Chapter 1

Introduction and Objectives

1.1 Introduction

Natural resources such as petroleum, coal are getting depleted for their increasing consumption. Besides, chemical compounds from these sources and the processes for their production are most often hazardous and add to environmental problems. Therefore, there is a need for searching environment friendly or 'green' processes and products, which will cause fewer hazards to the environment, consume less energy and will be renewable also. Oils from plant sources i.e., vegetable oils are perfect sources of such 'green' products as they are renewable in nature. Their products have the same quality compared to those from conventional sources but cause no harm to environment. Fatty acid, the main component of oleochemical industry, is one class of such industrially important compound. Various fatty acids are obtained by the hydrolysis of different vegetable oils. There are some conventional hydrolysis processes which are used in the oleochemical industry for quite a long time. But these processes have some serious disadvantages like disposal of by-products which are not eco-friendly in nature, consumption of high amount of energy and sometimes occurrence of unwanted properties like colour, odour in the final product. On the other hand, hydrolysis using biocatalysts like enzymes can be free from such disadvantages. Moreover, enzymes like 'lipase' sometimes possess excellent property of selectivity. This property helps to achieve excellent product purity which is impossible to attain in conventional processes. Two examples of such highly useful fatty acids are ricinoleic acid (a hydroxy monounsaturated fatty acid) and erucic acid (a monounsaturated fatty acid).

1.2 Conventional oil hydrolysis processes

Ricinoleic acid and erucic acid can be obtained by different methods of hydrolysis of castor oil and mustard oil, respectively.

Some conventional methods used for hydrolysis of oil are described below.

1.2.1 Base catalyzed oil hydrolysis

In this method, oil was hydrolyzed by water in presence of hot NaOH at temperature 70–100 °C. On completion of hydrolysis, HCl was slowly added for acidification. Product (fatty acid) from castor oil generated characteristic odour and coloration (Bednarcyk and Erickson, 1973). It required neutralization of excess mineral acid and disposal of large amount of salts (Johnson and Fritz, 1989).

1.2.2 High temperature steam process for oil hydrolysis

In this method oil was heated to temperature 150–350 °C at relatively low pressure, and then it was perturbed with heated steam. This method was not suitable as it was energy intensive and formed unwanted by-product like ricinoleic acid estolides during hydrolysis of castor oil (Puthli et al., 2006; Piazza and Farrell, 1991).

1.2.3 Colgate-Emery steam splitting process

In single stage splitting process, oil and water were fed in a countercurrent manner into a continuous flow column at temperature upto 260 ºC and pressure 60 bar. Sufficient dissolution of water occurred in oil phase without any mechanical agitation due to high operating temperature. The fat (oil) was introduced by a high pressure feed pump through a sparge ring placed about 3 feet from the bottom to top. Water, at a weight ratio of 40–50% of the oil, was introduced near the top of the column. The void volume of column was used as reaction chamber. The oil went upward through the hot glycerol-water collecting section and passed through the oil–water interface into the continuous phase where hydrolysis occurred. On injection of high pressure steam, temperature quickly increased to 260 ºC. On splitting glycerol was formed and dissolved in the water phase. Thus it went out from reaction zone. As a result, equilibrium shifted towards product and yield increased. It achieved 98–99% conversion in 1–3 h (Riegel and Kent, 2003).

But this process had certain disadvantages. High temperature and pressure could lead to possible denaturation of product and formation of unwanted side products in some cases. Enormous energy cost (790 MJ/Kg of oil split) due to high temperature and pressure was also a serious disadvantage of this process (Sonntag, 1989). Again, this process was not applicable to splitting of heat sensitive triacylglycerols like oils containing fatty acids with conjugated double bonds i.e., highly unsaturated triacylglycerols, unconjugated systems capable of thermal conjugation, hydroxy-containing fats and oils like castor oil (Vulfson, 1994; Sonntag, 1979).

1.2.4 Twitchell process

The mixture of oil and water was heated at temperature 150–250 ºC and 10–25 bar pressure. It took 6–10 h to split depending on nature of oil. Higher the average molecular weight of oil, slower was the splitting. Splitting was enhanced on increasing temperature to 225–260 ºC. It achieved 95% conversion in 8–24 h (Riegel and Kent, 2003). The long reaction time and possible denaturation of product were some of the disadvantages of this process.

These conventional processes along with some other specific processes were used by some researchers to obtain the two important fatty acids, namely, ricinoleic acid and erucic acid.

1.2.5 Some conventional processes for castor oil hydrolysis to ricinoleic acid

In earlier study, some conventional proceses were used to hydrolyze castor oil. A brief outline of each of these studies is given below.

1.2.5.1 Hydrolysis with Twitchell reagent

In castor oil hydrolysis by the Twitchell process, byproduct estolide made the catalyst less soluble than usual in the fat phase. Under these conditions 45% of the catalyst was detected in the aqueous phase. There was estolide formation during the splitting of castor oil using Twitchell reagent though there was 93% splitting in just 6 h

(Kallyanpur et al., 1967a). In presence of Twitchell reagent, rate of castor oil hydrolysis was much lower than those of other oils (Majid et al., 1980)**.**

1.2.5.2 High pressure splitting

In an earlier work (Kallyanpur et al., 1967b), non-catalytic high-pressure splitting of hydrogenated castor oil was studied at temperatures 225, 250, and 300 °C. At 225 °C, hydrolysis takes place followed by an inter-esterification reaction to form estolides. At 250 °C, monoethenoid acids also formed at a slow rate besides inter-esterification. At 300 °C, all the reactions occurred in shorter time resulting in products consisting predominantly of monoethenoid acids. Intramolecular dehydration (Priest and von Mikusch, 1940) and intermolecular dehydration at temperatures above 275 °C were examples of autoreactions of ricinoleic acid. In intermolecular dehydration, the hydroxyl groups condensed with a free carboxyl group to form estolide, ultimately leading to polycondensates.

In another work (Lakshminarayana et al., 1984a), variations in pressure (20–40 kg/cm^2), weight ratio of oil and water (1:0.4 to 1:1) and time (1–10 h) on hydrolysis of castor oil were studied. In case of high pressure reaction, 92% hydrolysis was achieved in 8 h at 40 kg/cm^2 pressure and 1:1 weight ratio. In two stage process, 96% hydrolysis was obtained at 20 kg/cm^2 pressure and 1:0.4 weight ratio in 10 h. Increase in pressure and reaction time was associated with decrease in weight ratio and increased extent of hydrolysis.

1.2.5.3 Cold saponification

Cold saponification of castor oil led to incomplete reaction (Hawke, 1949a; 1949b) and usual saponification with alkali under reflux yielded a partially interesterified product, coupled with insignificant dehydration (Hawke and Kohll, 1959a; 1959b).

1.2.6 Some conventional processes for oil hydrolysis yielding erucic acid

A brief description of some conventional proceses to hydrolyze cruciferae oils to isolate erucic acid is presented below:

1.2.6.1 Alkaline saponification followed by acidification

Alkaline saponification of high erucic acid (HEA) seed oil followed by acidification of the solution led to formation of a free fatty acid (FFA) mixture containing erucic acid as a component. In this study, expression, clarification, degumming and bleaching steps were used to prepare oil from crambe seed. The warm bleached oil (at 50 ºC) was saponified with 2M NaOH solution and refluxed at 108–112 ºC for 6 h. After this, the solution was cooled to obtain homogeneous, milky liquid with a heavy, white, soapy upper layer. Then the contents were further cooled at 23 °C for 1 h and at 6 °C for 4 h. The cake of soap stock was collected and acidified with 30% (w/w) hydrochloric acid until pH 1 was reached. A brownish yellow mixture of free fatty acid (FFA) and acid liquor was produced which was subsequently cooled for 1 h at 23 °C and 4 h at 6 °C to produce two well-separated phases without emulsion. The upper FFA layer was separated and dissolved in 95% ethanol (6 ml ethanol/g FFA) at 38–42 °C on a steam bath. Next, it was cooled to the crystallization temperature (-14 °C) and was allowed to crystallize overnight. The crystallization step was repeated. A final product of 365 g erucic acid per kg bleached oil with 87.1±1.4% purity was obtained (Vargas-Lopez et al., 1999).

1.2.6.2 Colgate-Emery steam splitting process followed by fractional distillation

Fat splitting process coupled with fractional distillation was earlier used as a method to isolate erucic acid from high-erucic acid oils (Hou, 2005). But it had certain disadvantages like high energy consumption. Colgate-Emery steam splitting process followed by fractional distillation was employed to produce erucic acid from high erucic acid yielding cruciferae oils. But, the reaction resulted in extensive degradation of product fatty acids. As for example, in fractional distillation, chain cleavage of erucic acid occurred on heating above ordinary distillation temperatures (Brady et al., 1988).

1.2.6.3 Crystallization

There are some other earlier crystallization methods specifically for preparation of erucic acid. Rapeseed oil acid in acetone (10% solution) was cooled for 16 h at -20 °C. Next, crystalline solids were separated by filtration. The filtrate was discarded while the

crystalline part was redissolved in acetone to make a 10% solution. The same crystallization procedure was repeated. The yield of erucic acid on the basis of total organic acids was 42.5% (Pasero et al., 1959).

In another method, solution containing 125 g mixed acids from rapeseed oil was dissolved in 1 litre acetone and cooled to 0 °C. After that, saturated acids were removed by filtration and the filtrate was cooled to -10 °C for 20 min. After filtration, the precipitate was collected. A 10% solution of this precipitate in acetone was prepared and then cooled to -15 °C. The precipitate from this crystallization was collected again and dissolved in sufficient acetone to make a 6% solution. This solution was again cooled to -15 °C to allow crystallization of erucic acid leading to 26 g erucic acid. As rapeseed oil contained 40–50% of erucic acid in its fatty acid profile, 26 g was equivalent to 42–52% of the desired acid (Taüfel et al., 1962).

Direct crystallization of the mixed acid soaps from 90% methanol followed by three recrystallization steps at 15 °C led to more than 90% erucic acid crystal. Up to 48% recoveries were reported depending on the original composition (Chobanov et al., 1965).

In another method, a low-temperature (−11 °C) crystallization from aqueous ethanol (ethanol/water, 3:1 v/v) was used and solvent requirement was 4.0–5.8 ml/g FFA. The third crystallization produced crystals containing 76% erucic acid with a recovery of 66% whereas starting material derived from crambe oil contained 56% erucic acid (Hagemann et al., 1962).

Processes like “silicalite” adsorption, aqueous surfactant separation and chromatography (Wilson and Sargent, 2001; Painuly and Grill, 1992; Sonntag, 1991; Breuer et al., 1987) were also employed for erucic acid production from vegetable oils.

1.3 Biocatalysis

1.3.1 Advantages of biocatalysis over conventional catalysis

In comparison with the conventional processes, biocatalysis has some distinct advantages (Bommarius and Riebel, 2004; Bailey and Ollis, 1986; Laidler and Peterman, 1979) which are given below:

i) Biocatalysts (enzymes) operate at or slightly above ambient temperature under atmospheric pressure and a moderate range of pH whereas conventional processes often involve high temperature or pressure or sometimes both. Most often biocatalysis occurs in aqueous media.

ii) Selectivity of enzyme is quite high leading to higher yield of desired product and lower amount of unwanted by-products or waste compared to conventional processes. This indicates that biocatalysis becomes an environmentally friendly alternative to the existing processes. Thus, biocatalysts are quite preferred for 'green chemistry'.

iii) It is observed that enzymes are more active than conventional catalysts, i.e., these lead to higher rate of reaction in most of the cases. Enzymes show high activity at ambient temperatures by catalyzing reactions faster than almost all synthetic catalysts. At increased reaction temperature, activity of synthetic catalysts may be comparable to highest activity of enzymes. With increasing temperature, enzyme activity does not increase continuously; rather at quite a low temperature, it actually loses nearly all of its activity.

iv) Enzyme catalysis is susceptible to be controlled by small molecules like detergents or the end product of a sequence of reactions in which it acts as a catalyst.

1.3.2 Similarities of biocatalysts with conventional catalysts

Biocatalysts possess certain similarities with conventional catalysts and these are (Bailey and Ollis, 1986):

i) Both biocatalysts and synthetic catalysts form complex with the reactant molecules. This leads to high similarity in rate expressions, sometimes these expressions are of identical forms.

ii) Both types of (synthetic and biological) catalysts gradually lose activity with progress in chemical reactions. Catalytic properties of enzymes can easily be destroyed by disturbing its native conformation through a moderate change in reaction conditions.

Among various enzymes, 'lipase' is the most suitable enzyme for hydrolysis of vegetable oil to produce valuable fatty acids.

1.4 Lipase

According to the 'Enzyme Commission (EC)' of 'International Union of Pure and Applied Chemistry (IUPAC)', enzymes are broadly classified based on their mode of action as (Kuchel, 1997):

i) Oxidoreductase: These catalyze oxidation-reduction reaction, where substrate is a hydrogen or electron donor.
ii) Transferase: These catalyze the transfer of a specific group of general form: $A + B\text{-}X \rightarrow A\text{-}X + B$
iii) Hydrolase: These catalyze hydrolytic cleavage of C-O, C-N, C-C bonds and other bonds.
iv) Lyase: These catalyze cleavage (non-hydrolytic) of C-O, C-N, C-C bonds and other bonds (except double bonds).
v) Isomerase: These catalyze change of geometrical (or spatial) arrangement of a molecule.
vi) Ligase: These catalyze joining of two molecules by formation of a covalent bond.

According to 'Enzyme Commission', lipase enzyme falls in the class of 'hydrolase' (i.e., class iii) and is designated as 'triacylglycerol acylhydrolase' (numbered as EC 3.1.1.3). Lipases are produced by living systems in order to hydrolyze mostly fats and oils. These can be defined as enzymes hydrolyzing the emulsified esters or

triacylglycerols at an oil–water interface (Desnuelle, 1961). Lipases can be of different origin like plant, animal, microbial (bacterial, fungal etc.) etc. It has wide range of properties depending on its source. Examples of 'plant lipase' are castor bean lipase, oat grains lipase, wheat grains lipase, cottonseed lipase, barley and malt lipase, rice bran lipase, corn lipase etc. (Kausar, 1981). Some examples of animal lipases are porcine pancreas lipase, lipoprotein lipase, etc. Examples of microbial lipases are lipases from *Candida rugosa, Pseudomonas cepacia, Aspergillus niger, Geotrichum candidum* etc.

Positional specificity, fatty acid specificity, thermostability are the main properties of lipase. Lipases can be enantioselective, chemoselective and regioselective (Vulfson, 1994). According to positional selectivity, lipase can be classified as regioselective (1, 3-selective) and non- regioselective. Some lipases have specific affinity towards specific fatty acids. Each lipase has its optimum pH. Thermostabilities of different lipases are different and so each lipase possesses optimum temperature (Yamane, 1987).

On the basis of inhibition study of lipase activity by chemical modofication, it is concluded that they can be classified as serine hydrolases i.e., all the lipases has 'serine' at its active site (Antonian, 1988). A wide range of reactions like hydrolysis, acidolysis, aminolysis, esterification, interesterification and alcoholysis are catalyzed by lipases. Lipases catalyze hydrolysis of vegetable oil or triacylglycerol (TAG) molecules to form free fatty acid(s) and glycerol. The reaction is reversible in nature and depends on water content of media. They catalyze esterification, interesterification and transesterification reactions when water content of medium is low (Sheldon, 1993).

The basic difference between lipase and esterase (another type belonging to 'hydrolase') is the unique ability of lipase in acting at the interface between aqueous and non-aqueous (lipid) phase (Schmidt and Verger, 1998; Verger, 1997). Its catalytic activity depends mainly on the aggregated state of substrates (particularly water immiscible substrates). Lipases are made and released from pancreas for mammals. The pancreatic lipase is released into small intestine, where it preferably attacks emulsified triacylglycerols leaving those present in the wall of pancreas itself. This supports the

hypothesis that lipase needs oil–water interface to get activated. From experimental data, it is quite clear that the activation involves opening of lid over active site by conformational changes which occurs only in presence of oil–water interface (Kazlauskas and Bornscheuer, 1998).

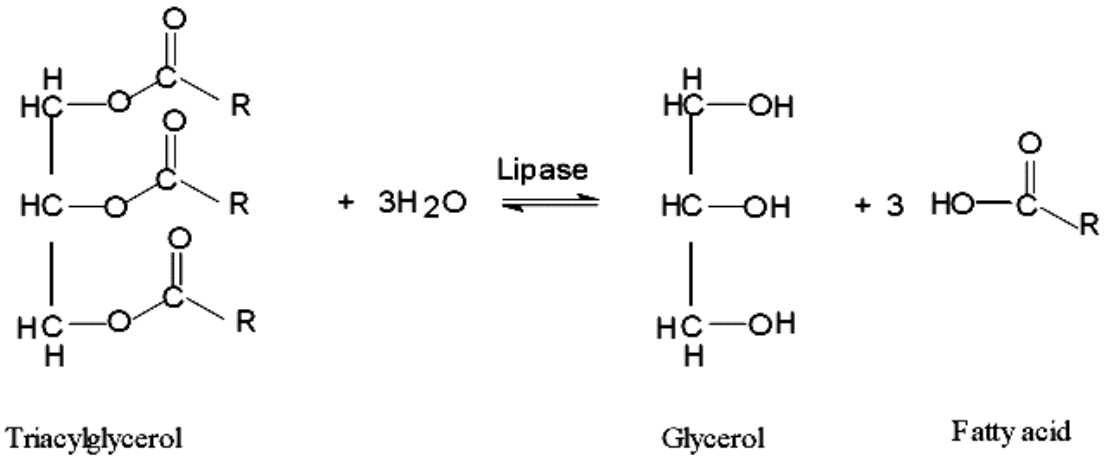

Fig. 1.1: Scheme of the hydrolysis reaction of triacylglycerol using lipase.

1.4.1 Advantages of lipase

There are several advantages of lipases over conventional catalysts (Paiva et al., 2000; Benjamin and Pandey, 1998; Gunstone, 1968).

i) Lipases possess higher catalytic efficiency due to much lower activation energy. So, it needs mild reaction conditions of temperature and pH leading to much lower energy requirements and thermal degradation of reaction products.

ii) Lipases have much higher ability than inorganic catalysts to discriminate among different types of compounds. This ability helps in producing high value added products. Besides, side reactions are fewer leading to cleaner products. Characteristics like substrate specificity, positional specificity and stereospecificity are responsible for such unusual ability.

a) Substrate specificity: Rate of lipolysis of triacylglycerol, diacylglycerol and monoacylglycerol are different. Some lipases have preference for specific fatty acids. As for example, *Candida rugosa* and *Geotrichum candidum* lipase have preference for

18-carbon fatty acids. Some lipases differentiate between fatty acids with different double bond positions. Production of Δ4, Δ5 and Δ6 fatty acids are significantly slower than Δ9 acids when *Geotrichum candidum* lipase is employed (Jensen, 1974) in both the cases (Δx means first double bond occurs at x-th carbon from carboxyl group of fatty acid).

b) Positional specificity: Some lipases like porcine pancreas lipase and *Rhizopus arrhizus* lipase prefer primary ester bonds for hydrolysis much more than secondary ester bonds. Some lipases like *Candida rugosa* hydrolyzes all three esters i.e., these lipases do not have positional specificity in hydrolysis.

c) Stereospecificity: This signifies faster hydrolysis of one primary sn-ester as compared to its counterpart. Fatty acids at different positions of glycerol backbone (sn-1 and sn-3 rather than sn-2) can be selectively cleaved. Hydrolysis at sn-1 position is faster than sn-3 and that at sn-2 is slowest.

1.4.2 Applications of lipase

Lipases have a variety of applications in different fields (Barredo, 2005; Villeneuve and Foglia, 1997; Kotting and Eibl, 1994; John and Abraham, 1991; Farooqui et al., 1987).These are:

i) It has wide application in oleochemical industries like in hydrolysis of fats and oils to produce fatty acids, glycerol, diacylglycerols and monoacylglycerols.
ii) It is used in transesterification to produce cocoa butter and margarine.
iii) In biosurfactant synthesis lipase has several applications.
iv) Food and dairy industry uses lipase in hydrolysis of milkfat, cheese ripening, modification of butterfat etc.
v) Pharmaceutical industry applies lipase in preparation of naproxen, ibuprofen.
vi) Lipase is used in agrochemical industry for preparation of insecticides and pesticides.
vii) Cosmetics industry applies lipase to synthesize emulsifiers and moisturizing agents.
viii) Lipase is used in beverages industry to improve aroma of beverages.
ix) The enantioselectivity property of lipase is in chemical industry to make chiral building blocks and chemicals.

x) Lipases are used to remove pitch (hydrophobic content of wood, mainly triacylglycerols and waxes). Lipase from *Candida rugosa* is used by Nippon Paper Industries to remove such compounds to the extent of 90%.

xi) Alkaline lipases from bacillus strains are used in leather industries to remove components causing environmental pollution.

xii) Lipase is used in meat and fish industries also.

xiii) Lipase is used in detergents to remove oily and greasy soils and contribute to making the detergent particularly useful at lower wash temperatures.

xiv) Lipase has medical relevance, particularly to atherosclerosis and hyperlipidemia.

xv) Lipases are used in resolution of racemic mixtures of organic compounds.

1.5 Limitations of earlier study

There are several studies of lipase catalyzed hydrolysis of vegetable oils among which some studies concentrated particularly on hydrolysis of castor oil and mustard oil. But systematic optimization of process variables to maximize production of ricinoleic acid from castor oil and erucic acid from mustard oil was not studied anywhere.

1.6 Limitations of lipase catalyzed hydrolysis of vegetable oil

There are certain limitations of lipase catalyzed hydrolysis of vegetable oil.

i) The accumulation of more surface active monoacylglycerol and fatty acid (products in different stages of hydrolysis) replace less surface active lipase (enzyme) and triacylglycerol (substrate) from oil–water interface (the place for reaction). This in turn decreases interaction between lipase and triacylglycerol leading to low conversion (Reis et al., 2009).

ii) With increasing speed of agitation, extent of denaturation of lipase increases leading to decrease in its activity and simultaneous decrease in hydrolysis (Sadana, 1991).

iii) At higher temperature and extreme pH, lipase gets denatured to a large extent (Laidler and Peterman, 1979; Tipton and Dixon, 1979).

1.7 Processes to overcome these limitations

There are certain processes to overcome the above mentioned limitations.

i) Immobilization of lipase on specific support can retain activity at adverse process conditions and make it suitable for reuse.
ii) Addition of metal ions can lead to formation of metal salts of fatty acids which gets solubilized in aqueous phase and thus lipase can avail higher interfacial area.
iii) Different additives and organic solvents can enhance conversion.
iv) Surfactant/organic solvent/buffer ternary system can form reverse micelle leading to enhanced conversion.
v) Systematic optimizations of lipase catalyzed hydrolysis processes can be carried out.
vi) Addition of surfactant can enhance production of fatty acid by forming emulsions or micelles.

1.7.1 Immobilization

There are different methods of immobilization of lipases and these are, i) physical adsorption method employing weak interaction forces; ii) mechanical containment of lipase like microencapsulation, containment in reverse micelles and entrapment in hollow fibre membrane; iii) chemical linkage in which covalent bond is formed between lipase and the membrane by active bridge molecules like cyanogen bromide (CNBr) and bifunctional reagents like glutaraldehyde. This method can further be classified as random or site specific. It can be performed by cross linking, ion-exchange or by covalent bonding. Denaturation of lipase may occur in case of random linkage. But, activity of lipase is increased in site specific immobilization as orientation of the active sites is uniform. Development of latter method needs techniques of molecular biology (Pugazhenthi and Kumar, 2004; Villeneuve et al., 2000; Bailey and Ollis, 1986). Various carriers like polyvinyl chloride (PVC), agarose, celite, silica gel, chitin, chitosan, kieselguhr, and poly (styrene-divinylbenzene) copolymer are used as support for immobilization (Villeneuve et al., 2000). On immobilization, a change in the microenvironment of lipase occurs leading to the inhibition or enhancement of the activity (Villeneuve et al., 2000).

Immobilization has certain advantages over free enzyme systems. Stability of immobilzed lipase is greatly enhanced with respect to free one. Recoverability and reusability of immobilized lipase is also higher than free lipase (Pugazhenthi and Kumar, 2004).

Immobilization has some disadvantages also. It can cause a decay of activity even upto 90%. Different reasons are postulated to explain these phenomena. Steric hindrance effect is one of them. It postulates that the active site of lipase may get distorted by immobilization. Another is interfacial limitation phenomena which suggest that the diffusion of substrates or products in the vicinity of the surface for immobilization may be very low (Rios et al., 2004). Free lipase is normally highly active to lipids. But when it is immobilized in a solid carrier, contact of lipase with water and lipid phases becomes very low. In case of hydrophobic carrier, contact of the water phase with the lipase gets limited. This is proved by a study showing an activity of 18.3% was retained after immobilization of lipase in a hydrophobic photo-cross-linkable resin (Kimura et al., 1983). Also, a hydrophilic carrier poses obstruction in front of the lipid phase from reaching the lipase (Lavayre and Baratti, 1982).

1.7.2 Addition of metal ions

The slow rate of oil hydrolysis is attributed mostly to inhibition by products like fatty acids. They have higher interfacial activity than lipase and largely replace lipase from interfacial area (Reis et al., 2009; Gilbert et al., 1991; Isobe et al., 1988). Metal ions remove fatty acids from the oil–water interface by forming metal salts such that the lipase can act freely on other oil molecules. This step involving fatty acid removal from reaction site thus becomes the rate controlling one (Wang et al., 1988).

1.7.3 Additives and organic solvents

The slow rate of hydrolysis can be increased by addition of organic solvents and different additives. Organic solvents can solubilize water insoluble substrates and thus can help in decreasing mass transfer limitations. Role of additives are not clear but these may enhance activity of lipase (Puthli et al., 2006).

1.7.4 Reverse micelles

Presently, reverse micelles act as reaction media for a large number of reactions catalyzed by enzymes like lipases. Reverse micelles are considered as microreactors within which enzyme can be safeguarded from detrimental effects of (organic) solvents. These are formed by certain combination of organic solvent, surfactant and water. Polar core of reverse micelle solubilize certain amount of water depending on reverse micellar systems. In this aqueous microenvironment, lipase catalyzed reactions take place though overall organic solvent remains dominant. Reverse micelles are dynamic entities, capable to interchange its constituents with another micelle or the bulk organic solvent. Forces like hydrophobic interaction, van der Waals interaction, electrostatic and hydrogen bond interactions play important role to keep structures of reverse micelles together. Lipases and in general enzymes are microencapsulated within reverse micelles by three methods and these are, i) injection method in which lipase dissolved in aqueous solution is added to the surfactant solution in organic solvent; ii) phase transfer method by which lipase is transferred from an aqueous phase to a micellar phase; iii) dissolution method in which lyophilized lipase is added to reverse micelles already having an aqueous phase (Carvalho and Cabral, 2000).

Advantages of reverse micelles particularly in case of lipase catalyzed hydrolysis processes are, i) substrates and products are mostly hydrophobic and so largely soluble in organic solvents; ii) these structures form spontaneously and takes pretty short time to reach equilibrium; iii) these structures solubilize both hydrophobic and hydrophilic compounds; iv) low reaction volume is attained; v) interfacial contact area is increased; vi) activity and/or stability may be increased; vii) higher temperature can be attained as thermal stability is achieved due to low water content; viii) it can be scaled up easily (Hochkoeppler and Palmieri, 1990; Walde and Luisi, 1990; Walde, 1990; Walde and Luisi, 1989).

Besides, reverse micellar system has certain disadvantages also and these are; i) surfactants used can have denaturation effects; ii) in case of non-lyophilized enzymes, low water content is quite difficult to achieve; iii) recovery of product and lipase is still difficult (Carvalho and Cabral, 2000).

1.7.5 Systematic optimization of process variables

The process variables like speed of agitation, pH of buffer solution, temperature, buffer concentration and enzyme concentration have immense effects on conversion in case of hydrolysis of vegetable oil to specific fatty acid. Systematic optimization of these variables can ultimately lead to higher conversion. This optimization can be carried out by single variable method in which one variable is changed at a time keeping all other variables constant. But, this method does not take into account effects of interaction of different variables (Potumarthi et al., 2008) which affects enzyme deactivation more than individual ones. On variation of solution pH, the sensitivity of a protein (enzyme) can be affected greatly at higher temperatures and the combined effect of temperature and pH may change enormously from one enzyme to another (Bailey and Ollis, 1986). So, conversion in lipase catalyzed hydrolysis process is also influenced severely by interactions among different variables. In order to take into account of these interactive effects, statistical optimization method using response surface methodology is to be applied.

1.7.6 Use of surfactants to form micelles or emulsions

Surfactants can have different effects on lipase catalyzed hydrolysis depending on their nature. While some surfactants may deactivate lipase, some others can enhance its activity. Surfactant decreases surface tension of the liquid and thus reduces droplet size leading to enhanced interfacial area (Noor et al., 2003). Consequently, there occurs much higher contact between lipase and triacylglycerol. Surfactant molecules accumulate at oil–water interface due to a balance between its hydrophilicity and hydrophobicity; changes interfacial physical properties and thus solubilizes hydrophobic substrates to a greater extent (van Os, 1998). Substrate reactivity also increases sometimes within micelle. Some surfactants stabilize lipase structure (mainly its secondary and tertiary structure) due to hydrophobic interaction and thus help in retaining lipase activity at higher values of speed of agitation and temperature and extreme values of pH (Fendler and Fendler, 1975). Sometimes specific surfactant enhances lipase activity and hence

overall production also (Yamamoto and Fujiwara, 1988). Surfactants often help in reducing consumption of buffer by forming emulsion.

1.8 Aims/Objectives of the current research

As discussed earlier, the processes like immobilization and formation of reverse micelle have been widely used to obtain higher conversion of vegetable oil to fatty acids. So, in this work, the effect of surfactants in the above reaction is studied in order to maximize conversion. Besides, systematic optimizations of process variables using either free lipase or free lipase coupled with surfactant are also explored for this purpose.

Now, as castor oil possesses ricinoleic acid in all three positions of its triacylglycerol structure, non-regioselective lipase is to be considered in this study. But, as mustard oil possesses erucic acid mainly in 1 and 3 position of its triacylglycerol structure, a regioselective lipase is to be chosen in this study. The aims/objectives of this work are summarized below:

1.8.1 Non-Regioselective lipase catalyzed hydrolysis of castor oil to ricinoleic acid

i) The study of systematic optimization of process variables like speed of agitation, initial pH of buffer, temperature, buffer concentration and enzyme concentration by single variable optimization method.

ii) Application of response surface methodology to study systematic optimization considering individual, cumulative and interactive effects of process variables like initial pH of buffer, temperature, buffer concentration and enzyme concentration at fixed speed of agitation (optimum from single variable method).

iii) To choose most effective surfactant for enhancement in conversion; to study systematic optimization considering various effects of process variables like speed of agitation, initial pH of buffer, temperature, buffer concentration, enzyme concentration and surfactant concentration in surfactant enhanced hydrolysis by response surface methodology.

1.8.2 Regioselective lipase catalyzed hydrolysis of mustard oil to erucic acid

i) Use of single variable optimization method for studying systematic optimization of process variables like speed of agitation, initial pH of buffer, temperature, buffer concentration and enzyme concentration.

ii) With fixed speed of agitation (optimum from single variable method), study of systematic optimization of variables considering different effects of process variables like initial pH of buffer, temperature, buffer concentration and enzyme concentration by response surface methodology.

iii) To choose best surfactant (or mixed surfactant system) for enhancement in conversion; application of response surface methodology to study systematic optimization considering different effects of process variables like speed of agitation, initial pH of buffer, temperature, buffer concentration, enzyme concentration and surfactant concentration(s) in surfactant enhanced hydrolysis.

1.8.3 Kinetics study

i) Hydrolysis catalyzed by free lipase.

ii) Hydrolysis catalyzed by free lipase with surfactant enhancement.

1.9 Organization of the thesis

In this thesis, it is aimed to maximize production of ricinoleic acid by castor oil hydrolysis and production of erucic acid by mustard oil hydrolysis with the help of lipase enzyme as catalyst. For this purpose, effective process variables are optimized in both the cases by conventional single variable method and then by response surface methodology to incorporate interactive effects of process variables. Further enhancement in conversion in presence of specific surfactant is also explored for the two cases. Chapter 1 describes the conventional hydrolysis processes in brief and the advantages of lipase (biocatalyst) catalyzed hydrolysis with respect to these conventional processes. Chapter 2 deals with hydrolysis of castor oil catalyzed by lipase. Initially, suitable lipase is chosen based on studies by previous workers. After that, the optimization of process variables is carried

out by the single variable optimization method. In order to observe the interaction of different process variables, statistically designed experiments are carried out. Experimental results are analyzed with the help of response surface methodology to obtain most preferable condition for maximum conversion to ricinoleic acid. Chapter 3 encompasses surfactant enhanced lipase catalyzed hydrolysis of castor oil. Initially, the most efficient surfactant is chosen based on some preliminary experiments. Then, optimum combination of process variables is obtained based on statistically designed experiments coupled with analysis of results by response surface methodology. Chapter 4 provides detailed discussion on lipase catalyzed hydrolysis of mustard oil. At the beginning, suitable lipase is selected on the basis of earlier studies. Then, single variable optimization method is employed to optimize the process variables. This is followed by statistically designed experiments and application of response surface methodology to analyze results to get optimum process conditions to maximize production of erucic acid. Chapter 5 deals with the lipase catalyzed hydrolysis of mustard oil enhanced by surfactant. The best surfactant system (a mixed surfactant system) is detected based on some initial experiments. Next statistically designed experiments coupled with analysis of results by response surface methodology are employed to determine optimum combination of process variables. Chapter 6 deals with kinetics study of hydrolysis of both oils using free lipase. Besides, it shows the effect of addition of surfactants in both cases on rate of hydrolysis.