ibuprofen and 1-propanol [33]. The most promising use of esterification in the 21st century, is that it could be utilized to produce higher quality biodiesel from low quality feedstocks [34].

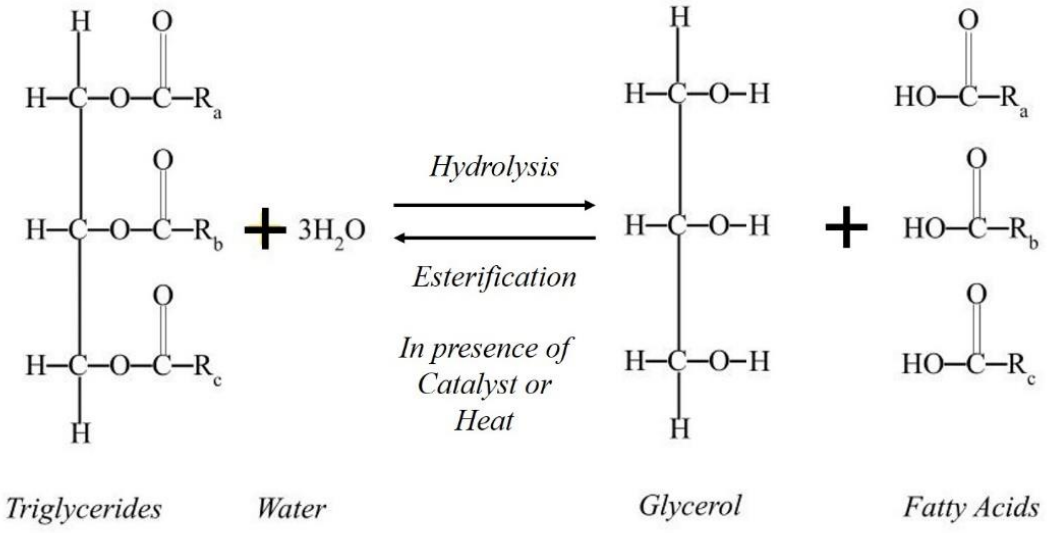


Figure 1.4. Chemical reaction showing the difference between hydrolysis and esterification reaction.

1.4.3. Transesterification

The process of exchanging the organic alkyl groups (R_a, R_b, R_c) of a triglyceride molecule in vegetable oils with a methyl group ($-CH_3$) is called transesterification as shown in **Figure 1.5**. The products obtained from the transesterification reaction includes various fatty acid methyl esters (FAMEs) and glycerol co-product. Transesterification reactions can be carried out in two pathways, using either catalytic (with acid or base as catalyst) route or non-catalytic (with high temperature and pressure processes) route [35]. The catalytic pathway includes large reaction times and catalyst separation which are disadvantageous. Hence, transesterification processes are mainly carried out industrially using supercritical alcohol [36]. However, there are drawbacks of using supercritical alcohol for transesterification process which includes the use and handling of high temperature and pressure equipment which are energy intensive and cost consuming [37]. So, in the past decade, much emphasis has been given to transesterification reactions that utilize lipases (enzymes) as biocatalysts. The main product obtained from transesterification include FAMEs which are the main constituents of biodiesel [38] and glycerol as co-product, which are purified and utilized as one of the raw materials in manufacturing of various cosmetics, personal care products and pharmaceutical drugs [39].

1.4.4. Interesterification

The term interesterification is broadly used to refer several reactions in which an ester of a fatty acid may react with fatty acids, alcohols, or other fatty acid esters to produce an ester differing in composition from its original. This process offers a means for improving oils and fats by redistribution of the fatty acids on the molecule. Alcoholysis, glyecerolysis, acidolysis and ester interchange are examples of interesterification processes [40]. Ester interchange is the most important of these interesterification reactions with most commercial value since it alters the

functional properties of the product [41]. Interesterified fats and oils finds their application in food products [42].

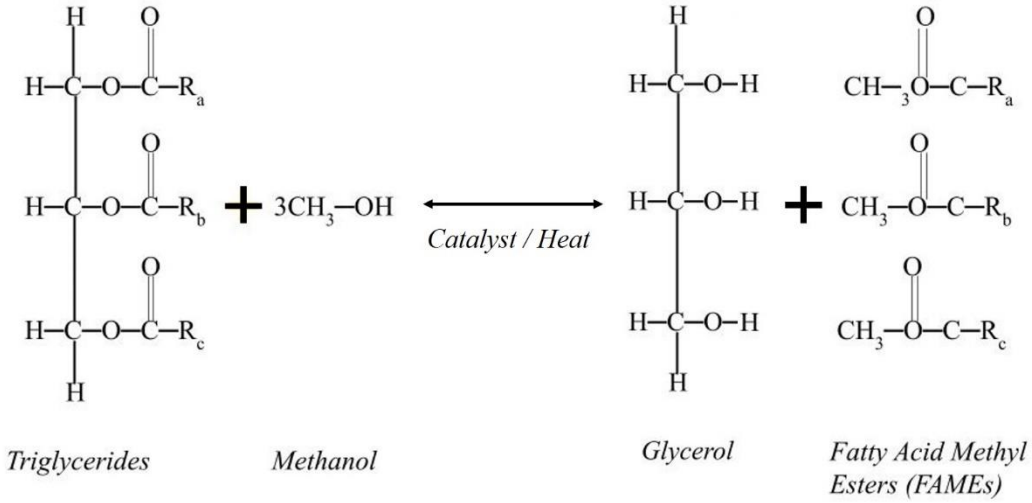


Figure 1.5. Chemical reaction showing transesterification process.

1.4.5. Hydrogenation

Oils containing unsaturated fatty acids (especially soybean oil) undergoes hydrogenation in the presence of high temperature, pressure, and nickel catalyst. This process is commonly known as hardening since it is concerned with producing oils with the desired solid properties. The hydrogenation process is a method of breaking the double bonds in the fatty acid chains by the addition of hydrogen. The hydrogenation reaction finds its application in the manufacturing process of vegetable ghee, which is one of the widely consumed edible product in India [43, 44].

1.4.6. Halogenation

Oils containing unsaturated fatty acids can be halogenated in the presence of reagents such as iodine or chlorine. Iodine number represents the percentage of iodine adsorbed by a fat or oil and it is useful to determine the degree of unsaturation [45]. Halogenated oils and fats find their

application in arthritis cures and as one of the raw material in the manufacturing of artificial rubber plasticizers [46].

1.5. Oleochemicals and importance oleochemical industry

Chemicals that are derived from plant-based oils or animal fats are called oleochemicals. Oleochemicals are considered as a green alternative to petroleum-based chemical products since they are derived from renewable resources such as oils and fats and the products derived causes less pollution and are non-toxic in nature [47]. **Figure 1.6** represents commonly used feedstocks in global oleochemical industries [48]. In the United states, due to large scale production of soybean oil, it is widely used as feedstock in oleochemicals manufacturing. Generally, oleochemicals are used as raw materials in the manufacture of personal care products, cosmetics, coatings, adhesives, sealants, lubricants, surfactants, grease, food, pharmaceuticals, and nutraceuticals [49]. In the last decade, there has been a boost to the oleochemical industry due to the increase in supply and demand of biodiesel, which is also derived in oleochemical industry via transesterification process [48].

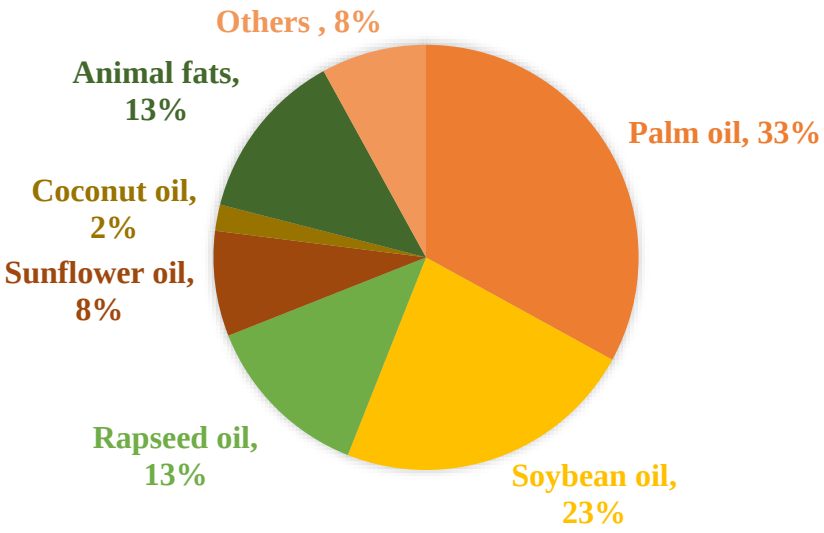


Figure 1.6. Commonly used feedstocks in global oleochemical industries.

Since the United Nation's World summit on sustainable development called for the promotion of a sustainable use of biomass in 2002, the annual global production of major vegetable oils has increased from 84.6 million tons in 2002 to 137.3 million tons in 2009 and to 209.7 million tons in 2020 [50]. Of the total amount of oil produced only 32% is available to the oleochemical industries, with much of the remainder being used for nutrition [30]. Currently, the global market value for oleochemical industry stands at 20.1 billion USD and is expected to grow at a rapid rate. The important companies that dominate production of oleochemicals includes, Emery Natural Oleochemical (Malaysia), Oleon NV (European), Wilmar International (Singapore), Kuala Lumpur Kepong Berhad (Malaysia), and Vantage Specialty Chemicals (United States). The two dominant processes carried out in the oleochemical industry are hydrolysis and transesterification to convert renewables to value added products. **Figure 1.7** represents key products derived from oleochemical industries. Fatty acids form the majority of those used in the production of oleochemicals [48].

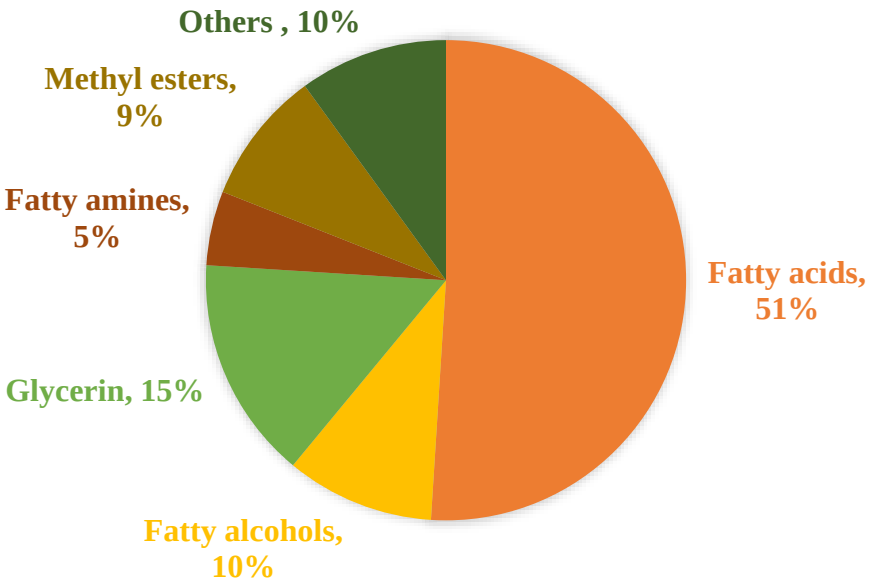


Figure 1.7. Key products derived from oleochemical industries using renewable feedstocks.

A summary of all the applications involved with respect to individual products derived from oleochemical industry are summarized below,

1.5.1. Fatty acids

Fatty acids which are produced from hydrolysis reaction are of extreme importance to the food industry as different fatty acids supply the different characteristic flavor and aroma to many different food products. However, the application of fatty acids extends far beyond the characteristic flavoring of food products. A large volume of fatty acids goes into the manufacturing of various soaps and detergents. Fatty acids are also used as raw material in the manufacture of personal care products, paints and coatings, surfactants, lubricants, polymers, and candles [26].

Research has shown that there are various fatty acids which are essential to humans for healthy living [51]. For example, linoleic acid (C18:2), an omega-3 fatty acid, is at the start of a pathway that our body chemistry uses to make arachidonic acid which appears in high proportions in our cell membranes. It is also the starting material for the metabolic production of prostaglandin which has been shown to have an involvement with blood clotting, heart function, control of blood pressure and has a function in the central nervous system. γ-linolenic acid and α-linolenic acid fatty acids are also indispensable to human nutrition [51]. Other omega-3 fatty acids include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [52].

Good sources of these omega-3 fatty acids containing oils are cold water fishes (salmon, herring, mackerel, sardines, and cod) as well as many seed crops including, linseed oil, soya oil and hemp seed oil. The market for these acids is expanding rapidly at present due to the growth of a more health-conscious society. In the past decade, the production of linoleic acid, γ-linoleic acid and other omega-3 fatty acids (from fish oils) as additives for oil-based health care products such as cod-liver oil, primrose and star-flower oil, has gained attention [53]. These omega-3 fatty acids

are also considered as therapeutics for cardiovascular disease, cyclosporin, hypercholesterolemia, hypertriglyceridemia, hypertension, and autoimmune conditions including osteoarthritis, rheumatoid arthritis, systemic lupus erythematosus, scleroderma, and multiple sclerosis [54].

1.5.2. Glycerin

Vegetable glycerin (or glycerol) which is derived as a co-product from oleochemical industry using hydrolysis and transesterification processes is mainly used as raw materials in the manufacturing of various food products, cosmetics, and pharmaceutical drugs. For example, in the food industry, glycerin is added to enhance mixing between oil and water-based ingredients or to sweeten food products or to prevent formations of ice crystals on frozen foods [55]. Many of the pharmaceutical drugs including heart medication, dietary supplements, and almost all the over-the-counter medications have glycerin as one of the raw materials. Apart from most of the cosmetics, it is also widely added in toothpastes to prevent from hardening. **Figure 1.8** shows pictorial representation of all the possible uses of glycerin [28, 56].

1.5.3. Fatty alcohols

Fatty alcohols are considered as intermediate products of oleochemical industry. They are used as raw material in the manufacture of printing inks or as plasticizers for the plastics industry. Trace amounts of fatty alcohols also finds its application in cosmetic, food, detergents, and surfactants industries as emulsion stabilizing agent [57].

1.5.4. Fatty acid methyl esters (FAMEs)

Fatty acid methyl esters (or FAMEs) are the primary constituents of biodiesel [58]. Biodiesel is considered as a green alternative to conventional diesel since they are non-toxic and biodegradable. Energy required to produce biodiesel is also considerably low compared to conventional diesel obtained from crude oil sources. Apart from biodiesel industries, FAMEs are

used in food applications as thickening and emulsifying agents and as a green solvent [59]. Major companies dominating FAMEs production includes, Biofuels Corporation (United States), Evonik Industries (Germany), Novaol (Italy) and GlycosBio (Malaysia).

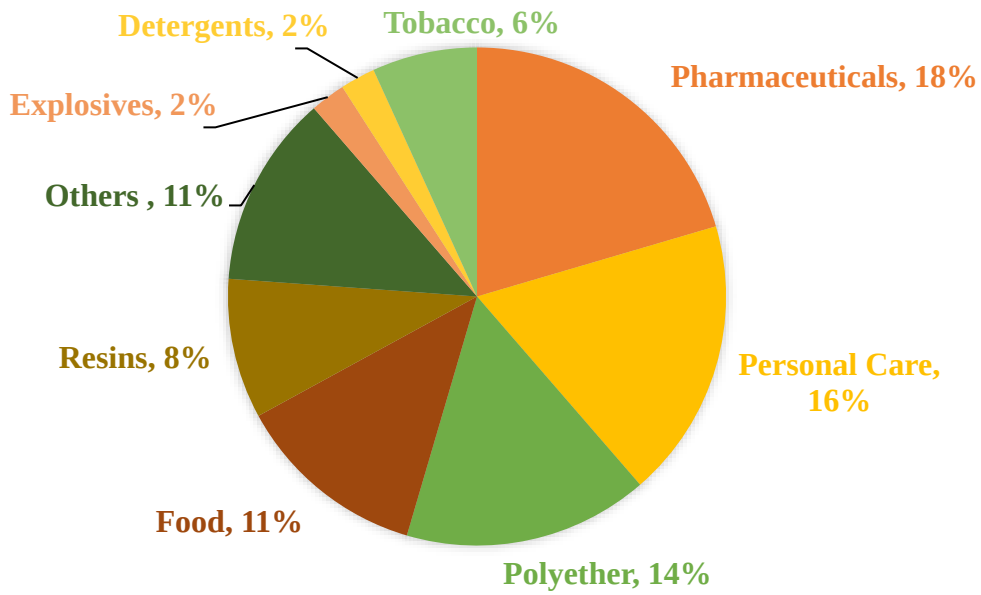


Figure 1.8. Industrial applications of glycerol (or glycerin).

1.5.5. Fatty amines

Fatty amines are made from the reaction between triglycerides (or fatty acids) and ammonia with elimination of two molecules of water at high temperature in the dehydrating catalyst such as silica gel or alumina or iron-based catalyst. Major application of fatty amines includes flotation agents, anticaking, and corrosion inhibitors [60]. **Figure 1.9** represents all the oleochemicals derived from oils and fats along with their respective modifications undergone [26].

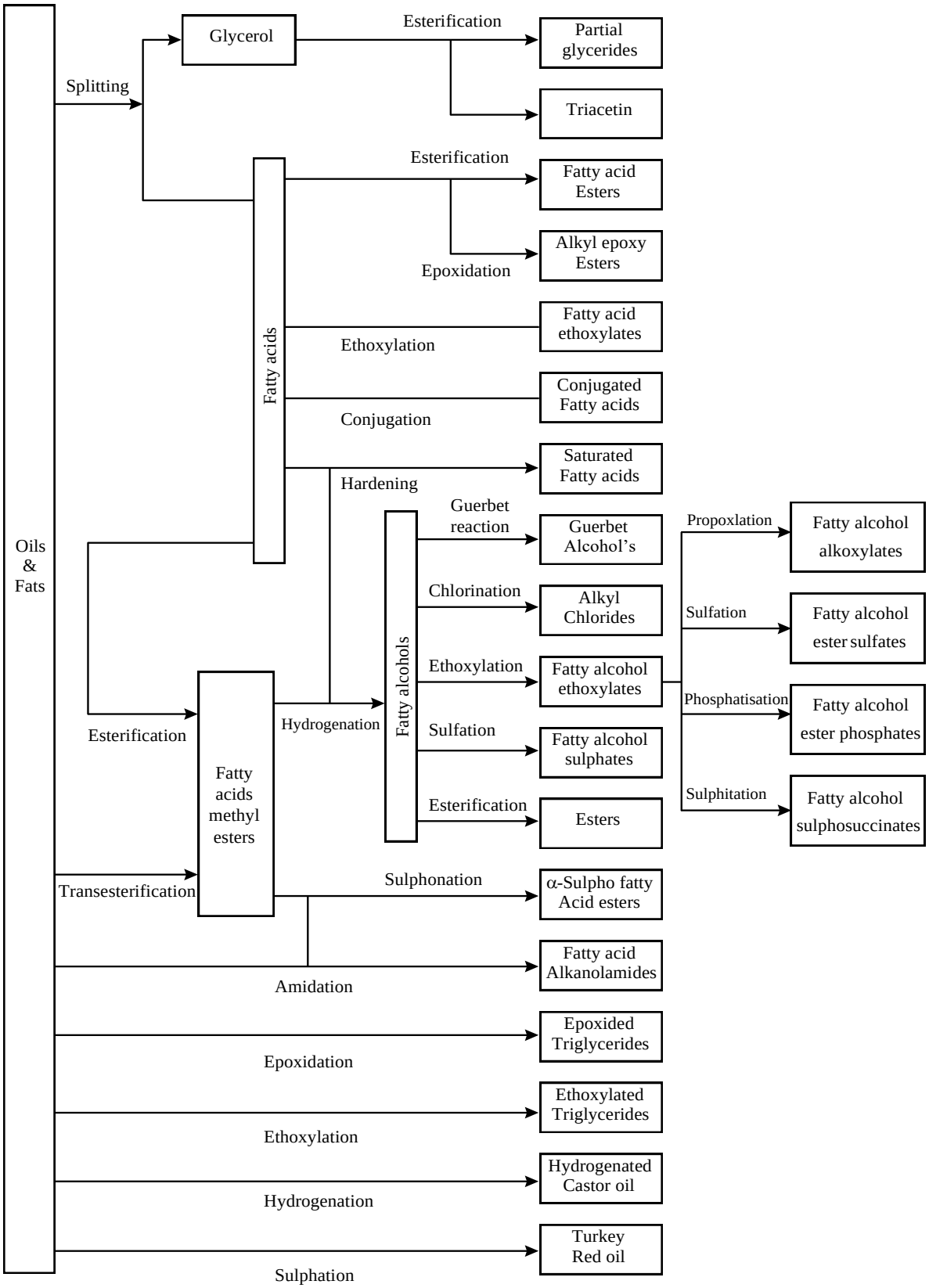


Figure 1.9. Oleochemicals derived from oils and fats.

1.6. Triglyceride hydrolysis (fat splitting): Basic chemistry and importance

Triglyceride hydrolysis is among the most used processes to produce free fatty acids and glycerol in various industries. The spectrum of triglycerides ranges from animal sourced tallows to many different edible and inedible vegetable oils. Another source of triglyceride is from algae and fish oils. All these represent an important suite of renewable sources for valorization into higher value-added products in the oleochemical industry. A single triglyceride molecule is composed of three fatty acid molecules joined together to a glycerol backbone, hence the stoichiometry of hydrolysis of triglyceride requires 3 mols of water and 1 mol of triglyceride to give 3 mols of free fatty acid (FFA) and 1 mol of glycerol (see **Figure 1.4**). The hydrolysis process follows a 3-stage mechanism with the formation of diglycerides, monoglycerides, and glycerol with liberation of free fatty acid in each stage as shown in **Figure 1.10**. However, oil and water are not miscible at room temperature and pressure hence, the mainstream production of this reaction requires high temperature and high-pressure processes which involves contacting oil with steam or superheated liquid water [29, 61, 62]. Alternative methods include using chemical catalysts [28, 63, 64]. Green alternative to these conventional methods for triglyceride hydrolysis includes deployment of microbial lipases (enzymes). The various free fatty acids and glycerol obtained from triglyceride hydrolysis are purified and separated using distillation type processes and are used as raw materials in various products as described in earlier section [65-67].

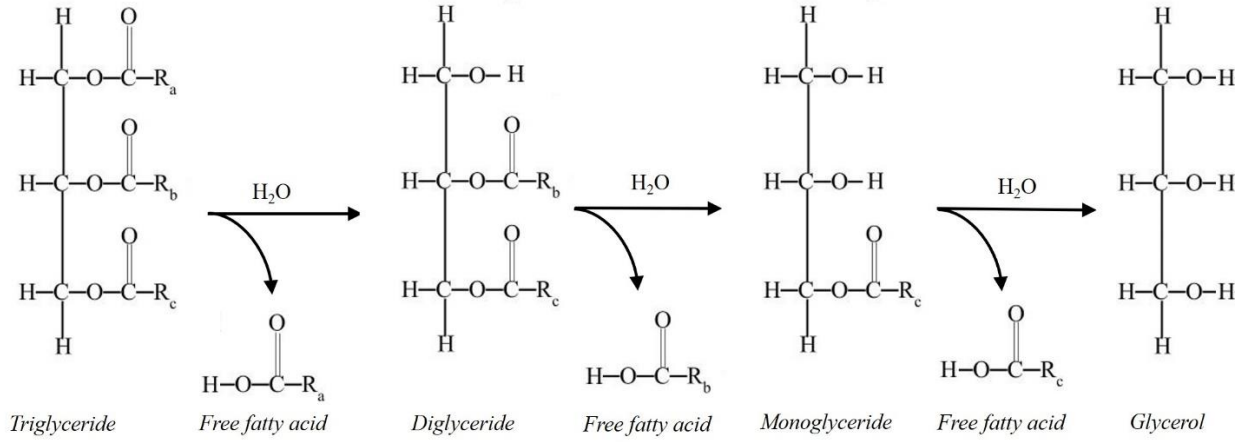


Figure 1.10. Triglyceride hydrolysis reaction.

1.7. Important conventional triglyceride hydrolysis (fat splitting) processes

All of the important processes to produce fatty acids from natural fats and oils involve the hydrolysis mechanism. Industrial processes for manufacturing of soap (saponification reaction) from natural fats and oils as raw material was revolutionized when Andrew Pears started marketing a high-quality transparent soap in the year 1789 [68]. Since then, research into soap manufacturing process is still being carried out in the 21st century. Procter & Gamble set up one of the first laboratories specifically structured for improving the soap manufacturing process in the year 1890. Indeed, soap production was one of the two founding processes of the company when James Gamble, an apprentice soap maker, joined with William Procter to sell soaps and candles to the population of Cincinnati in the year 1837. In the late 1930s and 1940s, major American companies developed much improved processes for the manufacture of soap by utilizing the fat splitting (hydrolysis) route since fatty acids and glycerol coproduct has abundant application apart from soaps. The modified process utilizing hydrolysis reaction also produced a more efficient recovery of glycerol and a much shorter processing time. Processes which carry out triglyceride hydrolysis can be divided into two groups; those which are carried out at normal atmospheric pressure, and

those carried out at high temperatures and pressures [69]. The commercial hydrolysis processes carried out at atmospheric pressure includes Twitchell type fat splitting processes and ecofriendly type fat splitting process using enzymes such as lipases as biocatalyst. However, the most common methods of large-scale production of different fatty acids are using continuous splitting type processes using high temperature, high pressure processes one of which is the Colgate-Emery process.

1.7.1. Twitchell fat splitting process

The Twitchell process, patented in 1897, is the earliest known processes developed for fat splitting [70]. This process was developed by Ernst Twitchell, who is latter known as the father of oleochemical industry. This method of fat splitting involves hydrolysis reaction carried out in large open type kettle reactors with fats or oils boiled along with water, 1-2 wt% of sulfuric acid, sulfonated mixture of oleic acid and naphthalene for 36-48 hours using open steam. The process has the advantage of requiring only relatively simple and inexpensive equipment as well as being easily installed and operated. The kettle reactors are made up of either large wooden or lead lined vessels into which water amounting to approximately half of the fat is added. The fat splitting process is usually repeated two to four times depending upon the degree of hydrolysis required. After each stage, the glycerol-water phase is removed and replaced with fresh process water. The tanks are generally covered to reduce contact with air as this causes discoloration. The long reaction periods and high steam consumption make this process one of the most energy intensive of methods and this is the reason for its limited use today. Despite these drawbacks the Twitchell process was in commercial use until the 1960s.

1.7.2. Continuous splitting process

The continuous countercurrent, high pressure and temperature process is currently the most efficient and widely used method for splitting fats and oils. This method is more commonly referred to as the Colgate-Emery process as referred to earlier [71]. Continuous splitting process makes use of intense processing conditions to increase the rate of reaction, however catalysts can also be used to further increase the reaction rates. Typically, reaction times achieved can be as low as two to three hours. Little discoloration of the fatty acids occurs and because of the efficient internal heat transfer, this process affords a high steam economy. **Figure 1.11** represents the process flow diagram for a continuous fat splitting process [72]. Although the figure represents a single stage continuous reactor, multi-stage versions are available depending upon the flow rates required.

The heart of the process consists of a column about 18 to 25 m long with 0.5 to 1 m in diameter. The column is constructed out of materials such as 316 stainless steel or Inconel alloy and is designed to resist pressures up to 10000 kPa and temperatures of more than 400^{o}C. The deaerated fats or oils are introduced by means of sparging rings, around one meter from the bottom of the column using a high-pressure pump. Water is introduced from the top of the column approximately at about half the rate of the fats or oils. The process is typically carried out at pressures of about 5000 kPa and a temperature of 260 ^{o}C. The use of high temperatures ensures a high degree of agitation within the column and at these high temperatures the fat/oil phase has an increased affinity for the water so adequate dissolution occurs ensuring no additional mechanical mixing is required. This method has proven to achieve >98% hydrolysis. The main manufacturers that utilize this continuous splitting process (Colgate – Emery process) and variations of this

process includes, Lurgi AG (Germany), Gianazza (Italy), LIPICO Technologies (Singapore) and Crown Asia Engineering (China).

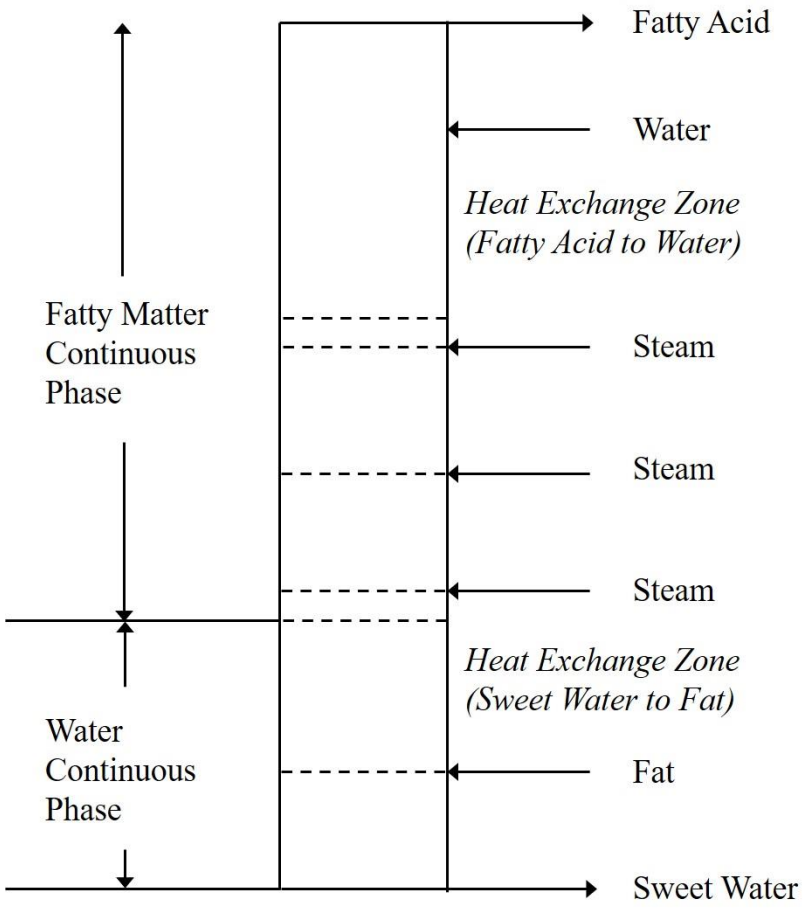


Figure 1.11. Process flow for continuous fat splitting method (Colgate-Emery Process)

The major drawbacks of these continuous fat splitting processes involve operating costs and the energy utilization required [56]. Additionally, every 12 to 18 months continuous fat splitting plants are required to shut down for maintenance and before re-starting, depending upon the feedstocks which may be expensive and sensitive (liable to denaturation) . The high temperature employed for steam splitting makes this process unsuitable for splitting sensitive triglycerides, unconjugated systems (which may undergo thermal degradation), hydroxylated fats

and oils (which may dehydrate) or polyunsaturated oils with high iodine numbers (which may polymerase). A green alternative to modify fats and oils without using these conventional processes which are energy intensive and environmentally harmful involve the use of lipase (enzyme) as a biocatalyst [73-75].

1.8. Conclusion

Oils and fats are mainly composed of triglyceride esters which contribute to one among several important sources of renewable raw materials utilized for manufacturing various edible and non-edible products. The global consumption and production rate of naturally occurring oils and fats are increasing at a rapid rate because the products obtained from modification of oils and fats using hydrolysis, esterification, interesterification and transesterification are considered as oleochemicals (green alternative to petrochemical) which are used as raw materials for the manufacturing of various value added products including soaps, cosmetics, personal care products, detergents, surfactants, pharmaceuticals, lubricants, paints, resins, and other antifreeze products and alkanoamides. Hydrolysis of triglyceride is one of the most utilized process in oleochemical industry. Complete hydrolysis of triglyceride ester yields fatty acids and glycerol. Classical hydrolysis (fat splitting) processes at industrial scale requires the reaction to be carried out at temperatures in the range 100 - 260°C and at pressures in the range 100 - 7000 kPa since oil and water are immiscible at room temperature or by using chemical catalysts. The major drawbacks of these processes are the long reaction times, operating costs and the energy utilization required. These adverse conditions used sometimes produce discolored fatty acids which needs further processing. Green alternatives to these conventional processes include deployment of enzymes (lipases) as biocatalysts.

1.9. References

[1] R. Micha, S. Khatibzadeh, P. Shi, S. Fahimi, S. Lim, K.G. Andrews, R.E. Engell, J. Powles, M. Ezzati, D. Mozaffarian, Global, regional, and national consumption levels of dietary fats and oils in 1990 and 2010: a systematic analysis including 266 country-specific nutrition surveys, British Medical Journal 348 (2014) g2272.

[2] J.B. Rattray, Biotechnology and the fats and oils industry—an overview, Journal of the American Oil Chemists' Society 61(11) (1984) 1701-1712.

[3] F. Taheripour, W.E. Tyner, US biofuel production and policy: implications for land use changes in Malaysia and Indonesia, Biotechnology for Biofuels 13(1) (2020) 1-17.

[4] A.A.H. Kadhum, M.N. Shamma, Edible lipids modification processes: A review, Critical Reviews in Food Science and Nutrition 57(1) (2017) 48-58.

[5] F. Shahidi, Bailey's Industrial Oil and Fat Products, Edible Oil and Fat Products: Processing Technologies, John Wiley & Sons (2005).

[6] H. Hager, Specific gravities of fats, resins, etc, Analyst 4(44) (1879) 206-207.

[7] H. Noureddini, B. Teoh, L. Davis Clements, Viscosities of vegetable oils and fatty acids, Journal of the American Oil Chemists' Society 69(12) (1992) 1189-1191.

[8] F. Gunstone, The chemistry of oils and fats: sources, composition, properties and uses, John Wiley & Sons (2009).

[9] W. Wei, C. Sun, W. Jiang, X. Zhang, Y. Hong, Q. Jin, G. Tao, X. Wang, Z. Yang, Triacylglycerols fingerprint of edible vegetable oils by ultra-performance liquid chromatography-Q-ToF-MS, Lebensmittel-Wissenschaft & Technologie 112 (2019) 108261.

[10] F. Hidalgo, R. Zamora, Triacylglycerols: Structures and properties, Semantic Scholar (2016).

[11] A.C. Rustan, C.A. Drevon, Fatty acids: structures and properties, Encylopedia of Life Sciences (2001).

[12] J.S. Negi, Nanolipid materials for drug delivery systems: A comprehensive Review, Characterization and Biology of Nanomaterials for Drug Delivery (2019) 137-163.

[13] C. Scrimgeour, Y. Gao, W.Y. Oh, F. Shahidi, Chemistry of fatty acids, Bailey's Industrial Oil and Fat Products (2005) 1-40.

[14] C. Mwanza, K. Zombe, Comparative Evaluation of Some Physicochemical Properties on Selected Commercially Available Soaps on the Zambian Market, Open Access Library Journal 7(3) (2020) 1-13.

[15] E.G. Giakoumis, Analysis of 22 vegetable oils' physico-chemical properties and fatty acid composition on a statistical basis, and correlation with the degree of unsaturation, Renewable Energy 126 (2018) 403-419.

[16] M. Zeece, Introduction to the Chemistry of Food, Academic Press (2020).

[17] L.M. Srivastava, Plant growth and development: hormones and environment, Elsevier (2002).

[18] M.W. Pariza, Y. Park, M.E. Cook, The biologically active isomers of conjugated linoleic acid, Progress in Lipid Research 40(4) (2001) 283-298.

[19] V. Marchand, C.P. Society, Nutrition, G. Committee, Trans fats: What physicians should know, Paediatrics & Child Health 15(6) (2010) 373-375.

[20] C. Schrock, W.E. Connor, Incorporation of the dietary trans fatty acid (C18: 1) into the serum lipids, the serum lipoproteins and adipose tissue, The American Journal of Clinical Nutrition 28(9) (1975) 1020-1027.

[21] A.M. Abdelghany, S. Zhang, M. Azam, A.S. Shaibu, Y. Feng, J. Qi, Y. Li, Y. Tian, H. Hong, B. Li, Natural variation in fatty acid composition of diverse world soybean germplasms grown in China, Agronomy 10(1) (2020) 24.
[22] M. Bockisch, Animal fats and oils, Fats and Oils Handbook (1998) 121-173.
[23] B. Naik, V. Kumar, Cocoa butter and its alterna-tives-a review, Journal of Bioresource Engineering Technology 2 (2014) 1-11.
[24] C. Montoya, B. Cochard, A. Flori, D. Cros, R. Lopes, T. Cuellar, S. Espeout, I. Syaputra, P. Villeneuve, M. Pina, Genetic architecture of palm oil fatty acid composition in cultivated oil palm (Elaeis guineensis Jacq.) compared to its wild relative E. oleifera (HBK) Cortés, Public Library of Science One 9(5) (2014) e95412.
[25] J.A. Kent, Kent and Riegel's handbook of industrial chemistry and biotechnology, Springer (2007) p.278.
[26] J. Salimon, N. Salih, E. Yousif, Industrial development and applications of plant oils and their biobased oleochemicals, Arabian Journal of Chemistry 5(2) (2012) 135-145.
[27] M. Bockisch, Fats and Oils Handbook, Elsevier (2015).
[28] H. Tan, A.A. Aziz, M. Aroua, Glycerol production and its applications as a raw material: A review, Renewable and Sustainable Energy Reviews 27 (2013) 118-127.
[29] H. Barnebey, A. Brown, Continuous fat splitting plants using the Colgate-Emery process, Journal of the American Oil Chemists' Society 25(3) (1948) 95-99.
[30] U. Biermann, U. Bornscheuer, M.A. Meier, J.O. Metzger, H.J. Schäfer, Oils and fats as renewable raw materials in chemistry, Angewandte Chemie International Edition 50(17) (2011) 3854-3871.
[31] Z. Zhen, T. Xi, Y. Zheng, Surface Modification of Magnesium and Its Alloys for Biomedical Applications, Elsevier (2015), pp. 301-333.
[32] H.E. Hoydonckx, D.E. De Vos, S.A. Chavan, P.A. Jacobs, Esterification and transesterification of renewable chemicals, Topics in Catalysis 27(1) (2004) 83-96.
[33] [33] M. Mittersteiner, B.L. Linshalm, A.P.F. Vieira, P.B. Brondani, D.R. Scharf, P.C. de Jesus, Convenient enzymatic resolution of (R, S)-2-methylbutyric acid catalyzed by immobilized lipases, Chirality 30(1) (2018) 106-111.
[34] M.J. Haas, Improving the economics of biodiesel production through the use of low value lipids as feedstocks: vegetable oil soapstock, Fuel Processing Technology 86(10) (2005) 1087-1096.
[35] D.Y. Leung, X. Wu, M. Leung, A review on biodiesel production using catalyzed transesterification, Applied Energy 87(4) (2010) 1083-1095.
[36] A. Demirbaş, Biodiesel fuels from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterifications and other methods: a survey, Energy Conversion and Management 44(13) (2003) 2093-2109.
[37] N.K. Patel, S.N. Shah, Biodiesel from plant oils, Food, Energy, and Water, Elsevier (2015), pp. 277-307.
[38] H. Fukuda, A. Kondo, H. Noda, Biodiesel fuel production by transesterification of oils, Journal of Bioscience and Bioengineering 92(5) (2001) 405-416.
[39] U. Schuchardt, R. Sercheli, R.M. Vargas, Transesterification of vegetable oils: a review, Journal of the Brazilian Chemical Society 9(3) (1998) 199-210.
[40] Z. Zhang, J. Ye, W.J. Lee, C.C. Akoh, A. Li, Y. Wang, Modification of palm-based oil blend via interesterification: physicochemical properties, crystallization behaviors and oxidative stabilities, Food Chemistry 129070.

[41] J. Orsavova, L. Misurcova, J.V. Ambrozova, R. Vicha, J. Mlcek, Fatty acids composition of vegetable oils and its contribution to dietary energy intake and dependence of cardiovascular mortality on dietary intake of fatty acids, International Journal of Molecular Sciences 16(6) (2015) 12871-12890.
[42] H. Rohm, C. Schäper, S. Zahn, Interesterified fats in chocolate and bakery products: A concise review, Lebensmittel-Wissenschaft & Technologie 87 (2018) 379-384.
[43] A. Dijkstra, G. Van Duijn, Vegetable oils: oil production and processing, Encylopedia of Food and Health(2016).
[44] A. Dijkstra, Soybean oil, Encylopedia of Food and Health (2016).
[45] W. Pocklington, Determination of the iodine value of oils and fats: results of a collaborative study, Pure and Applied Chemistry 62(12) (1990) 2339-2343.
[46] N. Soontag, Halogenation, Journal of the American Oil Chemists Society 40(5) (1963) 199-203.
[47] A. Zarli, Oleochemicals: all time players of green chemistry, Studies in Surface Science and Catalysis, Elsevier (2019), pp. 77-95.
[48] R. Tesser, R. Vitiello, V. Russo, R. Turco, M. Di Serio, L. Lin, C. Li, Oleochemistry Products, Industrial Oil Plant, Springer (2020), pp. 201-268.
[49] F.D. Gunstone, R.J. Hamilton, Oleochemical manufacture and applications, Chemical Rubber Company Press (2001).
[50] T. Laubner, World Summit on Sustainable Development, Johannesburg, South Africa, 26 August-4 September 2002: People, Planet and Prosperity, German Yearbook of International Law 45 (2002) 417.
[51] S.M. Innis, Essential fatty acids in growth and development, Progress in Lipid Research 30(1) (1991) 39-103.
[52] A.P. Simopoulos, Essential fatty acids in health and chronic disease, The American Journal of Clinical Nutrition 70(3) (1999) 560s-569s.
[53] K. Nivetha, A.S. Rao, A. Nair, Microbial production of Omega-3 fatty acids: An overview, Journal of Applied Microbiology (2021).
[54] J.E. Bauer, Therapeutic use of fish oils in companion animals, Journal of the American Veterinary Medical Association 239(11) (2011) 1441-1451.
[55] H.S. Muzammil, B. Rasco, S. Sablani, Effect of inulin and glycerol supplementation on physicochemical properties of probiotic frozen yogurt, Food & Nutrition Research 61(1) (2017) 1290314.
[56] R. Ciriminna, C.D. Pina, M. Rossi, M. Pagliaro, Understanding the glycerol market, European Journal of Lipid Science and Technology 116(10) (2014) 1432-1439.
[57] G.R. Kelm, R.R. Wickett, The role of fatty acids in cosmetic technology, Fatty Acids, Elsevier (2017), pp. 385-404.
[58] S.K. Hoekman, A. Broch, C. Robbins, E. Ceniceros, M. Natarajan, Review of biodiesel composition, properties, and specifications, Renewable and Sustainable Energy Reviews 16(1) (2012) 143-169.
[59] G. Wypych, Databook of Green Solvents, Elsevier (2019).
[60] S. Madbouly, C. Zhang, M.R. Kessler, Bio-based plant oil polymers and composites, Elsevier (2015).
[61] D. Rooney, L. Weatherley, The effect of reaction conditions upon lipase catalysed hydrolysis of high oleate sunflower oil in a stirred liquid–liquid reactor, Process Biochemistry 36(10) (2001) 947-953.

[62] R.L. Holliday, J.W. King, G.R. List, Hydrolysis of vegetable oils in sub-and supercritical water, Industrial & Engineering Chemistry Research 36(3) (1997) 932-935.
[63] K. Ngaosuwan, E. Lotero, K. Suwannakarn, J.G. Goodwin Jr, P. Praserthdam, Hydrolysis of triglycerides using solid acid catalysts, Industrial & Engineering Chemistry Research 48(10) (2009) 4757-4767.
[64] V.R. Murty, J. Bhat, P. Muniswaran, Hydrolysis of oils by using immobilized lipase enzyme: a review, Biotechnology and Bioprocess Engineering 7(2) (2002) 57-66.
[65] R. Swenson, Fatty Acids. Their Chemistry, Properties, Production, and Uses, Journal of the American Chemical Society 87(11) (1965) 2526-2527.
[66] K.S. Markly, Fatty Acids: Their Chemistry, Properties, Production and Uses, Fatty Acids(1961).
[67] C.A. Quispe, C.J. Coronado, J.A. Carvalho Jr, Glycerol: Production, consumption, prices, characterization and new trends in combustion, Renewable and Sustainable Energy Reviews 27 (2013) 475-493.
[68] S.H. Abbas, 7 Body-Cleansing Technology, Handbook of Detergents, Part E: Applications 141 (2008) 135.
[69] H.B. Routh, K.R. Bhowmik, L.C. Parish, J.A. Witkowski, Soaps: from the Phoenicians to the 20th century—a historical review, Clinics in Dermatology 14(1) (1996) 3-6.
[70] A. Langmuir, The Twitchell Process and the Glycerine Trade, Industrial & Engineering Chemistry 9(2) (1917) 195-196.
[71] H. Barnebey, A. Brown, Continuous fat splitting plants using the Colgate-Emery process, Journal of the American Oil Chemists' Society 25(3) (1948) 95-99.
[72] J. Chupa, S. Misner, A. Sachdev, P. Wisniewski, G.A. Smith, R. Heffner, Soap, fatty acids, and synthetic detergents, Handbook of Industrial Chemistry and Biotechnology, Springer(2017), pp. 979-1032.
[73] F. Hasan, A.A. Shah, A. Hameed, Industrial applications of microbial lipases, Enzyme and Microbial Technology 39(2) (2006) 235-251.
[74] M.R. Taskinen, T. Kuusi, 7 Enzymes involved in triglyceride hydrolysis, Bailliere's Clinical Endocrinology and Metabolism 1(3) (1987) 639-666.
[75] A. Valenzuela, S. Nieto, Biotechnology of lipids: the use of lipases for the structural modification of fats and oils, Grasas y Aceites (1994).

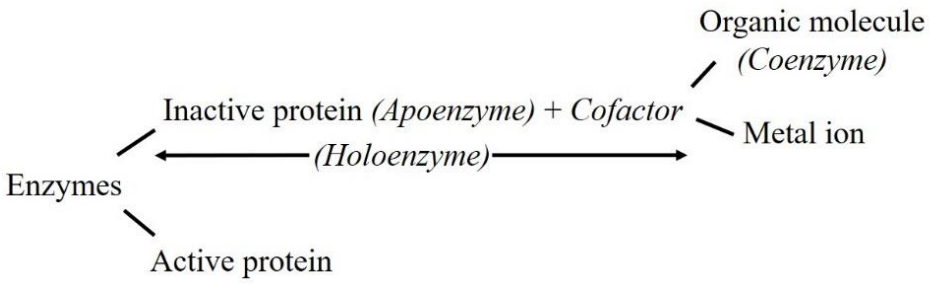
Chapter 2 : Enzymatic catalysis and lipase derived from *Candida rugosa.*

2.1. Understanding enzymes.

Enzymes are proteins capable of increasing the rate of chemical reactions taking place within living cells/organisms without themselves suffering any overall change or damage [1, 2]. In general, proteins consist of L-amino acid residues linked together by peptide bonds. The sequence of amino acids linked by each polypeptide chain constitutes to the primary structure of the target protein. These sequences can be determined by a systematic set of biochemical assays. The secondary structure constitutes of regular, repeating, three-dimensional (3-D) features. These secondary structures determine if a protein make up to fibrous and globular category. In fibrous, structural proteins, the secondary structures are largely uninterrupted but are disrupted at many points in globular, functional proteins. Fibrous proteins are insoluble in water whereas globular proteins are soluble in water. The overall 3-D structure of each polypeptide chain make up to form tertiary structure and proteins may consist of one or more polypeptide chains with the complete structure being called the quaternary structure [3]. The 3-D structures of proteins in fibers and crystals are determined using X-ray diffraction (XRD) analysis [4].

Enzymes are composed of globular, functional proteins which are capable of catalyzing many reactions and the reactants of enzyme-catalyzed reactions are called as substrates, and each enzyme is very specific in character which allows them to act on a specific substrate or substrates to produce a specific product or products. Enzymes are present in all living cells, where they are executing all vital functions including the metabolic processes. In fact, all living organisms produce thousands of different enzymes to catalyze thousands of cellular processes. However, without the aid of another component called a co-factor, many enzymes cannot function their catalytic activity [5]. The co-factor could be an organic or inorganic compound. When the co-

factor is composed of inorganic compound, the inactive protein component of an enzyme is called the apoenzyme, and the active enzyme along with the cofactor, is called the holoenzyme. The cofactors used to make the enzyme active are generally composed of metal ions which binds tightly to the enzyme. In some cases, an organic molecule can be used to activate the enzyme then the cofactor is called as a coenzyme. If the cofactor is tightly bound to the enzyme, then it becomes difficult to remove the cofactor without damaging the enzyme. In that case the enzyme and the bound cofactor is termed as a prosthetic group. To summarize diagrammatically,



Enzymes exhibit chemical and stereochemical specificity with respect to both substrates and products. To achieve such specificities, enzymes require at least three different points of interaction between the enzyme and the substate. Different substrates bound at different rates to the enzyme at specific sites to form an enzyme-substrate complex in which reacting groups are held near each other and to catalytic sites. That region of the enzyme's 3-D structure which contains the substrate-binding sites, and the catalytic sites is termed as the "active site".

The activity of an enzyme to act on a particular substrate is determined by direct consequence of the amino acid sequence involved in their makeup [6]. According to the Fischer lock-and-key hypothesis, the active site has rigid structural features which are complementary to those of each substrate. In contrast, the Koshland induced-fit hypothesis suggests that at least some

active sites are flexible, possessing a structure complementary to that of a substrate only when the latter is bound to the enzyme [7]. These models can explain some aspects of enzyme specificity, but do not suggest any mechanism for driving forward the enzyme-catalyzed reaction. The stability of enzyme-bound transition state which would have to be formed appears to be the most important factor in determining whether an enzyme will act on a particular substrate(s) to produce a product(s) [8].

2.2. Brief history of enzymes.

Human's use of enzymes to speed up processes' dates back to the earliest times of civilization. Important human activities in primitive communities such as the production of certain types of foods and beverages, and the tanning of hides and skins to produce leather for garments, involved the application of enzyme activities, albeit unknowingly. However, not until the 19th century with the development of biochemistry and the innovative work of several eminent scientists and engineers, the nature of enzymes and how they operate begin to be clarified [9]. In 1833, two scientists from France, Anselme Payen and Jean-Francois Persoz explained the isolation of an amylolytic type enzyme by incubating barley [10]. Shortly afterwards, Jons Jacob Berzelius, a chemist from Sweden coined the term catalysis in the year 1836 to describe the property of certain substances to increase the rate of chemical reactions [11]. In the year 1836, a German physiologist, Theodor Schwann discovered the digestive enzyme pepsin and soon after Wilhelm Kuhne coined the term 'enzyme' in the year 1877 [12, 13]. In the 1880's, Christian Hansen a chemist from Denmark succeeded in obtaining pure rennet from calves' stomachs. The use of which in cheese-making resulted in considerable improvements in both product quantity and quality. Shortly thereafter he industrialized the production of rennet thus leading to the first industrial production of enzymes [14]. In the year 1897, the brothers Hans and Eduard Buchner

from Germany demonstrated that the transformation of glucose into ethanol could be carried out by enzymes present in cell-free extracts of yeast [15]. Eduard Buchner won the Nobel prize in chemistry for his contribution towards fermentation in the year 1907.

The field of science advanced dramatically during the 20th century. James Sumner in the year 1926 crystalized urease from Jack-bean extracts which led the way to obtaining enzymes in purified and crystallized form [16]. He later won the Nobel prize in 1946. Once methods to obtaine pure enzymes were available, it paved the way for the development of procedures for their industrial production and use. The 1960's witnessed two major breakthroughs that had a major impact on the enzyme industry; (1) the commercialization of glucoamylase which catalyzes the production of glucose from starch with much greater efficacy than that of the chemical process involving acid hydrolysis, and (2) the launch of the first enzymatic detergents. The advancement of genetic engineering in the 1980's provided the tools necessary for the isolation, production and commercialization of new robust enzymes thus seeding a second explosive wave to the current billion-dollar enzyme industry [17, 18].

2.2.1 Biocatalysis in the 21st century

In order to address the challenge of increasing carbon footprint, the chemical industries of the 21st century intends to produce very complex reaction products in a sustainable way to take account of public demands in relation to pharmaceuticals, food, and fine chemistry companies. One such cleaner and sustainable approach is through deployment of enzymes as industrial biocatalysts [19]. Enzymes are considered as biocatalysts because they are biodegradable, operate at mild temperature and pH and the products derived are non-toxic in nature. Enzymes are also highly selective and specific. However, they exhibit disadvantageous behavior when it comes to large scale manufacturing processes on account of expense, stability, and inactivation due to

operating conditions [20]. **Table 2.1** represents all the milestones and major developments in enzymatic biocatalysis [13].

Recent advancement in X-ray crystallography and other analytical methods in the field of protein chemistry along with the ever-increasing amounts of biological information available from genomics programs and molecular techniques such as directed evolution and gene and genome shuffling, are bringing powerful means to bear on the study and manipulation of enzyme structure and function. The search for improvements in existing enzyme-catalyzed procedures, the need to develop new technologies and the increasing concern for responsible use and reuse of raw materials can be expected to stimulate not only the rational modification of enzymes to match specific requirements but also the design of new enzymes with totally novel properties [21].

Table 2.1. Major milestones timeline in enzymology and biocatalysis.

Year	Major developments
1833	Discovery of enzymes
1894	Lock and key model
1905	Discovery of cofactors
1913	Michaelis-Menten kinetics
1926	Discovery of enzymes are proteins
1950	First immobilization of proteins
1958	Induced fit model
1970	Production of high fructose corn syrup using immobilized glucose isomerase
1972	First immobilized enzyme based industry (Bayer)
1991	Directed evolution
1992	Enzyme nomenclature
1997	Lipase process to produce chiral amines
2002	Biodiesel production using enzyme as catalyst
2016	Engineered C-Si bond forming enzymes
2020	Nine enzyme cascade to produce islatravir

2.3. Enzymes: Market growth, general applications, and classification.

The global enzyme market size was valued at 9.9 billion USD in 2019 and is expected to grow at a steady compound annual growth of 7.1% from 2021 to 2027. These enzymes are mainly divided into three categories: industrial enzymes, food enzymes and animal enzymes [22, 23].

Applications of enzymes are diverse in nature; they are used mainly in biochemical and biotechnology systems. Various enzymes are used in the food industries to manufacture various food products including dairy products, bakery products, meat, and beverages such as fruit juices, beers, and wine. **Table 2.2** represents various enzymes used in the food industry with respect to their notable application [24]. Industrial enzymes find its application in cleaning products, production of ethanol, textiles, research, genetics, and paper industry [22]. Apart from these, enzymes are also used as markers for diseases, as tools for diagnosing diseases, as antioxidants, as therapeutic agents, as sources of biofuels, as analytical and clinical reagents, and in immunoassays and pharmaceuticals [25].

There are uncountable number of enzymes available in nature and about 75,000 exist in human body, but approximately only 2000 different enzymes have been identified and structured [26]. Due to the lack of consistency in the nomenclature, it became apparent as the list of known enzymes rapidly grew that there was a need for a systematic way of naming and classifying enzymes. An enzyme commission (E.C) in 1992 was appointed by the International Union of Biochemistry, where they classified enzymes into six main classes, based on the total reaction catalyzed [27]. Each enzyme was assigned a code number, consisting of four numbers, separated by dots. The first digit in the code shows to which of the main classes the enzyme belongs, as shown in **Table 2.3**.

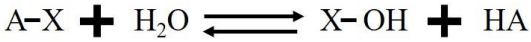
Table 2.2. Various application of enzymes in food industry.

Enzyme	Application
α-Amylase	Baking, brewing, starch liquefaction, quality improvement
Glucoamylase	Brewing, high glucose and high fructose syrups
Protease	Brewing, coagulation of milk
Lactase	Producing lactose free products
Lipase	Cheese production and flavor development
Esterase	Flavor and fragrance enhancer in fruit juices
Cellulase	Animal feeds and clarifying fruit juices
Glucose oxidase	Shelf-life improvement
Catalase	Food preservative
Peroxidase	Flavor and color development

Table 2.3. Different enzyme classes with respect to type of reaction catalyzed.

First digit	Enzyme class	Type of reaction catalyzed
1	Oxidoreductases	Oxidation/reduction reactions
2	Transferases	Transfer of an atom or group between two molecules (excluding reactions in other classes)
3	Hydrolases	Hydrolysis reaction
4	Lyases	Removal of a group from substrate (not by hydrolysis)
5	Isomerases	Isomerization reactions
6	Ligases	The synthetic joining of two molecules, coupled with the breakdown of pyrophosphate bond in a molecule

The second and third digits in the code further describe the kind of reaction being catalyzed. There is no general rule, because the meaning of these digits is defined separately for each of the main classes. Within the scope of the current topic, classification 3 (hydrolases) is the focus of this dissertation. These enzymes catalyze hydrolytic reactions of the form given below and are further branched according to the type of bond hydrolyzed as shown in **Table 2.4**.



The third digit further describes the type of bond hydrolyzed. Thus, E.C.3.1.1 enzymes are carboxylic ester hydrolases, E.C.3.1.2 enzymes are thiol ester hydrolases, E.C.3.1.3 enzymes are phosphoric monoester hydrolases and E.C.3.1.4 enzymes are phosphoric diester hydrolases.

Table 2.4. Significance of second digit with respect to bond hydrolyzed.

Second digit	Bond hydrolyzed
1	ester
2	glycosidic
4	peptide
5	C-N bonds other than peptides

2.4. Biocatalysis using lipases.

Lipases (triacylglycerol hydrolase E.C.3.1.1.3) are a class of serine hydrolases with excellent catalytic properties and have been well studied as biocatalysts for over 80 years because of their unique physiochemical behavior and ability to catalyze complex reactions at mild operating temperature and pressure [28]. They are remarkably effective in catalyzing versatile

reactions, such as hydrolysis [29-31], esterification [32], interesterification [33], transesterification [34], aminolysis [35], acidolysis [36], perhydrolysis [37], alcoholysis and C-C bond formation [38]. The natural substrates of lipases are long-chain triacylglycerols, which have low solubility in water; and the reaction is catalyzed at the lipid-water interface [39]. Lipases use water molecules to split triglycerides to create two daughter products (free fatty acid and glycerol) by fixing the hydroxyl group (-OH) and the hydrogen (-H) from water molecule. **Figure 2.1** demonstrates how lipases work.

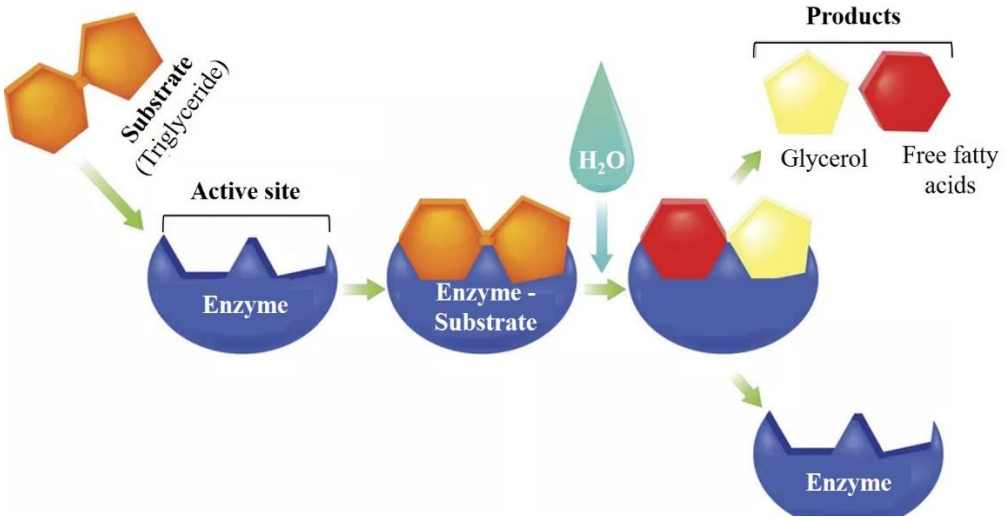


Figure 2.1. Lipase catalyzed triglyceride hydrolysis.

Currently, lipases dominate the market of industrial enzymes, accounting for 70% of all processes carried out in biotechnology applications [22, 40]. These include dairy products, detergents, pharmaceuticals, chemicals, agriculture products, oleochemicals and medical devices [41-45]. **Table 2.5** represents industrial applications of microbial lipases [46, 47]. Lipases as biocatalysts are highly selective, commercially available from many vendors and requires no cofactors to operate. They may be used to produce enantiomerically pure chemicals and synthetic

intermediates [48], therefore its production and utilization are better alternative to chemical catalysts.

Table 2.5. Various industrial applications of lipases.

Industry	Action	Product or application
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy foods	Hydrolysis of milk, cheese ripening, modification of butter	Flavoring
Bakery foods	Flavor improvement	Shelf-life prolongation
Beverages	Aroma improvement	Beverages
Food dressings	Quality improvement	Mayonnaise, dressings, whippings
Health foods	Transesterification	Healthy foods
Fats and oils	Transesterification and hydrolysis	Cocoa butter, margarine, fatty acids, glycerol
Chemicals	Enantioselectivity, synthesis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification and hydrolysis	Specialty lipids, digestive foods
Cosmetics	Synthesis	Emulsifiers, moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Quality improvement

Lipases are biocatalysts derived from plants, animals, molds, bacteria, or fungi [49] and are considered biodegradable and non-toxic to the environment [50]. **Table 2.6** compares the lipase

based splitting process to conventional splitting process, highlighting some of the advantages and disadvantages.

Table 2.6. Comparison of lipase process with conventional fat splitting processes.

Parameter	Lipase process	Conventional process	
		Alkaline process	Continuous splitting process
Triglyceride content in raw material	Triglycerides are converted to free fatty acids	Soap formation	Triglycerides are converted to free fatty acids
Water content in the raw material	It is not deleterious for lipase	More soaps formation	Does not affect
Yield	High, >80%	High, >96%	High, >98%
Reaction rate	Low	High	High
Glycerol recovery	Easy, high grade	Complex, low grade	Easy, low grade
Catalyst recovery and reuse	Easy if immobilized	Difficult, mostly lost in post processing steps	No catalyst involvdd
Energy costs	Low, 20-50°C	Medium, 60-80°C	High, >200°C
Catalyst cost	High	Low	No catalyst involved
Environmental impact	Low; wastewater treatment not required	High; wastewater treatment required	High; emissions

Microbial lipases are often preferred over enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, active in organic solvents, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media [22, 51-54]. Microbial lipases are often

divided into two based on regioselectivity with respect to the acyl chains of the triglycerides: Sn-1,3 regiospecific (Example: lipase derived from *Rhizomucor miehei*) or non-regiospecific (Example: lipase derived from *Candida rugosa*). **Table 2.7** represents some of the notable industrial microbial lipases and their respective application along with their enzyme manufacturing company [46, 55]. However, often standardizing methodologies for lipases derived from microorganisms become difficult since their properties depends on the genus and species of microorganism used to derive.

Table 2.7. Industrial application of microbial lipases.

Type	Source	Application	Enzyme manufacturing company
Fungal	*Candida rugosa*	Organic synthesis	Amano, Biocatalysts, Boehringer Mannheim, Fluka, Genzyme, Sigma
	Candida antarctica	Organic synthesis	Boehringer Mannheim, Novo Nordisk
	Thermomyces lanuginosus	Detergent additive	Boehringer Mannheim, Novo Nordisk
	Rhizomucor miehei	Food processing	Novo Nordisk, Biocatalysts, Amano
Bacterial	*Burkholderia cepacia*	Organic synthesis	Amano, Fluka, Boehringer Mannheim
	Pseudomonas alcaligenes	Detergent additive	Genencor
	Pseudomonas mendocina	Detergent additive	Genencor
	Rhododendron viscosum	Organic synthesis	Asahi, Biocatalysts

2.5. The concept of interfacial activation.

As previously described, the natural substrates are insoluble lipid compounds prone to aggregation in aqueous solution. Lipases exert their activity on the carboxyl ester bonds of triacylglycerol. In 1958, two scientists from France, Sarda and Desnulle described a steep increase in the lipase activity at substrate concentration exceeding their solubility threshold as being the major difference to the esterases [56]. Since then, the formation of an interface between aggregated substrates along with their respective aqueous solution has been recognized as necessary for the activation of lipases. This behavior is known as "interfacial activation" and the structural rationale behind this concept was elucidated years later after the first three-dimensional (3-D) structures of lipase were derived [57]. Studies conducted on the 3-D structures of lipases revealed that the enzyme active sites are shielded from the solvent by a mobile structure, called the "lid" or "flap", that must be displaced upon interaction with the substrate/water interface in order to yield an active enzyme conformation with the catalytic center accessible for the substrates to enter [58]. However, this movement due to the presence of a hydrophobic substrate does not change the final properties of lipases, such as specificity or selectivity. These properties can only be modulated by genetic manipulation or physiochemical modifications (including immobilization) [59]. The mechanism of lid opening varies between lipases but in all cases leads to the creation of an open, accessible active site and a large hydrophobic lipid binding site. Lipases without a lid or with a lid but no interfacial activation have also been described [42, 60]. To date, the broader definition of a lipase as carboxylesterase catalyzing hydrolysis and synthesis of long chain acylglycerols is generally accepted and seems to be adequate to describe all known lipases. It specifically refers to the behavior of enzymes on insoluble substrates, but it must be recalled the most lipases are active

also on soluble esterase substrates. In low-water conditions, the reverse synthetic reaction is favored, leading to esterification, alcoholysis and acidolysis [61].

Research has shown that, lipases in aqueous solution exhibit two conformation, the so called “open conformation” and “closed conformation” and are in an unstable equilibrium with each other favoring more towards the closed conformation. However, upon contacting with hydrophobic substrates (oil drops), the lipases get adsorbed onto the substrate favoring open conformations, permitting the substrates to enter [62]. **Figure 2.2** shows the conformational equilibrium in aqueous solution and interfacial activation of lipases [37].

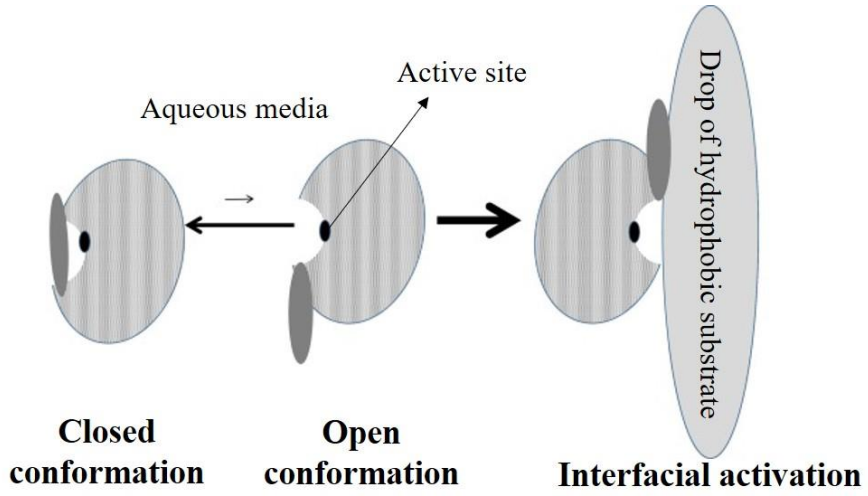


Figure 2.2. Conformational mobility and interfacial activation of lipases.

2.6. Structural fold of lipases.

All lipases display the same structural architecture, the so-called α/β hydrolase fold, and have identical catalytic machineries. The original description of this fold was based on the comparison of the 3-D structures of hydrolases mostly unrelated in the primary sequence and active on substrates very different in structure, one of which was fungal lipase [63]. All lipases whose 3D structures were later resolved were found to be existing similar structural fold. Lipases

of known 3D structure are currently classified by the Structural Classification of a Proteins (SCOP) database into 7 different families based on the elements of the basic fold that they contain: acetylcholinesterase-like, gastric lipase, lipase, fungal lipase, bacterial lipase, pancreatic lipase N-terminal domain, and cutinase-like [64]. Generally, in α/β hydrolases, the active site is composed of a catalytic triad containing a nucleophile, an acidic residue, and histidine, suggestive of that of serine proteases but with different order in the sequence of nucleophile-acid-histidine [63]. The catalytic triad of lipases is composed of serine (Ser), aspartate (Asp) or glutamate and histidine (His) (see **Figure 2.3**), with the serine enclosed in the consensus motif as previously described which forms a sharp turn (the nucleophile elbow) in a strand-turn-helix motif in strand β5 which forces the nucleophile to adopt unusual main chain Φ and ψ torsion angle [65].

Hydrolysis of triglyceride by lipases follows a two-step mechanism. The nucleophilicity of the active serine is enhanced by transferring a proton to the catalytic susceptible ester bond. A tetrahedral intermediate is formed carrying a negative charge on the carbonyl oxygen atom of the scissile bond and it is stabilized through hydrogen bonding to main-chain NH groups. The proton on the histidine is then transferred to the ester oxygen of the bond that is cleaved and a covalent intermediate is formed with the fatty acid from the substrate esterified to serine. The second step of the reaction corresponds to deacylation of the enzyme through a water molecule that hydrolyses the covalent intermediate. In this case, transfer of a proton from water to the active site (serine) produces a hydroxide ion (-OH) that attacks the carbonyl carbon atom in the substrate-enzyme covalent intermediate. In addition, the negatively charged tetrahedral intermediate is stabilized by hydrogen bonds (-H) to the oxyanion hole. Finally, histidine donates a proton to the oxygen atom of the active serine and the acyl component is released [66]. Figure 2.3 shows the mechanism of lipase catalyzed triglyceride hydrolysis with respect to the catalytic triad involved [65, 66].

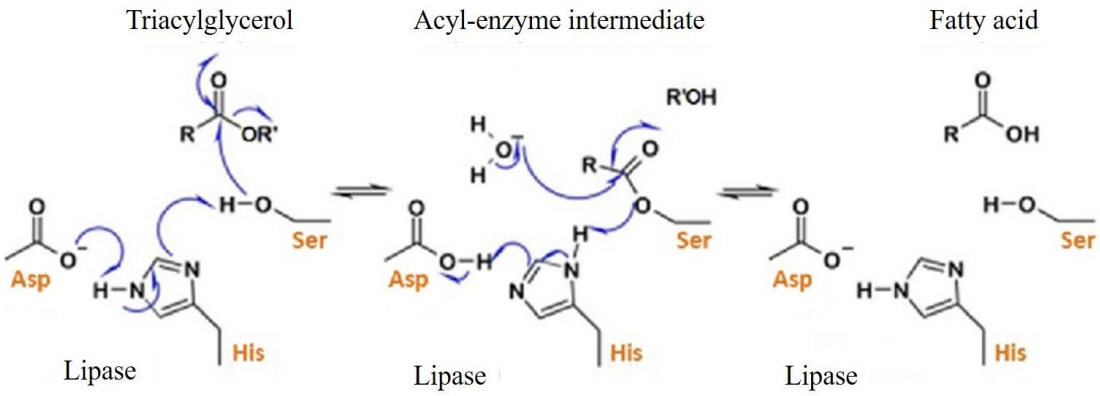


Figure 2.3. Two-step mechanism to demonstrate lipase catalyzed triglyceride hydrolysis.

2.7. Kinetics of lipase catalyzed reactions.

Most of the homogeneous enzyme catalyzed reactions follow Michaelis – Menten (M-M) kinetics [67]. Homogeneous reactions involving single substrate or pseudo single substrate enzyme catalyzed reactions follows the general equation given below,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Where, E is the enzyme, S is the substrate, P is the product and ES is the enzyme substrate complex. E is recovered at the end of the reaction, as expected from any catalytic reactions. The rate of formation of ES at any time t = k_1[E][S], where [E] is the concentration of free enzyme and [S] the concentration of free substrate at time t. Also, at time t, the rate of breakdown of ES back to E and S = k_{-1}[ES], where [ES] is the concentration of enzyme-substrate complex.

The assumption on which Michaelis-Menten kinetics is based is that an equilibrium between enzyme, substrate and enzyme-substrate complex is almost instantly set up and maintained, the breakdown of enzyme-substrate complex to products being too slow to disturb this equilibrium [68]. Based on this assumption, therefore, k_1[E][S] = k_{-1}[ES]. After a series of

mathematical modification and steady state assumption by Briggs-Haldane [69] the final equation was derived to be,

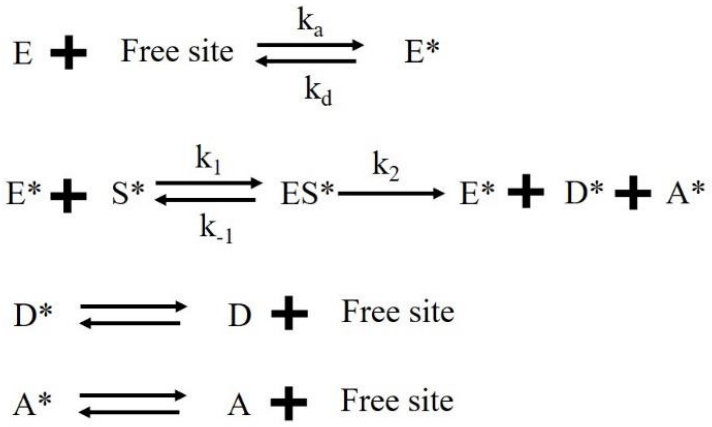
$$v = \frac{V_{max}[S]}{[S]+ K_m} \tag{2.7.1}$$

Where, *v* is the volumetric rate of reaction and K_m is the M-M constant, which is given by,

$$K_m = \frac{k_{-1}+ k_2}{k_1} \tag{2.7.2}$$

K_m values are calculated using linear plots of this equation mentioned above (Lineweaver-Burk plot). The main drawback of M-M equation is that it is derived with respect to single substrate homogeneous equations.

Lipase systems are complex systems with biphasic characteristics and the reaction is catalyzed at the interface. Hence, interfacial area plays a major role in determining lipase-based catalysis for biphasic systems. Historically, numerous models have been developed to predict kinetics of interfacial catalysis by lipase. The mechanism of lipase-catalyzed triglyceride hydrolysis at the triglyceride-water interface proceeds in these steps, 1) enzymes [E] gets adsorbed at the oil-water interface [I] following Langmuir type adsorption equilibrium followed by, 2) the formation of [ES] complex involving M-M mechanism and then 3) products of hydrolysis are formed followed by 4) desorption of products from the enzyme. Marangoni in 2003, proposed that rate-limiting step was the formation of products (step 3) and that step 2 was very fast compared to step 1 [70]. The reaction scheme for lipase-catalyzed triglyceride hydrolysis at low triglyceride concentrations follows [71, 72],



Where, E, D, and A stands for enzyme, diglyceride, and free fatty acid molecules at the bulk aqueous phase (E + A) or bulk oil phase (D). E*, ES*, S*, D* and A* represents the same at the oil-water interface. Free site is the available site at the oil-water interface and k_a, k_d, k_1, k_{-1}, k_2 are rate constants of enzyme adsorption and desorption, enzyme-substrate formation and dissociation and product formation, respectively. By making a mass balance for biphasic system with assumption of $[E_{tot}] = [E] + [E^*][IA]$ and serious mathematic deduction, Sunan et al., modified the M-M equation to [71],

$$v_i = \frac{V_{max,IA}\alpha}{\alpha + K_d^*} \tag{2.7.3}$$

Where, vi is the initial rate of hydrolysis, $V_{max,IA}$ is the maximum initial rate at given interfacial area, α is the free site concentration and K_d^* the interfacial affinity constant = k_d/k_a. At fixed interfacial area, the above equation is modified to,

$$v_i = \frac{V_{max,E}[E]}{[E] + K_d^*} \tag{2.7.4}$$

Where, $V_{max,E} = k_2[E^*]_{max}[IA]$ is the maximum initial rate corresponding to saturated oil-water interface. The above equation demonstrates that v_i as a function of $[E]_{tot}$ should reach an asymptotic maximum value which is directly proportional to $[E^*]_{max}$. It can be concluded from the

model that the reaction rate at the interface can be significantly increased by simply increasing the interfacial area in the oil-water emulsion. However, studies have shown that increasing interfacial area causes lipase to denature due to operational conditions and stress experienced since enzymes are susceptible to changes in environment, hence there should be an optimum between both [73]. Denaturization associated with enzymes due to changes in microenvironment is often associated with protein folding, protecting the active site or destruction of protein in adverse conditions [41, 42].

2.8. Lipase derived from *Candida rugosa.*

The lipase chosen for this study was derived from the microorganisms *Candida rugosa* and is capable of catalyzing fatty acids at all position (sn-1,2,3) of glycerol backbone at mild operating conditions (room temperature and neutral pH) [74]. They are of extreme importance to industrial applications involving enzymatic approaches since they exhibit high activity and versatility in its class [75]. Lipase derived from *Candida rugosa* are glycoproteins composed of 543 amino acid residues with molecular weight of 60kD and are encoded to the LIP gene family and have shown to exhibit 8 different isoenzymes (LIP1-LIP8) [76, 77]. Irrespectively, all LIP isoenzymes secreted shows activity towards most of the substrates of different chain length and in aqueous solutions exhibit, closed (“inactive”) and open (“active”) conformations which make them highly active at the oil-water interface [78]. Hence, a high interfacial surface area in the oil/water emulsion is desirable to encourage fast overall reaction rates as shown in section 2.6. **Figure 2.4** represents the structure of lipase derived from *Candida rugosa* showing the lid and the tunnel leading to the active site [75]. The lid is composed of α-helices and the active site is composed of a catalytic triad of Serine-Histidine-Glutamate (Ser-His-Glu) residues [79]. The hydrolysis reaction takes place

after the substrates are attached to the active site in two steps; acylation of Ser followed by diacylation of the lipase and release of fatty acids [80].

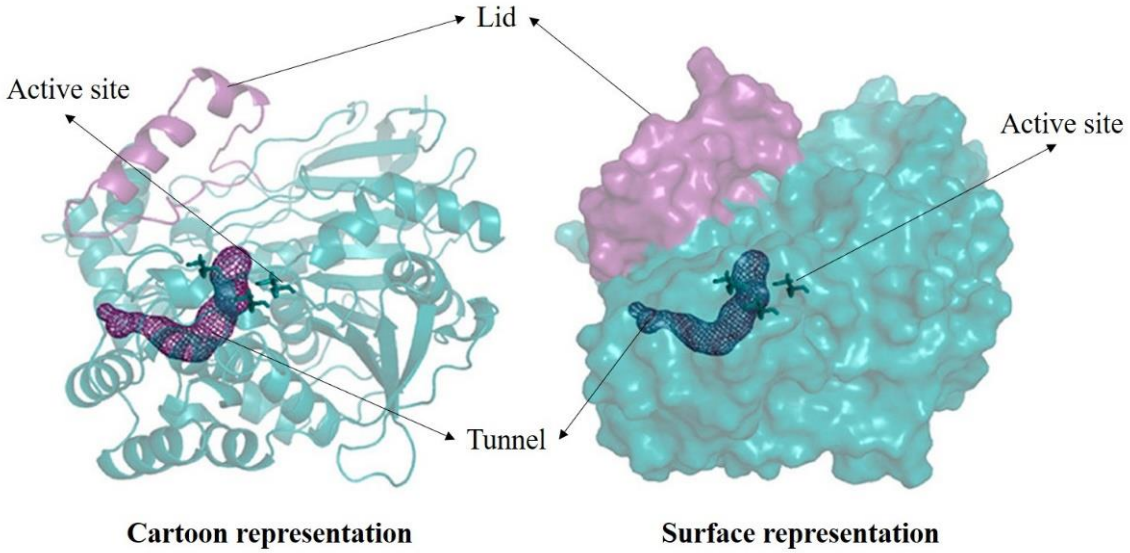


Figure 2.4. Structure of lipase derived from *Candida rugosa*.

Most of the commercially produced lipases derived from *Candida rugosa* consists of a mixture of three LIP isoenzymes (LIP1, LIP2 & LIP3) with LIP1 contributing to more than 80% of the makeup [79]. Lipase derived from *Candida rugosa* in aqueous media finds its application in hydrolysis reactions and synthesis of various esters and in low or no aqueous media they are used in applications involving esterification, alcoholysis, acidolysis, aminolysis, interesterification and transesterification reactions [75].

2.9. Industrial applications specific to lipase derived from *Candida rugosa.*

Currently, lipases dominate the market of industrial enzymes because the enzyme preparation involved, and the products derived are considered safe to human health and the environment. Commercially available lipase derived from *Candida rugosa* is the most utilized enzyme in biotechnology applications. Their industrial applications are diverse ranging from dairy

products (cheese recovery, flavor enhancement and the production of enzyme-modified cheese), detergents, pharmaceuticals (ibuprofen, naproxen), chemicals, agriculture products (pesticides, insects), oleochemicals (fat and oil hydrolysis, and the synthesis of bio-detergents), to medical devices (biosensors) [41-45]. **Table 2.8** represents notable industrial applications and other process that shows potential for industrial application of commercial lipase derived from *Candida rugosa* [22, 81-89].

Apart from the noted applications in Table 2.8, lipase derived from *Candida rugosa* are also extensively used in biosensors. Biosensors are devices composed of a Biological Recognition Element (BRE), in this case lipase derived from *Candida rugosa* and a sensor element. Lipase based biosensors are used to identify industrial contaminants such as pesticides and water pollutants [90]. Research into biosensors using lipase derived from *Candida rugosa* has successfully detected compounds such as diazinon [90], dimethyl phthalate [91], and other organo-chlorine pesticides [92].

Table 2.8. Industrial applications of commercial lipase derived from *Candida rugosa*.

Target compound	Reaction involved	Maximum possible conversions achieved	Application
Oleochemical industry			
Various fatty acids	Hydrolysis	88%	Soaps, detergents, personal care products, cosmetics
Glycerol	Hydrolysis	88%	Cosmetics, pharmaceuticals
Food and flavor industry			
Butyric acid	Hydrolysis	75%	Dairy flavor enhancer
Omega-3 fatty acids	Hydrolysis	84%	Health beneficial
Linoleic acid	Hydrolysis	47%	Flavor precursor
L-Menthol	Hydrolysis & esterification	42% & 48.8%	Flavor & fragrances in beverages, toothpaste, cosmetics
Geranyl propionate	Esterification	47%	Floral
Pharmaceutical industry			
S-Atenolol	Acetylation	42%	β - blocker
S-Ibuprofen	Esterification	31%	Anti-inflammatory drug
S-Ezetimibe	Hydrolysis	50%	Reduction of cholesterol
S-Naproxen	Hydrolysis	49%	Anti-inflammatory drug
Dihydropyridine	Hydrolysis	22%	Treatment of heart diseases
Other (not yet industrialized applications)			
L/D-Lactic acid	Hydrolysis	73%	Food, cosmetics
Methyl oleate	Esterification	79%	Emulsifiers, detergents
Fatty acid methyl esters	Transesterification	95%	Biodiesel

2.10. Specific aims.

Lipase derived from *Candida rugosa* are versatile in catalyzing various processes and they show promising potential for large scale commercialization for fat/oil modification through hydrolysis. However, when used in aqueous form, it exhibits low thermal stability, activity limited to a narrow pH range, and sensitivity to higher shear forces. These factors contribute to less than favorable process economics for lipase-based fat splitting processes. Hence, there is a need for enhancing lipase-catalyzed process using methods such as immobilization or process intensification techniques. The studies and results presented in this dissertation are specific to commercial lipase derived from *Candida rugosa* which was sourced from Sigma-Aldrich (United States) in crude lyophilized form, and it is not compared with any other commercially available lipases across the world. Sigma-Aldrich is one among the leading manufacturer and distributer for lipase derived from *Candida rugosa* as shown in Table 2.7. Before any studies, it was necessary to prepare uniform aqueous solutions from lyophilized lipase using solubilization technique since crude lyophilized lipase is prone to impurities. It was also important to establish a reliable technique for quantifying proteins in an aqueous solution.

2.11. Experimental section.

2.11.1. Materials.

Crude lipase from the yeast *Candida rugosa* (EC 3.1.1.3, triacylglycerol acylhydrolase) (Sigma-Aldrich, United States), was obtained in lyophilized form with a reported specific activity of ≥700 unit/mg solid based on triglyceride hydrolysis at pH 7.2 and 27°C. A protein assay kit with bovine serum albumin (BSA) (2 mg/mL) as standard with Coomassie G-250 dye (ThermoFisher Scientific, USA) using UV-Vis spectroscopy (Genesys 10s spectrometer, ThermoFisher Scientific, United States) were used to analyze total protein concentrations in the

aqueous solutions. Deionized water used in the study was produced from a WATER PRO\RO water system (Labconco^{TM}, United States). 0.1 M, pH 7.4 phosphate buffer saline was used as solvent for solubilizing crude lyophilized lipase. All experiments were performed in triplicates and the results are reported as the mean of this value and the standard deviation.

2.11.2. Determination of protein concentrations.

Historically, a variety of assays to quantify total proteins in biological samples have been incorporated. Colorimetric protein assays such as Bicinchoninic acid (BCA), Bradford, and Lowry have been commercially used to determine total protein concentrations in aqueous solutions [93]. The Bradford assay to quantify proteins has proven to be fast, effective, and cheap with a detection limit of 20-2000 µg/mL [94]. Hence, proteins were quantified using spectroscopy in accordance with the method outlined by Bradford (1976), using bovine serum albumin (BSA) as standard and commissive G-250 as a protein binding dye. This dye binds only to the proteins in solution via ionic interactions between the sulfonic acid groups of the dye and the positive protein amine groups. This shifts the UV absorbance to 595 nm. The amount of bound dye is proportional to the concentration of protein available in the aqueous solution and is not hindered by other components present in the solution. Hence, this dye can be used to quantify proteins even when other chemical agents are present [95].

2.11.3. Preparations of aqueous lipase solutions.

Aqueous solutions (%wt/v) of lipase were made up by mixing a known amount of lyophilized lipase powder into 0.1 M, 7.4 pH phosphate buffer saline (PBS) on an orbital shaker for 1 hour at room temperature (22 ± 2°C). The orbital shaker was preferred over magnetic or overhead stirrers in order to minimize enzyme denaturization upon aggregation due to shear effects [73, 96, 97]. It was also important to use extremely clean glassware since presence of

detergents/surfactants could denature the enzyme [98]. PBS was used throughout the study since literature [99] indicated lipase showed higher solubility in low molality phosphate buffer solutions. During the solubilization process, care was taken to avoid precipitation since formation of precipitates could be indicative of protein denaturation or aggregation [96].

2.12. Results and discussion.

2.12.1. Purification of proteins.

Lipase used in this study consisted of a mixture composed of 73% LIP1, 8% LIP2 and 19% LIP3 isoenzymes. Studies conducted using individual isoenzymes have shown that, LIP1 isoenzyme is twice as active compared with LIP2 and LIP3 for medium chain length substrates, whereas LIP3 is more active in hydrolyzing short chain length triglycerides. In order to purify these individual isoenzymes, various conventional protein purification and separation techniques such as ultrafiltration, precipitation or affinity chromatography are used [100]. However, these techniques are complicated, laborious, time-consuming and expensive. Hence, many industries avoid using these techniques and use commercial lipase without any purification.

During the dissolution procedure for the crude lipase there was evidence of some insoluble components as shown in **Figure 2.5 (a),** which were assumed to be insoluble impurities in the form of emulsifiers, stabilizers and other stabilizing compounds used for lyophilization requiring removal [101, 102]. These compounds also interfere with the Bradford assay when quantifying protein. Therefore, the aqueous lipase mixtures after solubilization were centrifuged at 8000 RPM for twenty minutes at 10°C (Centrifuge 5810 R, Eppendorf, Germany) to remove insoluble compounds to obtain uniform “semi-purified” form of aqueous lipase solution [103]. The resulting supernatant as shown in **Figure 2.5 (b)** was separated and used in subsequent experiments.

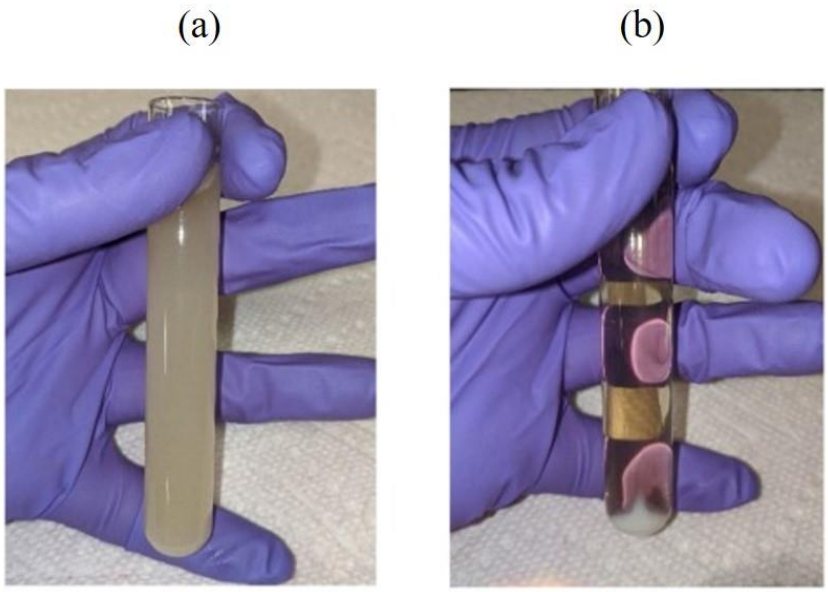


Figure 2.5. (a) Showing solubilization of the aqueous lipase solution in 0.1 M, 7.4 pH phosphate buffer saline solution at 22°C **(b)** Showing **s**emi-purified supernatant at 8000 RPM for 20 minutes at 10°C.

2.12.2. Protein quantification using the Bradford assay.

A standard curve as shown in **Figure 2.6** was established using BSA as standard by varying the concentration with stepwise dilution using 0.1 M, pH 7.4 PBS buffer. The absorbance data obtained for various concentrations of the standard were fit into a linear model and the unknown protein concentration of samples with different lipase loading was back calculated using the absorbance value obtained. During the lyophilized lipase solubilization step, the concentration of total protein in aqueous lipase solution showed variability. The variability depended on the orbital shaker speed, addition of lyophilized lipase rate, and time. Hence, all these parameters were kept consistent and monitored to avoid experimental errors. The Bradford assay was also conducted at various temperature in the range 20 – 60°C and showed similar total protein concentrations

suggesting that protein denaturization on account of temperature increase is not a limiting factor in determining total protein concentration.

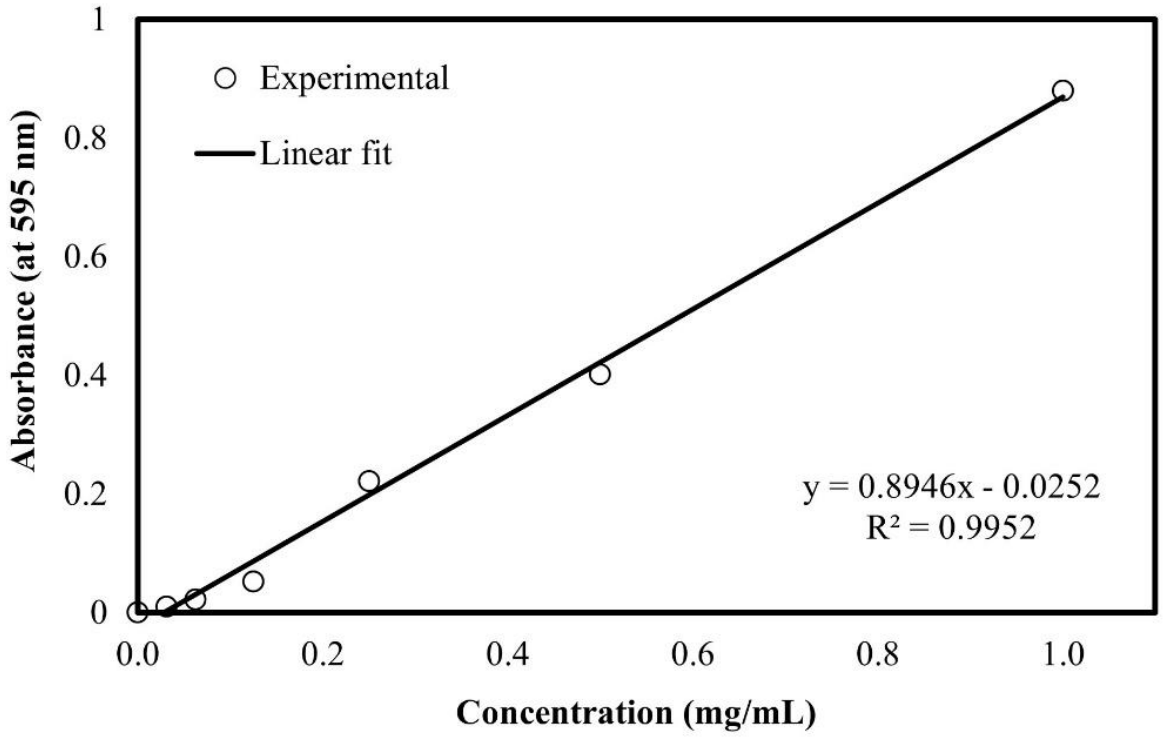


Figure 2.6. Linear model obtained for protein quantification using Bovine serum albumin (BSA) as standard using Bradford assay conducted at room temperature (22 ± 2°C) measured at 595 nm.

Total protein concentrations in aqueous lipase solutions obtained after semi-purification step using various lipase loadings are shown in **Table 2.9**. The protein concentrations increased linearly with respect to lipase loading.

Table 2.9. Total protein concentrations in aqueous lipase solution measured after semi purification using Bradford assay with respect to corresponding lipase loading.

Lipase loading (%wt/v)	0.25	0.5	0.75	1.00	1.25	1.50	2.00
Total protein concentration (mg/mL)	0.065	0.114	0.175	0.204	0.274	0.365	0.489

All the measurements were done in triplicate and the error was less than 5% in each case.

2.13. Conclusion.

Enzymatic processes are capable of catalyzing complex chemical reactions is an ecofriendly alternative to conventional chemical processes. Lipases show promising potential in catalyzing reactions such as hydrolysis, esterification, interesterification, transesterification, acidolysis and alcoholysis. The use of microbial lipases for the hydrolysis of natural oils such as triglyceride esters is a green alternative to conventional processes to produce high value-added chemicals. However, biocatalysis using lipases in aqueous solutions is limited often by instabilities, slow kinetics due to mass transfer limitations, and by challenges of economic lipase recycling. Hence, there is a need for enhancing lipase-catalyzed process using methods such as immobilization or process intensification. In the present study, commercially available lipase derived from *Candida rugosa* has been recognized as promising biocatalyst for modification of oils and fats. The sourced commercial lipase from Sigma-Aldrich was solubilized using 0.1 M, 7.4 pH phosphate buffer saline and quantified spectroscopically using Bradford assay.

2.14. References.

[1] A.K. Patel, R.R. Singhania, A. Pandey, Production, purification, and application of microbial enzymes, Biotechnology of Microbial Enzymes, Elsevier(2017), pp. 13-41.
[2] W. Aehle, Enzymes in industry: production and applications, John Wiley & Sons (2007).
[3] G.A. Petsko, D. Ringe, Protein structure and function, New Science Press (2004).
[4] T. Palmer, Understanding enzymes, E. Horwood (1991).
[5] G. Litwack, Human biochemistry, Academic Press (2017).
[6] C.L. Tsou, The role of active site flexibility in enzyme catalysis, Biochemistry-New York-English Translation of Biokhimiya 63(3) (1998) 253-258.
[7] D.E. Koshland Jr, The key–lock theory and the induced fit theory, Angewandte Chemie International Edition in English 33(23-24) (1995) 2375-2378.
[8] S.R. Miller, An appraisal of the enzyme stability-activity trade-off, Evolution 71(7) (2017) 1876-1887.
[9] D. Blow, So do we understand how enzymes work?, Structure 8(4) (2000) R77-R81.
[10] A. Payen, J.-F. Persoz, Mémoire sur la diastase, les principaux produits de ses réactions, et leurs applications aux arts industriels, Annales de Chimie et de Physique 53 (1833) 73-92.
[11] J. Moulijn, P. Van Leeuwen, R. Van Santen, History of catalysis, Studies in Surface Science and Catalysis 79 (1993).
[12] J.S. Fruton, A history of pepsin and related enzymes, The Quarterly Review of Biology 77(2) (2002) 127-147.
[13] C.M. Heckmann, F. Paradisi, Looking back: A short history of the discovery of enzymes and how they became powerful chemical tools, ChemCatChem 12(24) (2020) 6082.
[14] S.K. Garg, B.N. Johri, Rennet: current trends and future research, Food Reviews International 10(3) (1994) 313-355.
[15] G.F. Fuhrmann, B. Völker, Regulation of glucose transport in Saccharomyces cerevisiae, Journal of Biotechnology 27(1) (1992) 1-15.
[16] J.B. Sumner, Note. The recrystallization of urease, Journal of Biological Chemistry 70(1) (1926) 97-98.
[17] P. Thivend, C. Mercier, A. Guilbot, Determination of starch with glucoamylase, General Carbohydrate Method, Elsevier (1972), pp. 100-105.
[18] D. Basketter, J. English, S. Wakelin, I. White, Enzymes, detergents and skin: facts and fantasies, British Journal of Dermatology 158(6) (2008) 1177-1181.
[19] R.A. Sheldon, S. van Pelt, Enzyme immobilisation in biocatalysis: why, what and how, Chemical Society Reviews 42(15) (2013) 6223-6235.
[20] A. Illanes, Enzyme biocatalysis, Principles and Applications. Editorial Springer-Verlag New York Inc., United States (2008) 1-56.
[21] M. Smyth, J. Martin, x Ray crystallography, Molecular Pathology 53(1) (2000) 8.
[22] F. Hasan, A.A. Shah, A. Hameed, Industrial applications of microbial lipases, Enzyme and Microbial Technology 39(2) (2006) 235-251.
[23] R. Singh, M. Kumar, A. Mittal, P.K. Mehta, Microbial enzymes: industrial progress in 21st century, 3 Biotech 6(2) (2016) 1-15.
[24] S. Raveendran, B. Parameswaran, S. Beevi Ummalyma, A. Abraham, A. Kuruvilla Mathew, A. Madhavan, S. Rebello, A. Pandey, Applications of microbial enzymes in food industry, Food Technology and Biotechnology 56(1) (2018) 16-30.

[25] K. Soetan, O. Aiyelaagbe, C. Olaiya, A review of the biochemical, biotechnological and other applications of enzymes, African Journal of Biotechnology 9(4) (2010).
[26] H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne, The protein data bank, Nucleic Acids Research 28(1) (2000) 235-242.
[27] E.C. Webb, Enzyme nomenclature 1992. Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzymes, Academic Press (1992).
[28] K.E. Jaeger, T. Eggert, Lipases for biotechnology, Current Opinion in Biotechnology 13(4) (2002) 390-397.
[29] F. Tavares, J. Petry, P. Sackser, C. Borba, E. Silva, Use of castor bean seeds as lipase source for hydrolysis of crambe oil, Industrial Crops and Products 124 (2018) 254-264.
[30] S. Sun, J. Liu, X. Li, A novel and rapid method for fatty acid preparation by the lipase-catalyzed hydrolysis of Phoenix tree seeds, 3 Biotech 8(9) (2018) 1-8.
[31] C.H. Su, H.C. Nguyen, M.L. Nguyen, P.T. Tran, F.M. Wang, Y.L. Guan, Liquid lipase-catalyzed hydrolysis of gac oil for fatty acid production: Optimization using response surface methodology, Biotechnology Progress 34(5) (2018) 1129-1136.
[32] P.Y. Stergiou, A. Foukis, M. Filippou, M. Koukouritaki, M. Parapouli, L.G. Theodorou, E. Hatziloukas, A. Afendra, A. Pandey, E.M. Papamichael, Advances in lipase-catalyzed esterification reactions, Biotechnology Advances 31(8) (2013) 1846-1859.
[33] M.M. Soumanou, M. Pérignon, P. Villeneuve, Lipase-catalyzed interesterification reactions for human milk fat substitutes production: A review, European Journal of Lipid Science and Technology 115(3) (2013) 270-285.
[34] A. Bajaj, P. Lohan, P.N. Jha, R. Mehrotra, Biodiesel production through lipase catalyzed transesterification: an overview, Journal of Molecular Catalysis B: Enzymatic 62(1) (2010) 9-14.
[35] S. Zeng, J. Liu, S. Anankanbil, M. Chen, Z. Guo, J.P. Adams, R. Snajdrova, Z. Li, Amide synthesis via aminolysis of ester or acid with an intracellular lipase, ACS Catalysis 8(9) (2018) 8856-8865.
[36] M. Rychlicka, N. Niezgoda, A. Gliszczyńska, Lipase-catalyzed acidolysis of egg-yolk phosphatidylcholine with citronellic acid. New insight into synthesis of isoprenoid-phospholipids, Molecules 23(2) (2018) 314.
[37] X.P. Wang, P.F. Zhou, Z.G. Li, B. Yang, F. Hollmann, Y.-H. Wang, Engineering a lipase B from Candida antactica with efficient perhydrolysis performance by eliminating its hydrolase activity, Scientific Reports 7(1) (2017) 1-5.
[38] J. López-Fernández, M.D. Benaiges, F. Valero, Rhizopus oryzae lipase, a promising industrial enzyme: biochemical characteristics, production and biocatalytic applications, Catalysts 10(11) (2020) 1277.
[39] R. Gupta, N. Gupta, P. Rathi, Bacterial lipases: an overview of production, purification and biochemical properties, Applied Microbiology and Biotechnology 64(6) (2004) 763-781.
[40] S. Li, X. Yang, S. Yang, M. Zhu, X. Wang, Technology prospecting on enzymes: application, marketing and engineering, Computational and Structural Biotechnology Journal 2(3) (2012) e201209017.
[41] A. Houde, A. Kademi, D. Leblanc, Lipases and their industrial applications, Applied Biochemistry and Biotechnology 118(1-3) (2004) 155-170.
[42] R.D. Schmid, R. Verger, Lipases: interfacial enzymes with attractive applications, Angewandte Chemie International Edition 37(12) (1998) 1608-1633.

[43] D. Méndez-Sánchez, M. López-Iglesias, V. Gotor-Fernández, Hydrolases in organic chemistry. Recent achievements in the synthesis of pharmaceuticals, Current Organic Chemistry 20(11) (2016) 1186-1203.
[44] U.M. de Oliveira, L.J.L. de Matos, M.C.M. de Souza, B.B. Pinheiro, J.C. Dos Santos, L.R. Gonçalves, Efficient biotechnological synthesis of flavor esters using a low-cost biocatalyst with immobilized Rhizomucor miehei lipase, Molecular Biology Reports 46(1) (2019) 597-608.
[45] F.T.T. Cavalcante, F.S. Neto, I.R. de Aguiar Falcão, J.E. da Silva Souza, L.S. de Moura Junior, P. da Silva Sousa, T.G. Rocha, I.G. de Sousa, P.H. de Lima Gomes, M.C.M. de Souza, Opportunities for improving biodiesel production via lipase catalysis, Fuel (2020) 119577.
[46] R. Sharma, Y. Chisti, U.C. Banerjee, Production, purification, characterization, and applications of lipases, Biotechnology Advances 19(8) (2001) 627-662.
[47] E. Vulfson, Industrial applications of lipases, Lipases (1994).
[48] A. Basso, S. Serban, Industrial applications of immobilized enzymes—A review, Molecular Catalysis 479 (2019) 110607.
[49] V.M. Pahoja, M.A. Sethar, A review of enzymatic properties of lipase in plants, animals and microorganisms, Journal of Animal and Poultry Sciences 2(4) (2002) 474-484.
[50] K.G. Clarke, Bioprocess engineering: An introductory engineering and life science approach, Elsevier (2013).
[51] B. Andualema, A. Gessesse, Microbial lipases and their industrial applications, Biotechnology 11(3) (2012) 100-118.
[52] M. Laguerre, M.N. Mputu, B. Brıys, M. Lopez, P. Villeneuve, E. Dubreucq, Regioselectivity and fatty acid specificity of crude lipase, (2017).
[53] H. Treichel, D. de Oliveira, M.A. Mazutti, M. Di Luccio, J.V. Oliveira, A review on microbial lipases production, Food and Bioprocess Technology 3(2) (2010) 182-196.
[54] P.K. Robinson, Enzymes: principles and biotechnological applications, Essays in Biochemistry 59 (2015) 1.
[55] K.E. Jaeger, M.T. Reetz, Microbial lipases form versatile tools for biotechnology, Trends in Biotechnology 16(9) (1998) 396-403.
[56] L. Sarda, P. Desnuelle, Action of pancreatic lipase on emulsified esters, Biochimica et Biophysica Acta 30 (1958) 513-521.
[57] F. Winkler, A. d'Arcy, W. Hunziker, Structure of human pancreatic lipase, Nature 343(6260) (1990) 771-774.
[58] U. Derewenda, A.M. Brzozowski, D.M. Lawson, Z.S. Derewenda, Catalysis at the interface: the anatomy of a conformational change in a triglyceride lipase, Biochemistry 31(5) (1992) 1532-1541.
[59] A.A. Mendes, P.C. Oliveira, H.F. de Castro, Properties and biotechnological applications of porcine pancreatic lipase, Journal of Molecular Catalysis B: Enzymatic 78 (2012) 119-134.
[60] F.I. Khan, D. Lan, R. Durrani, W. Huan, Z. Zhao, Y. Wang, The lid domain in lipases: Structural and functional determinant of enzymatic properties, Frontiers in Bioengineering and Biotechnology 5 (2017) 16.
[61] A. Rajendran, A. Palanisamy, V. Thangavelu, Lipase catalyzed ester synthesis for food processing industries, Brazilian Archives of Biology and Technology 52(1) (2009) 207-219.
[62] R.C. Rodrigues, J.J. Virgen-Ortíz, J.C. Dos Santos, Á. Berenguer-Murcia, A.R. Alcantara, O. Barbosa, C. Ortiz, R. Fernandez-Lafuente, Immobilization of lipases on hydrophobic

supports: immobilization mechanism, advantages, problems, and solutions, Biotechnology Advances 37(5) (2019) 746-770.
[63] D.L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S.M. Franken, M. Harel, S.J. Remington, I. Silman, J. Schrag, The α/β hydrolase fold, Protein Engineering, Design and Selection 5(3) (1992) 197-211.
[64] A.G. Murzin, S.E. Brenner, T. Hubbard, C. Chothia, SCOP: a structural classification of proteins database for the investigation of sequences and structures, Journal of Molecular Biology 247(4) (1995) 536-540.
[65] J.D. Schrag, Y. Li, S. Wu, M. Cygler, Ser-His-Glu triad forms the catalytic site of the lipase from Geotrichum candidum, Nature 351(6329) (1991) 761-764.
[66] T. Ishida, S. Kato, Theoretical perspectives on the reaction mechanism of serine proteases: the reaction free energy profiles of the acylation process, Journal of the American Chemical Society 125(39) (2003) 12035-12048.
[67] A. Cornish-Bowden, One hundred years of Michaelis–Menten kinetics, Perspectives in Science 4 (2015) 3-9.
[68] L. Michaelis, M. Menten, Die kinetik der invertinwirkung Biochem Z 49: 333–369, Find this article online (1913).
[69] G.E. Briggs, J.B.S. Haldane, A note on the kinetics of enzyme action, Biochemical Journal 19(2) (1925) 338.
[70] A.G. Marangoni, Enzyme kinetics: a modern approach, John Wiley & Sons (2003).
[71] S. Saktaweewong, P. Phinyocheep, C. Ulmer, E. Marie, A. Durand, P. Inprakhon, Lipase activity in biphasic media: Why interfacial area is a significant parameter?, Journal of Molecular Catalysis B: Enzymatic 70(1-2) (2011) 8-16.
[72] S.W. Tsai, C.S. Chang, Kinetics of lipase-catalyzed hydrolysis of lipids in biphasic organic—aqueous systems, Journal of Chemical Technology & Biotechnology 57(2) (1993) 147-154.
[73] Y.K. Lee, C.L. Choo, The kinetics and mechanism of shear inactivation of lipase from Candida cylindracea, Biotechnology and Bioengineering 33(2) (1989) 183-190.
[74] M. Cygler, J.D. Schrag, Structure and conformational flexibility of Candida rugosa lipase, Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1441(2-3) (1999) 205-214.
[75] J. Barriuso, M.E. Vaquero, A. Prieto, M.J. Martínez, Structural traits and catalytic versatility of the lipases from the Candida rugosa-like family: A review, Biotechnology Advances 34(5) (2016) 874-885.
[76] X. Li, Z. Liu, G. Wang, D. Pan, L. Jiao, Y. Yan, Overexpression of Candida rugosa lipase Lip1 via combined strategies in Pichia pastoris, Enzyme and Microbial Technology 82 (2016) 115-124.
[77] E. Subroto, R. Indiarto, A. Pangawikan, S. Huda, V. Yarlina, Characteristics, immobilization, and application of Candida rugosa lipase, Food Research 4(5) (2020) 1391-1401.
[78] R. Verger, Enzyme kinetics of lipolysis, Methods in Enzymology, Elsevier (1980) pp. 340-392.
[79] P.D. De Maria, J.M. Sánchez-Montero, J.V. Sinisterra, A.R. Alcántara, Understanding Candida rugosa lipases: an overview, Biotechnology Advances 24(2) (2006) 180-196.

[80] P. Monecke, R. Friedemann, S. Naumann, R. Csuk, Molecular modelling studies on the catalytic mechanism of Candida rugosa lipase, Molecular Modeling Annual 4(12) (1998) 395-404.
[81] E. Vanleeuw, S. Winderickx, K. Thevissen, B. Lagrain, M. Dusselier, B.P. Cammue, B.F. Sels, Substrate-specificity of Candida rugosa lipase and its industrial application, ACS Sustainable Chemistry & Engineering 7(19) (2019) 15828-15844.
[82] W. Abdelmoez, N. Mostafa, A. Mustafa, Utilization of oleochemical industry residues as substrates for lipase production for enzymatic sunflower oil hydrolysis, Journal of Cleaner Production 59 (2013) 290-297.
[83] P. Anuradha, D.J. Rani, K. Vijayalakshmi, S.A. Pasha, S. Kamakshi, Microbial lipases: a potential tool for industrial applications, Journal of Pure and Applied Microbiology 3(1) (2009) 301-306.
[84] S. Sachan, A. Singh, Lipase enzyme and its diverse role in food processing industry, Everyman's Science (2015) 214.
[85] H.F.d. Castro, A.A. Mendes, J.C.d. Santos, C.L.d. Aguiar, Modification of oils and fats by biotransformation, Quimica Nova 27(1) (2004) 146-156.
[86] A. Sikora, D. Chełminiak-Dudkiewicz, M. Ziegler-Borowska, M.P. Marszałł, Enantioseparation of (RS)-atenolol with the use of lipases immobilized onto new-synthesized magnetic nanoparticles, Tetrahedron: Asymmetry 28(2) (2017) 374-380.
[87] A. Mustranta, Use of lipases in the resolution of racemic ibuprofen, Applied Microbiology and Biotechnology 38(1) (1992) 61-66.
[88] A. Singh, Y. Goel, A.K. Rai, U. Banerjee, Lipase catalyzed kinetic resolution for the production of (S)-3-[5-(4-fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one: An intermediate for the synthesis of ezetimibe, Journal of Molecular Catalysis B: Enzymatic 85 (2013) 99-104.
[89] C.S. Chang, S.W. Tsai, A facile enzymatic process for the preparation of (S)-naproxen ester prodrug in organic solvents, Enzyme and Microbial Technology 20(8) (1997) 635-639.
[90] N. Zehani, R. Kherrat, S.V. Dzyadevych, N. Jaffrezic-Renault, A microconductometric biosensor based on lipase extracted from Candida rugosa for direct and rapid detection of organophosphate pesticides, International Journal of Environmental Analytical Chemistry 95(5) (2015) 466-479.
[91] L. Mita, V. Sica, M. Guida, C. Nicolucci, T. Grimaldi, L. Caputo, M. Bianco, S. Rossi, U. Bencivenga, M.S.M. Eldin, Employment of immobilised lipase from Candida rugosa for the bioremediation of waters polluted by dimethylphthalate, as a model of endocrine disruptors, Journal of Molecular Catalysis B: Enzymatic 62(2) (2010) 133-141.
[92] S. Baali, R. Kherrat, S. Zougar, Z. Djeghaba, F. Benamia, N. Jaffrezic-Renault, N. Haddour, Comparative study of responses of two enzymatic biosensors based on lipase from Candida rugosa and Porcine pancreas for detection of diclofop-methyl, Sensor letters 11(11) (2013) 2021-2029.
[93] C.V. Sapan, R.L. Lundblad, N.C. Price, Colorimetric protein assay techniques, Biotechnology and Applied Biochemistry 29(2) (1999) 99-108.
[94] M. Johnson, Protein quantitation, Materials and Methods 2 (2012) 115.
[95] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry 72(1-2) (1976) 248-254.

[96] S. Kiese, A. Papppenberger, W. Friess, H.-C. Mahler, Shaken, not stirred: mechanical stress testing of an IgG1 antibody, Journal of Pharmaceutical Sciences 97(10) (2008) 4347-4366.
[97] C. Thomas, D. Geer, Effects of shear on proteins in solution, Biotechnology Letters 33(3) (2011) 443-456.
[98] V. Delorme, R. Dhouib, S. Canaan, F. Fotiadu, F. Carrière, J.-F. Cavalier, Effects of surfactants on lipase structure, activity, and inhibition, Pharmaceutical Research 28(8) (2011) 1831-1842.
[99] J.H. Lee, S.B. Kim, C. Park, S.W. Kim, Effect of a buffer mixture system on the activity of lipases during immobilization process, Bioresource Technology 101(1) (2010) S66-S70.
[100] R. Saxena, A. Sheoran, B. Giri, W.S. Davidson, Purification strategies for microbial lipases, Journal of Microbiological Methods 52(1) (2003) 1-18.
[101] W. Wang, Lyophilization and development of solid protein pharmaceuticals, International Journal of Pharmaceutics 203(1-2) (2000) 1-60.
[102] C.L.B. Reis, E.Y.A.d. Sousa, J.d.F. Serpa, R.C. Oliveira, J.C.S.d. Santos, Design of immobilized enzyme biocatalysts: Drawbacks and opportunities, Química Nova 42(7) (2019) 768-783.
[103] K. Veeraragavan, B.F. Gibbs, Detection and partial purification of two lipases from Candida rugosa, Biotechnology Letters 11(5) (1989) 345-348.

Chapter 3 : Immobilization of lipase derived from Candida rugosa.

3.1. Separation conundrum.

Given the classical Michalis-Menten (M-M) reaction kinetics, the removal of one or more of the reaction products will drive the reaction forward to produce more product(s) thereby, increasing the maximum possible obtainable conversion, as well as increasing the reaction rate. For the case of lipase-catalyzed triglyceride hydrolysis this involves either the removal fatty acids or the glycerol co-product [1]. Over the years, there have been several methods employed for such types of separations [2, 3]. However, it is crucial that the chosen method is not detrimental to any of the products obtained or to the biocatalyst incorporated. Due to the sensitive characteristics of enzymes and the nature of the fatty acids, the number of possible techniques for separation is often limited. The following is a brief discussion of potentially useful methods for effective separations.

Firstly, the extraction of fatty acids from the bulk oil phase by super critical carbon dioxide [4] and secondly, the use of membrane separation techniques for the recovery of lipase from glycerol [5].

3.1.1. Using super critical fluids.

One of the advantages of using high temperature, high pressure processes for the hydrolysis of triglycerides as mentioned in the first chapter, is that the products eluting are already hot [6]. Therefore, only a limited amount of additional energy is required for distillation type processes for separation. Whereas separation of fatty acids produced by a low temperature process such as lipase catalyzed hydrolysis, require a significant amount of energy to be added to obtain the same degree of fractionation. For those fatty acids, however, which are expected to be commercially produced by the natural enzyme route, conventional distillation would only serve to degrade them [7]. Modifications to these standard distillation columns, such as those used in low-pressure

distillation, can be used successfully if controlled properly. Such equipment is often used in the distillation of many natural products as in the removal of vitamins from fish oils [8].

One relatively new technology, which uses supercritical fluids such as ethylene, ethane, sulfur hexafluoride, near-critical propane and carbon dioxide is gaining increased attention for biochemical processes [9, 10]. These supercritical fluids are materials which are above their critical point and have represented a unique class of non-aqueous media for biocatalysis and bio separation in 21st century. The advantages of using enzymes in supercritical fluids includes the following [11]:

- Synthesis reactions, in which water is a product, can be driven to completion.
- The solubilities of hydrophobic substrates are increased relative to those in water.
- The thermostability of biomolecules in supercritical fluids is greater than in water.
- The solvent can be readily recycled.
- Biochemical reactions and separations can be integrated into a single step.

As mentioned above, reactions which produce water can be made to produce higher equilibrium conversion by the removal of water. Although this may not be suitable for hydrolysis. In general, supercritical fluids offer several advantages over conventional solvents as they have a low viscosity and high diffusivity which leads to significant increases in the rates of mass transfer [12]. Since the critical temperature of carbon dioxide is 31.3^{o}C problems with the thermal denaturation and decomposition of sensitive products and catalysts is avoided [13]. This means that this method is particularly suited to the types of sensitive fatty acids which lipase catalyzed hydrolysis can produce. Within the area of hydrolysis, supercritical carbon dioxide has been used successfully to extract fatty acids more selectively than triglycerides for the deacidification of olive oil when using pressures of 20 - 30 MPa and temperatures of 35 – 60°C [14].

3.1.2. Using membrane technology.

Membrane processes offer a useful method for separating the aqueous components composed of glycerol and lipase [15]. **Table 3.1** gives a list of commonly used methods in membrane separation technology, along with their application [16]. With respect to hydrolysis, the sweet water phase passes through a membrane while the catalyst (lipase) is retained. Sweetwater is then processed to recover glycerol [3, 17]. Within industry certain products which are extremely small are inherently difficult to separate. Such materials include finely dispersed solids, low molecular weight compounds and biological compounds. The processing of these materials has gained an increased importance for the industry, particularly for the newer biotechnological processes where product value is high.

Table 3.1. Description of commonly used membrane technology for separation processes.

Process	Driving force	Separation size	Materials separated
Microfiltration	Pressure gradient	10 – 1 mm	Small particles, large colloids
Ultrafiltration	Pressure gradient	< 0.1 mm – 5 nm	Emulsions, colloids, proteins
Reverse osmosis	Pressure gradient	< 5 nm	Dissolved salts, small organics
Electrodialysis	Electric field gradient	< 5 nm	Dissolved salts
Dialysis	Concentration gradient	< 5 nm	Dissolved salts

Ultrafiltration is a widely used process for separation of protein using pressure gradient [18]. The solutes retained or rejected by ultrafiltration membranes depends on the molecular

weights of protein and the membrane size used. This particular type of filtration is often chosen for the recovery of lipase under conditions which minimize denaturation [19]. A major drawback of use of membranes is the rapid decrease in flux, or flow per unit area, due to fouling [20, 21]. This is defined as material which is rejected by the membrane and fails to be removed from its proximity by the shear force the bulk flow imparts. Foulants can also gain entry to the internal structure of the membrane leading to pore blockage and foulants at the surface can lead to increased hydrodynamic shear hence fluid-membrane interface plays an influential role. The extent of membrane fouling depends on the nature of the membrane used and on the properties of the process feed [22]. It is therefore important to choose the proper membrane for the type of separation required. In the aspects of protein purification, cationic and anionic membranes can be incorporated to reduce fouling effects [23]. For biotechnological applications, pre-treatment may include prefiltration, ionic strength or pH adjustment or possibly pasteurization [23].

Gan et al., found that for lipase catalyzed hydrolysis, there was a significant drop in flux when the aqueous phase recovered after a reaction was treated in the ultrafiltration equipment [24]. It was assumed that some of the fatty acids which leached into the aqueous phase were being absorbed onto the membrane surface, which then became blocked and resulted in a dramatic loss of flux. It is therefore clear that it may be necessary to pre-treat the aqueous phase containing the lipase. This could be achieved by some sort of chemical pre-treatment or possibly by pre-filtration with a hydrophobic membrane. However, it is expected that as newer membranes become available that this may be unnecessary.

3.2. Immobilization of enzymes.

Currently, the best method of enzyme recovery and reuse is never to allow the enzyme to enter the aqueous phase by immobilizing the lipase on to a solid support [25]. Immobilization has

been used by many researchers and industries as a tool to improve activity, selectivity, specificity, resistance to inhibitors and product purification [26].

Immobilization is the process of physically confining and localizing the enzyme onto a solid support (carrier) which may provide a more stable physical environment for the enzyme, resulting in improved recyclability, thermal and chemical stability, reducing conformational changes. Since enzymes are proteins and they are soluble in water, it is difficult to separate them once the reaction is over for reuse in batch processes. To overcome this difficulty, enzymes are immobilized on the carrier materials or inside an insoluble matrix by various physical or chemical methods [27, 28].

Immobilization has the following distinct advantages,

- Expensive enzymes can be reused.
- The enzyme density in a particular location can be increased.
- Continuous operations are possible.
- Chemical and mechanical stability of enzymes are improved considerably.
- Products obtained are not contaminated with enzyme.
- Easy to incorporate process control techniques.
- Catalytic process route can be controlled more accurately.
- Allows development of multi-enzyme reaction systems.
- Effluent disposal problems are considered reduced.

In view of the above advantages, immobilized enzymes have become a potential source for industrial use. However, there are some disadvantages associated with using immobilized enzyme systems in the form of,

- Lower enzyme activity compared to native enzymes in aqueous solutions.

- Additional cost for carriers/supports and immobilization process.
- Lower reaction rates compared to native enzymes in aqueous solutions.
- Subject to fouling.
- Disposal or recycling cost of spent or damaged supports (carriers).

3.3. Lipase immobilization.

Lipase immobilization techniques are divided into five main groups as shown in the **Figure 3.1** [29, 30]. The divisions are mostly based on whether the enzymes are confined in a limited space (entrapment and encapsulation) or bound to support material or with each other (adsorption, covalent bonding and cross-linking) [27, 28, 31]. Lipase immobilization has been the topic of interest in the last decade for several researchers and industrial applications due to their ability to catalyze various reactions. There have been over 5000 publications (and increasing) on lipase immobilization using over 1000 different support materials in the past 10 years by utilizing various immobilization techniques [32].

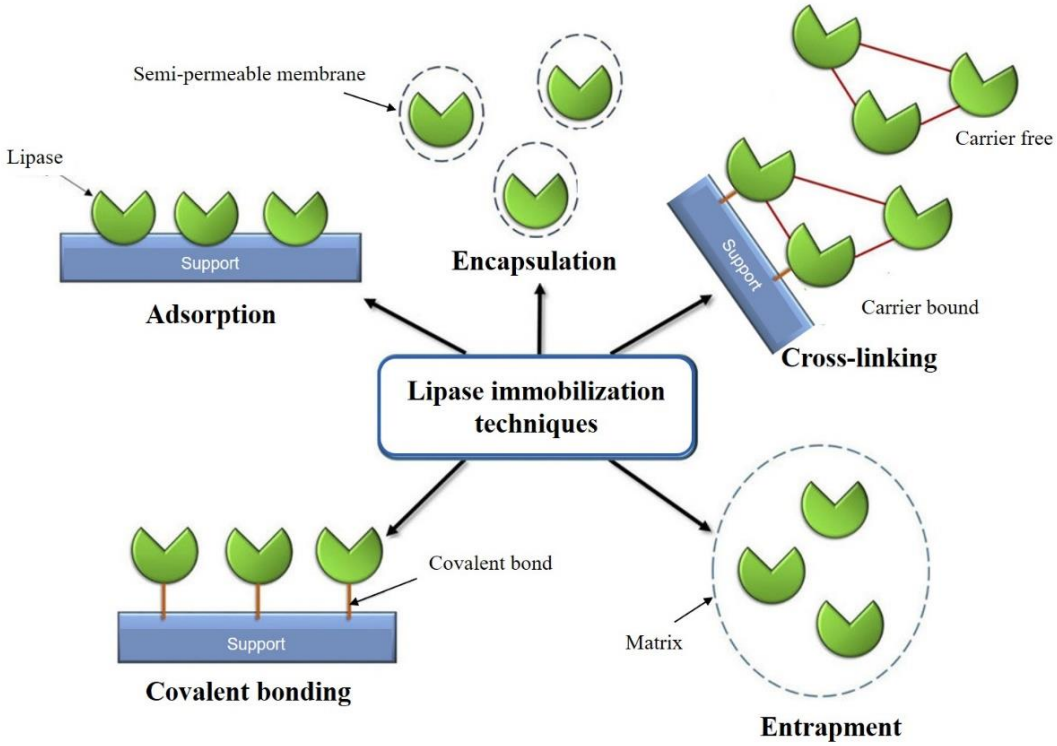


Figure 3.1. Various methods incorporated for immobilization of lipases.

3.3.1. Entrapment (or Occlusion).

Entrapment is one of the simplest irreversible physical process of enzyme immobilization [33]. The active lipase is entrapped using a support matrix (usually gels, foams and beads are used) that is insoluble in reaction media. In this method the support and enzyme does not interact with one another [34]. The support acts as a cage for lipase. **Table 3.2** summarizes some of the notable supports used for lipase immobilization using entrapment technique [35-40]. Immobilization of enzymes using the entrapment method is very simple and can be applied for most of the enzymes.

The advantages of this method include, (1) enzymes are not chemically modified and (2) enzyme properties are not altered. However, there are certain disadvantages in the form of enzyme deactivation during the immobilization protocol and leakage during continuous application, depending upon the type of support used. There are also diffusional limitations which cause

reduced accessibility for the substrate. The porosity of the support plays a vital role in this technique [41].

Table 3.2. Different supports employed for lipase immobilization using entrapment mechanism.

Support	Morphology	Lipase used	Immobilization efficiency (%)
Carrageenan	Particles	*Burkholderia cepacian*	43
Poly(vinyl alcohol)	Nanofibers	*Candida antarctica B*	75
Poly(vinyl alcohol) alginate	Gel	*Thermomyces lanuginosus*	94.5
Calcium alginate	Gel beads	*Candida rugosa*	97
Activated carbon	Particles	*Candida rugosa*	95
Celite 545	Particles	*Candida antarctica* B	94
Polyurethane	Foam	*Thermomyces lanuginosus*	94

3.3.2. Encapsulation.

In this method, the enzyme is trapped within a semipermeable membrane in the form of microscopic hollow spheres [42]. The entrapment method does not affect the activity of the enzyme. A further advantage of encapsulation is that each enzyme is in much closer contact with the surrounding solution than are those entrapped in the interior of gels [43]. However, in some instances, free radicals generated during the polymerization procedure may cause some loss of enzymatic activity. Also, since the encapsulated enzymes cannot escape because of their size, it follows that a very large substrate will not be able to diffuse in to reach the enzymes. Thus, this method of immobilization may not be suitable for proteolytic enzymes, or for macromolecular substrates hence there has not been many studies with regards to lipase immobilization using this technique [41]. Even though the micro encapsulation method is not free from some degree of

leakage of enzyme, the best advantage of this method is that each enzyme is in much closer contact with the substrate in the surrounding solution.

3.3.3. Covalent attachment.

In this method, the enzyme is attached to the surface by irreversible covalent bond formation via certain functional groups [25]. The enzyme functional groups most linked by covalent bonds to a carrier (support) are free α- or β- amino groups, but sulfhydryl, hydroxyl, imidazole or free carboxyl groups may also be involved [44]. **Figure 3.2** shows some of the commonly used water insoluble support materials for covalent immobilization of enzymes [45]. The covalent bonding method provides more permanent linkage between the enzyme and the support material. Covalent bonds can be formed under mild conditions, and the active site of enzyme must remain free from covalent attachments. There is still some possibility for loss of activity of the enzyme during bond formation mainly because of chemical reaction [46].

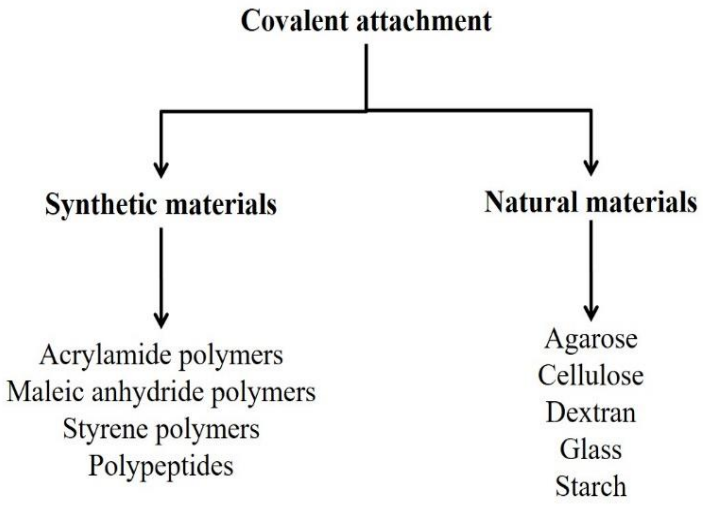


Figure 3.2. Commonly employed water insoluble supports for covalent immobilization.

In general, the conditions required for the covalent attachment of an enzyme to an insoluble support are such that some loss of activity is inevitable. However, very little activity change often takes place if a covalent attachment is brought about by means of chelation rather than by a chemical reaction; a wide range of supports such as cellulose, glass and nylon, if treated with salts of transition metals such as titanium, vanadium or iron chlorides, then washed and dried, can chelate enzymes; strong metal bridges are formed between hydroxyl oxygen atoms of the carrier and amino nitrogen atoms on the enzyme [47]. **Table 3.3** summarizes important supports used for lipase immobilization using covalent bond formation technique [40, 48-54].

Table 3.3. Different supports employed for lipase immobilization using covalent bond formation.

Support	**Morphology**	**Lipase used**	**Immobilization efficiency (%)**
Sepiolite	Particle	*Rhizopus oryzae*	79
Polyurethane	Foam	*Thermomyces lanuginosus*	83
Cellulose	Powder	*Candida rugosa*	94
Chitosan-silica	Nanocomposites	*Porcine pancreatic*	94
Carbon nanotubes	Nanotubes	*Thermomyces lanuginosus*	65
Glass beads	Beads	Candida rugosa	98
Octyl-glyoxyl agarose	Beads	*Rhizomucor miehei*	90
Divinyl sulfone agarose	Beads	*Candida antarctica B*	100
Superparamagnetic Fe_3O_4	Particles	*Candida rugosa*	100

3.3.4. Cross-linking.

Immobilization by cross-linking molecules of enzyme is most brought about by the action of glutaraldehyde, whose two aldehyde groups form Schiff's base linkages with free amino groups [55]. Since several free amino groups are likely to be present on each enzyme molecule, a cross-linked network will be formed. This procedure was first used by Quiocho and Richards, in 1964, to immobilize carboxypeptidase A [56]. Other reagents with two functional groups of relevance to enzyme immobilization include derivatives of bis-diazo benzidine, which act by means of diazo coupling .

Although cross-linking between identical enzyme molecules can result in immobilization at high enzyme concentrations, it is not an ideal method since many molecules simply act as supports for others. Hence, cross-linking is often performed in conjunction with other methods of immobilization. It can, for example be used to prevent the leakage of enzymes from a polymerized gel or to trap the enzyme around pre-formed polymer molecules [57]. Also, bifunctional reagents may be used, not only to link molecules of enzymes to each other, but also to link them to an inert support; thus, glutaraldehyde has often been used to attach enzymes to amino groups of carriers such as aminoethyl – cellulose or aminoalkylated porous glass [58].

Formation of three-dimensional networks as a result of intermolecular cross-linking is also a feature of glutaraldehyde cross-linkage, thus providing more secure bonds between enzyme and support surface. The role of glutaraldehyde as a bifunctional cross-linking agent and its ability to form stable intra and inter-sub-unit covalent bonds are also seen as positive features of glutaraldehyde behavior in this context. Formation of three-dimensional networks as a result of intermolecular cross-linking is also a feature of glutaraldehyde cross-linkage, thus providing more secure bonds between enzyme and support surface. The role of glutaraldehyde as a bifunctional

cross-linking agent and its ability to form stable intra and inter-sub-unit covalent bonds are also seen as positive features of glutaraldehyde behavior in this context [59]. As with any other method which involves the formation of covalent bonds by chemical reaction, cross-linking is usually performed under conditions which cause some loss of enzyme activity.

Historically, there have been many studies involving cross-linking for lipase catalyzed reactions using glutaraldehyde and other reagents. **Table 3.4** summarizes some of the notable studies on lipase immobilized using glutaraldehyde cross-linking techniques [32, 60-65]. Mbanjwa et al., studied glutaraldehyde and ethylene diamine in a carrier free system to immobilize *Pseudomonas flurescens* for the hydrolysis of p-nitrophenyl butyrate and showed 65% hydrolysis activity retention and stable for six cycles [66]. Manan et al., studied immobilization of *Rhizomucor miehei* on chitosan with 1-ethyl-2[3-(dimethylamino)propyl] carbodiimide hydrochloride as crosslinking agent for esterification reaction of eugenyl benzoate and achieved higher conversion than aqueous lipase solution [67, 68].

Table 3.4. Different studies associated with lipase immobilization using glutaraldehyde cross-linking mechanism.

Support	Morphology	Lipase used	Immobilization efficiency (%)
Collogen	Fibers	*Candida rugosa*	94
Alginate	Microspheres	*Candida antarctica B*	89
Chitosan	Nanoparticles	*Candida rugosa*	55.6
Support-free	-	*Porcine pancreatic*	40
Support-free	-	*Candida rugosa*	76
Octyl-silica-amino-glutaraldehyde	Particles	*Candida antarctica B*	98
Silica	Particles	*Candida rugosa*	80.5
Polyolefin	Particles	*Candida rugosa*	65

3.3.5. Adsorption.

Among various tested methods which use different enzyme-support interactions, immobilization of lipase via physical adsorption on to solid carrier (support) has appeared to be simple, cheap, effective and sustainable. All other methods involve chemical or enzyme modifications in order to increase binding. Adsorption involves binding of enzymes to an insoluble carrier or support (solids) by van der Waals forces of interaction, ionic interactions and hydrogen bonding [69]. Other parameters to be considered include enzyme desorption from the supports, formation of enzyme dimers on the support surface, one-point or multipoint interaction of enzyme with the support depending on the surface functional groups present, ease of support modification, support surface area and porosity, ability to perform in continuous operations, availability and cost [70]. **Table 3.5** shows a comparative summary of all the enzyme immobilization techniques [69].

In 1916, Nelson and Griffin showed that invertase could be adsorbed onto activated charcoal without any change in enzymatic activity, thus providing the first immobilized enzyme, although they made no subsequent use of it [71]. Since then, many solid supports have been accessed for enzyme immobilization using adsorption techniques.

Lipases have a unique mechanism of action called interfacial activation [72, 73]. In aqueous media, a large percentage of lipase molecules have their active center covered by a polypeptide chain referred to as a lid, which may isolate it from the reaction medium (closed form). In the presence of a hydrophobic surface, the enzyme becomes adsorbed on it, fixing a new structure (so-called open form) where the active center is fully exposed, thus enabling the lipase to catalyze the substrate. This idea has been exploited in the past ten years to selectively immobilize many lipases on a variety of hydrophobic supports by stabilizing their open forms [72, 74-76]. Physicochemical properties of the support including surface area, particle size, pore structure and type of functional group present on the surface also play a vital role in lipase immobilization [77].

Table 3.5. Highlighting the advantages and disadvantages associated with various lipase immobilization techniques incorporated.

Immobilization technique	Advantages	Disadvantages
Physical adsorption	• Simple and cheap • High catalytic activity • No changes to the native enzyme • No need of reagents • Expensive supports can be reused multiple times	• Low stability • Possible loss of biomolecules • Desorption due to weak bonds
Encapsulation and entrapment	• Protection of biocatalyst • Allows the transport of low molecular weight compounds • Enables continuous operation • Facilitates enzyme separation and simplified downstream processing • Allows controlled release of product	• Limitations on mass transfer • Low enzyme loading
Cross-linking	Strong biocatalyst binding Prevents leakage Decreased desorption Stability is increased	• Active site is altered • Diffusion limitations • Loss of enzyme activity
Covalent bonding	• Strong binding • Stability is drastically increased • Prevents elution of biocatalysts • Flexibility in design of support material and method	• Limited enzyme mobility causes decreased enzyme activity • Support materials are not renewable • Expensive

In general, supports for immobilizing lipase by adsorption using hydrophobic interaction can be classified into two categories, inorganic and organic supports. Inorganic supports are often made hydrophobic using chemical reagents. Minerals, ceramics, silicas, silica gels, and bentonite are the most studied class of inorganic support due to their excellent dispersive-morphological parameters and porous structure [78, 79]. They are often modified using various polymers to enhance immobilization. Metals such as gold [80], titanium, zirconium [81], and aluminum [82] have also been studied for lipase immobilization. Apart from minerals and metals, activated carbon as support has been extensively reported in the literature [83]. **Table 3.6** represents notable studies on lipase immobilization using adsorption technique for inorganic support systems [84-96].

Various naturally occurring and synthetic polymers have been deployed as supports for lipase immobilization. Chitosan obtained from chitin by deacetylation is a widely studied naturally occurring polymer support demonstrated for lipase immobilization and used industrially under the brand name Chitopearl [97-101]. Apart from chitosan, agarose a polysaccharide polymer, is also studied and used industrially for lipase immobilization marketed under the name Sepharose. **Table 3.7** lists some of the notable studies on lipase immobilization using adsorption techniques for various naturally occurring polymer supports [102-107].

Table 3.6. Various inorganic supports deployed for lipase immobilization using adsorption technique.

Support	Lipase used	Catalyzed reaction
Silica nanoparticles functionalized with octyl trimethoxysilane	*Thermomyces lanuginosus*	Synthesis of biodiesel
Composite bentonite	*Pancreatic lipase*	Hydrolysis of olive oil
Silica modified with organosilanes	*Candida antarctica B*	Oxidation of cyclic ketones to lactones
Nanostructured tin dioxide	*Candida rugosa*	Synthesis of isoamyl acetate
Mesoporous silica	*Mucor miehei*	Hydrolysis of 4-nitrophenyl acetate
Sol-gel based on propyltrimethoxysilane	*Candida rugosa*	Synthesis of vitamin E succinate
Mesoporous organosilica	*Candida antarctica B*	Esterification of levulinic acid
Silica nanowires with octadecyl groups	*Burkholderia cepacian*	Hydrolysis of 4-nitrophenyl palmitate
Hydrophobic magnetic particles	*Burkholderia cepacian*	Synthesis of biodiesel
Silica functionalized with cyclodextrin	*Burkholderia cepacian*	Transesterification of palm kernel oil with ethanol
Steric acid modified silica nanoparticles	*Candida rugosa*	Glycerolysis of olive oil
Silica aerogels modified with methyl group	*Candida rugosa*	Hydrolysis of olive oil
Mesoporous tin dioxide	*Burkholderia ambifaria*	Synthesis of cinnamyl acetate
Silica coated with octyl groups	*Pancreatic lipase*	Synthesis of fatty acid esters of D-xylose
Magnetic mesoporous silica	*Burkholderia cepacian*	Transesterification resolution of racemic aromatic secondary alcohols
Magnetic Fe_3O_4 nanoparticles	*Alcaligenes sp*	Kinetic resolution of (R,S)-2-(2-chloro-1-hydroxyethyl)thiophene

Table 3.7. Various naturally occurring organic supports deployed for lipase immobilization using adsorption technique.

Support	Lipase used	Catalyzed reaction
Chitosan functionalized different alkyl chains	*Candida antarctica B*	Hydrolysis of fish oil
Hydoxypropyl methyl cellulose	*Pseudomonas fluorescens*	Synthesis of amino esters
Octyl agarose	*Candida antarctica B*	Hydrolysis of R/S methyl mandelate
Silk fiber treated with polydimethylsiloxane	*Candida sp.99-125*	Hydrolysis of olive oil
Octyl agarose beads	*Thermomices lanuginosus*	Hydrolysis of methyl mandelate
Cellulose/lignin hydrogel beads	*Candida rugosa*	Hydrolysis of p-nitrophenyl butyrate
Hydrophobic silk fibers	*Candida sp.99-125*	Synthesis of tri-substituted trimethylolpropane esters

In addition to natural supports, synthetic polymers form a large and varied group of enzyme carriers with specific reference to immobilization of lipases because of their highly hydrophobic nature [108, 109]. Polypropylene, polystyrene, polyacrylamide, polyethylene and poly(N-methylolacrylamide) have all been identified and used commercially as suitable synthetic supports for lipase immobilization for various small scale industrial applications [110-112]. **Table 3.8** represents important commercial synthetic hydrophobic supports used for lipase immobilization with their respective application [113-120].

Table 3.8. Various synthetic organic supports deployed for lipase immobilization using adsorption technique.

Support	Lipase used	Catalyzed reaction
Low density polyethylene	*Candida rugosa*	Hydrolysis of sunflower oil
Epoxy activated acrylic beads	*Candida antarctica B*	Kinetic resolution of racemic (R/S)-propranolol
Mesoporous polymethacrylate	*Thermomyces lanuginosus*	Synthesis of lubricant ester, n-octyl oleate
Polypropylene beads	*Candida rugosa*	Production of bio lubricant base oils
Poly(urea-urethane) nanoparticles	*Candida antarctica B*	Synthesis of geranyl oleate and geranyl propionate
Polystyrene particles	*Candida antarctica B*	Esterification of oleic acid with ethanol
Macroporous acrylic	*Staphylococcus warneri*	Synthesis of ethyl butyrate
Porous styrene divinylbenzene	*Candida antarctica B*	Hydrolysis of triacetin
Divinylbenzene acrylate polymer	*Candida antarctica B*	Synthesis of n-3 polyunsaturated fatty acids-rich triacylglycerols
Octadecyl methacylate	*Rhizomucor miehei*	Synthesis of conjugated linoleic acid

In recent years, the rapid development in material science have resulted in exploitation of a range of functional groups such as epoxy, amide and aldehyde groups which are extensively used for covalent binding of lipases [121, 122]. Among them, the epoxy group was the most used functional group of carriers. Some epoxy functionalized carriers sold commercially such as Immobead 150, Eupergit C and other self-made carriers with epoxy group (polymeric microspheres, magnetic microspheres, silica nanoparticles) provide ideal matrices to enable immobilization of enzymes since they allow multipoint binding between enzyme and carrier [123-

125]. Another important commercially available immobilized lipase is Novozym 435 produced by Novozymes. Novozym 435 (N435) makes use of Lewait VP OC 1600 resin composed of poly(methyl methacrylate) crosslinked with divinylbenzene to immobilize lipase derived from *Candida antarctica* B using hydrophobic interactions. They are one among the most used immobilized enzyme in small scale industries for various applications. **Table 3.9** represents some of the industrial application of Novozym 435.

Novozym 435 is considered as the perfect immobilized lipase since it offers high activity, broad pH stability and shows versatility in reactions catalyzed [33, 126]. However, they are very expensive when sourced and do not offer easy recyclability. The spent support materials are either discarded or sent back to the company for regeneration. Enzyme leakage upon continuous operations for long periods of time has been reported. This was explained due to lipase immobilization mechanism being adsorption based on hydrophobic interactions [127]. It also demonstrated inhibitory effects to large size substrates since the open lid conformation of *Candida antarctica B* (CALB) is very narrow for substrates to enter and react. It may be noted that CALB by itself is a very expensive enzyme compared to some other enzymes in its class [126].

Table 3.9. Industrial application of Novozym 435 – *Candida antarctica B* immobilized onto poly(methyl methacrylate) resins crosslinked with divinylbenzene.

Reaction	Substrate(s)	Product(s)
Hydrolysis	Chiral alcohols and esters	Benzoxazole derivatives
Hydrolysis	Trans-2-phenylcyclopropyl azolid and methyl	Trans-2-phenylcyclopropyl 1,2,3-azolide
Hydrolysis	(R)-C5-lipidic dialkylnylcarbinols	(S)-C5-lipidic dialkylnylcarbinols
Esterification	Propionic acid and benzyl alcohol	Benzyl propionate
Esterification	Linoleic (LA), conjugated linoleic (CLA), and pinolenic acid (PLA)	Triacylglycerol
Esterification	Stearic acid and ethylene glycol	Ethylene glycol monostearate
Esterification	Stearic acid and cetostearyl alcohol	Cetostearyl stearate
Esterification	2-phenylpropionic acid and 1-octanol	Octyl-2-phenylpropionate
Transesterification-acidolysis	Canola oil with caprylic acid	Triacylglycerols with medium chain fatty acids
Transesterification-alcoholysis	Ethyl acetoacetate and 2-phenylethanol	2-phenylethyl acetoacetate
Aminolysis	Caffeic acid and (R,S)-α-phenylethylamine	Chiral caffeic acid amide
Aminolysis	Methyl esters and amines	N-acylethanol amines
Aminolysis	Methyl esters and with propargyl amine	Propargyl amides
Glycerolysis	Corn oil	Diacylglycerol
Aminolysis	Various bulky methyl esters and amines	Chiral amines (1-phenylethylamine)

3.4. Specific aims.

Considering the advantage of lipase and the quest for finding optimum supports for enzyme immobilization, three commercially available methacrylate-based polymer resins (LifeTech™ ECR1030M, ECR8285, ECR8806M) were identified and quantified for their ability to act as effective supports for lipase derived from *Candida rugosa* and their potential application in triglyceride hydrolysis were studied. These commercial polymer resins have proven to have good mechanical integrity, high surface area due to their porosity, ability to perform in continuous operation, high hydrophobicity, presence of surface functional groups which enhances multipoint attachments to prevent desorption, and are cheap and available in plenty [26, 128]. They also exhibit chemical resilience to most substrates and offers easy multipoint immobilization. Temperature, pH and ionic strength play a vital role during the immobilization. Immobilization conditions determine the final output performance of the resins and the lipase. Previous study has shown that lipase derived from *Candida rugosa* is highly active and soluble at low molarity phosphate buffer solutions at temperatures close to room temperatures [129]. Hence, these conditions will be utilized during the immobilization procedure.

The aim of this research was to immobilize lipase derived from *Candida rugosa* onto recently developed polymeric resins that are commercially available, and which promote hydrophobic interactions between enzyme and support, thus providing an optimal environment for catalytic hydrolysis. The performance of the resins immobilized with lipase derived from *Candida rugosa* was analyzed and compared by performing vegetable oil hydrolysis in three identical continuously stirred tank batch bioreactors. The thermal stability and recyclability of the immobilized lipase were also evaluated in each case. Protein sizing was studied using a dynamic light scattering technique. The physiological properties of the resins were determined using,

Fourier transform infrared spectroscopy (FT-IR), N_2 adsorption-desorption isotherms (BET) measurement and scanning electron microscopy (SEM). Contact angle measurements on the supports were made to study the hydrophobic and oleophilic nature of these resins. Furthermore, the effect of presence of chemical modifier agents such as glutaraldehyde, 3-aminopropyl triethoxysilane (APTES), and itaconic acid during the immobilization step was also investigated.

3.5. Materials and methods.

3.5.1. Materials.

Mesoporous methacrylate polymer resins (supports) were donated by LifetTech™ ECR Enzyme Immobilization Resins (Purolite® Corporation, UK). The physical properties of the resins supplied are summarized in **Table 3.10**. Aqueous lipase solutions were prepared and quantified using the method described in Chapter 2. Mazola vegetable oil used as purchased is the main substrate oil used for hydrolysis. Other chemicals used without further modification included, high purity phosphate buffer saline (pH 7.4), glutaraldehyde, 3-aminopropyl triethoxysilane (APTES), and itaconic acid (Sigma-Aldrich, United States). Deionized water used was produced in a WATER PRO\RO water system (Labconco™, United States) comprised the aqueous phase. All experiments were performed in triplicate and the results are reported as the mean of this value and the standard deviation.

3.5.2. Protein sizing.

Dynamic light scattering (DLS) using a NanoBrook Omni (Brookhaven Instrument Corporation, United States) particle sizer analyzer was used to measure the vesicle characteristics and the presence of agglomerates of lipase in aqueous solution. The instrument consisted of a standard 35 mW red diode laser, nominally providing light at a wavelength of 640 nm. The instrument was operated at a 90° scattering angle using 3 mL polystyrene cuvettes equipped with

temperature control module. Prior to DLS measurements, the aqueous lipase solutions were centrifuged at 8,000 RPM for 4 hours at 10°C [130].

Table 3.10. Properties of the three methacrylate polymer resins supplied by Purolite® Corporation used in the study.

Product Name	ECR1030M	ECR8285	ECR8806M
Functional groups	None	Epoxy & butyl	Octadecyl
Matrix	Methacrylate	Methacrylate	Methacrylate
Appearance	White spherical beads	White spherical beads	White spherical beads
Avg pore diameter (Å)	220 – 340	400 – 600	400 – 650
Particle size range (µ)	300 – 710	250 – 1000	300 - 1000

3.5.3. Lipase immobilization protocol.

Prior to immobilization the resins were sieved using a nested column of sieves to obtain a uniform particle size to assure reproducibility. Resins retained on the 510 µm diameter sieve were used in the experiments. Since, the surface of the resins is highly hydrophobic requiring that, the surface be "wetted" prior to immobilization. The wetting process involved suspending 1 g of the support particles in 5 ml of ethanol, at room temperature for 30 minutes. This procedure modified the polymer surface and facilitated immobilization [111, 113]. 10 mL of the aqueous lipase solution were added to 1 g of polymer resins along with 30 mL deionized water in a shake flask. The contents were agitated for 20 hours at 20ºC in an orbital shaker. Separation of loaded resins

and aqueous lipase was performed by vacuum filtration. The percent protein loading onto the resins was determined by analyzing the aqueous phase for lipase before and after the immobilization procedure using the Bradford protein quantification method. The uptake of protein was calculated by mass balance. The resins were then washed with deionized water. Considerable pressure was applied during washing and filtration step to remove weakly bound lipases (to avoid leaching) and untargeted proteins from the polymer surface. Measurements showed that up to 5% of the proteins were lost during the washing step. After washing, the loaded resins were dried in a vacuum oven operated at 30°C for 24 hours.

3.5.4. Polymeric support characterization.

Contact angle measurements, to study hydrophobic and oleophilic nature of these resins were carried out using a goniometer/tensiometer (150-UL Model, Ram*é-Hart Instrument Corporation, United States)*. Prior to measurement the resin particles were pressed between two heated stainless-steel plates held at around 150-200°C, in order to produce flat sheet of the polymer. Approximately, 7 µL drop of liquid (vegetable oil or deionized water) was metered onto the surface of the polymer using a hypodermic. The specific surface areas (S_{BET}) of each resin was measured by the Brunauer, Emmett and Teller (BET) adsorption isotherm (N_2) technique using an ASAP 2020 (Micrometrics Instrument Corporation, United States) analyzer. Before measurement, samples were degassed at 50ºC for 12 hours under vacuum to remove loosely held adsorbed species. The adsorption data were fitted to the BET model to obtain S_{BET} values. Pore size distribution were calculated using the desorption branches of the N_2 isotherms and fitted into Barrett, Joyner and Halenda (BJH) model. Fourier transform infrared (FT-IR) spectra were obtained on these polymer resins using a Vertex 70 (Bruker, United States) infrared spectrometer. The polymers were ground up and formed into pellets for FT-IR measurements. FT-IR spectra

were collected between 400 – 4000 cm^{-1} with a resolution of 4 cm^{-1}, averaging 64 scans. Surface morphology of the polymeric resins was studied using Versa 3D dual beam (FEI Company, United States) electron microscopy. The resins were sputter coated with gold prior to image processing using a Q150T ES (Quorom, United Kingdom) sputter coater.

3.5.5. Lipase-catalyzed triglyceride hydrolysis protocol.

The performance of immobilized lipase was measured based on the rate of release of free fatty acid [131] from triglyceride hydrolysis, using vegetable oil as substrate. Previous studies indicate that a 3:1 ratio of oil to water provided maximum conversion [132]. Hence in the current work, triglyceride hydrolysis was carried out by mixing oil, water and immobilized lipase in a 30:10:1 ratio (weight basis), in three 250 mL continuously stirred tank batch reactors, equipped with overhead stirrers (operated at 100 RPM), temperature and pH meters. Reactions were carried out in triplicate. At periodic time intervals, approximately 0.5 g of the emulsion in the reactor was taken out and solubilized in 20 mL of 50:50 v/v mixture of ethanol and acetone. An additional effect of adding the solvent was to denature lipase and stop any further reaction from occurring [132, 133]. The mixture was titrated against, 0.05 M NaOH using an Excellence T5 (Mettler-Toledo, United States) auto titrator equipped with an end point titration program using phenolphthalein as the acid-base indicator to measure the total FFA content. The number of moles of FFA produced is directly proportional to the amount of NaOH consumed. Lipase activity is expressed in terms of percentage conversion of substrate oil using an average molecular weight of vegetable oil ~900 g/mol (assumed) [134]. The uncertainty in the percent conversion was determined by assuming the uncertainty in the volume of titrant equals the standard deviation of three repeat measurements and then propagating this uncertainty through the calculation of

conversion. The equations leading up to the calculation of percent conversion in detail along with uncertainty calculation are shown in supplementary information.

3.5.6. Recycling studies.

The retained activity after each reaction cycle was determined by conducting a short hydrolysis reaction over 1 hour using lipase immobilized onto methacrylate-based resins. At the end of 1-hour reaction time, the polymer resins were filtered using vacuum filtration fitted with nylon mesh with an opening of 100 µm. The resins were then washed with deionized water for three cycles (750 mL total volume) to remove residues sticking onto the polymers from hydrolysis reaction. The resins were then dried overnight in a vacuum oven operated at 30°C after which they were used for the next reaction cycle. This procedure was repeated for a total of five cycles.

3.5.7. Immobilization in the presence of chemical modifiers protocol

The final set of experiments was performed to determine the effect of the addition of modifying agents during the immobilization procedure, on the percent yield of FFA. During the immobilization protocol described above, 0.1 g of the modifying agent, glutaraldehyde, APTES or itaconic acid were added 30 mL deionized water. Molar concentrations of the modifying agents during the immobilization protocol is 0.016 mol/L for glutaraldehyde, 0.015 mol/L for APTES and 0.025 for itaconic acid. When the modifying agents were added to the deionized water, the pH of the system substantially changed depending upon the agent that was added, hence it was important to monitor the pH of the system during the immobilization protocol. The subsequent performance of the lipase immobilized in the presence of a chemical modifier was determined in the continuously stirred tank batch reactors as described previously in section 3.5.5.

3.6. Results and discussion.

3.6.1. Protein characterization.

Protein vesicle size plays a major role in enzyme immobilization since it determines the final performance of the biocatalyst, in terms of stability and activity when immobilized onto the polymer supports. Pore diameter of the supports is another parameter that plays a major role, generally pore diameters 4-5 times greater than the size of the proteins is chosen to ensure conformational mobility and eliminate diffusion limitations taking place within the pores of the support [128]. Lipase derived from *Candida rugosa* in aqueous solution in highly purified form should have a hydrodynamic diameter size in the range of 50-80 Å in the un-agglomerated form [112, 135]. Lipase when highly purified should also show higher volumetric activity and minimize undesired side reactions catalyzed by contaminants. On the other hand, highly, purified lipase can be unstable and expensive to produce. Commercially available methacrylate-based polymers should serve as an effective support for lipase immobilization since they have an average pore diameter in the range of 220-650 Å as shown in **Table 3.10**. However, results of the DLS experiments shown in **Figure 2**, show the average vesicle size of the protein in aqueous solution for 0.2 wt% lipase loading to be approximately 27 nm and 69 nm for a 2 wt% lipase loading observed in the semi-purified form. DLS experiments were also conducted at varying temperature range (10-40°C), and using deionized water, which yielded similar results.

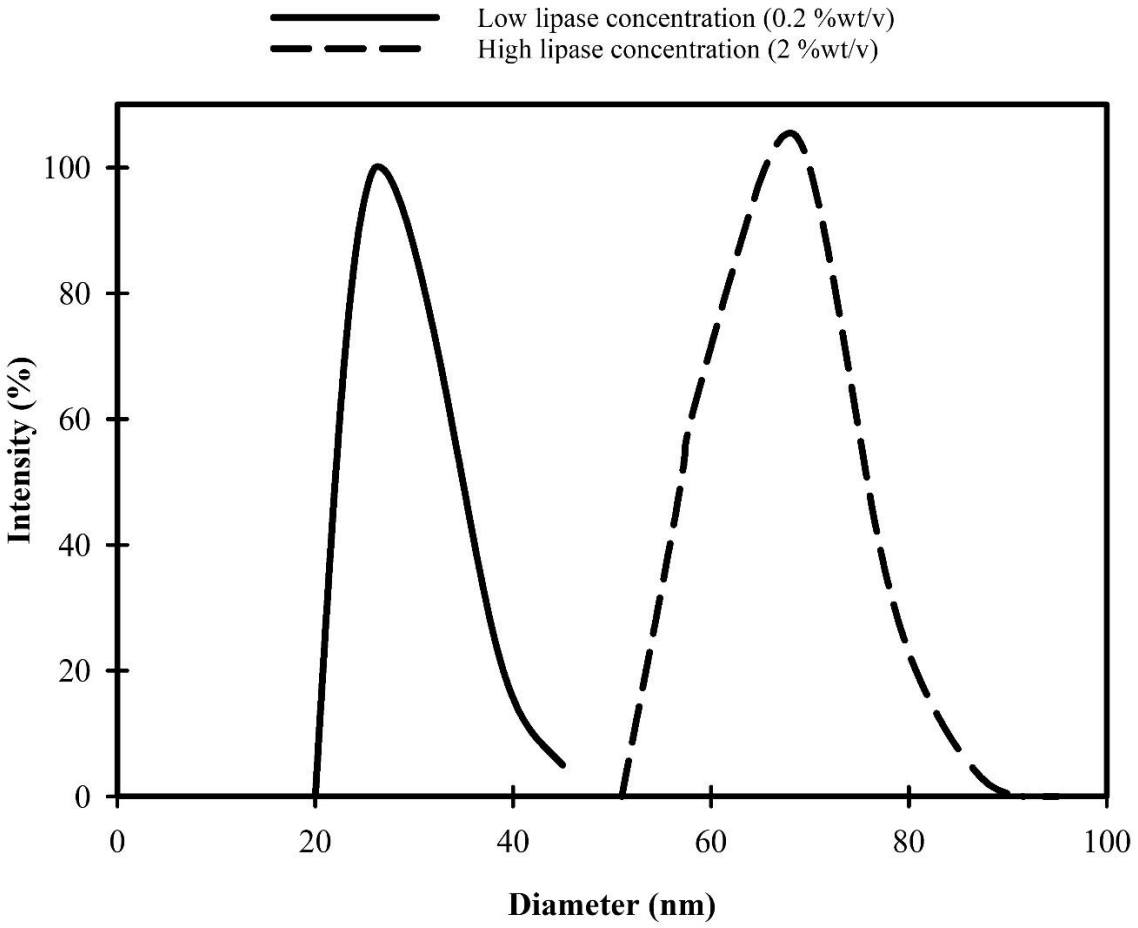


Figure 3.3. Variation of protein vesicle sizes as indicated by intensity measurements by dynamic light scattering for lipase solutions at a concentration of 0.2 wt%/v and 2.0 wt%/v.

Since it was observed that, vesicle diameter increased as the protein concentration increased in aqueous solution, immobilization could be occurring mostly on the external surface of the polymer support, preventing penetration into the interior pores of the supports [136]. **Figure 3.4** shows the results of percent protein loading on the polymer as a function of protein concentration in the external aqueous buffer solution. This could be indicative of proteins not being able to penetrate the surface of the polymer supports. It is also interesting to note that, all three supports adsorbed almost same amount of proteins (± 10% within error bars) which is promising

for comparison studies. **Table 3.11** represents amount of protein adsorbed by the resins with respect to per gram of the support for various lipase loading.

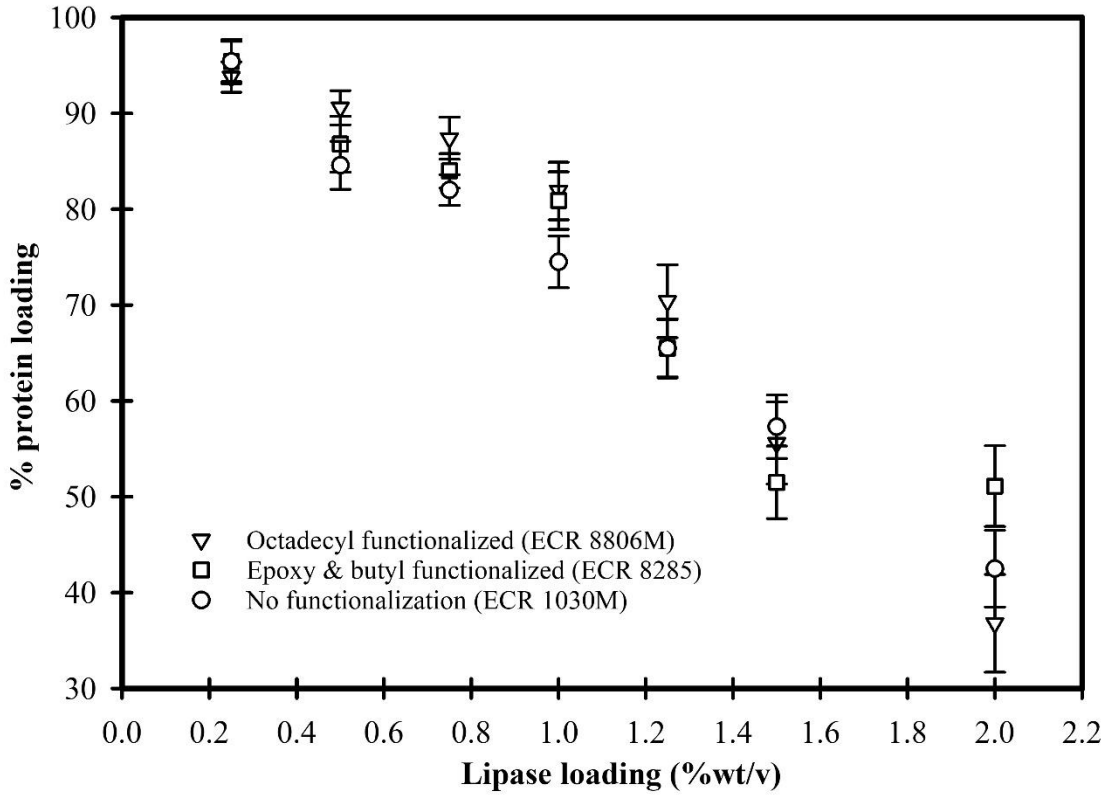


Figure 3.4. Experimental measurements of enzyme loading on each support as function of total protein uptake: Comparison the three-polymer support.

Table 3.11. Variation of lipase uptake with total protein uptake for polymers ECR8806M, ECR8285 and ECR1030M using 0.1 M, 7.4 pH phosphate buffer saline solution at 22°C.

Lipase loading (%wt/v)	0.25	0.50	0.75	1.00	1.25	1.50	2.00
No functionalization (ECR1030M) (mg of protein/g of support)	0.031	0.046	0.077	0.074	0.081	0.104	0.104
Epoxy & Butyl (ECR8285) (mg of protein/g of support)	0.031	0.051	0.077	0.820	0.081	0.094	0.125
Octadecyl (ECR8806M) (mg of protein/g of support)	0.030	0.054	0.077	0.083	0.086	0.101	0.109

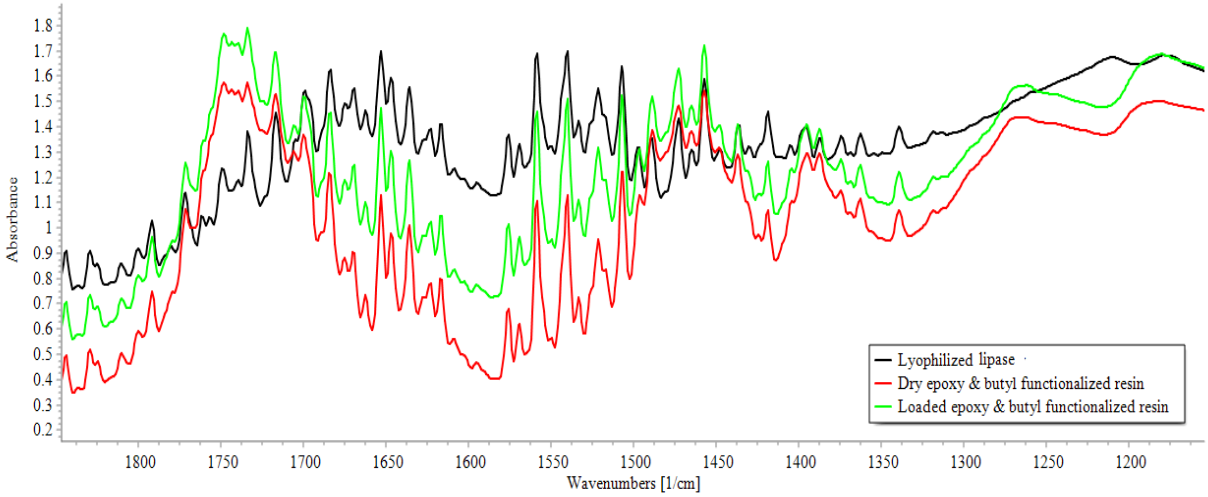
All the measurements were done in triplicate and the error was less than 5% in each case

3.6.2. Polymeric support characterization: Before and after lipase immobilization.

It was important to characterize the polymeric supports in the dried form to assure the presence of lipase after immobilization. Lipase could desorb during the washing process since the main mechanism of immobilization is via physical adsorption via hydrophobic interactions [137]. **Figure 3.5 [A]** shows FT-IR spectra for resins functionalized with epoxy and butyl groups. After lipase immobilization and following the washing process, spectra for the resins still showed an intense peak at wavenumber of 1652.9 (cm^{-1}), indicating the presence of -CONH- (amide I) stretching as seen in lyophilized lipase spectra. **Figure 3.5 [B]** shows FT-IR spectra for resins functionalized with octadecyl groups which shows intense peaks at 1652.9 (cm^{-1}) wavenumber and at 1540.3 (cm^{-1}) wavenumber due to amide I and amide II stretching. Similar results were seen for un-functionalized resins. This suggests the existence of lipase on polymeric resins after the immobilization and washing process, since these peaks were absent in polymers which are not

immobilized with lipase and the stretching's are consistent with results seen in literature for various immobilization supports [138-140]. Full FT-IR spectra can be seen in **Figure S1** of the supplementary information. This proves that the lipase immobilized onto the polymer resins is not easily desorbed. N_2 adsorption data in **Table 3.12**, show that, after lipase immobilization and washing, the BET surface area (m^2/g) and pore volume (cm^3/g) decreased. Close investigation of the BET plots shows that most of the pores less than 50 Å in diameter disappeared or were filled after lipase immobilization. Pore volume reduction appears to be less significant in the case of the larger pores. It is possible that the smaller pores are more readily filled with salts while the larger pores are more readily filled with semi-purified lipase. BET experiments also show that average pore size of the methacrylate resins increased but was not very significant after lipase immobilization. This could be due to expansion of the pores after immobilization.

[A] Epoxy and butyl functionalized methacrylate supports (ECR8285)



[B] Octadecyl functionalized methacrylate supports (ECR8806M)

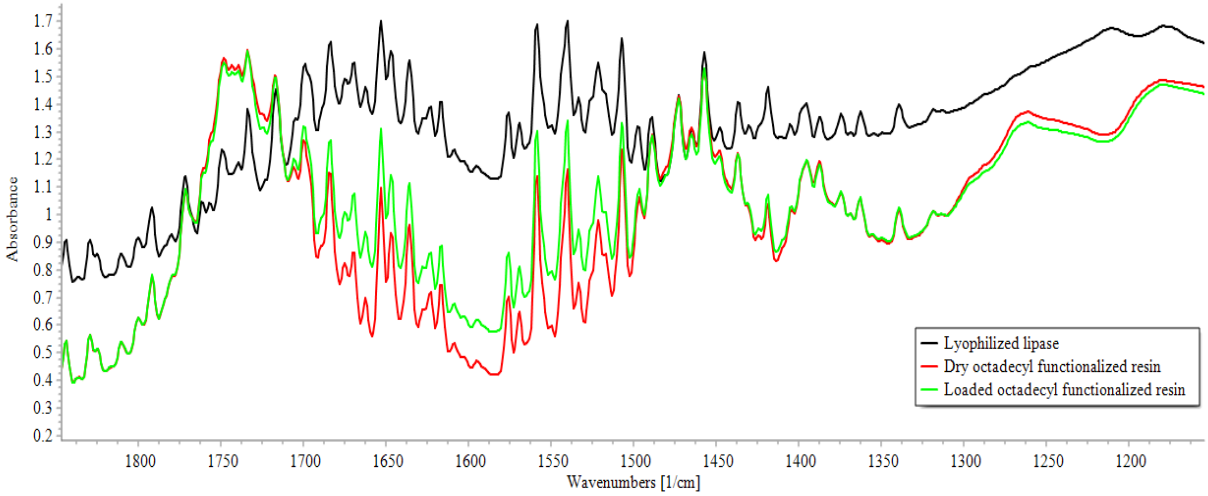


Figure 3.5. FT-IR spectra showing comparisons of lyophilized lipase, dry polymer resins and polymer resins at 0.25 %wt/v aqueous lipase loading.

Table 3.12. BET surface area measurements, pore volumes, and average pore size measurements at 0 wt%/v lipase loading and 1.0 wt%/v lipase loading for polymers ECR8806M, ECR8285, and ECR1030M.

Lipase loading (%wt/v)	BET surface area (S_{BET}) (m^2/g)	Pore volume (cm^3/g)	Avg Pore Size (Å)
Methacrylate resins with no functional groups (ECR1030M)			
0	133.6 ± 0.4	0.55	179
1	92.8 ± 0.3	0.42	195
Methacrylate resins with epoxy & butyl functional groups (ECR8285)			
0	194.5 ± 1.0	0.62	128
1	145.8 ± 0.6	0.53	145
Methacrylate resins with octadecyl functional groups (ECR8806M)			
0	113.9 ± 0.4	0.50	175
1	84.8 ± 0.3	0.42	198

We estimate the uncertainty on the pore volume at 2% of the reported value based on 6 repeat measurements of a certified standard

3.6.3. Performance of lipase upon immobilization on methacrylate polymer supports.

Lipase immobilized onto methacrylate-based resins using 0.25%wt/v of aqueous lipase loading were chosen to assess the initial comparative performance. Further, aqueous lipase solutions were prepared by adjusting with phosphate buffer saline to provide equivalent concentrations of protein adsorbed on the polymeric supports using the Bradford assay. **Figure 3.6** clearly shows faster reaction rates for lipase when immobilized onto polymer supports compared to free lipase in aqueous solution observed at room temperature (~22ºC). Lipase immobilized onto resins with no functionalization reached a maximum of 36 percent yield of FFA, functionalized

with epoxy and butyl groups reached a maximum of 53 percent yield of FFA and functionalized with octadecyl groups reached a maximum of 64 percent yield of FFA around 80-hour time point and achieved steady state yields over time. Observation with lipase in free solution showed a maximum of 27 percent yield of FFA with achievement of steady state yields of FFA at the 60-hour time point. Overall, better performance was noted for lipase when immobilized onto methacrylate resins functionalized with octadecyl groups.

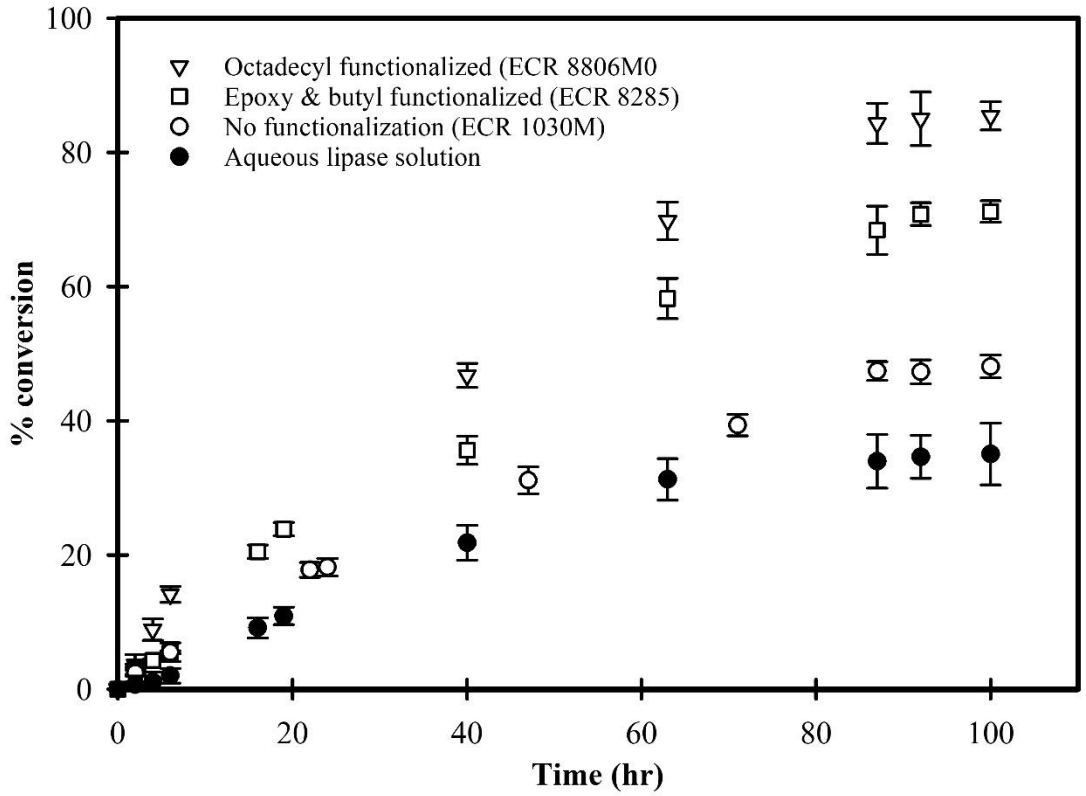


Figure 3.6. Hydrolytic performance of 0.25 %wt/v of aqueous lipase immobilized onto methacrylate-based polymer resins conducted at 22°C: Rate of free fatty acid production, comparing free lipase in solution, and for immobilized lipase on each of the polymers ECR8806M, ECR8285, and ECR1030M.

The poor performance of lipase in free solution is explained in terms of enzyme deactivation upon physical stirring, oil droplets size, temperature and pH of the system. Generally, lipase from *Candida rugosa* has an optimum temperature of 35-40°C and an optimum pH of 7-8, according to the literature [113, 141-143]. Since, the reaction was carried out at room temperature and with FFA forming as the products, the pH of the system tended to be lower with the intrinsic activity tending to decrease because of the pH gradient formed inside the reactor or within the support i.e not all lipase are at their optimum pH conditions because of FFA formation (This phenomenon also holds true in the case of immobilized supports system) [128]. The rates at which FFA and glycerol are formed also depends on the stirring rate which determines the oil droplet size, since lipase from *Candida rugosa* is more active at the oil-water interface [144]. However, excessive stirring or stirring over a long period of time will denature the enzyme in aqueous media [145]. These factors contribute to the low performance noticed in the case of lipase in free solution. Another factor that majorly influences enzyme activity is the immobilization pH. The reactivity of the protein groups with the support depends on the pH, and that way the orientation of the enzyme molecules on the support may be altered by changing the immobilization pH [146]. However, all the immobilizations was carried out at pH 7.4. Hence, it will be interesting to study the effect of change of immobilization pH. But it is beyond the scope of this study. Thus, underlining the potential advantage of immobilization. Better performance following immobilization on methacrylate supports may be potentially explained in terms of hydrophobicity, oleophilicity, presence of surface functional groups and enzyme desorption. Comparing the performance of different methacrylate-based immobilized resins, lipase immobilized onto resins with no surface functional groups showed the lowest yields of FFA. This may be due to the lack of more hydrophobic interaction between the enzyme and the support or by significant enzyme desorption.

Supports with functional groups on their surface tend to be more hydrophobic which causes more hydrophobic interaction between the support and the enzyme during the immobilization protocol [147]. Hence, it was important to measure the extent of hydrophobicity of these supports. **Table 3.13** shows the apparent contact angle measurements for the methacrylate-based polymer resins. Octadecyl functionalized resins showed the highest contact angle when measured with deionized water which confirms high hydrophobicity. The contact angle experimental images are shown in **Figure 3.7**. It is therefore possible that, during the immobilization step greater hydrophobic interaction between the lipase and the support occurs. This would promote more open conformations when lipase is immobilized with enhanced interfacial activation. This in turn could explain higher yields of FFA achieved when compared with the supports with no functional groups present. Further, there could be anchoring of enzymes due to strong bond formation between the functional groups present on the surface of the supports and the enzymes, thereby increasing activity and stability. For example, epoxy groups on the surface of methacrylate supports can react with amino or thiol groups associated with lipase and form strong bonds with the support. This may then cause conformational changes to the enzyme thereby enhancing its stability via formation of multipoint covalent bonding [123, 124]. However, it is unclear how the octadecyl groups on the surface of the polymer resins enhances the activity and stability other than by increasing the hydrophobic interactions.

Table 3.13. Contact angle measurements for methacrylate-based polymer resins conducted at 22°C: Comparison of contact angle values for each polymer in contact with (i) water and (ii) vegetable oil.

Functionalization	Water (°)	Vegetable oil (°)
No functionalization (ECR1030M)	81 ± 2	13 ± 2
Epoxy & butyl groups (ECR8285)	88 ± 2	16 ± 0
Octadecyl groups (ECR8806M)	92 ± 3	07 ± 1

From the contact angle experiments shown in **Table 3.13**, in the presence of vegetable oil it may be noted that the methacrylate-based polymer resins are highly oleophilic. This shows that lipase is likely to be surrounded by large concentrations of oil when immobilized compared with the lipase in aqueous solution. This could explain the faster reaction rates observed in the case of immobilized supports with enhanced oil-water partitioning at the interface playing a significant role. During the 100-hour reaction time (see **Figure 3.6)**, significant polymer decomposition or breakage due to physical stirrings was not observed. Hence, the mechanical integrity of these supports is exceptional. From literature, it can also be noted that yields of FFA can also be increased by addition of water at mid-way during the reaction which will shift the equilibrium conversion [132]. Another possible explanation for lower FFA yields could be lipase desorbing inside the reactor during the reaction. Hence, a set of hydrolysis experiments was conducted using immobilized lipase but stopping the experiment at 10 hours. The supports were filtered and removed from the system. The hydrolysis was then continued for another 24 hours using just the

filtered emulsion to check for any changes in percent yield of FFA. The experiments with supports having no functional groups on the surface (ECR1030M) showed a 16 percent increase in FFA yield which is indicative of enzyme desorption. Methacrylate supports functionalized with octadecyl groups and epoxy and butyl groups showed less than 2 percent increase in yield FFA which could be indicative that enzymes are not desorbing during the hydrolysis reaction.

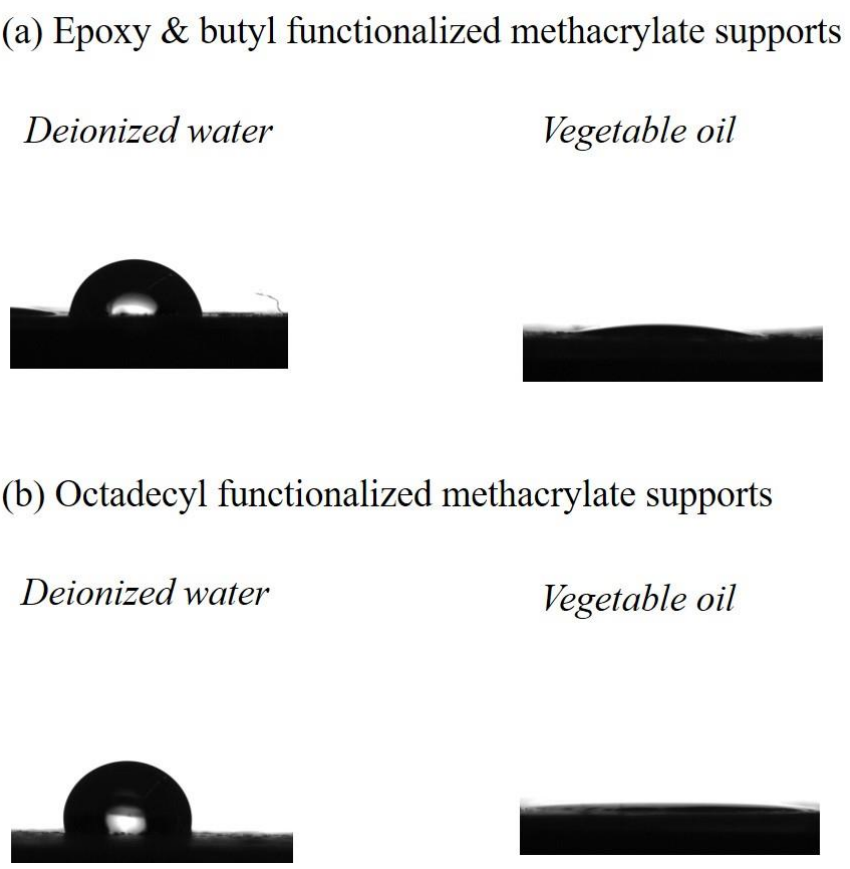


Figure 3.7. Apparent contact angle measurements images for methacrylate polymer resins measured with sample liquid volume of 7 µL (Veg oil or deionized water) at room temperature (~23°C).

3.6.4. Thermal stability.

Thermal stability for the lipase immobilized onto the methacrylate resins was determined by conducting a series of batch hydrolysis reactions, operated for one hour at different

temperatures in the range 20 – 95°C. The results are shown in **Figure 3.8** with activity reflected in percent yield of FFA. The optimum temperature for lipase derived from *Candida rugosa* when immobilized onto methacrylate resins appears to close to 60°C. This suggests increase in thermal stability, possibly explained by changes in physical and chemical properties of the enzyme upon immobilization. For all three-supports, the activity decreased considerably above 60°C which may be accounted by a combination factors for example, enzyme denaturation at higher temperatures or due to protein folding causing irregular enzyme structure formations on the surface of the polymer [148]. Some portion of the enzymes may still be acting as ion-exchanger [149].

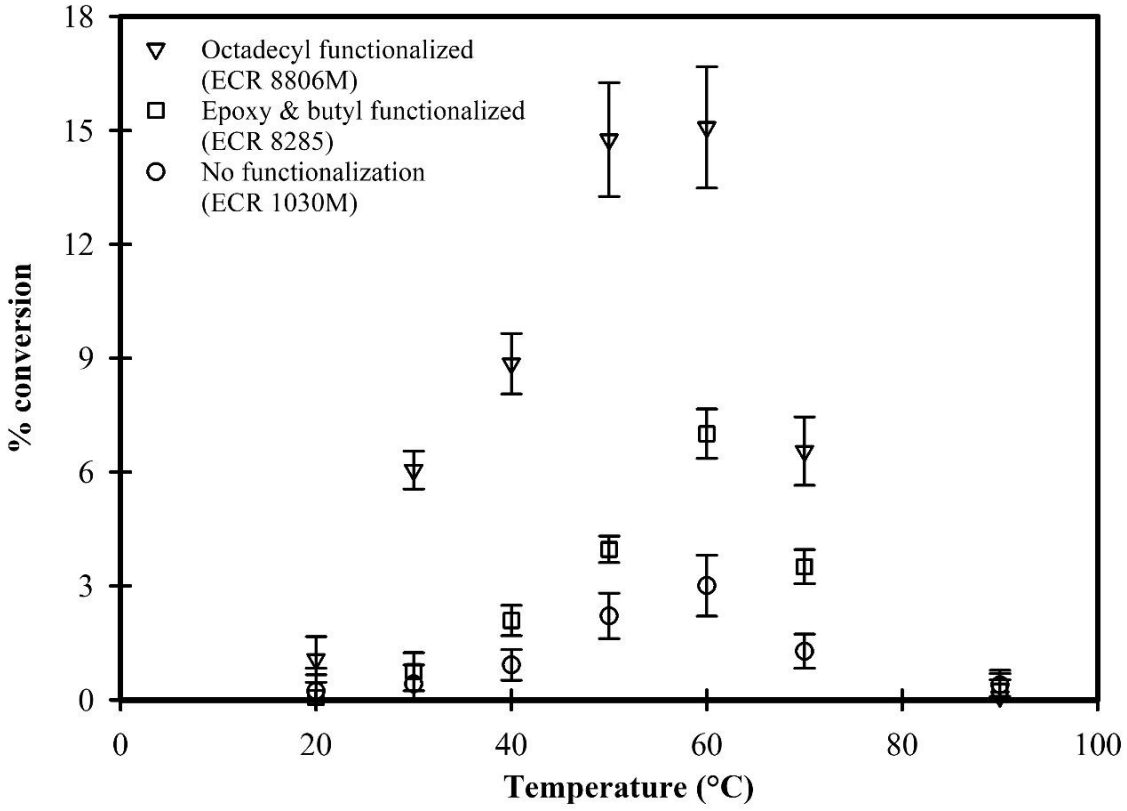


Figure 3.8. Hydrolytic performance of 0.25 %wt/v of aqueous lipase immobilized onto methacrylate-based polymer resins conducted at different temperatures in the range 20°C – 90°C: Rate of fatty acid production, comparing the performance of immobilized lipase on each of the polymers ECR8806M, ECR8285, and ECR1030M.

3.6.5. Recycling.

One of the properties that is significantly improved when lipase is immobilized is the ability to be recycled and used in continuous reactors [27]. The recycling experiments were conducted at 55°C where the highest yields of FFA were observed during the initial runs as shown in **Figure 3.9**. The percentage yield of FFA (an indicator of activity) followed a bell-shaped trend with maximum percent yield of FFA seen at the end of third hydrolysis cycle.

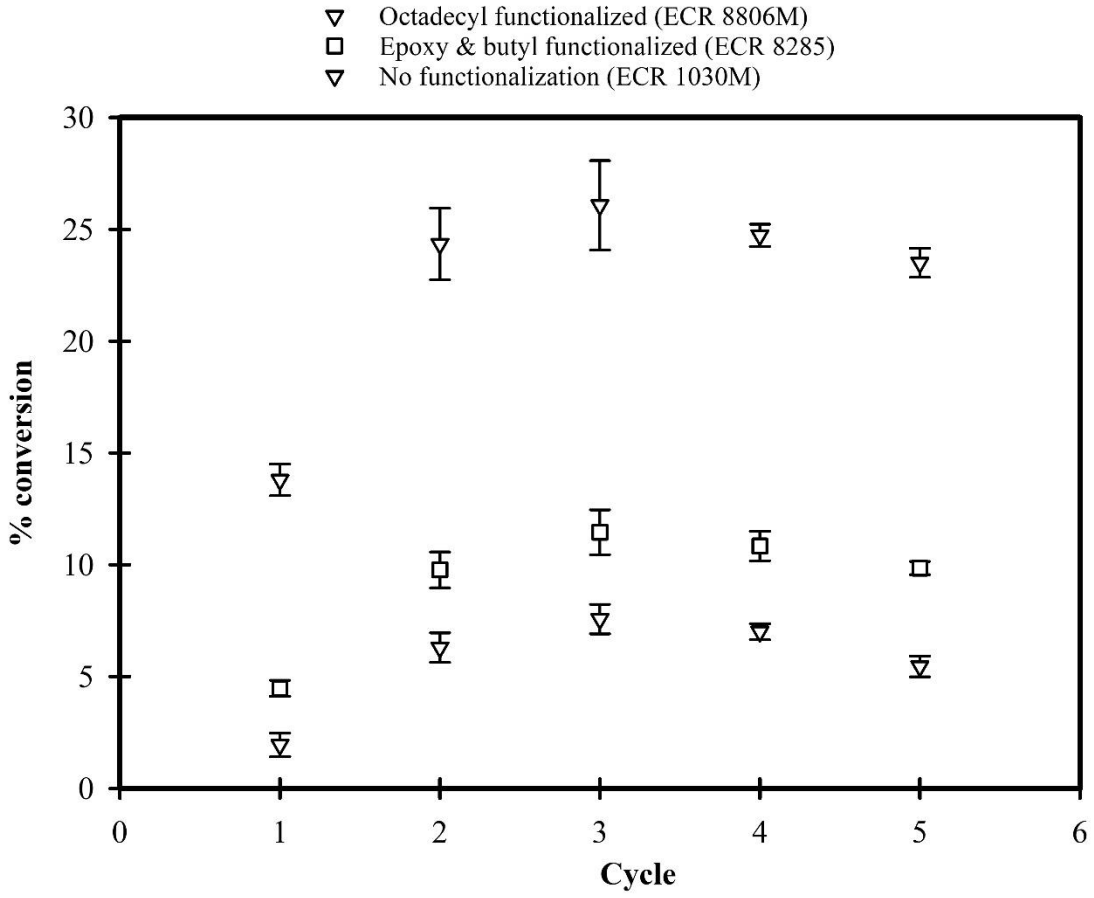


Figure 3.9. Hydrolytic performance of 0.25 %wt/v of aqueous lipase immobilized onto methacrylate-based polymer resins conducted after 1-5 cycles of reaction, separation, washing, and recycling : Rate of fatty acid production at 55°C, comparing the performance of immobilized lipase on each of the polymers ECR8806M, ECR8285, and ECR1030M.

This was not consistent with the results seen in literature, which in most case show that activity decreases with the number of cycles [150]. The bell-shaped trend observed here could be explained by the oleophilic nature of the polymer supports, as evidenced by contact angle measurements (see Table 3.13). A possible explanation is the presence of residual triglycerides and FFAs inside the pores of the polymer after the wash, which continue to diffuse out cycle by cycle since the wash solvent used is water. This may explain the apparent faster reaction rates observed in cycles 1 to 3. This explanation was further supported by observed increases in the weight of the polymer resins (on a dry basis) after each hydrolysis cycle shown in **Figure 3.10**. After the third hydrolysis cycle the activity decreased after each subsequent hydrolysis cycle, reflecting trends reported in the literature. The decrease in observed activity could be because of combined effects of denaturation and desorption.

Stable mechanical integrity of the polymer support after repeated use is also an important consideration. Scanning electron microscopy (SEM) was used to investigate changes in surface morphology and to look for evidence of damage in the resins. Since these supports are highly oleophilic, there still may be substantial residues from the hydrolysis reaction inside the pores of the resins even after washing with deionized water. Hence, the resins were washed multiple cycles using acetone as wash solvent and dried overnight before SEM examination. Examination revealed no evidence of significant breakage, cracking or swelling of supports. However, closer inspection of the surface of the resins indicated slight changes in morphology but these were not considered to be significant. This was observed in all the methacrylate-based resin supports. The changes in surface morphology of octadecyl functionalized methacrylate resin supports are shown in **Figure 3.11** (**a)** initial dry surface morphology and **(b)** surface morphology after immobilization, washing

process and at the end of 5-cycle hydrolysis reaction. This suggest that the supports are amenable to recycling and can be used in different types of bioreactors and adverse conditions .

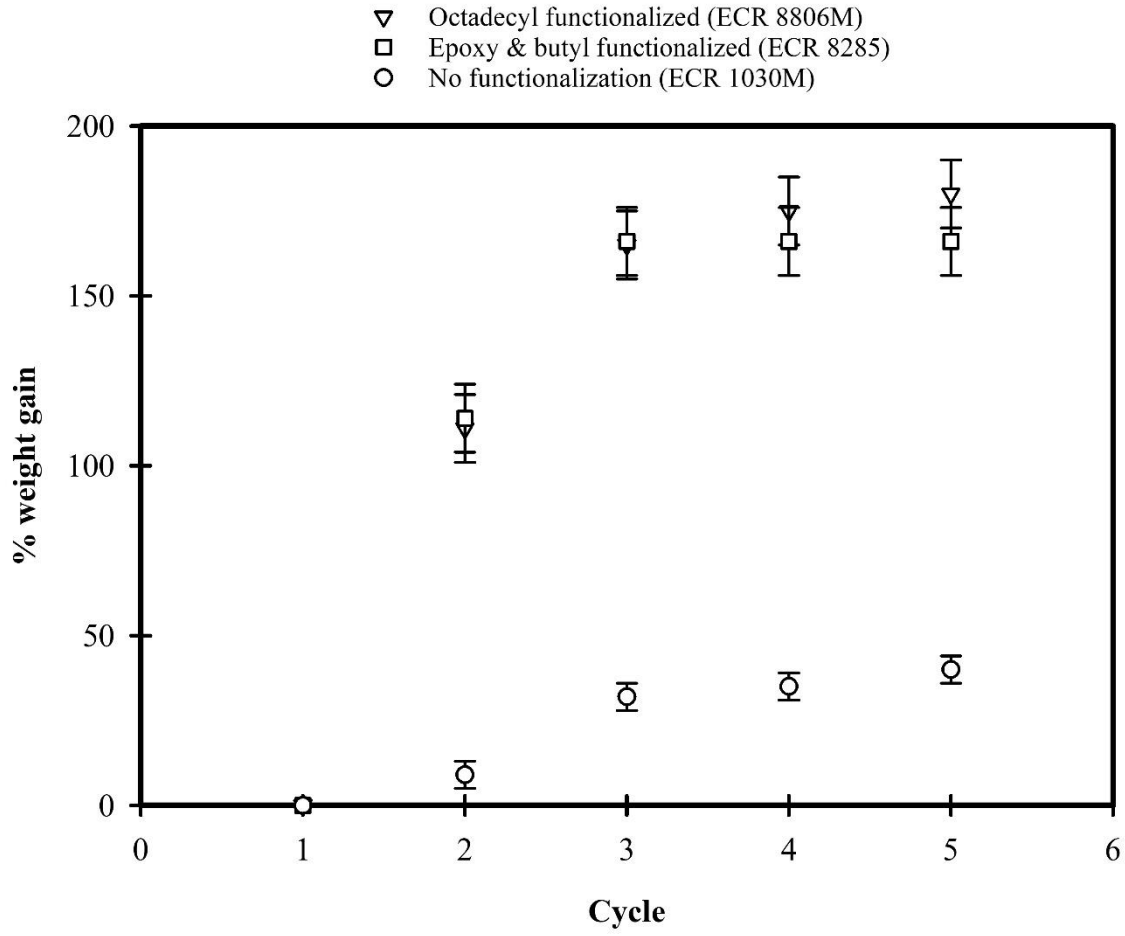
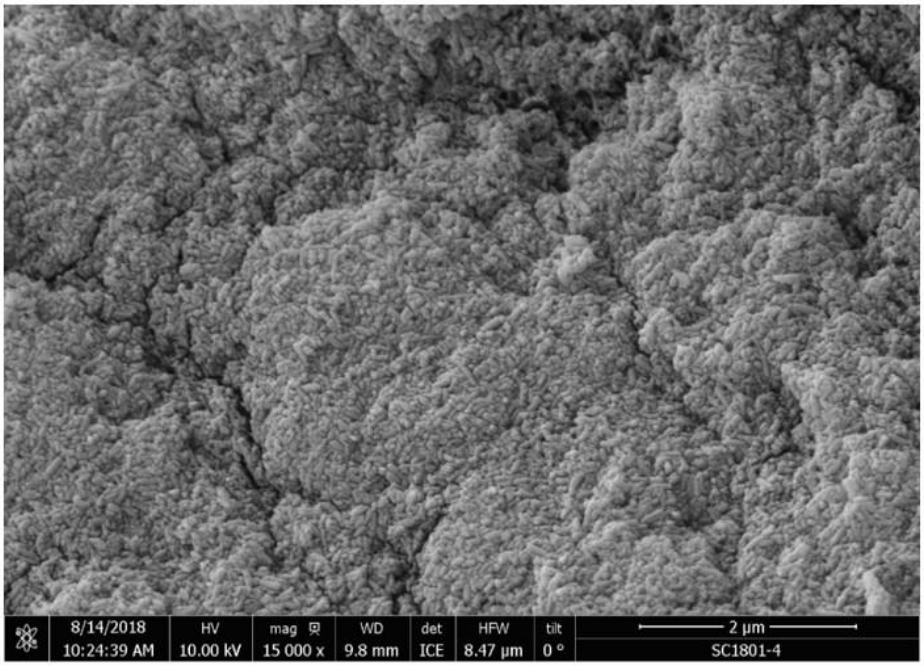


Figure 3.10. Percent weight gain noted after washing as a function of hydrolysis cycle for methacrylate polymer resins.

(a)



(b)

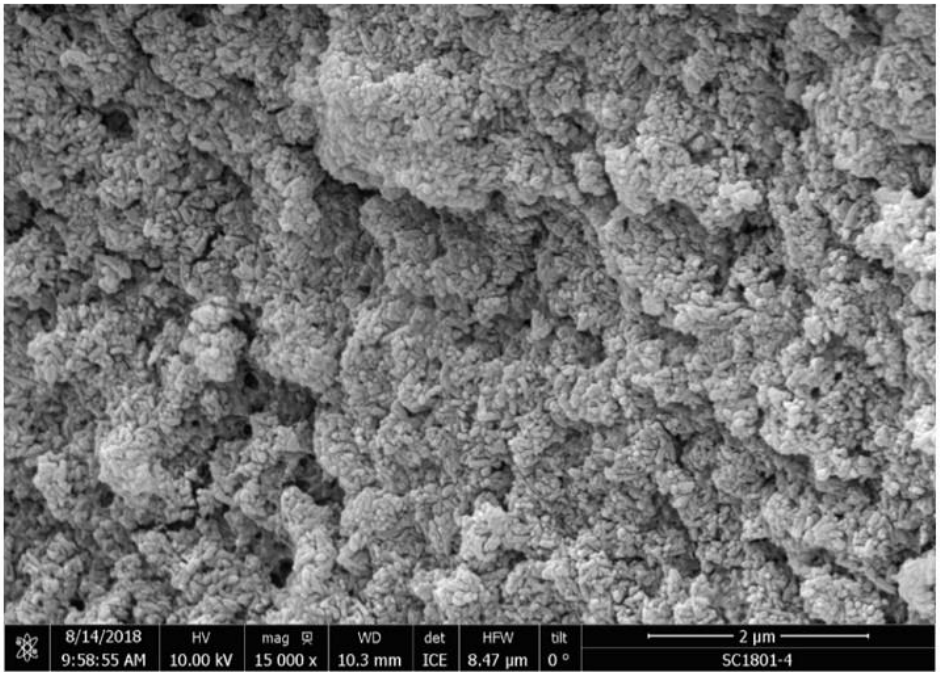


Figure 3.11. Scanning electron microscopy images showing the details of the surface morphology of octadecyl (ECR8806M) functionalized methacrylate polymer resins, **(a)** new polymer **(b)** polymer after immobilization, washing process and at the end of 5 batches of hydrolysis reaction.

3.6.6. Effect of presence of chemical modifying agents during immobilization protocol.

Hydrolysis reactions were conducted using lipase immobilized on the functionalized methacrylate resins in the presence of chemical modifying agents. Glutaraldehyde and (3-Aminopropyl)triethoxysilane (APTES) are chosen because they are among the many modifying agents studied in literature [151, 152]. Itaconic acid was recently identified to be a co-monomer in methacrylate resins and was also examined [153, 154]. **Figure 3.12 & 3.13** shows hydrolytic performance of 0.25 %wt/v of lipase when immobilized onto epoxy and butyl functionalized and octadecyl functionalized methacrylate resins in the presence of various modifying agents.

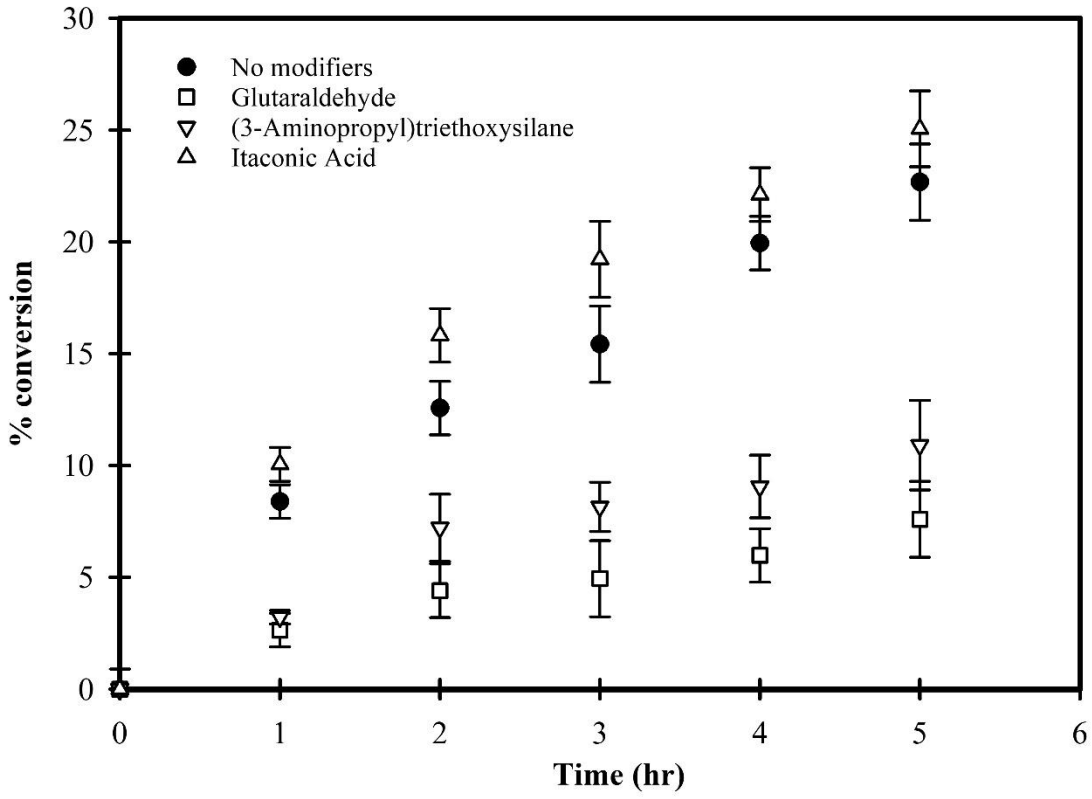


Figure 3.12. Hydrolytic performance of 0.25 %wt/v of aqueous lipase immobilized onto methacrylate-based polymer resins at 55°C: Rate of fatty acid production, comparing the performance of immobilized lipase on the unmodified polymer ECR8285M, with polymer treated respectively with glutaraldehyde, (3-amino-propyl) triethoxysilane, and itaconic acid.

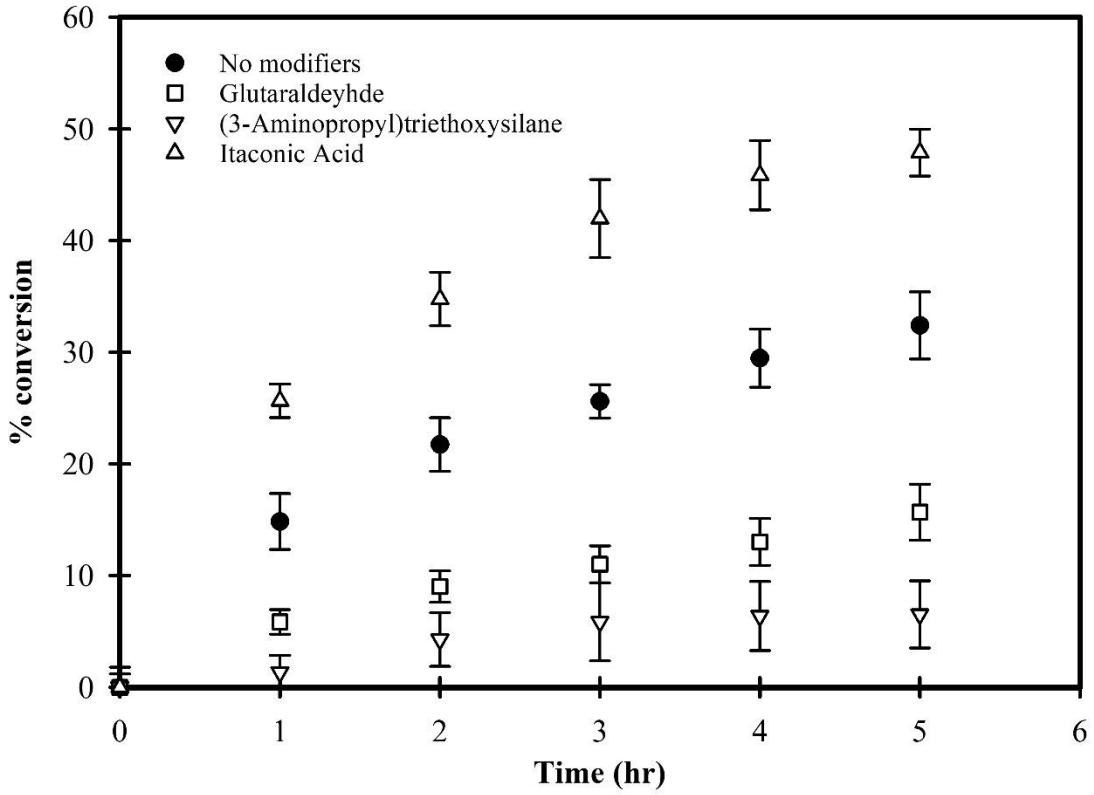


Figure 3.13. Hydrolytic performance of 0.25 %wt/v of aqueous lipase immobilized onto methacrylate-based polymer resins at 55°C: Rate of fatty acid production, comparing the performance of immobilized lipase on the unmodified polymer ECR8806M, with polymer treated respectively with glutaraldehyde, (3-amino-propyl) triethoxysilane, and itaconic acid.

Immobilization of lipase conducted in the presence of glutaraldehyde and APTES onto the functionalized methacrylate resins in presence of deionized water resulted in poorer reaction performance compared with reaction using the supports that were immobilized in the absence of the modifiers. Possible explanations of diminished lipase activity in the case of APTES or glutaraldehyde presence during the immobilization protocol could be due to conformational changes causing substrate inaccessibility, changes in overall immobilization mixture pH, or enzyme inhibition due to the presence of chemical modifiers. 0.1 g of APTES or glutaraldehyde in 30 mL deionized water recorded a pH of 10.5 and 8. Hence, the pH of the overall immobilization

mixture system was no longer maintained at 7.4. The overall immobilization mixture pH was recorded to be 9 and 7.2 in the presence of APTES and glutaraldehyde, respectively. Hence, in the case of APTES the changes in pH are suggested as the dominant mechanism for enzyme deactivation since lipase derived from *Candida rugosa* has shown higher performance when immobilized at a pH of 7.4. But, in the case of glutaraldehyde, the modifying agent could be covalently anchoring the enzymes onto the polymer causing conformational changes leading to substrate inaccessibility. 0.1 g of itaconic acid in 30 mL deionized water recorded a pH of 2.5. At this pH, the lipase derived from *Candida rugosa* should show no activity or minimal activity according to the literature [113, 141-143].

However, it showed conclusive evidence of higher performance in the case of functionalized methacrylate polymer resins. Hence a series of control experiments were performed, and the results are shown in **Figure 3.14**. Reaction conducted in the presence of itaconic acid but without lipase showed no enhancement of reaction rates proving that it is not performing as a catalyst. Furthermore, reaction conducted with free aqueous lipase in the presence of itaconic acid also showed no improvement in reaction rates. This further suggested that itaconic acid improves the support-enzyme chemistry and leading to enhance reaction rates. However, the mechanism is still unclear and further investigation is needed.

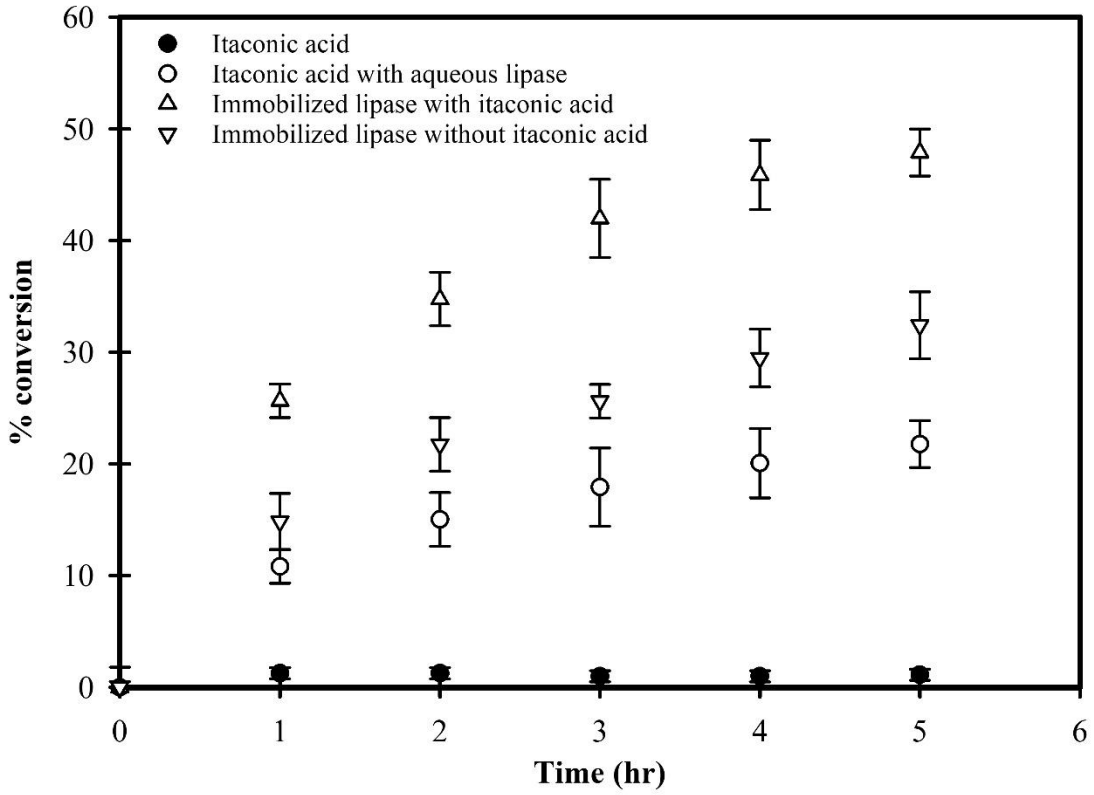


Figure 3.14. Comparison of aqueous hydrolysis rates of tri-glyceride ester: (i) in the presence of aqueous itaconic acid only, (ii) in the presence of itaconic acid and lipase in free aqueous solution, (iii) in the presence of lipase immobilized onto methacrylate-based polymer resin ECR 8806M at 55°C in the presence of itaconic acid (iv) in the presence of lipase immobilized onto methacrylate-based polymer resin ECR 8806M at 55°C in the absence of itaconic acid.

3.7. Conclusion.

Lipase derived from *Candida rugosa* was immobilized onto three Purolite Lifetech™ methacrylate-based polymer resins (ECR8806M, ECR8285, ECR1030M) and their performance compared with each other and with free lipase in aqueous solution using triglyceride hydrolysis as a model reaction. Dynamic light scattering measurements revealed surface adsorption was dominant with minimal internal penetration for lipase immobilization on methacrylate-based polymer resins. This is supported by the observation that percentage protein loading onto the methacrylate-base resins decreases as the enzyme loading in aqueous solution increases. FT-IR

analysis further confirmed the presence of lipase on the polymer resins after immobilization. N_2 adsorption isotherm experiments showed that the average pore size in each polymer increased upon immobilization. Contact angle measurements revealed octadecyl functionalized methacrylate polymer resins are more hydrophobic and oleophilic compared to epoxy & butyl functionalized methacrylate resins which could explain the higher reaction rates observed due to oil-water partitioning at the interface. Octadecyl functionalized resins (ECR8806M) showed superior performance when lipase was immobilized onto them was explained in terms of high hydrophobic interactions and oleophilic nature. Epoxy & butyl groups functionalized resins (ECR8285) performed better than un-functionalized resins (ECR1030M) when lipase was immobilized. This was explained in terms of multi-point covalent bond formations, less hydrophobicity and enhance oleophilic nature compared to octadecyl functionalized resins. Conclusive evidence of enzyme desorption and denaturation inside the reactor during hydrolysis was noticed in the case of un-functionalized methacrylate resins. The optimum operating temperature shifted closer to 60°C when lipase was immobilized onto methacrylate-based resins. It is concluded that this difference represents a significant advantage for the immobilized systems as transport rates and reaction kinetics will be enhanced at higher temperatures. All resins showed excellent recycling behavior with high retention of activity, cycle to cycle at 55°C operating temperature. Examining the octadecyl functionalized methacrylate polymer resins using scanning electron microscopy revealed changes in surface morphology, however they are not significant which makes them excellent for recycling or continuous operations. Presence of itaconic acid during the immobilization protocol revealed enhanced performance for functionalized methacrylate-based resins and had no effects on un-functionalized resins.

3.8. References.

[1] A. Gog, M. Roman, M. Toşa, C. Paizs, F.D. Irimie, Biodiesel production using enzymatic transesterification–current state and perspectives, Renewable Energy 39(1) (2012) 10-16.
[2] J. Borensztajn, M. Reddy, A. Gladstone, A simple method for the separation of triacylglycerols from fatty acids released in lipase assays, Journal of Lipid Research 29(11) (1988) 1549-1552.
[3] K. Belafi-Bako, P. Bakonyi, Integration of Membranes and Bioreactors, Biotechnology and Bioengineering, IntechOpen2019.
[4] S.D. Manjare, K. Dhingra, Supercritical fluids in separation and purification: A review, Materials Science for Energy Technologies 2(3) (2019) 463-484.
[5] M. Ardi, M. Aroua, N.A. Hashim, Progress, prospect and challenges in glycerol purification process: A review, Renewable and Sustainable Energy Reviews 42 (2015) 1164-1173.
[6] H. Barnebey, A. Brown, Continuous fat splitting plants using the Colgate-Emery process, Journal of the American Oil Chemists' Society 25(3) (1948) 95-99.
[7] R. Potts, F. White, Fractional distillation of fatty acids, Journal of the American Oil Chemists' Society 30(2) (1953) 49-53.
[8] O. Catchpole, J. Grey, K. Noermark, Fractionation of fish oils using supercritical CO2 and CO_2+ ethanol mixtures, The Journal of Supercritical Fluids 19(1) (2000) 25-37.
[9] A.S. Teja, C.A. Eckert, Commentary on supercritical fluids: research and applications, Industrial & Engineering Chemistry Research 39(12) (2000) 4442-4444.
[10] C.L. Phelps, N.G. Smart, C.M. Wai, Past, present, and possible future applications of supercritical fluid extraction technology, Journal of Chemical Education 73(12) (1996) 1163.
[11] S.V. Kamat, E.J. Beckman, A.J. Russell, Enzyme activity in supercritical fluids, Critical Reviews in Biotechnology 15(1) (1995) 41-71.
[12] A.A. Clifford, J.R. Williams, Introduction to supercritical fluids and their applications, Supercritical fluid methods and Protocols, Springer (2000) pp. 1-16.
[13] Y. Fan, J. Qian, Lipase catalysis in ionic liquids/supercritical carbon dioxide and its applications, Journal of Molecular Catalysis B: Enzymatic 66(1-2) (2010) 1-7.
[14] L. Brunetti, A. Daghetta, E. Fedell, I. Kikic, L. Zanderighi, Deacidification of olive oils by supercritical carbon dioxide, Journal of the American Oil Chemists Society 66(2) (1989) 209-217.
[15] K. Nath, Membrane separation processes, PHI Learning Pvt. Ltd (2017).
[16] K. Scott, R. Hughes, R. Hughes, Industrial membrane separation technology, Springer Science & Business Media (1996).
[17] M.J. Ko, H.J. Park, S.Y. Hong, Y.J. Yoo, Continuous biodiesel production using in situ glycerol separation by membrane bioreactor system, Bioprocess and Biosystems Engineering 35(1) (2012) 69-75.
[18] R. van Reis, A. Zydney, Bioprocess membrane technology, Journal of Membrane Science 297(1-2) (2007) 16-50.
[19] V. Orr, L. Zhong, M. Moo-Young, C.P. Chou, Recent advances in bioprocessing application of membrane chromatography, Biotechnology Advances 31(4) (2013) 450-465.
[20] E. Bukusoglu, H. Koku, P.Z. Çulfaz-Emecen, Addressing challenges in the ultrafiltration of biomolecules from complex aqueous environments, Current Opinion in Colloid & Interface Science 46 (2020) 52-64.
[21] A. Alkhudhiri, N. Darwish, N. Hilal, Membrane distillation: A comprehensive review, Desalination 287 (2012) 2-18.

[22] P. Prádanos, A. Hernández, J. Calvo, F. Tejerina, Mechanisms of protein fouling in cross-flow UF through an asymmetric inorganic membrane, Journal of Membrane Science 114(1) (1996) 115-126.
[23] F. Meng, S.-R. Chae, A. Drews, M. Kraume, H.-S. Shin, F. Yang, Recent advances in membrane bioreactors (MBRs): membrane fouling and membrane material, Water research 43(6) (2009) 1489-1512.
[24] Q. Gan, H. Rahmat, L. Weatherley, Simultaneous reaction and separation in enzymatic hydrolysis of high oleate sunflower oil–evaluation of ultrafiltration performance and process synergy, Chemical Engineering Journal 71(2) (1998) 87-96.
[25] R.A. Sheldon, S. van Pelt, Enzyme immobilisation in biocatalysis: why, what and how, Chemical Society Reviews 42(15) (2013) 6223-6235.
[26] R.C. Rodrigues, J.J. Virgen-Ortíz, J.C. dos Santos, Á. Berenguer-Murcia, A.R. Alcantara, O. Barbosa, C. Ortiz, R. Fernandez-Lafuente, Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions, Biotechnology Advances (2019).
[27] L. Cao, L. van Langen, R.A. Sheldon, Immobilised enzymes: carrier-bound or carrier-free?, Current Opinion in Biotechnology 14(4) (2003) 387-394.
[28] L. Cao, Immobilised enzymes: science or art?, Current Opinion in Chemical Biology 9(2) (2005) 217-226.
[29] L.Y. Jun, L.S. Yon, N. Mubarak, C.H. Bing, S. Pan, M.K. Danquah, E. Abdullah, M. Khalid, An overview of immobilized enzyme technologies for dye and phenolic removal from wastewater, Journal of Environmental Chemical Engineering 7(2) (2019) 102961.
[30] D. Gonçalves Filho, A.G. Silva, C.Z. Guidini, Lipases: sources, immobilization methods, and industrial applications, Applied Microbiology and Biotechnology 103(18) (2019) 7399-7423.
[31] H.H. Nguyen, M. Kim, An overview of techniques in enzyme immobilization, Applied Science and Convergence Technology 26(6) (2017) 157-163.
[32] B.R. Facin, M.S. Melchiors, A. Valério, J.V. Oliveira, D.b.d. Oliveira, Driving immobilized lipases as biocatalysts: 10 years state of the art and future prospects, Industrial & Engineering Chemistry Research 58(14) (2019) 5358-5378.
[33] R.A. Sheldon, Enzyme immobilization: the quest for optimum performance, Advanced Synthesis & Catalysis 349(8-9) (2007) 1289-1307.
[34] A. Tomin, D. Weiser, Z. Bata, L. Corici, F. Peter, L. Poppe, Entrapment of lipases in novel sol-gel systems, (2009).
[35] T. Tosa, T. Sato, T. Mori, K. Yamamoto, I. Takata, Y. Nishida, I. Chibata, Immobilization of enzymes and microbial cells using carrageenan as matrix, Biotechnology and Bioengineering 21(10) (1979) 1697-1709.
[36] D. Balogh-Weiser, C. Németh, F. Ender, B. Gyarmati, A. Szilágyi, L. Poppe, Electrospun nanofibers for entrapment of biomolecules, Electrospinning Method Used to Create Functional Nanocomposites Films, IntechOpen2018, pp. 135-147.
[37] K. Won, S. Kim, K.-J. Kim, H.W. Park, S.-J. Moon, Optimization of lipase entrapment in Ca-alginate gel beads, Process Biochemistry 40(6) (2005) 2149-2154.
[38] M. Cea, M.E. González, M. Abarzúa, R. Navia, Enzymatic esterification of oleic acid by Candida rugosa lipase immobilized onto biochar, Journal of Environmental Management 242 (2019) 171-177.

[39] A. Ursoiu, C. Paul, T. Kurtán, F. Péter, Sol-gel entrapped Candida antarctica lipase B—A biocatalyst with excellent stability for kinetic resolution of secondary alcohols, Molecules 17(11) (2012) 13045-13061.
[40] B.R. Facin, A. Valério, D. Bresolin, G. Centenaro, D. de Oliveira, J.V. Oliveira, Improving reuse cycles of Thermomyces lanuginosus lipase (NS-40116) by immobilization in flexible polyurethane, Biocatalysis and Biotransformation 36(5) (2018) 372-380.
[41] C.L.B. Reis, E.Y.A.d. Sousa, J.d.F. Serpa, R.C. Oliveira, J.C.S.d. Santos, Design of immobilized enzyme biocatalysts: Drawbacks and opportunities, Química Nova 42(7) (2019) 768-783.
[42] N. Guajardo, K. Ahumada, P.D. de María, Immobilized lipase-CLEA aggregates encapsulated in lentikats® as robust biocatalysts for continuous processes in deep eutectic solvents, Journal of Biotechnology 310 (2020) 97-102.
[43] M. Nasseau, Y. Boublik, W. Meier, M. Winterhalter, D. Fournier, Substrate-permeable encapsulation of enzymes maintains effective activity, stabilizes against denaturation, and protects against proteolytic degradation, Biotechnology and Bioengineering 75(5) (2001) 615-618.
[44] M.L. Verma, M. Puri, C.J. Barrow, Recent trends in nanomaterials immobilised enzymes for biofuel production, Critical Reviews in Biotechnology 36(1) (2016) 108-119.
[45] Z. Zhao, M.-C. Zhou, R.-L. Liu, Recent developments in carriers and non-aqueous solvents for enzyme immobilization, Catalysts 9(8) (2019) 647.
[46] F.L. Oliveira, A. de S. França, A.M. de Castro, R.O. Alves de Souza, P.M. Esteves, R.S.B. Gonçalves, Enzyme Immobilization in Covalent Organic Frameworks: Strategies and Applications in Biocatalysis, ChemPlusChem 85(9) (2020) 2051-2066.
[47] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, Enzyme and Microbial Technology 40(6) (2007) 1451-1463.
[48] C. Luna, C. Verdugo, E.D. Sancho, D. Luna, J. Calero, A. Posadillo, F.M. Bautista, A.A. Romero, Biocatalytic behaviour of immobilized Rhizopus oryzae lipase in the 1, 3-selective ethanolysis of sunflower oil to obtain a biofuel similar to biodiesel, Molecules 19(8) (2014) 11419-11439.
[49] X.J. Huang, P.C. Chen, F. Huang, Y. Ou, M.R. Chen, Z.K. Xu, Immobilization of Candida rugosa lipase on electrospun cellulose nanofiber membrane, Journal of Molecular Catalysis B: Enzymatic 70(3-4) (2011) 95-100.
[50] X. Xiang, S. Ding, H. Suo, C. Xu, Z. Gao, Y. Hu, Fabrication of chitosan-mesoporous silica SBA-15 nanocomposites via functional ionic liquid as the bridging agent for PPL immobilization, Carbohydrate Polymers 182 (2018) 245-253.
[51] N. Matuoog, K. Li, Y. Yan, Immobilization of Thermomyces lanuginosus lipase on multi-walled carbon nanotubes and its application in the hydrolysis of fish oil, Materials Research Express 4(12) (2017) 125402.
[52] E. Yilmaz, K. Can, M. Sezgin, M. Yilmaz, Immobilization of Candida rugosa lipase on glass beads for enantioselective hydrolysis of racemic Naproxen methyl ester, Bioresource Technology 102(2) (2011) 499-506.
[53] L. Fernandez-Lopez, N. Rueda, R. Bartolome-Cabrero, M.D. Rodriguez, T.L. Albuquerque, J.C. dos Santos, O. Barbosa, R. Fernandez-Lafuente, Improved immobilization and stabilization of lipase from Rhizomucor miehei on octyl-glyoxyl agarose beads by using CaCl2, Process Biochemistry 51(1) (2016) 48-52.

[54] J.C. dos Santos, N. Rueda, A. Sanchez, R. Villalonga, L.R. Gonçalves, R. Fernandez-Lafuente, Versatility of divinylsulfone supports permits the tuning of CALB properties during its immobilization, RSC Advances 5(45) (2015) 35801-35810.
[55] O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R.C. Rodrigues, R. Fernandez-Lafuente, Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization, RSC Advances 4(4) (2014) 1583-1600.
[56] F. Quiocho, Immobilized proteins in single crystals, Methods in enzymology, Elsevier (1976), pp. 546-558.
[57] T. Palmer, Understanding enzymes, E. Horwood (1991).
[58] H. Ashraf, Q. Husain, Stabilization of DEAE cellulose adsorbed and glutaraldehyde crosslinkedwhite radish (Raphanus sativus) peroxidase, Journal of Scientific & Industrial Research (2010).
[59] Y. Wine, N. Cohen-Hadar, A. Freeman, F. Frolow, Elucidation of the mechanism and end products of glutaraldehyde crosslinking reaction by X-ray structure analysis, Biotechnology and Bioengineering 98(3) (2007) 711-718.
[60] M. Ziegler-Borowska, D. Chelminiak-Dudkiewicz, T. Siódmiak, A. Sikora, K. Wegrzynowska-Drzymalska, J. Skopinska-Wisniewska, H. Kaczmarek, M.P. Marszałł, Chitosan–collagen coated magnetic nanoparticles for lipase immobilization—New type of "enzyme friendly" polymer shell crosslinking with squaric acid, Catalysts 7(1) (2017) 26.
[61] W. Jin, Y. Xu, X.-W. Yu, Formation lipase cross-linked enzyme aggregates on octyl-modified mesocellular foams with oxidized sodium alginate, Colloids and Surfaces B: Biointerfaces 184 (2019) 110501.
[62] T.-C. Hung, R. Giridhar, S.-H. Chiou, W.-T. Wu, Binary immobilization of Candida rugosa lipase on chitosan, Journal of Molecular Catalysis B: Enzymatic 26(1-2) (2003) 69-78.
[63] S. Velasco-Lozano, F. López-Gallego, J.C. Mateos-Díaz, E. Favela-Torres, Cross-linked enzyme aggregates (CLEA) in enzyme improvement–a review, Biocatalysis 1(open-issue) (2016) 166-177.
[64] V. Vescovi, W. Kopp, J.M. Guisán, R.L. Giordano, A.A. Mendes, P.W. Tardioli, Improved catalytic properties of Candida antarctica lipase B multi-attached on tailor-made hydrophobic silica containing octyl and multifunctional amino-glutaraldehyde spacer arms, Process Biochemistry 51(12) (2016) 2055-2066.
[65] S. Gao, Y. Wang, X. Diao, G. Luo, Y. Dai, Effect of pore diameter and cross-linking method on the immobilization efficiency of Candida rugosa lipase in SBA-15, Bioresource Technology 101(11) (2010) 3830-3837.
[66] M.B. Mbanjwa, K.J. Land, T. Windvoel, P.M. Papala, L. Fourie, J.G. Korvink, D. Visser, D. Brady, Production of self-immobilised enzyme microspheres using microfluidics, Process Biochemistry 69 (2018) 75-81.
[67] F.M. Abd Manan, N. Attan, Z. Zakaria, A.S.A. Keyon, R.A. Wahab, Enzymatic esterification of eugenol and benzoic acid by a novel chitosan-chitin nanowhiskers supported Rhizomucor miehei lipase: Process optimization and kinetic assessments, Enzyme and Microbial Technology 108 (2018) 42-52.
[68] F.M. Abd Manan, N. Attan, Z. Zakaria, N.A. Mahat, R.A. Wahab, Insight into the Rhizomucor miehei lipase supported on chitosan-chitin nanowhiskers assisted esterification of eugenol to eugenyl benzoate, Journal of Biotechnology 280 (2018) 19-30.
[69] T. Jesionowski, J. Zdarta, B. Krajewska, Enzyme immobilization by adsorption: A review, Adsorption 20(5-6) (2014) 801-821.

[70] J. Zdarta, A.S. Meyer, T. Jesionowski, M. Pinelo, A general overview of support materials for enzyme immobilization: characteristics, properties, practical utility, Catalysts 8(2) (2018) 92.
[71] J. Nelson, E.G. Griffin, Adsorption of invertase, Journal of the American Chemical Society 38(5) (1916) 1109-1115.
[72] R. Verger, 'Interfacial activation'of lipases: facts and artifacts, Trends in Biotechnology 15(1) (1997) 32-38.
[73] R.D. Schmid, R. Verger, Lipases: interfacial enzymes with attractive applications, Angewandte Chemie International Edition 37(12) (1998) 1608-1633.
[74] L. Brady, A.M. Brzozowski, Z.S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J.P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskov, A serine protease triad forms the catalytic centre of a triacylglycerol lipase, Nature 343(6260) (1990) 767.
[75] F. Hasan, A.A. Shah, A. Hameed, Industrial applications of microbial lipases, Enzyme and Microbial Technology 39(2) (2006) 235-251.
[76] K.E. Jaeger, T. Eggert, Lipases for biotechnology, Current Opinion in Biotechnology 13(4) (2002) 390-397.
[77] E.A. Manoel, J.C. dos Santos, D.M. Freire, N. Rueda, R. Fernandez-Lafuente, Immobilization of lipases on hydrophobic supports involves the open form of the enzyme, Enzyme and Microbial Technology 71 (2015) 53-57.
[78] H. Castro, M. Silva, G. Silva, Evaluation of inorganic matrixes as supports for immobilization of microbial lipase, Brazilian Journal of Chemical Engineering 17(4-7) (2000) 849-858.
[79] V. Minovska, E. Winkelhausen, S. Kuzmanova, Lipase immobilized by different techniques on various support materials applied in oil hydrolysis, Journal of the Serbian Chemical Society 70(4) (2005) 609-624.
[80] I. Venditti, C. Palocci, L. Chronopoulou, I. Fratoddi, L. Fontana, M. Diociaiuti, M.V. Russo, Candida rugosa lipase immobilization on hydrophilic charged gold nanoparticles as promising biocatalysts: Activity and stability investigations, Colloids and Surfaces B: Biointerfaces 131 (2015) 93-101.
[81] Y.Z. Chen, C.B. Ching, R. Xu, Lipase immobilization on modified zirconia nanoparticles: Studies on the effects of modifiers, Process Biochemistry 44(11) (2009) 1245-1251.
[82] D. Yildirim, E. Baran, S. Ates, B. Yazici, S.S. Tukel, Improvement of activity and stability of Rhizomucor miehei lipase by immobilization on nanoporous aluminium oxide and potassium sulfate microcrystals and their applications in the synthesis of aroma esters, Biocatalysis and Biotransformation 37(3) (2019) 210-223.
[83] N.A. Kabbashi, N.I. Mohammed, M.Z. Alam, M.E.S. Mirghani, Hydrolysis of Jatropha curcas oil for biodiesel synthesis using immobilized Candida cylindracea lipase, Journal of Molecular Catalysis B: Enzymatic 116 (2015) 95-100.
[84] D. Cazaban, A. Illanes, L. Wilson, L. Betancor, Bio-inspired silica lipase nanobiocatalysts for the synthesis of fatty acid methyl esters, Process Biochemistry 74 (2018) 86-93.
[85] M. Guncheva, M. Dimitrov, D. Zhiryakova, Novel nanostructured tin dioxide as promising carrier for Candida rugosa lipase, Process Biochemistry 46(11) (2011) 2170-2177.
[86] H. Dong, Y. Li, G. Sheng, L. Hu, The study on effective immobilization of lipase on functionalized bentonites and their properties, Journal of Molecular Catalysis B: Enzymatic 95 (2013) 9-15.

[87] Y. Hu, X. Jiang, S. Wu, L. Jiang, H. Huang, Synthesis of vitamin E succinate by interfacial activated Candida rugosa lipase encapsulated in sol-gel materials, Chinese Journal of Catalysis 34(8) (2013) 1608-1616.
[88] Y. Jiang, H. Liu, L. Wang, L. Zhou, Z. Huang, L. Ma, Y. He, L. Shi, J. Gao, Virus-like organosilica nanoparticles for lipase immobilization: characterization and biocatalytic applications, Biochemical Engineering Journal 144 (2019) 125-134.
[89] Q. Jin, X. Li, C. Deng, Q. Zhang, D. Yi, X. Wang, Y. Tang, Y. Wang, Silica nanowires with tunable hydrophobicity for lipase immobilization and biocatalytic membrane assembly, Journal of Colloid and Interface Science 531 (2018) 555-563.
[90] C.-H. Liu, C.-C. Huang, Y.-W. Wang, D.-J. Lee, J.-S. Chang, Biodiesel production by enzymatic transesterification catalyzed by Burkholderia lipase immobilized on hydrophobic magnetic particles, Applied Energy 100 (2012) 41-46.
[91] L.S. Martin, A. Ceron, P.C. Oliveira, G.M. Zanin, H.F. de Castro, Different organic components on silica hybrid matrices modulate the lipase inhibition by the glycerol formed in continuous transesterification reactions, Journal of Industrial and Engineering Chemistry 62 (2018) 462-470.
[92] A.K. Singh, M. Mukhopadhyay, Immobilization of lipase on carboxylic acid-modified silica nanoparticles for olive oil glycerolysis, Bioprocess and Biosystems Engineering 41(1) (2018) 115-127.
[93] S. Gao, W. Wang, Y. Wang, G. Luo, Y. Dai, Influence of alcohol treatments on the activity of lipases immobilized on methyl-modified silica aerogels, Bioresource technology 101(19) (2010) 7231-7238.
[94] R.C. Rodrigues, J.J. Virgen-Ortíz, J.C. Dos Santos, Á. Berenguer-Murcia, A.R. Alcantara, O. Barbosa, C. Ortiz, R. Fernandez-Lafuente, Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions, Biotechnology Advances 37(5) (2019) 746-770.
[95] V. Vescovi, J.B.C. dos Santos, P.W. Tardioli, Porcine pancreatic lipase hydrophobically adsorbed on octyl-silica: A robust biocatalyst for syntheses of xylose fatty acid esters, Biocatalysis and Biotransformation 35(4) (2017) 298-305.
[96] L. Xu, G. Cui, C. Ke, Y. Fan, Y. Yan, Immobilized Burkholderia cepacia lipase on pH-responsive pullulan derivatives with improved enantioselectivity in chiral resolution, Catalysts 8(1) (2018) 13.
[97] E. Magner, Immobilisation of enzymes on mesoporous silicate materials, Chemical Society Reviews 42(15) (2013) 6213-6222.
[98] M. Hartmann, X. Kostrov, Immobilization of enzymes on porous silicas–benefits and challenges, Chemical Society Reviews 42(15) (2013) 6277-6289.
[99] S.B. Hartono, S.Z. Qiao, J. Liu, K. Jack, B.P. Ladewig, Z. Hao, G.Q.M. Lu, Functionalized mesoporous silica with very large pores for cellulase immobilization, The Journal of Physical Chemistry C 114(18) (2010) 8353-8362.
[100] B. Krajewska, Application of chitin-and chitosan-based materials for enzyme immobilizations: a review, Enzyme and Microbial Technology 35(2-3) (2004) 126-139.
[101] J.C. dos Santos, H.L. Bonazza, L.J. de Matos, E.A. Carneiro, O. Barbosa, R. Fernandez-Lafuente, L.R. Gonçalves, H.B. de Sant'Ana, R.S. Santiago-Aguiar, Immobilization of CALB on activated chitosan: Application to enzymatic synthesis in supercritical and near-critical carbon dioxide, Biotechnology Reports 14 (2017) 16-26.

[102] P. Urrutia, R. Arrieta, L. Alvarez, C. Cardenas, M. Mesa, L. Wilson, Immobilization of lipases in hydrophobic chitosan for selective hydrolysis of fish oil: The impact of support functionalization on lipase activity, selectivity and stability, International Journal of Biological Macromolecules 108 (2018) 674-686.
[103] K.C. Badgujar, P.A. Pai, B.M. Bhanage, Enhanced biocatalytic activity of immobilized Pseudomonas cepacia lipase under sonicated condition, Bioprocess and Biosystems Engineering 39(2) (2016) 211-221.
[104] B. Chen, C. Yin, Y. Cheng, W. Li, Z.-a. Cao, T. Tan, Using silk woven fabric as support for lipase immobilization: The effect of surface hydrophilicity/hydrophobicity on enzymatic activity and stability, Biomass and Bioenergy 39 (2012) 59-66.
[105] S. Arana-Peña, C. Mendez-Sanchez, N.S. Rios, C. Ortiz, L.R. Gonçalves, R. Fernandez-Lafuente, New applications of glyoxyl-octyl agarose in lipases co-immobilization: Strategies to reuse the most stable lipase, International Journal of Biological Macromolecules 131 (2019) 989-997.
[106] D.B. Hirata, T.L. Albuquerque, N. Rueda, J.J. Virgen-Ortíz, V.G. Tacias-Pascacio, R. Fernandez-Lafuente, Evaluation of different immobilized lipases in transesterification reactions using tributyrin: Advantages of the heterofunctional octyl agarose beads, Journal of Molecular Catalysis B: Enzymatic 133 (2016) 117-123.
[107] S. Park, S.H. Kim, J.H. Kim, H. Yu, H.J. Kim, Y.-H. Yang, H. Kim, Y.H. Kim, S.H. Ha, S.H. Lee, Application of cellulose/lignin hydrogel beads as novel supports for immobilizing lipase, Journal of Molecular Catalysis B: Enzymatic 119 (2015) 33-39.
[108] B. Young, W. Pitt, S. Cooper, Protein adsorption on polymeric biomaterials I. Adsorption isotherms, Journal of Colloid and Interface Science 124(1) (1988) 28-43.
[109] B. Young, W. Pitt, S. Cooper, Protein adsorption on polymeric biomaterials: II. Adsorption kinetics, Journal of Colloid and Interface Science 125(1) (1988) 246-260.
[110] O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R.C. Rodrigues, R. Fernandez-Lafuente, Strategies for the one-step immobilization–purification of enzymes as industrial biocatalysts, Biotechnology Advances 33(5) (2015) 435-456.
[111] J.A. Bosley, A.D. Peilow, Immobilization of lipases on porous polypropylene: reduction in esterification efficiency at low loading, Journal of the American Oil Chemists' Society 74(2) (1997) 107-111.
[112] A. Salis, E. Sanjust, V. Solinas, M. Monduzzi, Characterisation of Accurel MP1004 polypropylene powder and its use as a support for lipase immobilisation, Journal of Molecular Catalysis B: Enzymatic 24 (2003) 75-82.
[113] A. Anand, L.R. Weatherley, The performance of microbial lipase immobilized onto polyolefin supports for hydrolysis of high oleate sunflower oil, Process Biochemistry 68 (2018) 100-107.
[114] O. Barbosa, C. Ariza, C. Ortiz, R. Torres, Kinetic resolution of (R/S)-propranolol (1-isopropylamino-3-(1-naphtoxy)-2-propanolol) catalyzed by immobilized preparations of Candida antarctica lipase B (CAL-B), New Biotechnology 27(6) (2010) 844-850.
[115] J.J. Bassi, L.M. Todero, F.A. Lage, G.I. Khedy, J.D. Ducas, A.P. Custódio, M.A. Pinto, A.A. Mendes, Interfacial activation of lipases on hydrophobic support and application in the synthesis of a lubricant ester, International Journal of Biological Macromolecules 92 (2016) 900-909.
[116] E.D. Cavalcanti, É.C. Aguieiras, P.R. da Silva, J.G. Duarte, E.P. Cipolatti, R. Fernandez-Lafuente, J.A.C. da Silva, D.M. Freire, Improved production of biolubricants from soybean oil

and different polyols via esterification reaction catalyzed by immobilized lipase from Candida rugosa, Fuel 215 (2018) 705-713.
[117] V. Chiaradia, N.S. Soares, A. Valério, D. de Oliveira, P.H. Araújo, C. Sayer, Immobilization of Candida antarctica lipase B on magnetic poly (urea-urethane) nanoparticles, Applied Biochemistry and Biotechnology 180(3) (2016) 558-575.
[118] B. Chen, M.E. Miller, R.A. Gross, Effects of porous polystyrene resin parameters on Candida antarctica lipase B adsorption, distribution, and polyester synthesis activity, Langmuir 23(11) (2007) 6467-6474.
[119] L. de Abreu, R. Fernandez-Lafuente, R.C. Rodrigues, G. Volpato, M.A.Z. Ayub, Efficient purification-immobilization of an organic solvent-tolerant lipase from Staphylococcus warneri EX17 on porous styrene-divinylbenzene beads, Journal of Molecular Catalysis B: Enzymatic 99 (2014) 51-55.
[120] K. Hernandez, C. Garcia-Galan, R. Fernandez-Lafuente, Simple and efficient immobilization of lipase B from Candida antarctica on porous styrene–divinylbenzene beads, Enzyme and Microbial Technology 49(1) (2011) 72-78.
[121] J.C. Santos, G.F. Nunes, A.B. Moreira, V.H. Perez, H.F. de Castro, Characterization of Candida rugosa Lipase Immobilized on Poly (N-methylolacrylamide) and Its Application in Butyl Butyrate Synthesis, Chemical Engineering & Technology: Industrial Chemistry-Plant Equipment-Process Engineering-Biotechnology 30(9) (2007) 1255-1261.
[122] F. López-Gallego, L. Betancor, C. Mateo, A. Hidalgo, N. Alonso-Morales, G. Dellamora-Ortiz, J.M. Guisán, R. Fernández-Lafuente, Enzyme stabilization by glutaraldehyde crosslinking of adsorbed proteins on aminated supports, Journal of Biotechnology 119(1) (2005) 70-75.
[123] L. Thudi, L.S. Jasti, Y. Swarnalatha, N.W. Fadnavis, K. Mulani, S. Deokar, S. Ponrathnam, Enzyme immobilization on epoxy supports in reverse micellar media: Prevention of enzyme denaturation, Journal of Molecular Catalysis B: Enzymatic 74(1-2) (2012) 54-62.
[124] M. Mihailović, M. Stojanović, K. Banjanac, M. Carević, N. Prlainović, N. Milosavić, D. Bezbradica, Immobilization of lipase on epoxy-activated Purolite® A109 and its post-immobilization stabilization, Process Biochemistry 49(4) (2014) 637-646.
[125] C.R. Matte, C. Bordinhão, J.K. Poppe, E.V. Benvenutti, T.M. Costa, R.C. Rodrigues, P.F. Hertz, M.A. Ayub, Physical-chemical properties of the support immobead 150 before and after the immobilization process of lipase, Journal of the Brazilian Chemical Society 28(8) (2017) 1430-1439.
[126] C. Ortiz, M.L. Ferreira, O. Barbosa, J.C. dos Santos, R.C. Rodrigues, Á. Berenguer-Murcia, L.E. Briand, R. Fernandez-Lafuente, Novozym 435: the “perfect” lipase immobilized biocatalyst?, Catalysis Science & Technology 9(10) (2019) 2380-2420.
[127] S. Arana-Peña, Y. Lokha, R. Fernández-Lafuente, Immobilization on octyl-agarose beads and some catalytic features of commercial preparations of lipase a from Candida antarctica (Novocor ADL): Comparison with immobilized lipase B from Candida antarctica, Biotechnology Progress 35(1) (2019) e2735.
[128] J.C.S.d. Santos, O. Barbosa, C. Ortiz, A. Berenguer-Murcia, R.C. Rodrigues, R. Fernandez-Lafuente, Importance of the support properties for immobilization or purification of enzymes, ChemCatChem 7(16) (2015) 2413-2432.
[129] E.B. Pereira, H.F. De Castro, F.F. De Moraes, G.M. Zanin, Kinetic studies of lipase from Candida rugosa, Twenty-Second Symposium on Biotechnology for Fuels and Chemicals, Springer (2001), pp. 739-752.

[130] S. Amin, G.V. Barnett, J.A. Pathak, C.J. Roberts, P.S. Sarangapani, Protein aggregation, particle formation, characterization & rheology, Current Opinion in Colloid & Interface Science 19(5) (2014) 438-449.
[131] R. Micha, S. Khatibzadeh, P. Shi, S. Fahimi, S. Lim, K.G. Andrews, R.E. Engell, J. Powles, M. Ezzati, D. Mozaffarian, Global, regional, and national consumption levels of dietary fats and oils in 1990 and 2010: a systematic analysis including 266 country-specific nutrition surveys, British Medical Journal 348 (2014) g2272.
[132] D. Rooney, L. Weatherley, The effect of reaction conditions upon lipase catalysed hydrolysis of high oleate sunflower oil in a stirred liquid–liquid reactor, Process Biochemistry 36(10) (2001) 947-953.
[133] M.Z. Kamal, P. Yedavalli, M.V. Deshmukh, N.M. Rao, Lipase in aqueous-polar organic solvents: Activity, structure, and stability, Protein Science 22(7) (2013) 904-915.
[134] M. Refvik, R. Larock, The chemistry of metathesized soybean oil, Journal of the American Oil Chemists' Society 76(1) (1999) 99-102.
[135] S. Sabbani, E. Hedenström, O. Nordin, The enantioselectivity of Candida rugosa lipase is influenced by the particle size of the immobilising support material Accurel, Journal of Molecular Catalysis B: Enzymatic 42(1-2) (2006) 1-9.
[136] A.G. Marangoni, Studies on the interaction of Rhizopus arrhizus lipase with dipalmitoylphosphatidylcholine liposomes, Colloids and Surfaces B: Biointerfaces 1(3) (1993) 167-176.
[137] M.M. Zheng, Y. Lu, L. Dong, P.M. Guo, Q.C. Deng, W.L. Li, Y.Q. Feng, F.H. Huang, Immobilization of Candida rugosa lipase on hydrophobic/strong cation-exchange functional silica particles for biocatalytic synthesis of phytosterol esters, Bioresource Technology 115 (2012) 141-6.
[138] E.Y. Ozmen, M. Sezgin, M. Yilmaz, Synthesis and characterization of cyclodextrin-based polymers as a support for immobilization of Candida rugosa lipase, Journal of Molecular Catalysis B: Enzymatic 57(1-4) (2009) 109-114.
[139] N.S. Rios, D.M.A. Neto, J.C.S. dos Santos, P.B.A. Fechine, R. Fernández-Lafuente, L.R.B. Gonçalves, Comparison of the immobilization of lipase from Pseudomonas fluorescens on divinylsulfone or p-benzoquinone activated support, International Journal of Biological Macromolecules 134 (2019) 936-945.
[140] Y. Wei, F. Mei, s.T. Dong, S. Ping, X. Tian, Z. Chun, Immobilization of Candida rugosa lipase on hexagonal mesoporous silicas and selective esterification in nonaqueous medium, Biochemical Engineering Journal 70 (2013) 97-105.
[141] P. Bailie, S. McNerlan, E. Robinson, W. Murphy, Immobilization of lipases for the hydrolysis of fats and oils, Food and Bioproducts Processing: Transactions of the Institution of Chemical Engineers, Part C (1995).
[142] L. Weatherley, D. Rooney, M. Niekerk, Clean synthesis of fatty acids in an intensive lipase-catalysed bioreactor, Journal of Chemical Technology & Biotechnology 68(4) (1997) 437-441.
[143] M. Murray, D. Rooney, M. Van Neikerk, A. Montenegro, L. Weatherley, Immobilization of lipase onto lipophilic polymer particles and application to oil hydrolysis, Process Biochemistry 32(6) (1997) 479-486.
[144] L.R. Weatherley, D. Rooney, Enzymatic catalysis and electrostatic process intensification for processing of natural oils, Chemical Engineering Journal 135(1-2) (2008) 25-32.

[145] Y.K. Lee, C.L. Choo, The kinetics and mechanism of shear inactivation of lipase from Candida cylindracea, Biotechnology and Bioengineering 33(2) (1989) 183-190.
[146] J.C. dos Santos, N. Rueda, O. Barbosa, M. del Carmen Millán-Linares, J. Pedroche, M. del Mar Yuste, L.R. Gonçalves, R. Fernandez-Lafuente, Bovine trypsin immobilization on agarose activated with divinylsulfone: Improved activity and stability via multipoint covalent attachment, Journal of Molecular Catalysis B: Enzymatic 117 (2015) 38-44.
[147] A. Basso, M. Hesseler, S. Serban, Hydrophobic microenvironment optimization for efficient immobilization of lipases on octadecyl functionalised resins, Tetrahedron 72(46) (2016) 7323-7328.
[148] J. Polaina, A.P. MacCabe, Industrial enzymes, Springer (2007).
[149] A. Sanchez, J. Cruz, N. Rueda, J.C. dos Santos, R. Torres, C. Ortiz, R. Villalonga, R. Fernandez-Lafuente, Inactivation of immobilized trypsin under dissimilar conditions produces trypsin molecules with different structures, RSC Advances 6(33) (2016) 27329-27334.
[150] J. Fraga, A. Penha, A. da S Pereira, K. Silva, E. Akil, A. Torres, P. Amaral, Use of Yarrowia lipolytica Lipase immobilized in cell debris for the production of lipolyzed milk fat (LMF), International journal of molecular sciences 19(11) (2018) 3413.
[151] M. Falahati, L. Ma'mani, A.A. Saboury, A. Shafiee, A. Foroumadi, A.R. Badiei, Aminopropyl-functionalized cubic Ia3d mesoporous silica nanoparticle as an efficient support for immobilization of superoxide dismutase, Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 1814(9) (2011) 1195-1202.
[152] M. Delvaux, S. Demoustier-Champagne, Immobilisation of glucose oxidase within metallic nanotubes arrays for application to enzyme biosensors, Biosensors and Bioelectronics 18(7) (2003) 943-951.
[153] T. Klement, J. Büchs, Itaconic acid–a biotechnological process in change, Bioresource Technology 135 (2013) 422-431.
[154] G. Bayramoglu, I. Gursel, M. Yilmaz, M.Y. Arica, Immobilization of laccase on itaconic acid grafted and Cu (II) ion chelated chitosan membrane for bioremediation of hazardous materials, Journal of Chemical Technology & Biotechnology 87(4) (2012) 530-539.

Chapter 4 : Influence of oriented external electric field on lipase-catalyzed hydrolysis.

4.1. Electrostatic process enhancement.

The application of electrical fields for the intensification of chemical processes for multiphase systems is well known [1]. Techniques used to disrupt interfaces and facilitate surface interactions includes electrostatic spraying [2-7], electrostatic pumping [8, 9], electrostatic mixing [9] and electro-osmosis [10]. Electrical fields can also enhance mass transfer rates [11, 12] and can be effectively utilized for electro-sorption [13], electro-oxidation [14], the enhancement of phase transfer reactions [11, 15] and to control phase inversion in liquid-liquid systems [16-18]. Blankenship et al., used batch distillation technique to separate binary mixture and showed 10% increase in efficiency under the influence of electrical fields [19]. This suggest that vapor-liquid equilibrium of chemical compounds can be altered under the influence of electrical fields and the enhanced performance is observed due to the enhanced formation of microdroplets. Hence, thermodynamic properties of substances can also be altered using electrical fields [19,20].

Electrostatic catalysis has been well known since Pocker and co-workers observed rate enhancements for heterolysis reactions using an external electric field (EEF) [21]. Since then, EEF has been proposed to enhance rate and selectivity of many chemical reactions [22-24]. The presence of electrical fields enhances the reactivity and selectivity in polarizable systems including processes involving methanol synthesis, reactions involving water, reactions with aromatics and metal/metal oxide supports [23]. However, orientation and magnitude of local field strength plays significant role in determining the extent of these enhancements [22,23]. Recent studies have shown that oriented external field (OEEF) can act as an invisible green co-catalyst and improve the thermodynamics of chemical reactions [25, 26]. However, the possibility of rate enhancement

due to the application of OEEFs on lipase catalyzed reactions has hitherto not been considered or studied in the literature.

In the case of electrostatic spraying, high voltages are applied to droplets as they form, for example from a single/multiple nozzle. Subject to appropriate electrical properties of the drop phase, and of the liquid into which the drops are dispersing, the interface between the two liquids at the drop surface may become destabilized by the electrical forces which build up at the interface resulting in disruption of the interface and ultimately breakage into very fine drops [27, 28]. A second significant phenomenon which can occur at the liquid-liquid interface in the presence of electric fields is the promotion of interfacial flows, referred to as the Marangoni instabilities [29]. These may be promoted by gradients of electrical charge on the surface of an electrically charged drop located (either stationary or moving) in an externally applied electrical field [30]. The gradients of charge are thought to result in enhanced interfacial tension gradients which in turn enhance interfacial flows. The net effect is enhancement of mass transfer flux. The enhancement of mass transfer in liquid-liquid extraction by electrostatic spraying and by electrically enhanced Marangoni effects have been the subject of several studies [12, 27, 31-37]. The intensifications achieved are impressive with increases in overall mass transfer up to an order of magnitude. One example is that described by He et al., [38] for the extraction of benzoic acid from water into mineral oil in a spray column in the presence of a steady DC voltage.

A related area of study is the application of electrostatic intensification to reacting systems involving liquid-liquid mixtures. The reaction of interest in the current study is the enzymatically catalyzed hydrolysis of triglyceride esters in an oil / water system resulting in the reaction products of FFAs and glycerol. Earlier research showed that electrostatic dispersion of aqueous solutions of microbial lipases into natural oils resulted in enhanced rates of hydrolysis [39]. The enhancements

in rate increased with respect to the magnitude of the applied external electrical field. It was assumed that the observed increases were explained only in terms of the increased interfacial area of the dispersed drops resulting in faster conversion rates [40]. Later analysis of rate data, together with independent measurement of drop sizes and dispersed phase hold-up in a spray reactor allowed calculation of the specific rate of reaction [2, 41-43]. Although the data show scatter, there was evidence that the electrical charge on the drops in the reactor enhances the specific rate of reaction (rate expressed independent of interfacial area) in addition to the overall rate [44, 45]. Three possible hypotheses for the observed difference are proposed: (1) Enhanced mass transport adjacent to the interface, altering reactant and product compositions together with pH at the interface. This could alter reaction equilibria and lipase activity. (2) Interfacial disturbances which may involve electrically induced enhanced Marangoni disturbances. (3) Other changes in the environment at the interface which enhance lipase activity such as enzyme orientation and binding.

Hence, in this study the possibility of performance enhancement due to the application of oriented external electric field (OEEF) for lipase-catalyzed triglyceride hydrolysis is exploited using lipase in aqueous media. Three batch reactor set ups were incorporated (1) using stagnant tank (fixed interface), (2) continuously stirred tank and (2) recirculated tubular flow mixed reactors. The stagnant tank system was used to evaluate the influence of electrical voltage when no external disturbances to substrates are present and flow mixed type is used for more practical application of this system. It was also important to analyze the results when lipase is immobilized onto solid support since lipase immobilized onto solid support exhibit far superior stability in terms of chemical and thermal compared with lipase in aqueous media [39].

4.2. Specific aims.

The goal of the current work was to determine the possible effect of electrostatic enhancement on lipase catalyzed reaction specific to triglyceride hydrolysis using the application of oriented external electrical field (OEEF). The lipase derived from *Candida rugosa* was used as a solution in aqueous buffer solution (homogenous system) and secondly immobilized onto solid support (heterogenous system). In the case of the heterogenous system, the system comprised of liquid-liquid-solid with lipase as the biocatalyst immobilized onto solid methacrylate resins (1) functionalized with octadecyl group (ECR8806M) and (2) functionalized with epoxy & butyl groups (ECR8285) supplied by Purolite® Corporation (United Kingdom). The effects of variable voltage, polarity changes and electrode distance on reaction performance were studied in three different situations: (1) in a rectangular batch reactor with a fixed and stable liquid-liquid interface with both substrates quiescent, (2) in mechanically stirred reactors operating in batch mode, and (3) in a recirculated tubular flow reactor operated with continuous flow of the two liquids also operated in batch mode. The rectangular batch reactor was designed to maintain a quiescent oil / aqueous interface of constant area in order to eliminate changes in area such as would occur in a dispersed system involving hydrodynamic turbulence, and thus allow study of electric field effect alone. The stirred reactor was designed to promote dispersion by mechanical shear alone, but with application of the electrical field across the resulting oil/aqueous emulsion. The aim was to minimize mass transfer limitations. The tubular reactor was designed to gain preliminary insights into the behavior of the reaction under plug flow conditions close to those of a continuous reactor. The tubular reactor aimed to allowed study of the immobilized biocatalyst under plug flow conditions.

4.3. Materials and methods.

4.3.1. Materials.

Aqueous lipase solutions were prepared and quantified using the method described in Chapter 2. Mazola vegetable oil used as purchased is the main substrate oil used for hydrolysis. Mesoporous methacrylate polymer resins (supports) functionalized with octadecyl groups (ECR8806M) and epoxy & butyl (ECR8285) were donated by LifeTech™ Resins (Purolite® Corporation, UK). The physiochemical properties of these resins are detailed in Chapter 3, section 3.5.1. Other chemicals used without further modification included, high purity phosphate buffer saline (pH 7.4), glutaraldehyde, 3-aminopropyl triethoxysilane (APTES), and itaconic acid (Sigma-Aldrich, United States). Deionized water used was produced in a WATER PRO\RO water system (Labconco™, United States) comprised the aqueous phase. A power supply (PASCO - Model No. SF-9585A) with capabilities to provide DC power output of 0-500 V was used to generate the oriented external electrical field.

4.3.2. Preparations of aqueous lipase solutions.

Batches of aqueous lipase solutions of 0.25 %wt/vol were prepared prior to each experimental run using the method detailed in Chapter 2. In summary, 0.25 g batches of lyophilized lipase were added to 250 mL beakers each containing 100 mL of 0.1 M, 7.4 pH phosphate buffer solution at room temperature (22 ± 2°C). These were placed on an orbital shaker for a period of 1 hour. The aqueous lipase solutions were centrifuged at 8000 RPM for 20 mins at 10°C (Centrifuge 5810 R, Eppendorf, Germany) to remove insoluble. The resulting supernatants were quantified for their protein content using the Bradford assay with Coomassie G-250 brilliant blue dye and BSA as standards [46]. The total protein concentration in the centrifuged aqueous buffered 0.25 %wt/vol lipase solution was 0.065 mg/mL (± 5%).

4.3.1. Preparation of immobilized support.

The details of the immobilization procedure are detailed in Chapter 3. In summary, 1 g of support particles (methacrylate resins) in the size range 510 – 660 μm were contacted with 10 mL of centrifuged lipase solution mixed with 30 mL of deionized water with 0.1 g (0.025 mol/L) of itaconic acid added. The mixture was shaken on an orbital shaker operated at 120 rpm for 20 h at 22°C. The resin supports, loaded with lipase were then separated from the solution using vacuum filtration followed by water washing and further vacuum filtration to ensure removal of weakly bound lipase or free lipase. The resin particles were then dried for 24 hours in a vacuum oven at 30°C and stored at 4°C prior to use. The amount of protein uptake per gram of support is shown in previous work [47].

4.3.2. Electrostatic batch bioreactors fabrications and set up.

Three types of reactor were used as listed in section 1.4. In each case, the reactors were fabricated with electrodes in order to expose the reacting mixtures to the externally applied electrical field. In the case of immobilized lipase, the tubular flow reactor equipped with a pre-mixer was used.

4.3.2.1. Quiescent batch electrostatic reactor.

The reactor was fabricated from clear-type acrylic polymer with a fixed rectangular geometry using height adjustable planar electrodes with dimensions shown in **Figure 4.1(a)**. The rectangular electrodes were fabricated of nickel-alloy (Monel 400) material 63.5 mm length x 38.1 mm width with a mesh opening size of 0.3 cm. The base electrode was fixed 12.7 mm above the bottom of the reactor. The vertical position of the upper electrode relative to the lower electrode was adjustable. The location of the liquid phases for the hydrolysis is shown in **Figure 4.1(b),** with the upper organic phase comprising 100 g of vegetable oil and the lower aqueous phase comprising

of 50 g deionized water mixed with 10 mL of centrifuged aqueous lipase solution. The organic phase was pipetted carefully onto the top of the aqueous layer taking great care to avoid disturbance at the interface. The electrodes were then connected to a DC power supply.

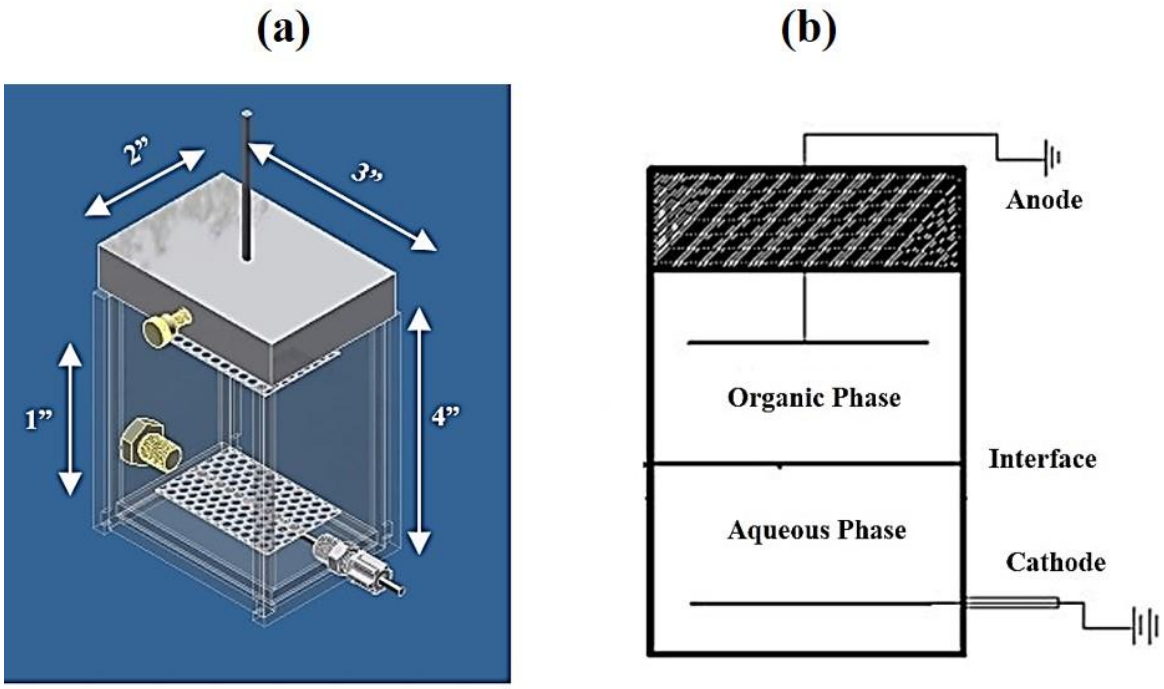


Figure 4.1. Quiescent batch electrostatic reactor: **(a)** graphical illustration with dimensions and **(b)** showing the reactor set up for hydrolysis reaction.

4.3.2.2. Stirred tank batch electrostatic reactor.

The stirred batch reactor was of cylindrical geometry of 250 mL volume and fabricated in polypropylene material. The reactor was equipped with two rectangular planar electrodes as shown in **Figure 4.2**. The electrode dimensions, mesh opening, and material were the same as described in section 2.4.1 for the quiescent batch electrostatic reactor. An overhead stirrer was located such that the impellor on the vertical drive shaft was located between the electrodes as shown in Figure 2. The drive shaft was located through centrally placed openings, 12.7 mm in diameter, on the

mesh electrode. The two electrodes were placed 25.4 mm apart. The stirrer was 3D printed using methacrylate polymer in order to be non-conductive to avoid disturbances to the electric fields. The electrodes were connected to the DC power supply. Previous studies showed that a 3:1 ratio of oil to water on a weight basis resulted in equilibrium conversion to FFA of 90% [48, 49]. 90 g of vegetable oil and 30 g of deionized water were mixed in the reactor for 30 mins to form a uniform emulsion. 10 mL of the aqueous lipase solution were then added to the emulsion in the reactor.

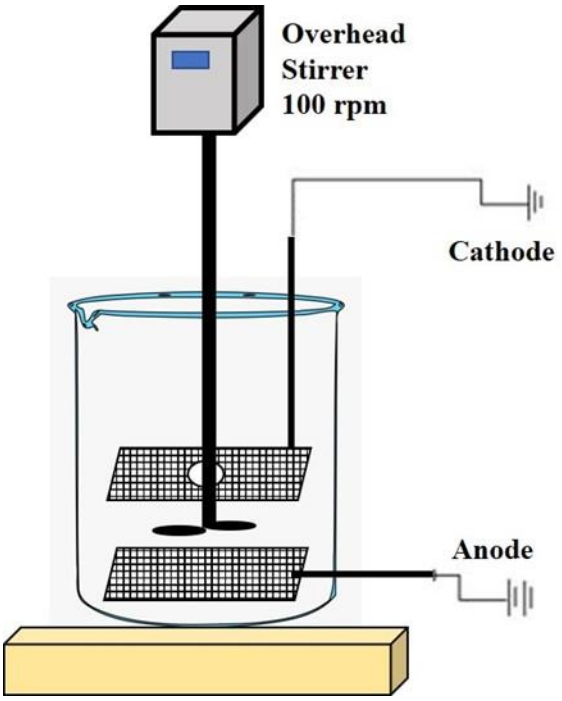


Figure 4.2. Stirred tank batch electrostatic reactor graphical illustration.

4.3.2.3. Recirculating tubular-flow batch electrostatic reactor.

Fabrication and general set up:

The reactor comprised of two 114.3 mm glass columns of 12.7 mm diameter, held together by an O-ring and pinch clamp. The reactor was equipped with two openings of 6.35 mm diameter,

one at each end. A circular nickel-alloy (Monel 400) mesh of 12.7 mm diameter and 100 μm opening was placed in between the two openings on both ends in such a way that two circulation loops (major and minor loop) are formed as shown in **Figure 4.3**. The major loop was connected to a 250 mL glass beaker reservoir containing 90 g vegetable oil and 30 g deionized water via tubing (Tygon® XL-60) with the fluids driven against gravity inside the reactor using a peristaltic pump (Watson-Marlow Fluid Technology, United Kingdom) and recirculated. The minor loop is also connected to a peristaltic pump operated in co-current mode which helped to achieve either uniform emulsion (well mixed) in the case of aqueous lipase or to recirculate resins and enhance mixing in the case of immobilized lipase. The electrodes were placed 203.2 mm apart from each other and connected to a variable DC power supply.

Aqueous lipase (Free enzyme):

In the experiments using aqueous solutions of free lipase, the peristaltic pumps were operated at the same speeds (2 seconds/revolution) providing a volumetric flowrate of 215 ± 5 mL/min for each pump. Since, both pumps were operated at high speeds, the fluids were well mixed (free from phase separation) throughout the system. 10 mL of aqueous lipase solution were added after 30 minutes once the oil-water mixture was well mixed. The mean residence time of the lipase experiencing the electric field inside the reactor was calculated to be 11% (±3%) of the effective residence times in either the quiescent batch electrostatic reactor or in the stirred tank batch electrostatic reactor.

Immobilized lipase:

A 2 g portion of immobilized lipase (lipase + support) was loaded into the reactor through the opening at the center of the column located between the two electrodes. The pumps were operated at lower speeds in the presence of immobilized lipase in order to avoid restriction

of the liquid flow due to accumulation of solid polymer particles in the upper section of the reactor. In order to avoid build-up of particles in the top section, the emulsion / particle slurry was recirculated as it approached the top of the column. The minor loop peristaltic pump was operated at a speed of 2 seconds/revolution showing a volumetric flowrate of 215 ± 5 mL/min with the main loop peristaltic pump running at a speed of 20 seconds/revolution showing a volumetric flowrate of 15 ± 2 mL/min. In order to ensure there was no phase separation throughout the system and eliminate mass transfer resistance, the reservoir was connected to an overhead stirrer operated at 300 rpm. The approximate residence time of the lipase experiencing the electric field inside the reactor was calculated to be 41% (±11%) of that in either the quiescent batch reactor or the stirred tank batch reactor.

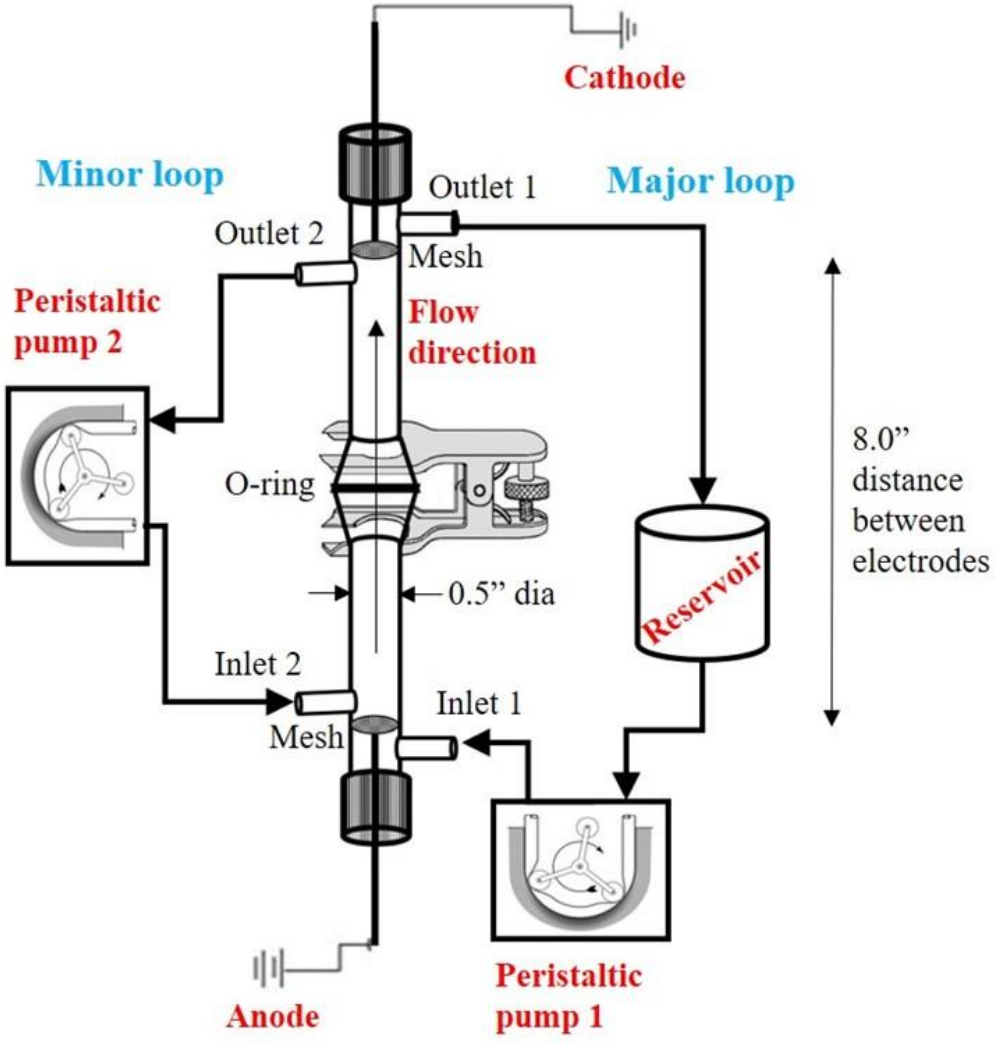


Figure 4.3. Recirculating tubular-flow batch electrostatic reactor. Pictorial representation of reactor set up and dimensions.

4.3.3. Sampling and activity measurements.

The influence of the applied oriented external electric field on the performance of lipase activity in both the free aqueous form and in immobilized form was measured based on the rate of release of FFA during triglyceride hydrolysis. All the experiments were conducted at room temperature (~22°C). Periodically, 0.25 ± 0.01 g of sample was taken from each reactor and solubilized in 20 mL mixture of solvent containing 50:50 v/v mixture of ethanol and acetone [44,

47, 48, 50-52]. This procedure denatured the enzyme and prevented further reaction from occurring. In the case of the quiescent batch reactor, samples were syringed from the oil phase. In the cases of the stirred tank batch reactor and the recirculating tubular flow reactor, samples in the form of emulsion were withdrawn for analysis. The treated samples were titrated against, 0.05 M sodium hydroxide (NaOH) using an Excellence T5 (Mettler-Toledo, United States) auto-titrator equipped with end point program along with phenolphthalein as the acid–base indicator to measure the total FFA content. The back-calculated value for the concentration of the NaOH solution was then used to determine the extent of hydrolysis. Lipase activity is expressed in terms of percentage conversion of substrate oil using an average molecular weight of vegetable oil ~900 g/mol (assumed) [53]. The uncertainty in the percent conversion was determined by assuming the uncertainty in the volume of titrant equals the standard deviation of three repeat measurements and then propagating this uncertainty through the calculation of conversion. The equations leading up to the calculation of percent conversion for all the three reactor systems along with uncertainty calculation are shown in detail in supplementary information.

For the quiescent batch electrostatic reactor experiments, four identical reactors were fabricated (see **Figure 4.4**), and the experiments were conducted in quartets. In the case of the stirred tank batch reactor (see **Figure 4.5**), experiments were conducted in three identical reactors. Experiments in the recirculating tubular flow reactor (see **Figure 4.6**) were conducted in triplicate in a single reactor. In all cases, the averaged values of volume of sodium hydroxide consumed are reported along with standard deviations.

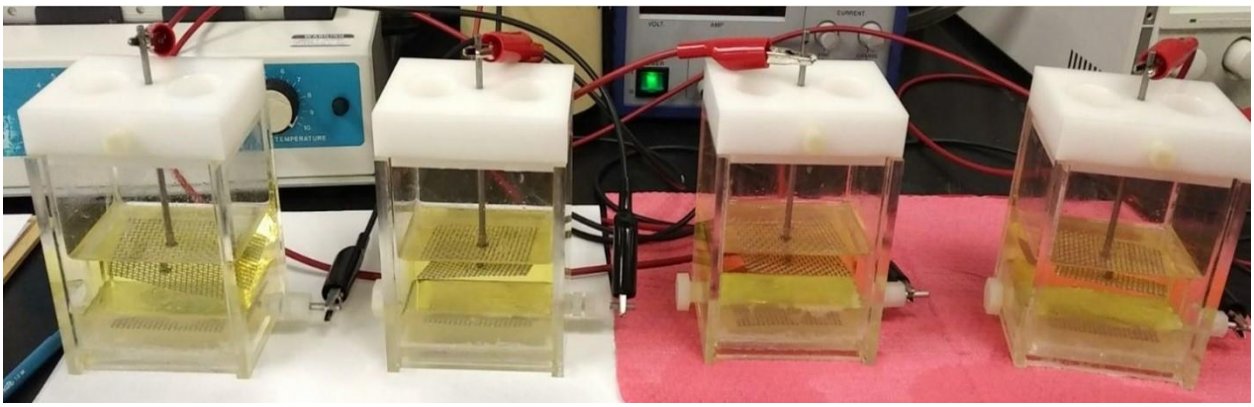


Figure 4.4. Quiescent batch electrostatic reactor set up.

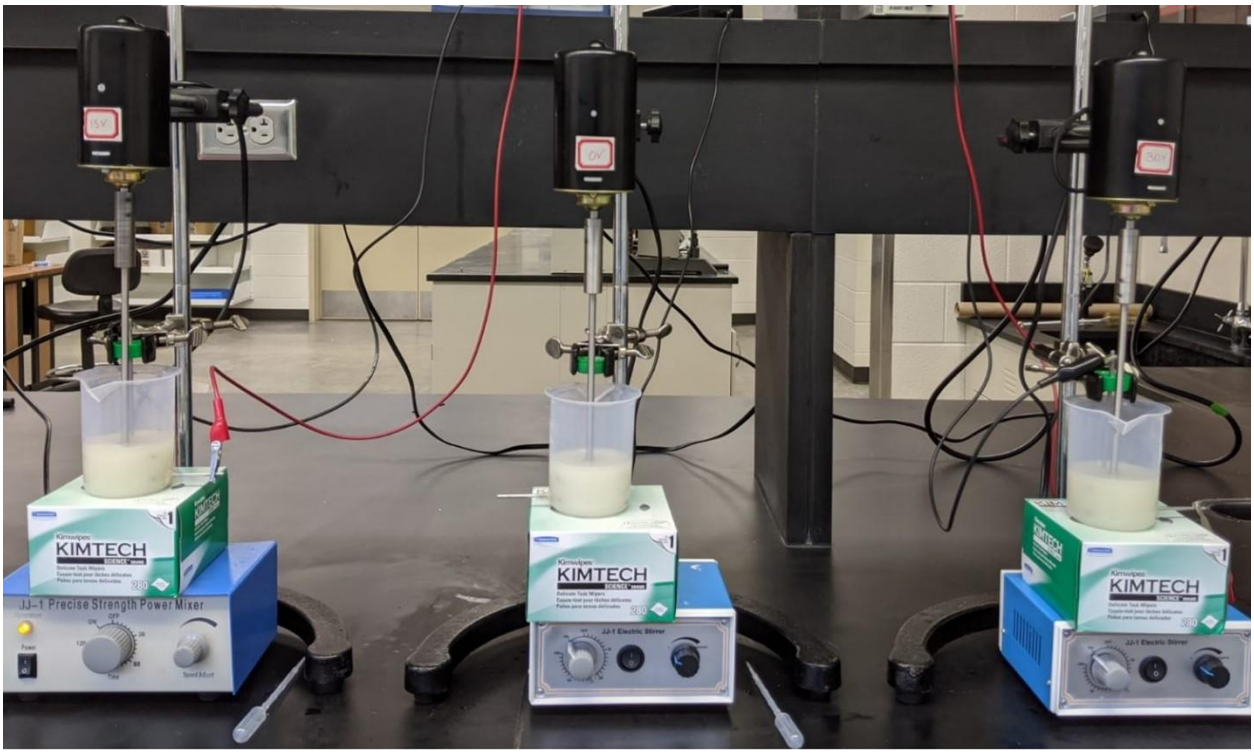


Figure 4.5. Stirred tank batch electrostatic reactor set up.

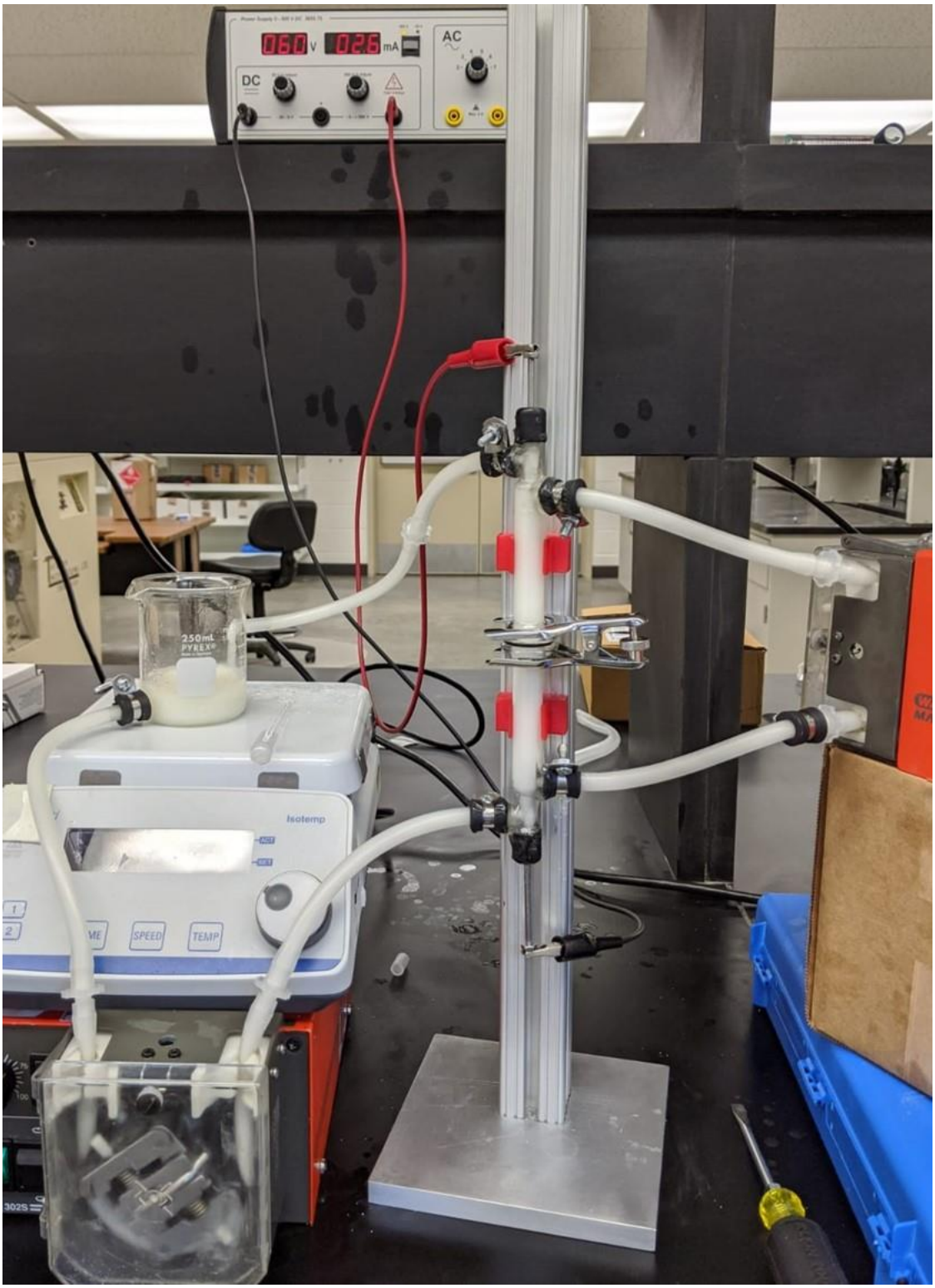


Figure 4.6. Recirculating tubular-flow batch electrostatic reactor for aqueous lipase set up.

4.4. Results and discussion.

4.4.1. Quiescent batch electrostatic reactor system.

4.4.1.1. Effect of sampling.

One of the concerns in measurement of the change in composition of the upper organic phase was the potential presence of composition gradients between the oil/water interface and the top surface (bulk) of the liquids in the reactor. In order to establish the extent to which this may have been the case, an initial rate experiment was conducted in which sampling of the oil phase was conducted close to the interface and also at the top of the reactor adjacent to the surface of the oil. **Figure 4.7** shows the results of this comparison with percentage conversion with respect to reaction time based on reaction conducted using aqueous lipase solution with an applied OEEF of 15 V across the interface.

The data in **Figure 4.7**, confirms that there was no significant difference between the composition close to the interface and that in the bulk organic phase. Therefore, sampling throughout the remainder of the experiments relied on samples being removed from the bulk part of the organic phase. Adoption of this approach minimized the risk of creating hydrodynamic disturbances close to the interface which could be induced by sampling close to the interface.

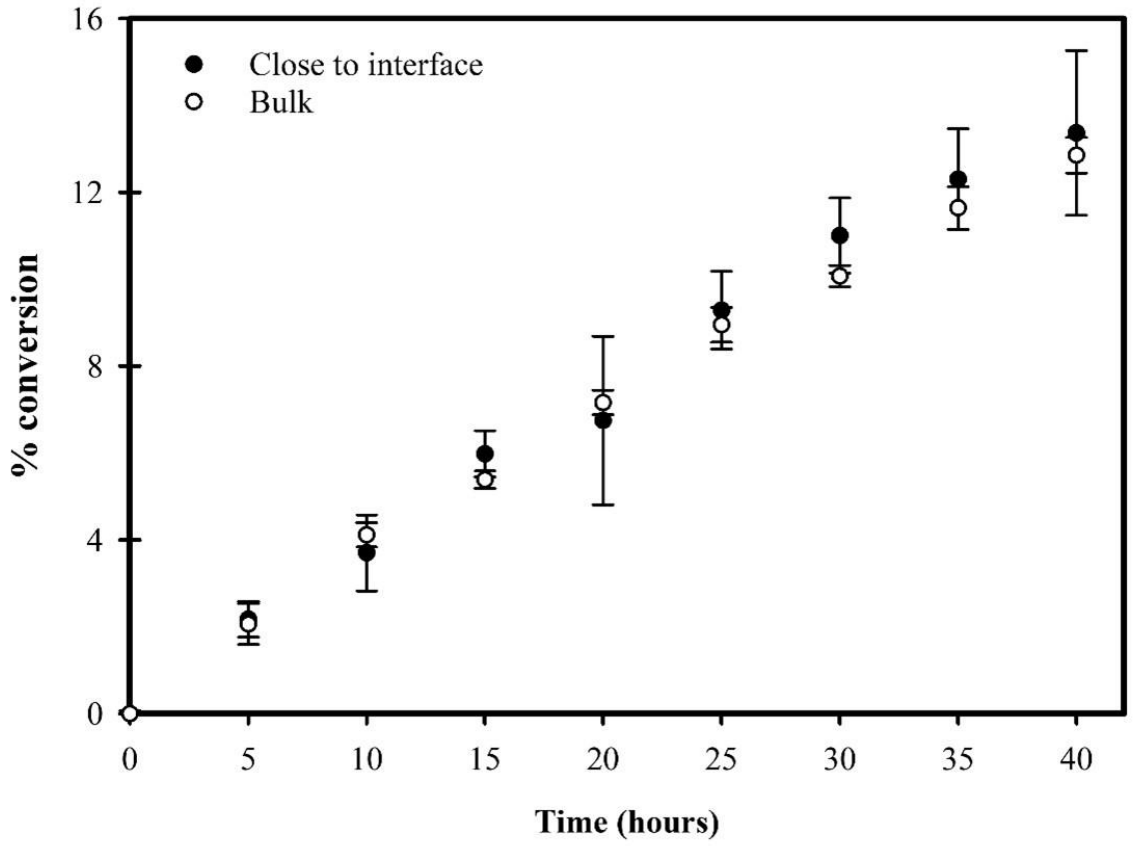


Figure 4.7. Effect of sampling position in quiescent batch electrostatic reactor using aqueous lipase solution (0.065 mg/mL) with applied oriented external electric field of 15 V and 25.4 mm electrode distance conducted at room temperature (22 ± 2°C).

4.4.1.2. Effect of varying applied oriented external electric field voltage.

The experimental results for the quiescent batch reactor are displayed in **Figure 4.8,** showing the influence of varying applied OEEF on reaction performance. Percentage conversion against reaction time using aqueous lipase solution with voltages of 15 V and 30 V applied are compared. Two control experiments were also conducted, one with no lipase at 0 V and another with lipase present but with 0 V applied. These results show that the application of a low voltage across the organic/aqueous interface resulted in enhanced performance. In both cases (15 V and 30 V applied) the reaction proceeded faster compared with the two controls. The reaction rate

increased as the applied external voltage increased. The values of electrical current were observed to be negligible which eliminated any significant electrical heating effect, enzyme inhibition due to electrical current flow [54] or electrolytically induced side reaction.

The possible reasons for the enhancement due to increased voltage of could be: (1) micro disturbances happening at the oil/water interface resulting in enhanced mass transfer across the interface, (2) migration of lipase towards the interface which enhances the equilibrium concentration of lipase at the interface, or (3) lipase undergoing conformational changes caused by the applied electric field favoring more open-lid formations. However, the design of the quiescent reactor was such that reaction in each case was conducted with a constant interfacial area. There was no visible evidence of either interfacial disturbance or changes in the shape of the interface which would enhance contact area and overall mass transfer.

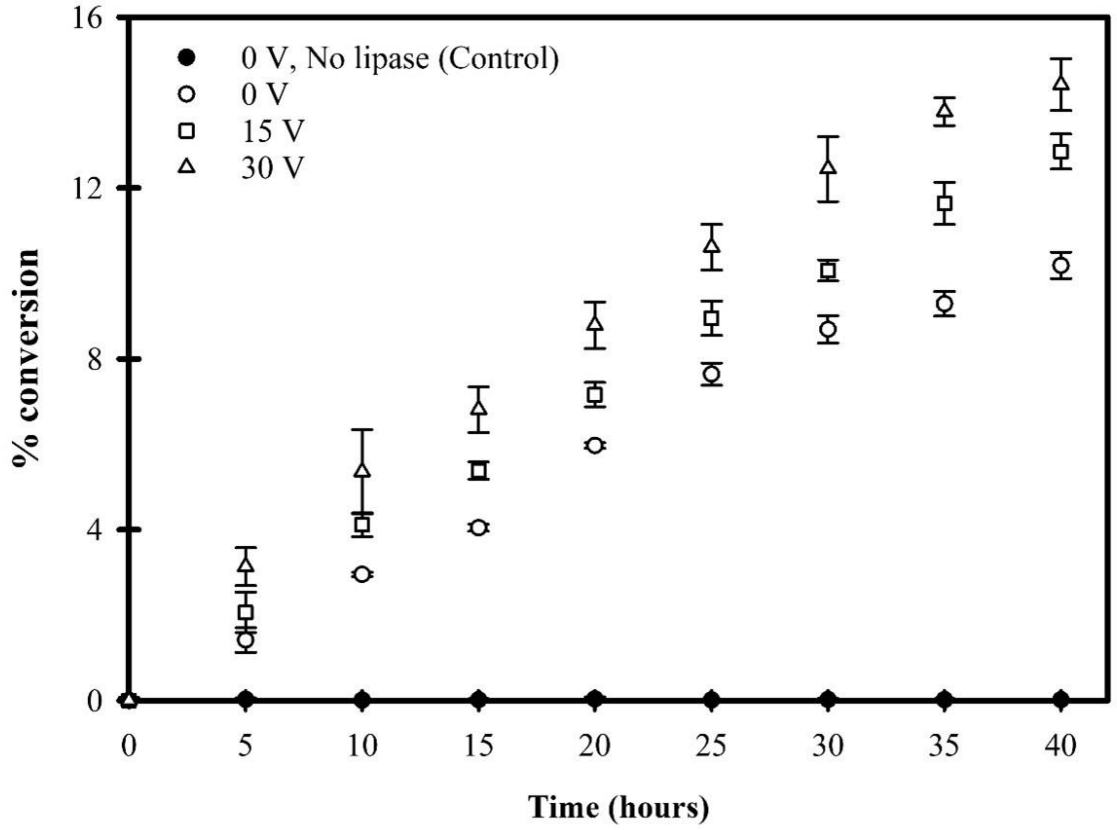


Figure 4.8. Effect of varying oriented external electric field voltage on the hydrolytic performance of aqueous lipase solution (0.065 mg/mL) in the quiescent batch electrostatic reactor using 25.4 mm electrode distance conducted at room temperature (22 ± 2°C).

4.4.1.3. Effect of change in polarity and electrode distance.

The possibility of enhanced migration of lipase to the interface under the influence of the electric field was evaluated by reversing the polarity of the two electrodes in the quiescent batch electrostatic reactor. Comparison of reactions conducted at +15 V (straight) and -15 V (reversed) externally applied voltage is shown in **Figure 4.9**. The results show no significant difference when the electric field direction is reversed even though the reaction is not carried out at the isoelectric point of the lipase (pH 5.6 - 5.8) [55]. This suggest that charge associated with lipase does not play a significant contributor in the present study. Total protein concentrations measured close to the

interface and in the bulk aqueous phase using the Bradford assay did not detect protein concentration gradients in the aqueous phase.

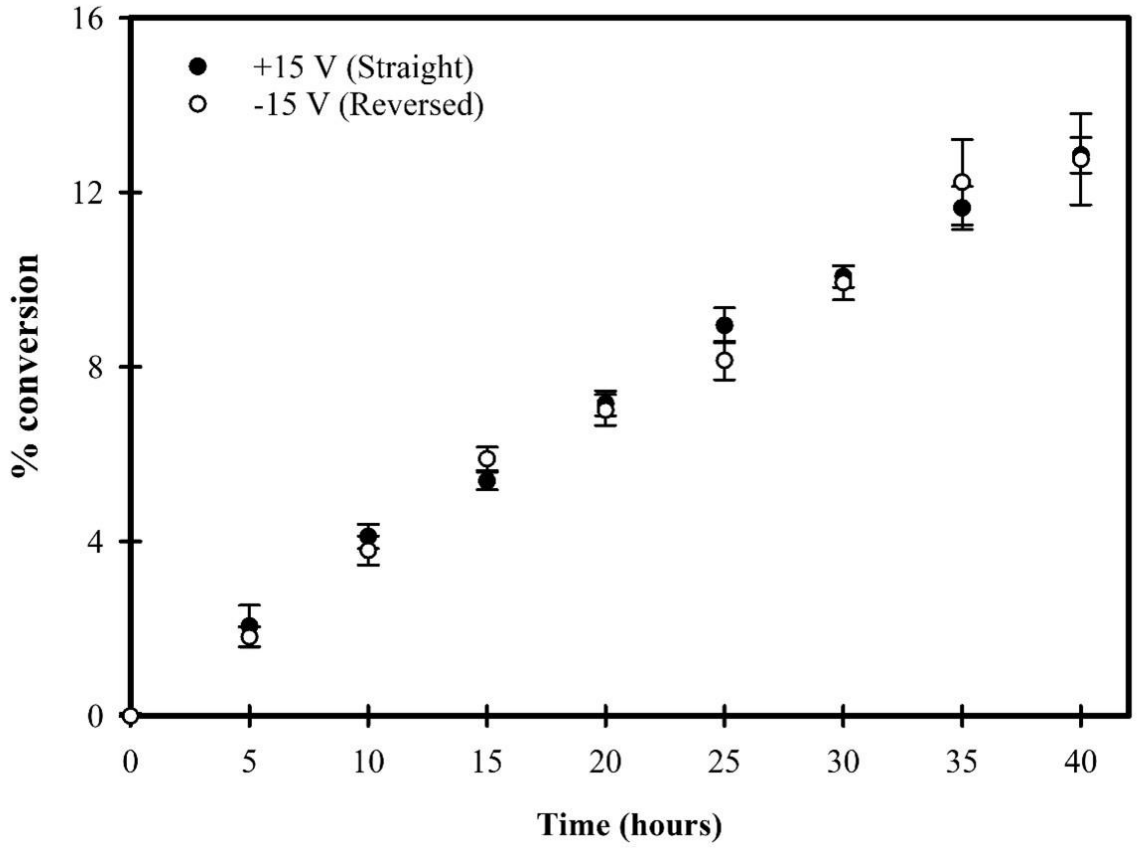


Figure 4.9. Effect of change in polarity on the hydrolytic performance of aqueous lipase solution (0.065 mg/mL) in the quiescent batch electrostatic reactor using externally applied voltage of 15 V with 25.4 mm electrode distance conducted at room temperature (22 ± 2°C).

Figure 4.10 shows the comparison of the quiescent batch reactor performance at two different electrode distances, 25 mm and 50 mm respectively. The results show no difference in reaction performance. One factor to be considered is the possible influence of the inter-electrode distance on the nominal field strength (voltage/distance). At the voltages studied, the differences in the nominal field strengths are unlikely to impact transport of species due to electro-kinetic

effects [56]. Another factor is the relatively high conductivity of the aqueous phase on account of the presence of buffering salts suggesting that the voltage drop across the reactor is mostly due to the electrical resistance of the oil phase. Since the volumes and levels of the oil phase in the case of the two sets of experiments shown in **Figure 4.10**, are identical, the voltage drop will be nearly the same. Although the effect of nominal field strength observed was negligible, at significantly higher voltages it would likely be a factor affecting reaction performance.

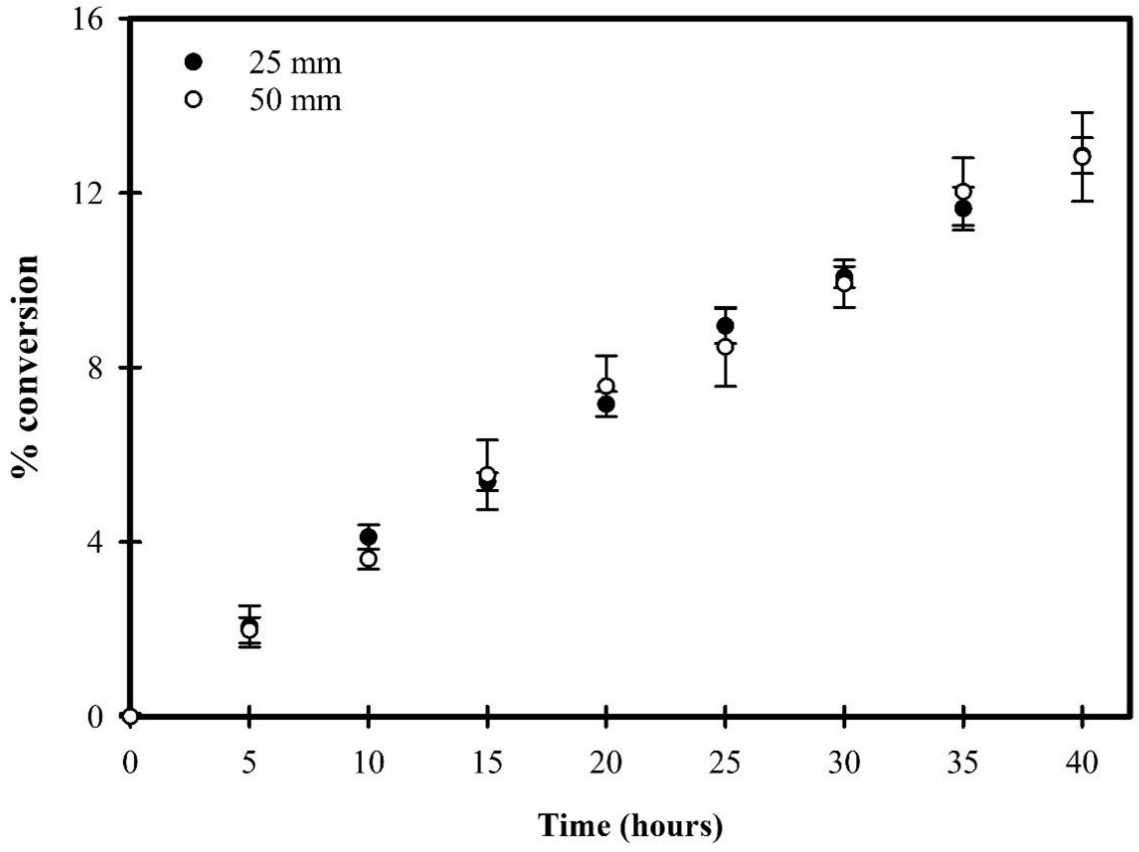


Figure 4.10. Effect of change in electrode distance on the hydrolytic performance of aqueous lipase solution (0.065 mg/mL) in the quiescent batch electrostatic reactor using externally applied voltage of 15 V conducted at room temperature (22 ± 2°C).

4.4.2. Stirred tank batch electrostatic reactor system.

A practical hydrolysis process would likely involve forced mixing and accordingly the performance in a stirred tank batch electrostatic reactor was evaluated, **Figure 4.11.** The general relationship between reaction performance and the magnitude of the applied voltage is comparable with that observed in the quiescent batch electrostatic reactor. However, comparison of the percent change in enhancements, show a significant difference. In the case of the stirred tank reactor, the percent change in conversion with respect to 0 V and 30 V at 24 hours reaction time was 29%. In contrast, at the same reaction time in the quiescent reactor it was 46% for the same increase in voltage, see Figure 5. The decreased performance in the stirred tank system is likely on account of increased hydrodynamic shear and its detrimental effect on the lipase activity. Another possibility is disturbance of the uniformity of the electric field due to stirring.

However, even when the mass transport limitations on reaction rate have been reduced or eliminated by stirring in the stirred tank batch electrostatic reactor, a notable electric field-induced rate enhancement is observed, supporting the hypothesis that the electric field induces a conformational change in the lipase activity rather than facilitating mass transport.

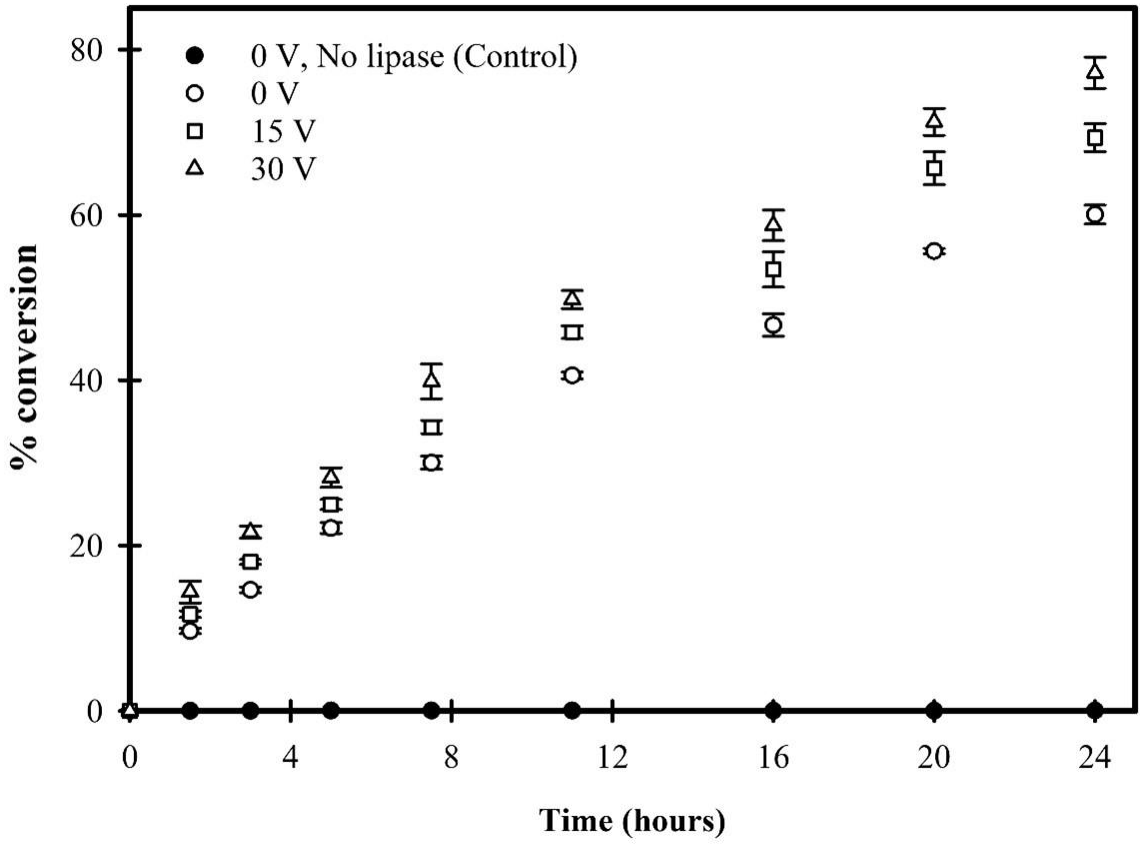


Figure 4.11. Effect of varying oriented external electric field voltage on the hydrolytic performance of aqueous lipase solution (0.065 mg/mL) in the stirred tank batch electrostatic reactor operated at 100 rpm using 25.4 mm electrode distance conducted at room temperature (22 ± 2°C).

4.4.3. Recirculating tubular-flow batch electrostatic reactor.

4.4.3.1. Aqueous lipase system.

The rates of fatty acid production in the co-current recirculating batch reactor are presented in **Figure 4.12,** showing the influence of applied OEEF voltage across the main section of the reactor. The trends shown are comparable with those from those seen in both the quiescent batch electrostatic reactor and in the stirred tank batch electrostatic reactor (**see Figure 4.8 and Figure 4.11**). The most striking aspect of the data in **Figure 4.12** is the significant increase in reaction performance when a voltage of 30 volts is applied, compared with the 0 V control and the case of

60 V. For example, at 210 minutes reaction time a conversion of 24% was observed at 30 V compared with 17% at 0 V. In contrast, at the higher voltage of 60 V, the conversion was measured at 20% which coincides with the conversion obtained at that reaction time when a voltage of 15 V was applied. At applied voltages in the range 0-30 V there was no measurable current flowing in the reactor. In contrast, at 60 V, current flows in the range 2.5 – 7 mA (**see Figure 4.6**) were measured. Literature on lipase behavior in response to pulsed electric fields suggests that the significant current flow is a cause of lipase inhibition [54, 57, 58]. Another possible explanation for decreased performance noticed at 60 V suggest the possibility of an optimum voltage for the enhancement of enzyme activity. This is consistent with data by Adibzadeh et al., who studied the effect of low tension electric current on bacterial lipase activity [59]. Though the paper was mostly concerned with electrostimulation of biological COD removal from oily wastewater, a set of enzyme activity measurements relevant to the current work, performed on lipase solutions from the supernate of the biologically active wastewater was presented. The activities of lipase solutions exposed to electrical currents in the range 0-20 mA were determined in a small cylindrical cell equipped with concentric cylindrical electrodes. Increase of current from 1 mA upwards clearly showed an increase in the lipase activity. At a current of 10 mA, lipase activity reached a maximum rate. Above 10 mA, lipase activity decreased on further increasing of current. There is evidence that an electric field can be used under certain circumstances to enhance enzyme activity and that there appears to be an optimum value of applied electrical field. This is consistent with the findings presented here.

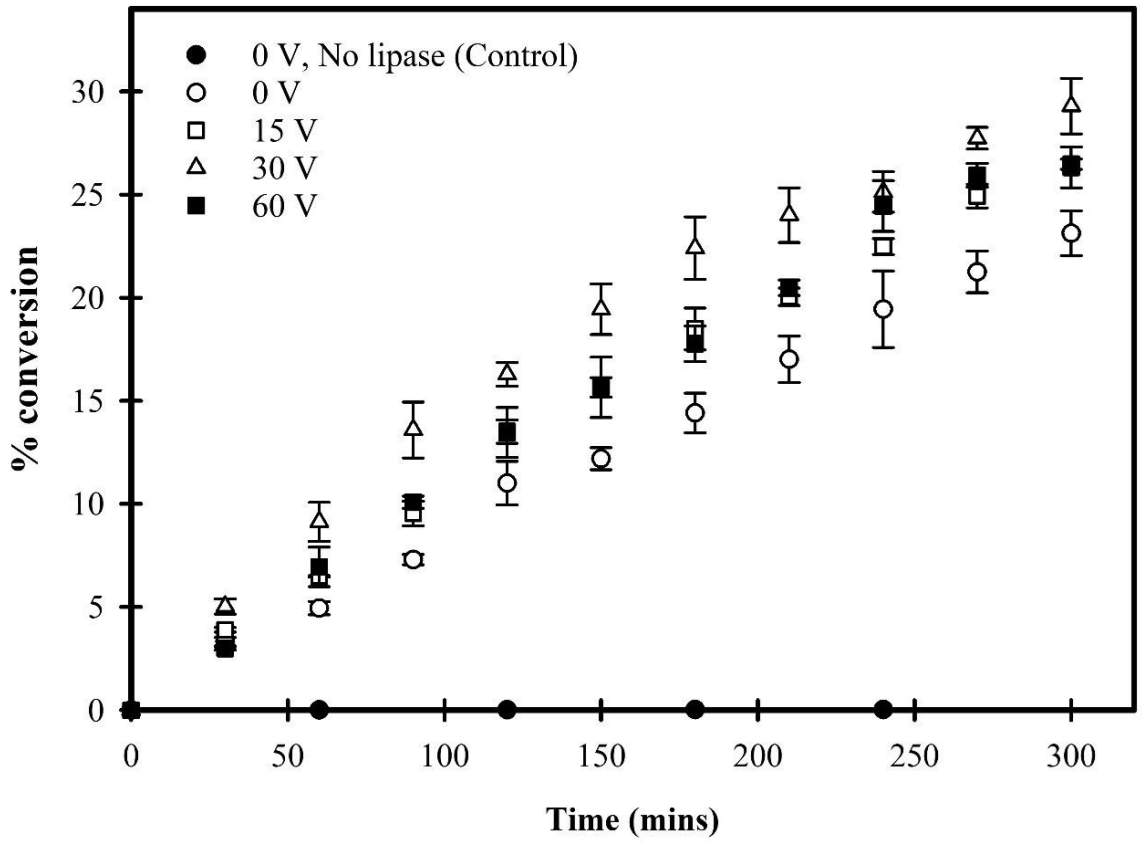


Figure 4.12. Effect of varying oriented external electric field voltage on the hydrolytic performance of aqueous lipase solution (0.065 mg/mL) in the recirculating tubular-flow batch electrostatic reactor conducted at room temperature (22 ± 2°C).

4.4.3.2. Immobilized lipase system.

The effect of OEEF on reaction performance in the cases of lipase immobilized onto methacrylate supports functionalized with epoxy & butyl groups (ECR8285) and octadecyl groups (ECR8806M) is shown in **Figure 4.13**. With reference to the two methacrylate polymers, lipase immobilization is based on hydrophobic interaction between the support and lipase by adsorption [60]. Immobilization may be enhanced by exploiting the functional groups present on the surface and introducing a cross-linking agent such as itaconic acid [47, 61-65]. Overall, lipase immobilized onto methacrylate supports with octadecyl functionalization performed better than the lipase

immobilized onto methacrylate supports with epoxy & butyl functionalization. This was consistent with literature data and is explained by the hydrophobic nature of these resins [47]. The results in **Figure 4.13** show no clear evidence of enhancement due to applied electric field in either case. These suggest that lipase when immobilized has already undergone conformational changes favoring towards the active "open lid" form [60].

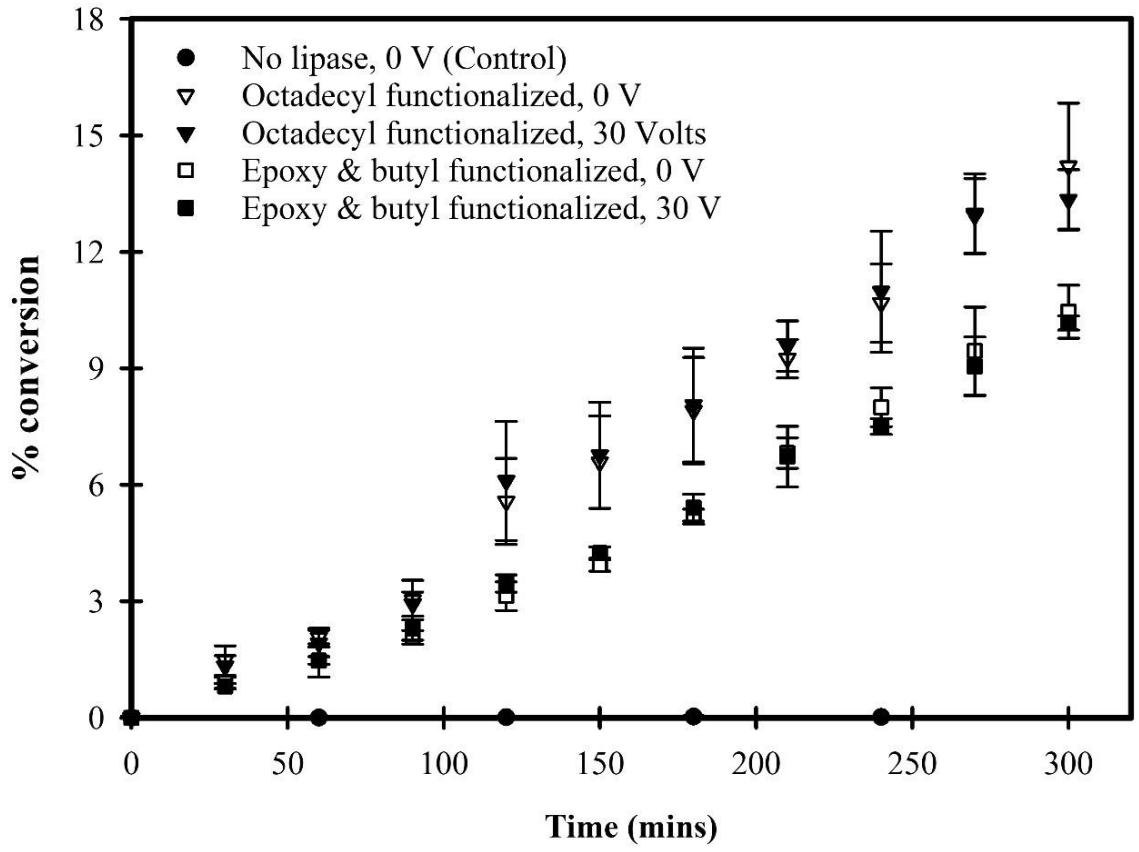


Figure 4.13. Effect of varying oriented external electric field voltage on the hydrolytic performance of lipase immobilized on functionalized methacrylate supports in the recirculating tubular-flow batch electrostatic reactor conducted at room temperature (22 ± 2°C).

4.4.4. Further discussion of influence of electric field on the reaction.

The possible reasons behind the observed influences of applied electrical field on reaction performance and in particular, enzyme activity is discussed in more depth. The interactions of electrical field and protein structure have been the subject of several studies on the molecular structure using crystallography and simulation [66-68]. Interfacial activation and the role of the so called open and closed "lid mechanism" in defining the catalytic activity of the enzyme according to its macro-environment have been described [69], as improved understanding of the three dimensional (3-D) structure of enzymes became available. The opened or closed lid status of the protein is important in determining the activity of the enzyme. Therefore, the nature of the physical and chemical environments surrounding the enzyme which influence this status are of interest in determining the key factors which may be controlled to optimize catalytic performance. The relationship between interfacial activation, a key factor in determining the opened or closed status of the lid, and hydrophobicity of the surface is described by Grochulski et al, showing that the open (active) state of the lid is associated with a hydrophilic surface, and the closed (inactive) state associated with a hydrophobic surface [68].

The above proposition is further supported by Peterson et al, who reported on the impact of the distribution of hydrophobic residues on enzyme activity [66]. More importantly, it was determined that electrostatic potential distribution on the molecular surface of the protein has a significant influence on the enzyme's pH activity profile. In their work, mapping of the electrostatic potential distribution for nine different lipases and esterases revealed a strong correlation between potential, pH, and optimum enzymatic activity. Peterson et al, also proposed the concept of an electrostatic catapult mechanism which further underlines the role of electrostatic forces in enzymatically catalyzed reactions. The concept may be summarized as follows. The

hydrolytic reaction of the triglyceride ester releases the free fatty acid which in a basic or neutral environment will become de-protonated and therefore carry a negative charge. Since in the pH range for optimum catalytic performance the active site of the enzyme will also carry negative charge. According to the proposed "electrostatic catapult mechanism" the de-protonated fatty acid is repulsed and ejected from the proximity of the site. It is assumed that the negative potential at the bottom of the active site increases the efficiency of repulsion of the charged free fatty acid, shifting reaction equilibrium, thus enhancing deacylation.

4.5. Conclusion.

The results of this study show that low voltage oriented external electric fields can enhance the rate of reaction for lipase catalyzed hydrolysis of a triacyl glycerol ester. This was confirmed in three reactor systems: a quiescent liquid-liquid cell reactor; a mechanically stirred tank batch reactor, and in a recirculated tubular-flow reactor using an applied oriented external electric field voltage in the range of 0-30 V in each case. The observed enhancements in the quiescent reactor of up to 60% based on conversion at 40 hours reaction time, strongly suggested that mass transfer enhancement does not explain the increase in conversion rate, and that the behavior of lipase in response to the oriented external electric field is a major factor contributing towards enhanced hydrolytic performance. This conclusion was drawn from the series of experiments considering the effect of oriented external electric field voltages, changes in polarity and electrode distance. Similar trends and behavior were noted in the stirred tank batch electrostatic reactor and in the recirculating tubular-flow reactor when external electric fields were applied with difference in overall conversion percentages in each reactor. Reaction performance in the stirred tank batch electrostatic reactor showed improved reaction performance of up 40% based conversion at 24 hours compared with that at 0 V. Reaction performance in the recirculating tubular-flow batch

reactor at 60 V field showed lower reaction performance of 5% based on conversion at 3 hours than at 30 V. The lower performance at 60 V is explained by lipase inhibition associated with current flow. Overall, the applied oriented external electric field showed enhancement reaction performance in the three reactor systems studied. The proposed mechanism is based on the lipase undergoing field induced conformational change resulting in the open lid state and associated greater catalytic activity. Reaction performance using lipase immobilized on functionalized methacrylate supports in the tubular reactor showed no significant difference when compared with aqueous lipase system.

4.6. References.

[1] Y. Kim, L.K. Park, S. Yiacoumi, C. Tsouris, Modular chemical process intensification: a review, Annual Review of Chemical and Biomolecular Engineering 8 (2017) 359-380.

[2] L. Weatherley, D. Rooney, M. Niekerk, Clean synthesis of fatty acids in an intensive lipase-catalysed bioreactor, Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology 68(4) (1997) 437-441.

[3] D.W. DePaoli, C. Tsouris, J.Q. Feng, Method for electrically producing dispersions of a nonconductive fluid in a conductive medium, Google Patents, 1998.

[4] J.Q. Feng, D.W. DePaoli, C. Tsouris, T.C. Scott, Spraying fine fluid particles in insulating fluid systems by electrostatic polarization forces, Journal of Applied Physics 78(4) (1995) 2860-2862.

[5] E.N. Kaufman, J.B. Harkins, M. Rodriguez Jr, C. Tsouris, P.T. Selvaraj, S.E. Murphy, Development of an electro-spray bioreactor for crude oil processing, Fuel Processing Technology 52(1-3) (1997) 127-144.

[6] W.T. Shin, S. Yiacoumi, C. Tsouris, Experiments on electrostatic dispersion of air in water, Industrial & Engineering Chemistry Research 36(9) (1997) 3647-3655.

[7] C. Tsouris, S. Neal, V. Shah, M. Spurrier, M. Lee, Comparison of liquid-liquid dispersions formed by a stirred tank and electrostatic spraying, Chemical Engineering Communications 160(1) (1997) 175-197.

[8] C. Tsouris, W.T. Shin, S. Yiacoumi, D.W. DePaoli, Electrohydrodynamic velocity and pumping measurements in water and alcohols, Journal of Colloid and Interface Science 229(2) (2000) 335-345.

[9] C. Tsouris, W.T. Shin, S. Yiacoumi, Pumping, spraying, and mixing of fluids by electric fields, The Canadian Journal of Chemical Engineering 76(3) (1998) 589-599.

[10] M. Bazhal, E. Vorobiev, Electrical treatment of apple cossettes for intensifying juice pressing, Journal of the Science of Food and Agriculture 80(11) (2000) 1668-1674.

[11] N. Wang, K. Kaminski, J. Petera, A.M. Allgeier, L.R. Weatherley, Electrostatically enhanced catalytic phase transfer hydrogenation of acetophenone under low external electric field, Chemical Engineering Journal 374 (2019) 1096-1101.

[12] L. Weatherley, J. Petera, Z. Qiu, Intensification of mass transfer and reaction in electrically disturbed liquid-liquid systems, Chemical Engineering Journal 322 (2017) 115-128.

[13] K. Sharma, R.T. Mayes, J. Kiggans Jr, S. Yiacoumi, J. Gabitto, D.W. DePaoli, S. Dai, C. Tsouris, Influence of temperature on the electrosorption of ions from aqueous solutions using mesoporous carbon materials, Separation and Purification Technology 116 (2013) 206-213.

[14] Z. Liu, Y. Li, M. Chen, A. Nueraihemaiti, J. Du, X. Fan, C.-Y. Tao, Enhanced leaching of vanadium slag in acidic solution by electro-oxidation, Hydrometallurgy 159 (2016) 1-5.

[15] N. Wang, A.M. Allgeier, L.R. Weatherley, Controlling reaction rate of phase transfer hydrogenation of acetophenone by application of low external electric field, AIChE Journal e17079.

[16] C. Tsouris, J. Dong, Effects of electric fields on phase inversion of liquid-liquid dispersions, Chemical Engineering Science 55(17) (2000) 3571-3574.

[17] C. Tsouris, J. Dong, Methods to control phase inversions and enhance mass transfer in liquid-liquid dispersions, Lockheed Martin Energy Research Corporation, 2002.

[18] J. Dong, C. Tsouris, Phase inversion of liquid–liquid dispersions under applied electric fields, Journal of Dispersion Science and Technology 22(1) (2001) 57-69.

[19] K. Blankenship, V. Shah, C. Tsouris, Distillation under electric fields, Separation Science and Technology 34(6-7) (1999) 1393-1409.
[20] K. Blankenship, D. DePaoli, J. Hylton, C. Tsouris, Effect of electrode configurations on phase equilibria with applied electric fields, Separation and Purification Technology 15(3) (1999) 283-294.
[21] Y. Pocker, R.F. Buchholz, Electrostatic catalysis of ionic aggregates. I. Ionization and dissociation of trityl chloride and hydrogen chloride in lithium perchlorate-diethyl ether solutions, Journal of the American Chemical Society 92(7) (1970) 2075-2084.
[22] E. Carbonell, M. Duran, A. Lledos, J. Bertran, Catalysis of Friedel-Crafts reactions by electric fields, The Journal of Physical Chemistry 95(1) (1991) 179-183.
[23] F. Che, J.T. Gray, S. Ha, N. Kruse, S.L. Scott, J.-S. McEwen, Elucidating the roles of electric fields in catalysis: A perspective, ACS Catalysis 8(6) (2018) 5153-5174.
[24] G. Náray-Szabó, Electrostatic catalysis in enzymes, Journal of Molecular Catalysis 47(2-3) (1988) 281-287.
[25] J. Joy, T. Stuyver, S. Shaik, Oriented external electric fields and ionic additives elicit catalysis and mechanistic crossover in oxidative addition reactions, Journal of the American Chemical Society 142(8) (2020) 3836-3850.
[26] S. Shaik, D. Mandal, R. Ramanan, Oriented electric fields as future smart reagents in chemistry, Nature Chemistry 8(12) (2016) 1091.
[27] K. Kamiński, M. Krawczyk, J. Augustyniak, L. Weatherley, J. Petera, Electrically induced liquid–liquid extraction from organic mixtures with the use of ionic liquids, Chemical Engineering Journal 235 (2014) 109-123.
[28] M. Krawczyk, K. Kamiński, J. Petera, Experimental and numerical investigation of electrostatic spray liquid-liquid extraction with ionic liquids, Chemical and Process Engineering (2012) 167-183.
[29] A.R. Ye, Y.S. Ryazantsev, M.G. Velarde, Drop motion and the Maragoni effect. Interaction of modes, Physica Scripta 1994(T55) (1994) 115.
[30] G. Stewart, J. Thornton, Charge and velocity characteristics of electrically charged droplets. Part I: theoretical considerations; Part II: preliminary measurements of droplet charge and velocity, Institution of Chemical Engineers Symposium Series, 1967, pp. 29-42.
[31] D. Tam, V. von Arnim, G. McKinley, A. Hosoi, Marangoni convection in droplets on superhydrophobic surfaces, (2009).
[32] P. Bailes, Solvent extraction in an electrostatic field, Industrial & Engineering Chemistry Process Design and Development 20(3) (1981) 564-570.
[33] L. Austin, L. Banczyk, H. Sawistowski, Effect of electric field on mass transfer across a plane interface, Chemical Engineering Science 26(12) (1971) 2120-2121.
[34] P. Iyer, H. Sawistowski, Effect of electric field on mass transfer across a plane interface, Proceedings of the International Solvent Extraction Conference, 1974, pp. 1029-1046.
[35] R. Wham, C. Byers, Mass transport from single droplets in imposed electric fields, Separation Science and Technology 22(2-3) (1987) 447-466.
[36] A. Glitzenstein, A. Tamir, Y. Oren, Mass transfer enhancement of acetic acid across a plane kerosene/water interface by an electric field, The Canadian Journal of Chemical Engineering 73(1) (1995) 95-102.
[37] G. Gneist, H.-J. Bart, Influence of high-frequency AC fields on mass transfer in solvent extraction, Journal of Electrostatics 59(2) (2003) 73-86.

[38] W. Hem, J. Chang, M. Baird, Enhancement of interphase mass transfer by a pulsed electric field, Journal of Electrostatics 40 (1997) 259-264.
[39] J. Petera, L. Weatherley, D. Rooney, K. Kaminski, A finite element model of enzymatically catalyzed hydrolysis in an electrostatic spray reactor, Computers & Chemical Engineering 33(1) (2009) 144-161.
[40] M. Van Niekerk, L. Weatherley, Electrostatically enhanced enzymatic hydrolysis of vegetable oil, Food and Bioproducts Processing: Transactions of the Institution of Chemical Engineers, Part C (1996).
[41] L. Weatherley, D. Rooney, M. Niekerk, Interfacial charge effects during natural oil hydrolysis, Studies in Environmental Science, Elsevier1997, pp. 107-117.
E. Jones, L.R. Weatherley, Modelling the kinetics of lipase-catalysed oil hydrolysis in an electrically enhanced liquid–liquid system, Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology 78(2-3) (2003) 194-198.
[42] L.R. Weatherley, D. Rooney, Enzymatic catalysis and electrostatic process intensification for processing of natural oils, Chemical Engineering Journal 135(1-2) (2008) 25-32.
[43] A. Anand, Enhanced Lipase-Catalyzed Hydrolysis and Modification of Fats and Oils, 2018 AIChE Annual Meeting, AIChE, 2018.
[44] A. Anand, Enhanced Lipase-Catalyzed Hydrolysis and Modification of Fats and Oils, 2019 North American Catalysis Society Meeting, NAM, 2019.
[45] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry 72(1-2) (1976) 248-254.
[46] A. Anand, P. Gnanasekaran, A.M. Allgeier, L.R. Weatherley, Study and deployment of methacrylate-based polymer resins for immobilized lipase catalyzed triglyceride hydrolysis, Food and Bioproducts Processing (2020).
[47] L. Weatherley, D. Rooney, M. Niekerk, Clean synthesis of fatty acids in an intensive lipase-catalysed bioreactor, Journal of Chemical Technology & Biotechnology 68(4) (1997) 437-441.
[48] D. Rooney, L. Weatherley, The effect of reaction conditions upon lipase catalysed hydrolysis of high oleate sunflower oil in a stirred liquid–liquid reactor, Process Biochemistry 36(10) (2001) 947-953.
[49] A. Anand, P. Gnanasekaran, A.M. Allgeir, L. Weatherley, Immobilization and kinetic studies of a microbial lipase from the fungal organism Candida rugosa on methacrylate polymer resins, 2019 AIChE Annual Meeting, AIChE, 2019.
[50] L. Weatherley, A. Anand, The Performance of Microbial Lipase Immobilized onto Ion Exchange Resins and onto the Natural Zeolite Clinoptilolite, International Journal of Chemical Separation Technology 2(2) (2016) 24-32.
[51] A. Anand, L.R. Weatherley, The performance of microbial lipase immobilized onto polyolefin supports for hydrolysis of high oleate sunflower oil, Process Biochemistry 68 (2018) 100-107.
[52] M. Refvik, R. Larock, The chemistry of metathesized soybean oil, Journal of the American Oil Chemists' Society 76(1) (1999) 99-102.
[53] S. Min, G.A. Evrendilek, H.Q. Zhang, Pulsed electric fields: processing system, microbial and enzyme inhibition, and shelf life extension of foods, IEEE Transactions on Plasma Science 35(1) (2007) 59-73.

[54] J. Vakhlu, Yeast lipases: enzyme purification, biochemical properties and gene cloning, Electronic Journal of Biotechnology 9(1) (2006) 0-0.
[55] M.A. Saucedo-Espinosa, P.S. Dittrich, In-Droplet Electrophoretic Separation and Enrichment of Biomolecules, Analytical Chemistry 92(12) (2020) 8414-8421.
[56] J.Y. Qian, Y.P. Gu, W. Jiang, W. Chen, Inactivating effect of pulsed electric field on lipase in brown rice, Innovative Food Science & Emerging Technologies 22 (2014) 89-94.
[57] S. Ho, G. Mittal, J. Cross, Effects of high field electric pulses on the activity of selected enzymes, Journal of Food Engineering 31(1) (1997) 69-84.
[58] A. Adibzadeh, A. Rezaee, Z. Salehi, Enhancement of lipase activity for the oily wastewater treatment by an electrostimulation process, RSC Advances 6(116) (2016) 115290-115297.
[59] E.A. Manoel, J.C. dos Santos, D.M. Freire, N. Rueda, R. Fernandez-Lafuente, Immobilization of lipases on hydrophobic supports involves the open form of the enzyme, Enzyme and Microbial Technology 71 (2015) 53-57.
[60] L. Thudi, L.S. Jasti, Y. Swarnalatha, N.W. Fadnavis, K. Mulani, S. Deokar, S. Ponrathnam, Enzyme immobilization on epoxy supports in reverse micellar media: Prevention of enzyme denaturation, Journal of Molecular Catalysis B: Enzymatic 74(1-2) (2012) 54-62.
[61] M. Mihailović, M. Stojanović, K. Banjanac, M. Carević, N. Prlainović, N. Milosavić, D. Bezbradica, Immobilization of lipase on epoxy-activated Purolite® A109 and its post-immobilization stabilization, Process Biochemistry 49(4) (2014) 637-646.
[62] X. Li, D. Li, W. Wang, R. Durrani, B. Yang, Y. Wang, Immobilization of SMG1-F278N lipase onto a novel epoxy resin: characterization and its application in synthesis of partial glycerides, Journal of Molecular Catalysis B: Enzymatic 133 (2016) 154-160.
[63] A. Basso, M. Hesseler, S. Serban, Hydrophobic microenvironment optimization for efficient immobilization of lipases on octadecyl functionalised resins, Tetrahedron 72(46) (2016) 7323-7328.
[64] J.M. Palomo, G. Muñoz, G. Fernández-Lorente, C. Mateo, R. Fernández-Lafuente, J.M. Guisán, Interfacial adsorption of lipases on very hydrophobic support (octadecyl–Sepabeads): immobilization, hyperactivation and stabilization of the open form of lipases, Journal of Molecular Catalysis B: Enzymatic 19 (2002) 279-286.
[65] M.T.N. Petersen, P. Fojan, S.B. Petersen, How do lipases and esterases work: the electrostatic contribution, Journal of Biotechnology 85(2) (2001) 115-147.
[66] P. Hanoian, C.T. Liu, S. Hammes-Schiffer, S. Benkovic, Perspectives on electrostatics and conformational motions in enzyme catalysis, Accounts of Chemical Research 48(2) (2015) 482-489.
[67] P. Grochulski, Y. Li, J.D. Schrag, M. Cygler, Two conformational states of Candida rugosa lipase, Protein Science 3(1) (1994) 82-91.
[68] R. Verger, 'Interfacial activation'of lipases: facts and artifacts, Trends in Biotechnology 15(1) (1997) 32-38.

Chapter 5 : Conclusions and future directions

Hydrolysis of triglycerides is one of the most utilized processes for the modification of oils and fats. Complete hydrolysis of triglyceride esters yields fatty acids and glycerol. Fatty acids and glycerol are used as raw materials for the manufacturing of various value-added products including soaps, cosmetics, personal care products, detergents, surfactants, pharmaceuticals, lubricants, paints, resins, and other antifreeze products and alkanoamides [1]. The use of microbial lipases as biocatalyst for the hydrolysis of natural oils composed of triglyceride esters provides a green alternative to conventional chemical processes [2]. The natural substrates of lipases are long chain triacylglycerols which have low solubility in water, forming a two-phase liquid-liquid system in which the presence of the liquid-liquid interface is known to activate the lipases which catalyze the hydrolysis (interfacial enzymes). Lipases as biocatalysts are highly selective, are commercially available and requires no cofactors to operate.

The lipase chosen for this study was derived from the microorganisms *Candida rugosa* which express the enzyme which is capable of catalyzing fatty acids at all positions (sn-1,2,3) on the glycerol backbone under mild operating conditions (room temperature and neutral pH) [3, 4]. However, industrial biocatalysis application of lipases derived from *Candida rugosa* in aqueous solutions is limited often due to enzyme instabilities, slow kinetics due to mass transfer limitations, and by challenges in economic lipase (enzyme) recycling [5]. Hence, there is a need for enhancing lipase-catalyzed processes using methods such as immobilization or other process intensification techniques [6, 7]. The sourced commercial lyophilized lipase was solubilized using 0.1 M, 7.4 pH phosphate buffer saline, purified and quantified spectroscopically prior to any experimentation.

5.1. Immobilization of lipase derived from *Candida rugosa*.

Separation of unsupported lipase from reaction products and unreacted substrate requires techniques including phase separation and ultrafiltration, both of which present significant economic challenges if considered for industrial scale processes. Immobilization has been used by many researchers and industries as a tool to improve activity, selectivity, specificity, resistance to inhibitors and product purification [8]. Immobilization is the process of physically confining and localizing the enzyme onto a solid support (carrier) which may provide a more stable physical environment for the enzyme, resulting in improved recyclability, thermal and chemical stability, reducing conformational changes [9]. There are four principal methods which can be used to immobilize lipase onto solid supports: adsorption, covalent cross linking, entrapment, and membrane confinement. Among various tested methods which use different enzyme-support interactions, immobilization of lipase via physical adsorption on solid support has appeared to be simple, cheap, effective, and sustainable.

Lipases have a peculiar mechanism of action called interfacial activation. In aqueous media, a large percentage of lipase molecules have their active center covered by a polypeptide chain referred to as a lid, which may isolate it from the reaction medium (closed form). In the presence of a hydrophobic surface, the enzyme becomes adsorbed on it, fixing a new structure (so-called open form) where the active center is fully exposed, thus enabling the lipase to catalyze the substrate. This idea has been exploited to selectively immobilize many lipases on a variety of hydrophobic supports by stabilizing their open forms using hydrophobic interactions [10, 11]. Temperature, pH, and ionic strength determines the efficacy of immobilization and physicochemical properties of the support including surface area, particle size, pore structure and type of functional group present on the surface also play a vital role in lipase immobilization.

Synthetic polymers form a large and varied group of enzyme carriers with specific reference to immobilization of lipases because of their highly hydrophobic nature. Considering these aspects, three commercially available methacrylate-based polymer resins (LifeTech^{TM} ECR 1030M, ECR8285, ECR8806M) were identified and quantified for their ability to act as effective supports for lipase derived from *Candida rugosa* and their potential application in triglyceride hydrolysis were studied. **Figure 5.1** shows pictorial representation of the conclusion from the study [12].

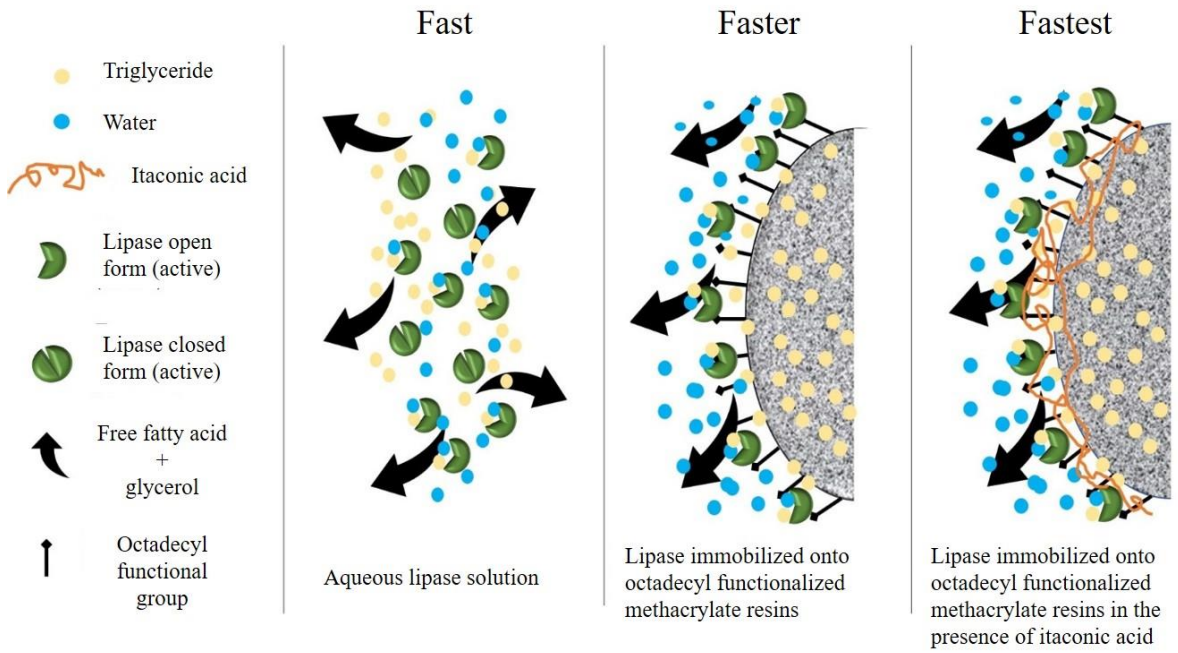


Figure 5.1. Comparative performance of lipase derived from *Candida rugosa* for triglyceride hydrolysis in aqueous solution, immobilized onto methacrylate polymer resins functionalized with octadecyl groups and in the presence of itaconic acid.

5.1.1. The main conclusions of the immobilization study are summarized as follows:

1) Octadecyl functionalized resins (ECR8806M) showed superior performance when lipase was immobilized onto them was explained in terms of high hydrophobic interactions and high oleophilic nature.
2) Epoxy & butyl groups functionalized resins (ECR8285) performed better than un-functionalized resins (ECR1030M) when lipase was immobilized. This was explained in terms of multi-point attachments via hydrophobic interaction and reactions due to epoxy groups present. Epoxy & butyl functionalized resins also displayed less hydrophobicity and oleophilic nature compared to octadecyl functionalized resins. In the case of un-functionalized methacrylate resins (ECR 1030M) significant lipase desorption and denaturation were observed during reaction.
3) All reins showed excellent recycling behavior with high cycle-cycle retention of activity at operating temperatures up to 55°C operating temperature with the exception of the un-functionalized methacrylate resins.
4) The presence of itaconic acid during the immobilization protocol revealed enhanced performance for functionalized methacrylate-based resins but had minimal effect on the performance of the un-functionalized resins [12].

5.2. Electrostatic process intensification of lipase-catalyzed triglyceride hydrolysis.

The reaction of interest in the study was lipase catalyzed hydrolysis of triglyceride esters to free fatty acids and glycerol in the oil / water system. Earlier research showed that electrostatic dispersion of aqueous solutions of microbial lipases into natural oils resulted in enhanced rates of hydrolysis. The enhancements in rate increased with respect to the magnitude of the applied external electrical field. It was assumed in previous research using electrostatically sprayed lipases

that the observed increases in hydrolysis rate were explained only in terms of the increased interfacial area of the dispersed drops resulting in faster conversion rates. Later analysis of rate data, together with independent measurement of drop sizes and dispersed phase hold-up in a spray reactor allowed calculation of the specific rate of reaction. Although the data show scatter, there was evidence that the electrical charge on the drops in the reactor enhances the specific rate of reaction (rate expressed independent of interfacial area) in addition to the overall rate. Hence, the possibility of performance enhancement due to the application of oriented external electric field (OEEF) for lipase-catalyzed triglyceride hydrolysis was exploited using lipase in aqueous media [13, 14].

5.2.1. The main conclusions of the electrostatic intensification study are summarized as follows:

1) The results of the study showed that low voltage oriented external electric fields can enhance the rate of reaction for lipase catalyzed hydrolysis of a triacyl glycerol ester. This was confirmed in three reactor systems: a quiescent liquid-liquid cell reactor; a mechanically stirred tank batch reactor, and in a recirculated tubular-flow reactor using an applied oriented external electric field voltage in the range of 0-30 V in each case.
2) The observed enhancements in the quiescent reactor (see **Figure 5.2**) of up to 60% based on conversion at 40 hours reaction time, strongly suggested that mass transfer enhancement alone does not explain the increase in conversion rate, and that the behavior of lipase in response to the oriented external electric field is a major factor contributing towards enhanced hydrolytic performance. This conclusion was drawn from the series of experiments considering the effect of oriented external electric field voltages, changes in polarity and electrode distance. Similar trends and behavior were noted in reaction data collected in the stirred tank batch electrostatic reactor and in the recirculating tubular-flow reactor when external electric fields were applied.

The observed rate data although different on account of the differences in reactor configuration, confirmed the same overall trend in terms of enhancements of overall conversion percentages.

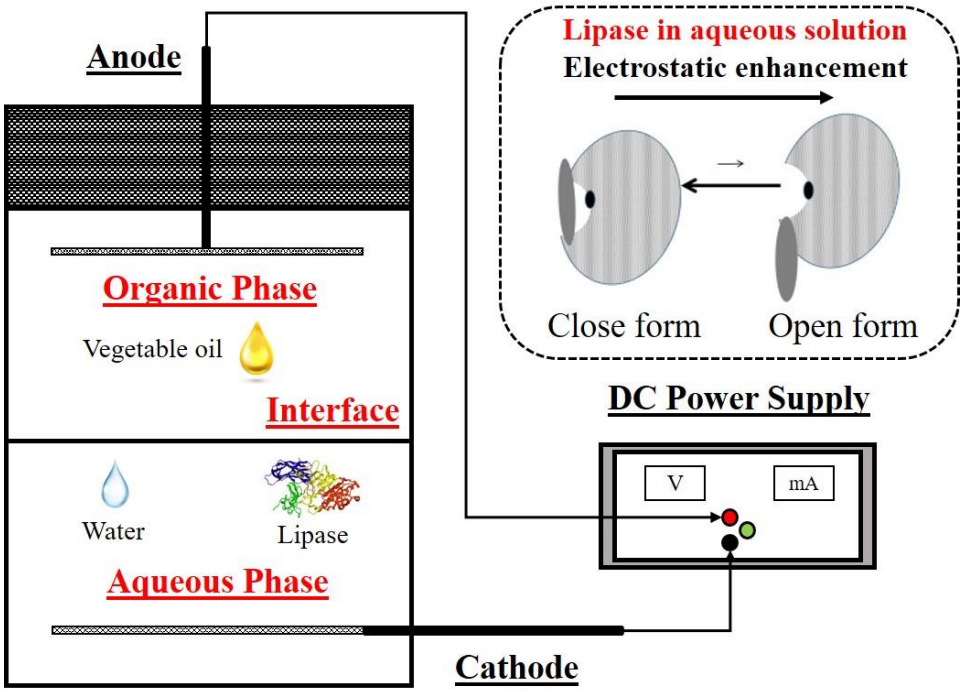


Figure 5.2. Pictorial representation of quiescent reactor showing electrostatic rate enhancement due to lipase undergoing conformational change for aqueous lipase catalyzed triglyceride hydrolysis.

3) Reaction performance in the stirred tank batch electrostatic reactor showed improved reaction performance of up 40% based conversion at 24 hours compared with that at 0 V. Reaction performance in the recirculating tubular-flow batch reactor at 60 V field showed lower reaction performance of 5% based on conversion at 3 hours than at 30 V. The lower performance at 60 V is explained by lipase inhibition associated with current flow. Overall, the applied oriented external electric field showed enhanced reaction performance in the three reactor systems studied.

4) The proposed mechanism is based on the premise that lipase undergoing field induced conformational change results in the open lid state and associated greater catalytic activity. Reaction performance using lipase immobilized on functionalized methacrylate supports in the tubular reactor showed no significant difference when compared with aqueous lipase system. Further in-depth investigation of the proposed mechanism is recommended for the future.
5) Reaction performance using lipase immobilized on functionalized methacrylate supports in the tubular reactor showed no significant difference when compared with aqueous lipase system.

5.3. Future directions.

The current study demonstrated the successful incorporation of methacrylate-based polymer resins for immobilization of lipase derived from *Candida rugosa* and the possibility of reaction enhancement due to the application of oriented external electric field (OEEF) for aqueous lipase catalyzed triglyceride hydrolysis. However, there are certain aspects and new directions that could be considered for future studies. A summary of possible future directions to the current research is listed below,

1) Hydrolysis of triglycerides was the basis for evaluating the performance of the immobilized and aqueous lipase. However, literature data suggests that lipase derived from *Candida rugosa* have shown to catalyze other reactions including transesterification and interesterification which are valuable for industrial applications [15]. The main challenge with regards to transesterification reaction is maintaining lipase stability and activity under different alcohols. Previous studies indicate that only 15% conversion was achieved when methanol was used as reactant and 72% conversion when ethanol was used as reactant [16]. Literature on interesterification using lipase derived from *Candida rugosa* is scarce. Tarnowska et al.,

interesterified goose fat and rapeseed oil mixture and showed that interesterified fats had reduced oxidative stability [17].

2) All the studies conducted were using soybean oil as the main substrate oil. However, the potential possibility of using other cheap quality oils including rapeseed oil and waste cooking oil as raw materials to convert them to value-added products can be explored [18]. Waste cooking oil costs about 60% less than fresh oils. Zaharudin et al., showed successful incorporation of immobilized lipase system for hydrolysis of waste cooking oil for environment-friendly utilization of used oils [19].
3) Previous studies on aqueous lipase catalyzed hydrolysis suggest that reaction performance and equilibrium conversions can be improved by adding water step-wise to the system [20]. This type of enhancement can also be studied for the immobilized system demonstrated in this work.
4) Presence of detergents have shown to have inhibitory effects on aqueous lipase catalyzed hydrolysis process, however immobilized systems have been shown to have negligible effects [21]. The possible effect of presence of various detergents and other inhibitory chemicals can be tested for immobilized lipase catalyzed processes using methacrylate functionalized resins.
5) Addition of enzyme-friendly solvents including ionic liquids to modify properties of the oil phase to enhance the reaction [22]. Stability issues is one of the major concerns in using ionic liquids as solvent for lipase-based processes. Nascimento et al., showed successful incorporation of lipase derived from *Burkholderia cepacian* in obtaining aroma esters using organic solvent/ionic liquid [23].
6) Droplet size plays a major role in determining the performance of lipase-catalyzed reactions. In the presented work, the droplet size has been kept consistent however techniques such as

phase doppler particle analysis (PDPA) and laser diffraction could be used to analyze and change droplet size and the reaction performance could be evaluated [24].

7) The possibility of using zeolites [25], activated carbon [26] or ion-exchange resins [27] for continuous in-situ removal of fatty acids to shift equilibrium conversion thereby increasing the reaction performance can be explored. Khedkar et al., showed effective selective batch adsorptive separation of fatty acids including oleic acid, linoleic acid and linolenic acid using silver ion-chromatography [28].
8) The quest for finding an alternative support should be continued by analyzing various ecofriendly adsorbents such as activated carbon in the form of coconut shells, wood, and bamboo and naturally occurring zeolites [29].
9) The main mechanism driving the positive performance enhancement for lipase catalyzed triglyceride hydrolysis using oriented external electric field (OEEF) was noted to be conformational change leading to more open lid formations. All the study demonstrated was under 60 V using 0.25 %wt/v of aqueous lipase solution. The effect of higher voltages (above 60 V) and varying concentrations of aqueous lipase to be studied and optimized.
10) Evaluation of other lipases or combination of lipases in the presence of electric fields with additional focus on isomeric and chirality selectivity could be performed and compared [30].

5.3.1. Kinetic modeling, technoeconomic analysis and lifecycle assessment.

Fundamental mathematical kinetic modeling using first order reaction kinetics with the help of Michalis-Menten kinetics and Lineweaver-Burk plot are to be incorporated and the rate constants are to be calculated for both immobilized systems and systems involving external electric field. Other models that make use of bisubstrates such as ternary complex random order model and Ping-Pong bi-bi model are to be investigated and applied for the systems presented in this

dissertation [31]. Comparison of the research with commercial process will provide insights into optimization of support performance, operating design, and process design. This can be used as a basis in evaluating the process economics i.e., to calculate total capital investment and total production investment which are based on equipment, utility, supports and chemical cost. Life cycle assessment and energy audit of potential scale-up of the systems described in the dissertation are to be compared with current thermal technology.

5.4. References.

[1] A. Dijkstra, G. Van Duijn, Vegetable oils: oil production and processing, Encylopedia of Food and Health (2016).

[2] P. Anuradha, D.J. Rani, K. Vijayalakshmi, S.A. Pasha, S. Kamakshi, Microbial lipases: a potential tool for industrial applications, Journal of Pure and Applied Microbiology 3(1) (2009) 301-306.

[3] S. Benjamin, A. Pandey, Candida rugosa lipases: molecular biology and versatility in biotechnology, Yeast 14(12) (1998) 1069-1087.

[4] P.D. De Maria, J.M. Sánchez-Montero, J.V. Sinisterra, A.R. Alcántara, Understanding Candida rugosa lipases: an overview, Biotechnology Advances 24(2) (2006) 180-196.

[5] A. Anand, L.R. Weatherley, The performance of microbial lipase immobilized onto polyolefin supports for hydrolysis of high oleate sunflower oil, Process Biochemistry 68 (2018) 100-107.

[6] A. Anand, Enhanced Lipase-Catalyzed Hydrolysis and Modification of Fats and Oils, 2018 AIChE Annual Meeting, AIChE, 2018.

[7] A. Anand, P. Gnanasekaran, A.M. Allgeir, L. Weatherley, Immobilization and kinetic studies of a microbial lipase from the fungal organism Candida rugosa on methacrylate polymer resins, 2019 AIChE Annual Meeting, AIChE, 2019.

[8] P. Bailie, S. McNerlan, E. Robinson, W. Murphy, Immobilization of lipases for the hydrolysis of fats and oils, Food and Bioproducts Processing: Transactions of the Institution of Chemical Engineers, Part C (1995).

[9] S. Datta, L.R. Christena, Y.R.S. Rajaram, Enzyme immobilization: an overview on techniques and support materials, 3 Biotechnology 3(1) (2013) 1-9.

[10] A. Basso, M. Hesseler, S. Serban, Hydrophobic microenvironment optimization for efficient immobilization of lipases on octadecyl functionalised resins, Tetrahedron 72(46) (2016) 7323-7328.

[11] E.A. Manoel, J.C. dos Santos, D.M. Freire, N. Rueda, R. Fernandez-Lafuente, Immobilization of lipases on hydrophobic supports involves the open form of the enzyme, Enzyme and Microbial Technology 71 (2015) 53-57.

[12] A. Anand, P. Gnanasekaran, A.M. Allgeier, L.R. Weatherley, Study and deployment of methacrylate-based polymer resins for immobilized lipase catalyzed triglyceride hydrolysis, Food and Bioproducts Processing (2020).

[13] L. Weatherley, D. Rooney, M. Niekerk, Clean synthesis of fatty acids in an intensive lipase-catalysed bioreactor, Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology 68(4) (1997) 437-441.

[14] J. Petera, L. Weatherley, D. Rooney, K. Kaminski, A finite element model of enzymatically catalyzed hydrolysis in an electrostatic spray reactor, Computers & Chemical Engineering 33(1) (2009) 144-161.

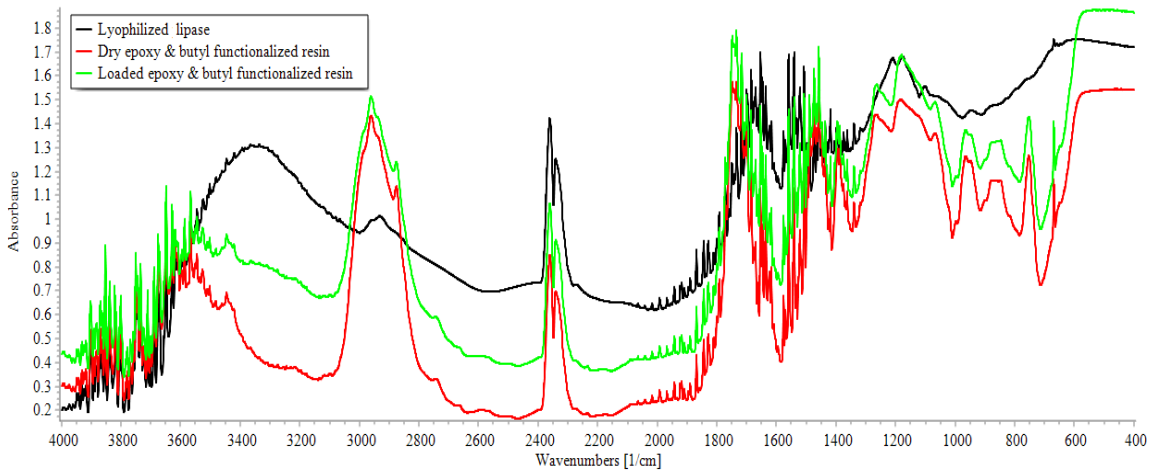
[15] E. Vanleeuw, S. Winderickx, K. Thevissen, B. Lagrain, M. Dusselier, B.P. Cammue, B.F. Sels, Substrate-specificity of Candida rugosa lipase and its industrial application, ACS Sustainable Chemistry & Engineering 7(19) (2019) 15828-15844.

[16] R. Abigor, P. Uadia, T. Foglia, M. Haas, K. Jones, E. Okpefa, J. Obibuzor, M. Bafor, Lipase-catalysed production of biodiesel fuel from some Nigerian lauric oils, Portland Press Ltd., 2000.

[17] K. Tarnowska, M. Kostecka, A. Piotrkowicz, M. Łobacz, B. Kowalski, Interesterification of goose fat and rapeseed oil mixture using Candida rugosa lipase immobilized in alginate beads, Riv Ital Sostanze Grasse 90 (2013) 95-105.
[18] M. Lopes, S.M. Miranda, J.M. Alves, A.S. Pereira, I. Belo, Waste cooking oils as feedstock for lipase and lipid-rich biomass production, European Journal of Lipid Science and Technology 121(1) (2019) 1800188.
[19] N.A. Zaharudin, R. Rashid, L. Azman, S.M.M. Esivan, A. Idris, N. Othman, Enzymatic hydrolysis of used cooking oil using immobilized lipase, Sustainable Technologies for the Management of Agricultural Wastes, Springer (2018) pp. 119-130.
[20] R. Sharma, Y. Chisti, U.C. Banerjee, Production, purification, characterization, and applications of lipases, Biotechnology Advances 19(8) (2001) 627-662.
[21] D. Kübler, A. Bergmann, L. Weger, K.N. Ingenbosch, K. Hoffmann-Jacobsen, Kinetics of detergent-induced activation and inhibition of a minimal lipase, The Journal of Physical Chemistry B 121(6) (2017) 1248-1257.
[22] J. Gorke, F. Srienc, R. Kazlauskas, Toward advanced ionic liquids. Polar, enzyme-friendly solvents for biocatalysis, Biotechnology and Bioprocess Engineering 15(1) (2010) 40-53.
[23] M. da Graça Nascimento, J.M.R. da Silva, J.C. da Silva, M.M. Alves, The use of organic solvents/ionic liquids mixtures in reactions catalyzed by lipase from Burkholderia cepacia immobilized in different supports, Journal of Molecular Catalysis B: Enzymatic 112 (2015) 1-8.
[24] R. Sijs, S. Kooij, H. Holterman, J. van de Zande, D. Bonn, Drop size measurement techniques for sprays: Comparison of image analysis, phase Doppler particle analysis, and laser diffraction, AIP Advances 11(1) (2021) 015315.
[25] O. Ilgen, H.S. Dulger, Removal of oleic acid from sunflower oil on zeolite 13X: Kinetics, equilibrium and thermodynamic studies, Industrial Crops and Products 81 (2016) 66-71.
[26] M. Vadi, E. Ghaseminejhad, Comparative study of isotherms adsorption of Oleic acid by activated carbon and multi-wall carbon nanotube, Oriental Journal of Chemistry 27(3) (2011) 973.
[27] O. Ilgen, Adsorption of oleic acid from sunflower oil on Amberlyst A26 (OH), Fuel processing technology 118 (2014) 69-74.
[28] M.A. Khedkar, S.R. Satpute, S.B. Bankar, P.V. Chavan, Adsorptive Removal of Unsaturated Fatty Acids Using Ion Exchange Resins, Journal of Chemical & Engineering Data (2020).
[29] J. Liu, R.T. Ma, Y.P. Shi, "Recent advances on support materials for lipase immobilization and applicability as biocatalysts in inhibitors screening methods"-A review, Analytica chimica acta 1101 (2020) 9-22.
[30] R.C. Rodrigues, M.A.Z. Ayub, Effects of the combined use of Thermomyces lanuginosus and Rhizomucor miehei lipases for the transesterification and hydrolysis of soybean oil, Process Biochemistry 46(3) (2011) 682-688.
[31] D. Mukesh, S. Jadhav, A.A. Banerji, K. Thakkar, H.S. Bevinakatti, Lipase-Catalysed Esterification Reactions—Experimental and Modelling Studies, Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology 69(2) (1997) 179-186.

Supplementary Information

[A] Epoxy and butyl functionalized methacrylate supports (ECR8285)



[B] Octadecyl functionalized methacrylate supports (ECR8806M)

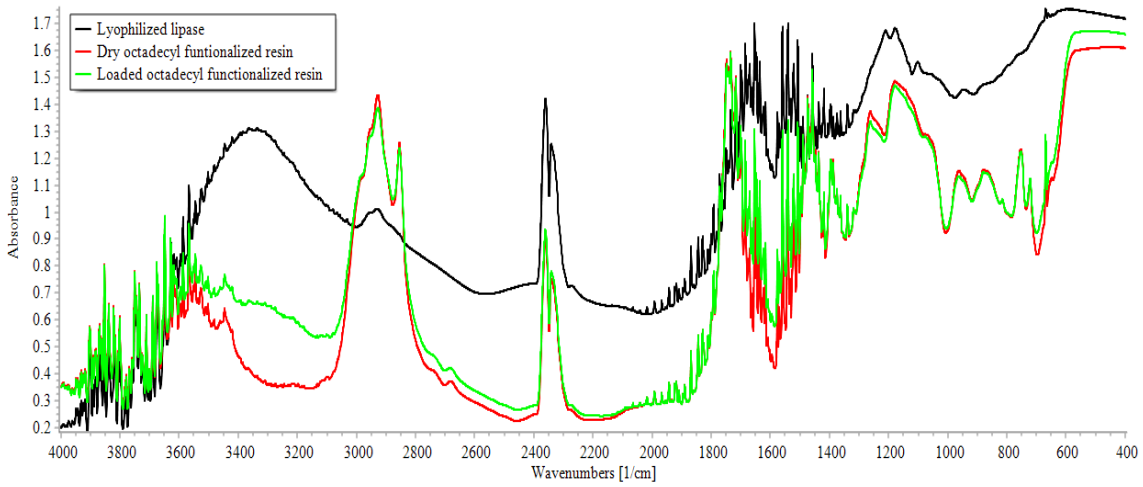


Figure S1**.** FT-IR spectra for surface functionalized methacrylate polymer supports compared with lyophilized lipase, dry polymer supports, and polymer supports loaded with 0.25 %wt/v immobilized lipase.

Calculation leading up to percent conversion and uncertainty.

Nomenclature:

Dimensions are in terms of mass (M), time (t), length (L) and temperature (T)

FFA – free fatty acid

NaOH – sodium hydroxide

VO – vegetable oil

H_2O – water

l – lipase

S – sample

R – reactor

r – reactants

i – initial

t – time

M_α – total number of moles of species α

m_α – total mass of species α

MW_α – molecular weight of species α, M/mole

c_α – molar concentration of species α, moles/L^3

V_α – volume of species α, L^3

Equations:

The moles of free fatty acid produced is directly proportional to the amount of sodium hydroxide consumed,

$$M_{FFA_{t=t}}(S) = V_{NaOH_{t=t}}(S) * c_{NaOH}$$

$$M_{VO_{t=i}}(R) = m_{VO_{t=i}}(R)/MW_{VO}$$

For quiescent batch reactor,

$$M_{VO_{t=t}}(R) = M_{VO_{t=i}}(R) - [(^{M_{FFA_{t=t}}(S)}/_{3}) * (m_{VO_{t=i}}(R)/m_{S_{t=t}})]$$

Note: In this case only the oil phase is sampled

For stirred tank and recirculating tubular flow batch reactor,

$$M_{VO_{t=t}}(R) = M_{VO_{t=i}}(R) - [(^{M_{FFA_{t=t}}(S)}/_{3}) * (m_{r_{t=i}}(R)/m_{S_{t=t}})]$$

$$m_{r_{t=i}}(R) = m_{VO_{t=i}}(R) + m_{H2O_{t=i}}(R)$$

Note: In this case the weight of catalyst is assumed to be negligible

The overall percent conversion for quiescent, stirred tank and recirculating tubular-flow batch reactors is given by,

$$\%\ conversion = \left(1 - \frac{Mc_{VO_{t=t}}(R)}{Mc_{VO_{t=i}}(R)}\right) * 100$$

Uncertainty is calculated based under the assumption that the only contribution is the titration volume,

For quiescent batch reactor,

$$uncertainity = [{}^{(M_{FFA_{t=t}}(S)_{STDev} * m_{VO_{t=i}}(R)/3 * m_{S_{t=t}})}\Big/_{M_{VO_{t=i}}(R)}] * 100$$

For stirred tank and recirculating tubular flow batch reactor,

$$uncertainity = [{}^{(M_{FFA_{t=t}}(S)_{STDev} * m_{r_{t=i}}(R)/3 * m_{S_{t=t}})}\Big/_{M_{VO_{t=i}}(R)}] * 100$$