LIPASES: ENZYMES OF PARAMOUNT IMPORTANCE WITH COMMERCIAL APPLICATIONS

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Abstract

Lipases (E.C.3.1.1.3) enzymes are involved in catalyzing the hydrolysis of carboxylic ester bonds in triglycerides or fats. Lipasesthat come under the canopy of carboxylesterases and can be classified, based on their sources, as well as their specificity into different types. These enzymes are of immense importance mostly because of their widespread and easy availability. A range of chemical reactions involves the use of lipases as biocatalysts. Lipases have a wide range of industrial applications such as in the production and processing of dairy products and other food products, in the pharmaceutical industry for drug development chemical synthesis along with many other applications. The cosmetic industry is another major industry that is working on the use of lipases to produce better quality skincare and cosmetic products. Researches are being conducted to explore the full potential of lipases and to devise novel methods of more efficient lipase production and purification.

Keywords: Commercialization, Industrial application, lipases

Introduction

Carboxylesterases include two groups namely lipases (EC 3.1.1.3) and non-specific esterases(EC 3.1.1.1) (Chahiniana and Sarda, 2009). Lipases, also known as triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are enzymes that are involved in the catalysis of the fat and oil hydrolysis, releasing, free fatty acids, in addition to diglycerides, monoglycerides, and glycerol (Sharma *et al*., 2001). Some of their properties include the ability to act under mild conditions, their stability in organic solvents, their broad substrate specificity, and their ability to show high regio and/ or stereoselectivity when catalyzing reactions. It is because of these versatile properties that lipases form a very important group of biocatalysts for processes in biotechnology (Jaeger & Reetz, 1998).

When excess water is present, lipases cause the catalysis of the hydrolytic breakage of the carboxylate ester bonds, resulting in the production of free fatty acid molecules as well as alcohols. They do this by attacking the organic-aqueous interface (Borelli & Trono, 2015). A range of reactions is catalyzed by lipases (Fig.1). The reactions catalyzed by lipases are grouped into two major categories, namely hydrolysis and synthesis. The synthesis reactions can further be classified into alcoholysis (an ester and alcohol exchange their groups), acidolysis (ester and acid exchange their groups), and interesterification (two esters exchange their groups), that can together be referred to as the transesterification reactions. Moreover, aminolysis and esterification (that occurs under the conditions of limiting water activity) are also grouped under the synthesis reactions. These enzymatic reactions have a wide range of usage on the industrial scale, especially in bioprocess industries that produce fertilizers, flavored foods, and other products like biodiesel (Sarmah *et al*., 2018; Borelli & Trono, 2015).

Lipases can be produced from various sources that include plants, animals/insects and microorganisms, the last one being the most widely used source, on the industrial scale, as discussed before. Lipases find their applications in the production of food and dairy products, chemicals and detergents, cosmetics, leather products etc. (Javed *et al.*, 2012).

Structure of lipases

Lipases are widely distributed in nature and belong to the superfamily having α/β hydrolase folds in them, which have usually 8 β strands that lie in parallel to each other inside their core. The β sheets formed consequently are then surrounded by many α helices (Fig. 1). Aspartic acid that are linked by hydrogen bonds (Javed *et al*., 2018) which are responsible for their catalytic function. Some of the main components of the lipase structure include the disulfide bonds and lids in addition to the oxyanion hole and binding pockets. While the lids are responsible for the exposure of hydrophobic patches in the enzymes, the binding pockets are, as the name indicates binding sites of the enzyme. The disulfide bonds between cysteine residues, stabilize the structure of lipids. Lastly, the oxyanion hole determines the efficiency of the catalysis. Moreover, when hydrolysis takes place, it results in the generation of an intermediate, and the oxygen ion thus

produced is stabilized by the residues of this hole (Sarmah *et al*., 2018).

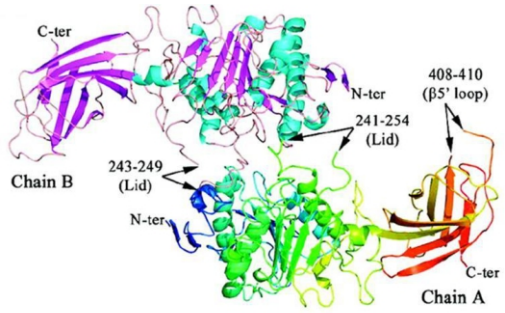

Fig. 1. Structure of Human Pancreatic Lipase (HPLP2) showing two monomer observed in the crystal unit (PDB ID 2PVS (adopted from Eydoux *et al*., 2008)

Mechanism of action of lipases

Lipases can catalyze various types of reactions like esterification, hydrolysis and transesterification and aminolysis, however, recently the importance of lipases in organic reactions like aldol condensations has also been documented (Dwivedee *et al*., 2018).

1. Hydrolysis:

Hydrolysis involves the lipase-catalyzed breakdown of fats into its major components namely fatty acids and glycerol. Hydrolysis reactions can be written as:

$RCOOR' + H_2O \rightarrow RCOOH + R'OH$

2. Synthesis:

The synthesis reactions can be grouped into the esterification, interesterification, alcoholysis and acidolysis reactions, the equations for which have been shown in Fig. 2.

The mechanism of action of lipases is such that first of all, a deprotonation step activates the serine residue, with the aid of aspartate and histidine residues (Fig. 3). Then due to the improved nucleophilicity of the OH^- of the serine residue, it gets the ability to attack the substrate carbonyl group, thereby forming a tetrahedral intermediate. This acyl-lipase intermediate is then stabilized by the oxyanion hole. Lastly, in the deacylation step, an attack on the acyl-enzyme is carried out by a nucleophile such as water molecules. This results in the release of the formed product and thus makes the catalytic site free for another substrate. In many lipases, the exposure to the active site is regulated by the lid.

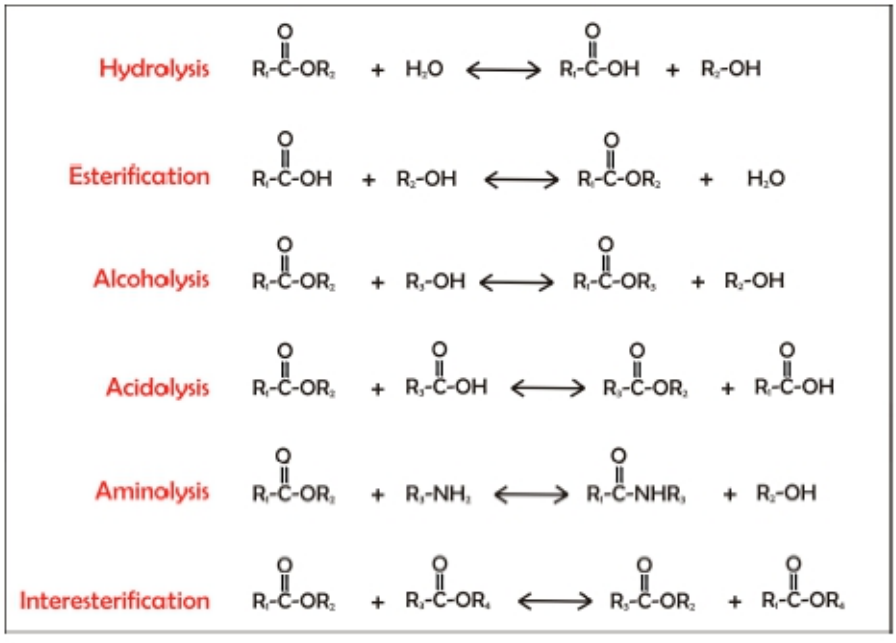

Fig. 2. Reactions catalyzed by lipases (adopted from Borelli & Trono, 2015)

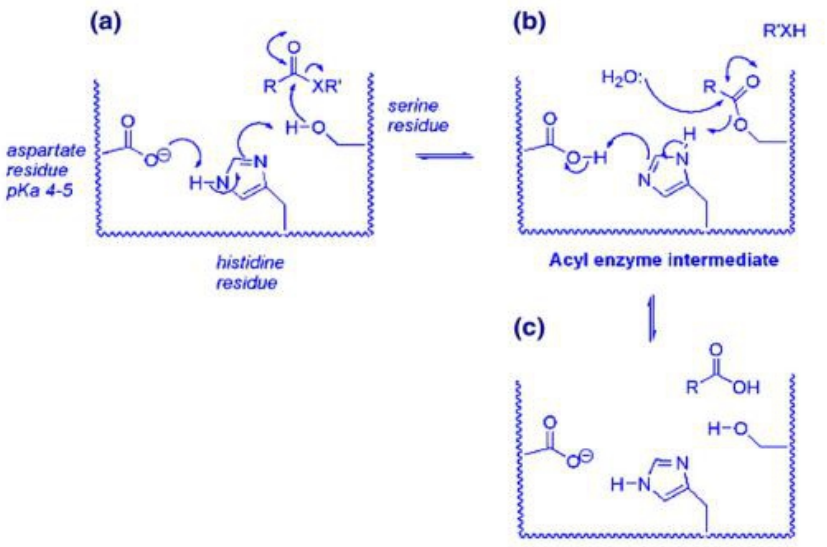

Fig. 3: Mechanism of action of lipases (adopted from Reis *et al*., 2009).

Classification of lipases

Lipases can be classified based on their specificity as well as their sources (Fig. 4).

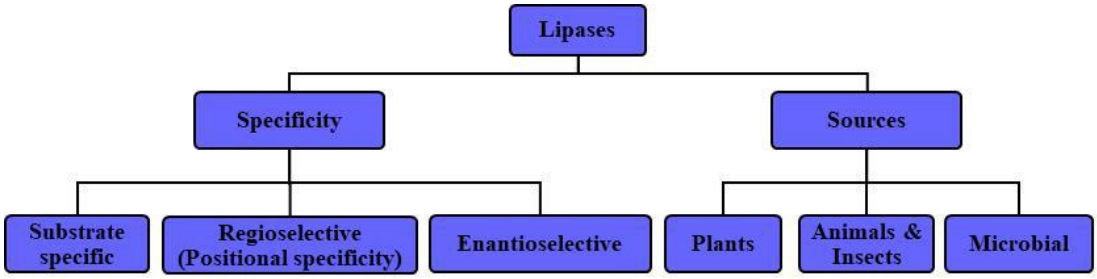

Fig. 4: Lipases classification based on specificity and source (adopted from Sarmah *et al*., 2018)

Specificity

Based on their specificity the lipases can be classified into substrate-specific, regioselective (positional specificity) and enantioselective lipases.

1. **Substrate Specific Lipases**:

According to the name these lipases are specific for specific substrates. This group of lipases is very useful in the reactions where instead of a pure substrate, a crude mixture of substrates is available, and the desired product needs to be synthesized using

only a specific, selective substrate. (Rebeiro *et al*., 2012). Therefore, to synthesize certain specific substrates only, these lipases can be used.

- **Phospholipases:**

Phospholipases are a class of lipases that hydrolyze phospholipids breaking them down into products. Activation of phospholipases requires an interface of the aqueous and organic phase. The phospholipases are grouped, according to the specific cleavage site on their substrate into A1 and A2, with their respective cleavage sites being the ester bonds at sn1, sn2. The products of this reaction are a free fatty acid molecule and an acyl lysophospholipid. Other groups include Phospholipase B (that hydrolyze the fatty acids at both these sites), phospholipase C (a phosphodiesterase, whose cleavage site is the glycerophosphate bond), and last but not the least phospholipase D (a phosphodiesterase, whose cleavage site is the terminal phosphodiester bond) (Borelli & Trono, 2015).

2. Regioselective Lipases

Regioselectivity is the property of lipases that allows them to distinguish between primary and secondary ester functional groups present in a molecule of triacylglycerol that allows them to move the direction of reaction towards the favorable reaction, rather than unwanted side reactions (Kumar *et al*., 2016). This property is useful on the industrial scale, in the reactions involving the production of chemicals and pharmaceutical products that require the optimization of isomeric compounds. An example of the use of regioselective lipases is the use of *Rhizopus oryzae* lipase for the acylation of ferulic acid with quercetin for the synthesis of flavonoid derivatives (Kumar *et al*., 2016). Another example is the use of regioselective lipase from C. *rugosa* to produce 30-OH-50-O-Acthymidine (Rivero & Palomo, 2016).

Based on selective functionality, the regioselective lipases can be further classified into:

Non-specific lipases: As the name indicates, non-specific can act on a variety of substrates. An example is the lipases produced by *Mucor meihei* that catalyze a wide range of reactions. They generally produce intermediates (mono and diacylglycerols), along with the main products (glycerol and fatty acid) by catalyzing the hydrolysis of triacylglycerols (Rebeiro *et al*., 2012).

1,3 specific lipases: This group includes the enzymes that are involved in the catalysis of the hydrolysis of triacylglycerols at C1 and C3 positions, resulting in the synthesis of fatty acids, along with monoacylglycerols and either 1,3 or 2,3 diacylglycerols (Barros *et al*., 2010).

Fatty acid specific lipases: Fatty acid specific lipases catalyze the hydrolysis of specific long-chain fatty acid molecules that have a double bond at the carbon number 9 (Rebeiro *et al*., 2012). According to research conducted on this group of lipases, several lipases were identified that were specific substrates having fatty acids of varying chain lengths, having different numbers of double bonds and distinct groups attached to those chains. According to this study, the lipases showing high specificity for short-chain esters include those produced b *Penicillium citrinum*, MJ2 *A. oryzae, Geotrichum candidum*, and a few others (Song *et al*., 2008). Others specific for medium length and long-chain esters were also reported.

3. Enantioselective Lipases:

The enantioselective lipases are specific for certain enantiomers, and can, therefore, specifically hydrolyze only those that they are specific for, from a mixture of enantiomers. This property allows them to be used to differentiate between various racemates (Barros *et al*., 2010). Transesterification of secondary alcohols in the pharmaceutical industry, menthol benzoate hydrolysis in food industry (Dhaake *et al*., 2013) and glycidic acid methyl ester hydrolysis in the medicine industry (Su *et al*., 2014) are a few examples of the use of enantiospecific lipases.

Sources of lipases

One of the reasons why so much research is being conducted on lipases is the fact that lipases can be extracted and purified from several sources. Therefore, the second system of lipase classification is the sources from which they are produced. A wide variety of lipases may be produced by plants, animals/insects and a variety of microbes (Patil *et al*., 2011).

Common sources of lipases

1. Plant sources:

Plants are one of the sources of lipases. Some parts of the plants that are important for lipase production. A majority of plant lipases are available from seed sources which are due to the saturation of triacylglycerols in these, that act as an efficient energy source for plants. Therefore, when plants germinate, these triacylglycerols are utilized to produce simple sugars, and this reaction is catalyzed by lipases. Lipase activity in plant seeds increases during germination as the triacylglycerols are converted to soluble sugars by the action of lipase (Patil *et al*., 2011). Plants however are not a very efficient source of lipases as they are not

very stable when the enzyme extraction and purification are performed by traditional means (Seth *et al*., 2014).

2. Animal and insect sources

Animals are also a source of lipases however, just like plants they too, are not a very efficient source because of complicated culture handling procedures and difficulty in the purification of enzymes. These are the least studied source of lipases. The most widely studied animal lipases are the pancreatic lipases, that find a wide range of applications in biochemical research (Pahoja & Sethar, 2002).

3. Microbial sources

Microbes are the most important source of lipases. Fungi, bacteria and yeast, are all sources of lipases, with most of these lipases being produced extracellularly. The factors that determine lipase production from microbes include the composition of the medium, along with the conditions of temperature, PH and concentration of dissolved oxygen (Thakur, 2012).

Fungal Lipases: Fungi are a very widely studied source of both extracellular and intracellular lipases due to certain factors that prove them to be better than all the other sources. These factors include their stability to heat and pH, high substrate specificity as well as efficient and cost-effective extraction. Moreover, many are soluble in organic solvents (Patil *et al*., 2011).

Yeast Lipases: Yeasts also produce lipases are an efficient source because it is comparatively easy to culture them. Many industries such as chemical and biodiesel industries make use of this property of yeasts, using mainly *Candida* spp. Such as C. *rugosa,* C. *utilis and* C. *antarctica* and various species of Saccharomyces for lipase production (Kobayashi *et al*., 2012).

Bacterial Lipases: Many species of bacteria are also producers of many extracellular (some are lipoproteins), intracellular and membrane-bound lipases, most of which are glycoproteins. Bacterial lipases mostly lack substrate specificity. Also, most are heat-stable. 51,70 Some of the lipases produced from bacteria such as those produced from *Bacillus licheniformis* and *Bacillus pumilus*, are heat stable and also important industrial enzymes (Sarmah *et al*., 2012).

Methods for the production of lipases

Lipases can be produced using various types of fermentation techniques.

Solid state fermentation (SSF): This process uses a solid matrix and is done in the presence of very little or no free water molecules. The substrate not only should provide support but also moisture and nutrients to the lipase-producing microorganism to grow on. Lipase production has been done from *Aspergillus niger* using SSF that used industrial waste solids from the palm oil industry as the support (Narasimha *et al*., 2011). In another study an archeon called *Natronococcus spp.* also showed a good lipase production under solid state fermentation conditions (Martin del Campo *et al*., 2015).

Submerged fermentation (SmF): This is the most used industrial lipase production method, because of its ability to be monitored easily (Silveira *et al*., 2016). *Pseudomonas spp.* has shown good lipase production under SmF in research conducted by (Narasimha *et al*., 2011). Another benefit of this type of fermentation is that cheaper substrate can be used in this method.

Fermentation using immobilized cell: Yet another technique of fermentation to produce lipases is the usage of immobilized lipase-producing cells. This allows the usage of these cells for catalyzing the reactions without disturbance. This also allows the avoidance of tedious purification and recovery processes associated with immobilizing the enzymes instead of the whole cells (He *et al.,* 2008).

Overall, each production method has its pros and cons. While the submerged fermentation allows the usage of comparatively cheaper substrates, the solid state fermentation is observed to have shown better results for the production of lipases from fungal sources and the fermentation using immobilized whole-cell allows the complicated purification steps to be skipped.

Conventional methods of lipase purification

For the purification of lipases biomass and insoluble particles are filtered or centrifuged in the first step, followed by the concentration of the supernatant by the process of ultrafiltration. Precipitation (used in 80% procedures) or extraction by an organic solvent may also be used for this purpose. For precipitation, about 60 percent of the procedures make use of $(NH_4)_2SO_3$, while 35% of the procedures use ethanol or an acid. In most cases, the precipitation step is first done and then chromatography is used as a separation technique. The precipitation technique usually gives higher yields compared to chromatography. A combination of

chromatography steps is usually required to achieve complete purity.

Chromatography is among the most popular conventional methods of extracellular lipase purification. Three commonly used chromatography methods include ion-exchange chromatography, gel filtration chromatography, and affinity chromatography, with ion-exchange chromatography being the most used method, followed by gel filtration chromatography. In ion-exchange chromatography, diethylaminoethyl (anionic exchanger) and carboxymethyl groups (cationic exchanger) are used. and the affinity chromatography, where hydrophobic interaction chromatography is an important and widely used affinity chromatography method. Hydroxyapatite is a commonly used adsorbent (Saxena *et al*., 1994).

Novel methods of lipase purification

Some of the novel methods of lipase production are as follows:

Reverse micellar extraction (RME): Surfactants are compounds that decrease the surface tension of a liquid. A micelle is a mass of molecules of a surfactant having hydrophilic heads and hydrophobic tails. The tails are located inside the core of the micelles. These micelles are present in a polar solvent in producing a colloidal solution (Saxena *et al*., 2003). Reverse micelles are, however, contrary to micelles, dispersed in a non-polar solvent, in which the heads are in the core while the tails are in contact with the solvent owing to the thermodynamic favorability of this. The cores of the reverse micelles entrap water, causing the hydrophilic organic compounds to dissolute. DNA, proteins, and enzymes can all be disrupted by this method. An increase in the charge of the headgroup means a decrease in the production of the reverse micelles (Levashov & Klyachko, 2001). Surfactants increase the yield and purification of the enzymes along with the specific activity of the enzymes (Saxena *et al*., 2003). The recovery of lipases by this method is done in two steps. The overall recovery process consists of two basic steps, proteins are first taken up into the reverse micelles. The proteins move from the organic solvent into the entrapped water, in an extraction process. This prevents the proteins from being denatured. This process is followed by a back-extraction process in which the proteins are recovered by moving from the reverse micelles into another aqueous solvent. The aqueous phase PH should be such that it allows the protein that has to be extracted, to maintain a charge that is opposite to that of the groups on the hydrophilic heads of the surfactant molecules so that the proteins can be extracted. In the second step, that is the back extraction, however, the PH of the organic phase should be such that the net charge on the protein equals that of the surfactant. This should be done to ensure that the protein is forced out of the reverse micelles (Tan *et al*., 2015).

Use of membranes: In research done on this method, a double membrane was made. One layer was formed from Chitosan bound Poly ultrafiltration fiber, while the other one was made from 1-ethyl-3 carbodiimide hydrochloride *N*-hydroxyl succin-imide. Purification of lipases was done using the membrane attached to glyceraldehyde, from *Candida rugosa* (Ye *et al*., 2005).

Immunopurification method: Affinity chromatography can be used to purify proteins by an antibody-antigen system. This highly selective method is called as immunopurification. Thousand to ten-thousand-fold protein purification have been achieved by this process (Harlow & Lane, 1988). In a study conducted by Rahimi and co-workers, two monoclonal antibodies called BF11A and VNH9 were tested to purify *Candida rugosa* lipase. The activity of lipase was found to be 99% and 92% respectively, which makes this method important for use in the future (Rahimi *et al*., 2004).

Applications

Synthesis of chemicals: Chirality of molecules is a very important property for drug development as the efficacy of many drugs is dependant on that, with mostly only one enantiomer showing bioactivity. Lipases are important industrially, as biocatalysts are used for the production of chiral building blocks. Many pesticides, chemicals and pharmaceutical products are synthesized with the help of lipases. Lipases not only increase the efficiency and selectivity but are also advantageous because of their ability to work under mild conditions (Hasan *et al*., 2006). An example of the industrial use of lipases is in the synthesis of Solketal (a chiral molecule used to synthesize various compounds like prostaglandins, diglycerides b- blockers, and glycerol phosphates.

Moreover, substantial amounts of optically active intermediate molecules are also being synthesized using lipases. Other than that, many fragrances and flavours (esters) are prepared efficiently by the action of lipases from short-chain fatty acids. Acetyl esters having an orangy odour, and butyl acetate having pineapple flavour are both prepared by the action of lipases. These fragrances and flavours are of good quality. Other than that, lipases are also used to catalyze the synthesis of monoacyl glycerols, that have widespread use as

emulsifiers in both the pharmaceutical and cosmetic industry. The benefit of using lipases instead of synthesizing them chemically is that the burnt taste and intense colour of the chemically synthesized monoacylglycerols can be avoided (Borelli & Trono, 2015).

Food Industry: Hydrolysis of fats and oils in the food production and processing industry to produce fatty acids using lipases is a common practice, on an industrial scale (Barros et. al 2010). Hundreds of food and bakery products are synthesized using lipases, and the selectivity of the lipases helps them perform their function efficiently. Many foods and bakery products can also be synthesized using lipids owing to their efficiency and selectivity. Removal of unnecessary fats from meat, synthesis of alternatives of cocoa butter (Panizza *et al*., 2013), production of triglycerides (with fish oil fatty acids), synthesis of esters of L-ascorbic acid, modification of Vitamin E and esterification of other vitamins, treatment of biomaterials, increasing the shelf life of bakery products, synthesis of fruit juices and dairy products, mayonnaise and edible lubricant production, fermented vegetable production and noodle softening all use lipases. Some lipases like those from *Bacillus subtilis* also make the bakery products such as bread appear good by improving their textures. Some phospholipases also emulsify lipids making the bread loafs more firm, whereas some endogenous lipids help in increasing the bread loaf volume. It is because of these properties that the lipases are replacing many traditional methods in the industry (Borelli & Trono, 2015).

Pharmaceutical industry: Many enzymes play significant roles in the pharmaceutical industry. Lipases are the main enzymes used for the hydrolytic reactions involving fats. The drug development industry uses mainly microbial lipases for various enantiomeric and regiospecific reactions for the production of chiral compounds, due to their ability to distinguish between different enantiomeric compounds in racemic mixtures. An example of drugs using lipases is anti-inflammatory drugs called Profens that are activated only when they undergo catalysis by lipases produced by *Candida antarctica and Candida rugosa* (Sikora *et al*., 2014). Many life-saving drugs have also been made by using lipase-catalyzed reactions (Ray, 2012). Many chiral substances in the medical industry are also produced by aminolysis reactions that use lipases for catalysis. Lipase concentration in serum also functions as a diagnostic tool for the recognition of pancreatic and heart diseases. Tumour necrosis factor activated naturally by pancreatic lipase, Bacterial lipases can be used as alternatives of pancreatic lipases and can therefore participate in treating the malignant tumors. Moreover, a lipase from the bacterium *Serratia marcescens* is involved in catalyzing a major step in the production of another drug called diltiazem (calcium blocker), that is used against Angina, and high blood pressure. Monoglyceride lipases are potential drug targets that make them important in the pharmaceutical industry (Pan *et al*., 2016) showed that the drug delivery process can be improved utilizing using phospholipids that are lipase labile (Sarmah *et al*., 2018).

Cosmetic Industry: Enzymes are generally performing various roles in skincare e.g. enzymes activate many inactive molecules in the skin. As an example, protease enzymes catalyze the hydrolysis of proteins and melanin synthesis is aided by tyrosinase. Moreover, in addition to the modulation of keratinization, many natural defense systems of the skin are activated by the enzymes. Lipases act as natural active means of producing cosmeceuticals or skincare products. Lipases have roles in the surface cleansing of the skin as well as nose, in which they act as active compounds. Moreover, its protentional in anti-cellulite treatment is also being studied. along with their role in the slimming of the body by degrading excess fat and removing dead skin cells. For this, they may work in conjunction with proteases. Makeup and hair care products all involve the use of lipases. Nowadays, lipase is being studied a lot for its potential use in cosmetic products (Schumacher & Thum, 2013).

Biodiesel: Biodiesel production is being widely studied because of its potential to replace the fuel in the future. This production is being done by trans esterifying edible and non-edible oils with alcohols. These reactions involve the use of lipases as biocatalysts. The use of lipases as biocatalysts is increasing due to various factors that make them better for use than chemicals. These factors include the ease of obtaining purified glycerol with very little production of waste and minimized use of energy (Lukovic´ *et al*., 2011). The major focus of biodiesel production is on their production from non-edible oils so that the availability of edible oils in the food sector is not compromised. An example of using non-edible oil is that the bacterium, *Burkholderia cepacia* has been fermented to produce lipases for catalyzing the transesterification of castor oil. (Baron *et al*., 2014).

A study conducted by (Aguieiras *et al*., 2015) shows the effect that different raw materials and alcohols have on the lipases used for biodiesel production. Other studies focus on how the parameters for biodiesel production using lipases can be

optimized to increase their production as well as the efficacy of the process (Ghaly *et al*., 2010) studied that oils that have phospholipids in them can increase the speed of the process of biodiesel production three times.

Conclusion

Lipases are, therefore, enzymes of paramount importance, both at a small scale and at an industrial scale. Different conventional and novel methods of lipase production and purification are being studied, to produce the maximum yield cost-effectively. Their properties such as their stability in organic solvents, broad substrate specificity, and their ability to act under mild conditions make them very useful for various purposes. Moreover, their ubiquitous presence and easy availability increase the potential of lipases to be studied. It is because of these properties that lipases are slowly replacing the chemically synthesized substances for producing various industrial products.

Future aspects

Lipases are being continuously studied for their potential use in pharmaceutical and cosmetic industries as biocatalysts. With the increasing demand for more and more makeup and skincare products, the interest in producing lipase-catalyzed reactions is increasing. This is also because the use of natural products as alternatives to chemical products is gaining importance. Scientists are, however, still investigating how the lipases can be engineered for their use in specific applications.

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