# *Review of Literature*

Global warming and depletion of fossil fuels, in recent years, had led to intensive investigations for providing the greater use of sustainable biofuels instead of fossil fuels. Biomass, which various biofuels are produced from, has an important role among other alternative energy sources including wind energy, solar energy, geothermal energy, etc. Biodiesel is one of the important biofuels and a clean energy source as an alternative to petroleum- based diesel fuels.

**Microbial lipase**

The use of enzyme-mediated processes can be traced to ancient civilizations. After the proteases and amylases, lipases are considered as the third group in volume of sales, moving billions of dollars, showing their application versatility which makes them especially attractive for industrial applications (Freire *et al*., 1997; Saxena *et al*., 2003; Mahadik *et al*., 2004; Vakhlu and Kour, 2006). Today, nearly 4000 enzymes are known, and of these, about 200 are in commercial use. The majority of the industrial enzymes is of microbial origin.

Many microorganisms and higher eukaryotes produce lipases. Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oil seeds, and decaying

food (Sztajer *et al*., 1988), compost heaps, coal tips, and hot springs (Wang *et al*., 1995). Lipase-producing microorganisms include bacteria, fungi, yeasts, and actinomycetes. Lipase producing strains have been isolated from industrial wastes, oil and fats processing factories, soil (Sharma *et al*., 2001; Colen *et al*., 2006). Fungal species are preferably cultivated in solid-state fermentation (SSF), while bacteria and yeast are cultivated in submerged fermentation (Dutra *et al*., 2008). Many bacterial, fungal and yeast lipases produced and available on commercial scale.

**Bacterial lipases**

A relatively smaller number of bacterial lipases have been well studied compared to plant and fungal. Table 2 describes the list of bacteria producing lipases. Bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins. *Staphylococcal* lipases are lipoprotein in nature (Brune and Gotz, 1992). Most of the bacterial lipases reported so far are constitutive and are nonspecific in their substrate specificity, and a few bacterial lipases are thermostable (Macrae and Hammond, 1985).

Maximum activity was observed at pH > 7.0 for *Pseudomonas fragi* (Nashif and Nelson, 1953) and at pH 9.0 for *P. aeruginosa* (Nadkarni, 1971). High production of lipase by *P. fragi* occurred in peptone-supplemented medium, although different peptones varied in their effectiveness (Nashif and Nelson, 1953). Though *Pseudomonas sp*. grow in a basal medium with ammonium sulfate, glucose citrate or pyruvate, it required an organic nitrogen source for lipase production (Alford and Pierce, 1963). They also observed the mixture of arginine, lysine and glutamic acid in the medium was effective for lipase production.

**Table 1: List of bacteria producing Lipases**

| Source | Genus | Species | References |
|---|---|---|---|
| BACTERIA (Gram Positive) | *Bacillus* | *B. Megaterium* | Godtfredsen, 1990 |
| | | *B. cereus* | El-Shafei and Rezkallah, 1997 |
| | | *B. stearothermophilus* | Gowland *et al*., 1987 and Kim *et al*., 1998 |
| | | *B. subtilis* | Kennedy and Rennarz, 1979 |
| | | *Recombinant B. subtilis 168* | Lesuisse *et al*., 1993 |
| | | *B. brevis* | Hou,1994 |
| | | *B. thermocatenulatus* | Rua *et al*., 1998 |
| | | *Bacillus sp. IHI-91* | Beeker *et al*., 1997 |
| | | *Bacillus strain W Al 28A5* | Janssen *et al*., 1994 |
| | | *Bacillus sp.* | Helisto and Korpela, 1998 |
| | | *B. coagulans* | EI-Shafei and Rezkallah, 1997 |
| | | *B. acidocaldarius* | Manco *et al*., 1998 |
| | | *Bacillus sp. RS-12* | Sidhu *et al*., 1998a; Sidhu *et al*., 1998b |
| | | *B. thermoleovorall S ID-J* | Lee *et al*., 1999 |
| | | *Bacillus sp. J 33* | Nawani and Kaur, 2000 |
| | *Staphylococcus* | *S. canosus* | Tahoun el al., 1985 |
| | | *S. aureus* | Lee and Yandolo, 1986 |
| | | *S. hyicus* | Kampen *et al*., 1998; Meens *et al*., 1997; Oort *et al*., 1989 |
| | | *S. epidermidis* | Farrell *et al*., 1993; Simons *et al*., 1998 |
| | | *S. warneri* | Talon *et al*., 1995 |
| | *Lactobacillus* | *L. delbruckii sub sp. bulgaricus* | EI-Sawah *et al*., 1995 |
| | | *Lactobacillus sp.* | Meyers *et al*., 1996 |
| | *Streptococcus* | *Streptococcus lactis* | Sztajer *et al*., 1988 |
| | *Micrococcus* | *M. freudenreichii* | Hou,1994 |
| | | *M. luteus* | Hou,1994 |

| Source | Genus | Species | References |
|---|---|---|---|
| | *Propionibacterium* | *Propionibacterium acne* | Sztajer *et al*., 1988 |
| | | *P. granulosum* | Sztajer *et al*., 1988 |
| | *Burkholderia* | *Burkholderia sp.* | Yeo *et al*., 1998 |
| | | *B. glumae* | El-Khattabi *et al*., 2000 |
| | *Pseudomonas* | *P. aeruginosa* | Aoyama *et al*., 1988; Holl, 1994; Ito *et al*., 2001 |
| | | *P. fragi* | Mencher and Alford, 1967 |
| BACTERIA (Gram Negative) | | *P. mendocina* | Jaeger and Reetz, 1998 |
| | | *P. putida 3SK* | Lee and Rhee, 1993 |
| | | *P. glumae* | Frenken *et al*., 1993; Noble *et al*., 1994 |
| | | | Hsu *et al*., 2000; Lang *et al*., 1998 |
| | | *P. cepacia* | Lacointe *et al*., 1996; Marangoni, 1994 |
| | | *P. fluorescens* | Sharon *et al*., 1998 |
| | | *P. aeruginosa KKA-5* | Lin *et al*., 1995; Lin *et al*., 1996 |
| | | *P. pseudoalcaligenes F - 111* | Sin *et al*., 1998; Dong *et al*., 1999; Miyazawa *et al*.,1998 |
| | | *Pseudomonas sp.* | Reetz and Jaeger, 1998 |
| | | | GuiIlou *et al*., 1995 |
| | | *P. fluorescens MFO* | Yang *et al*., 2000 |
| | *Chromobacterium* | *Pseudomonas sp. KWI56* | Diogo *et al*., 1999; HeJisto and Korpela, 1998; |
| | | *C. viscosum* | Reetz and Jaeger, 1998; Rees and Robinson, 1995 |
| | *Acinetobacter* | *A. pseudoalcaligenes* | Sztajer *et al*., 1988 |
| | *Aeromonas* | *A. radioresislells* | Chen *et al*., 1999 |
| | | *A. hydrophila* | Anguita *et al*., 1993 |
| | | *A. sorbia LP004* | Lotrakul and Dharmsthiti, 1997 |

**Table 2: List of fungi producing Lipases**

| Source | Genus | Species | References |
|---|---|---|---|
| FUNGI | *Rhizopus* | *R. delemar* | Klein *et al*., 1997; Espinosa *et al*., 1990; Hass *et al*., 1992; Lacointe *et al*., 1996 |
| | | *R. oryzae* | Salleh *et al*., 1993; Coenen *et al*., 1997; Beer *et al*., 1998; Essamri *et al*., 1998; Takahashi *et al*., 1998; Hiol *et al*., 2000 Hou,1994 |
| | | *R. arrhizus* | Sztajer and Maliszewska, 1989; Elibol and Ozer, 2001 |
| | | *R. nigricans* | Ghosh *et al*., 1996 |
| | | *R. nodosus* | Nakashima *et al*., 1988 |
| | | *R. microsporous* | Ghosh *et al*., 1996 |
| | | *R. chinensis* | Ghosh *et al*., 1996 |
| | | *R. japonicas* | Nakashima *et al*., 1988 |
| | | *R. niveus* | Kohno *et al*., |
| | *Aspergillus* | *A. flavus* | Long *et al*., 1996, 1998 |
| | | *A. niger* | Chen *et al*., 1995 |
| | | *A. japonicas* | Satyanarayan and Johri, 1981 |
| | | *A. awamori* | Satyanarayan and Johri, 1981 |
| | | *A. fumigatus* | Satyanarayan and Johri, 1981 |
| | | *A. oryzae* | Ohnishi *et al*., 1994a, b |
| | | *A. carneus* | Helisto and Korpela, 1998 |
| | | *A. repens* | Kaminishi *et al*., 1999 |
| | | *A. nidulans* | Mayordomo *et al*., 2000 |
| | *Penicillium* | *P. cyclopiun* | Chahinian *et al*., 2000 |
| | | *S. hyicus* | Van oort *et al*., 1989; Meens *et al*., 1997 Van Kampen *et al*., 1998 |
| | | *S. epidermidis* | Farrel *et al*., 1993; Simons *et al*., 1998 |
| | | *S. warneri* | Talon *et al*., 1995 |

A variety of lipases are produced from both Gram-positive and Gram-negative bacteria. Greater part of bacterial lipases comes from Gram-negative bacteria and the most important of them belonged to *Pseudomonas* sp. Alford and Pierce (1963) investigated a number of media for lipase production by *P. fragi*. Salts of unsaturated fatty acids inhibited lipase production by *P. fragi* (Smith and Alford, 1966), whereas tributyrin and trioctanoin had no effect on lipase production by *P. fragi* and *Micrococcus freudenreichii* (Lawrence *et al*., 1967). A strain of *Pseudomonas roqueforti* produced the maximum amount of lipase when grown in 0.5% casitone and 1% proflobroth (Eitenmiller *et al*., 1970).

The pH of the growth medium is also important for lipase production. Maximum lipase production at acidic pH 4.0-7.0 was reported from *Macrococcus caseolyticus* (Jonsson and Snygg, 1974) and *Bacillus licheniformis* (Akhtar *et al*., 1980). Breuil and Kushner (1975) studied on lipase production by *Acinobacter sp*. Growth and lipase production by *Micrococcus sp*. were unaffected by peptone of 0.5% to 2%, but lipase production by *Pseudomonas sp.* was stimulated by peptone (Akhtar *et al*., 1980). They also demonstrated milk is a good medium for growth of psychotropic bacteria and for lipase production.

Lipase synthesis by two strains of *P. fluorescens* (psychotropic), stimulated in milk medium at 7°C, was immediately preceded by a decrease in O2 tension which resulted in earlier production of lipase (Rowe and Gilmour, 1982). Various environmental factors such as temperature, pH, nitrogen, carbon and lipid sources, agitation, and dissolved oxygen concentration play an influence on

lipase production (Nahas, 1988). Besides this, free fatty acids, hydrolysable esters, bile salts, and glycerol also stimulate lipase production Lipase production is generally stimulated by lipids (Suzuki *et al*., 1988).

Physiological regulation of lipase activity by thermostolerant strain of *P. aeruginosa* EF2 under various conditions in batch, fed-batch, and continuous cultures support the contention that nitrate generally stimulates production of lipase (Gilbert *et al*., 1991).The ratio of surface area to volume variation and hence, aeration of cultures of *Pseudomonas fragi* had no effect on the quantity of lipase produced per cell; but increasing aeration by shaking resulted in both increased growth and lipase production, followed by a rapid decrease of lipase activity as shaking continued (Lawson *et al*., 1994). Seven species of *Pseudomonas viz P. aeruginosa, P. alcaligenes, P. fragi, P. glumae, P. cepacia, P. fluorescens* and *P. putida* were found to be lipase producing microbes (Jaeger *et al*., 1994). Gram-positive lipase producers are *Staphylococcus* (especially, *S. aureus* and *S. hyicus*), *Streptomyces* and *Bacillus species*. The most useful lipase producer genus used in industry is *Bacillus* among gram-positive bacteria (Jaeger, *et al*., 1994).

Triglycerides such as olive oil, groundnut oil and cottonseed oil, and fatty acids such as oleic acid, linoleic acid and linolenic acid stimulated lipase production by *P. mephitica* (Saxena *et al*., 1999). Riedel *et al*. (2001) carried out the analysis of *Serratia liquefaciens* MG1'lux AB insertion mutants, which were responsible for the secretion of extracellular lipase. Woods *et al*. (2001) reported the extracellular lipase production by psychotropic strains of *P. flourescens* (B52).

Most bacterial species studied for lipase production are non-pathogenic, mainly because these lipases were aimed to be used for biotechnological applications. It is a fact that extracellular lipases are important microbial virulence factors in addition to their industrial usage. In connection with this aspect, human pathogenic bacteria have been examined recently. One of them is *Staphylococcus epidermidis,* which is an opportunistic pathogen. During infection, it secretes two lipases that are considered to play a role in the colonization on the skin by breaking serum-derived triacylglycerols.

Besides *Pseudomonas* species among gram-negative bacteria *Achromobacter, Alcaligenes, Burkholderia and Chromobacterium* strains are the most common lipase produces (Gupta and Rathi, 2004). Ghosh *et al*. (2005) also reported a lipase producing bacterial strain which was isolated from phospholpid enriched media of *Pseudomonas* group and cultured in peptone, yeast extract and beef extract containing media. Al-Qodah *et al*. (2007) studied the enzyme production by thermophilic bacterium isolated and identified from a hot spring in Jordan and designated as *Bacillus sphaericus*. Among 57 heterotrophic bacterial strains isolated from marine sponge, *Pseudomonas* MS 1057 was observed to be the best producer with maximum lipase yield 750U/mL (Kiran *et al*., 2008). A B*acillus* strain, *B. stearothermophilus* isolated from air showed a maximum lipase activity 1585 U/mL, under optimized submerged fermentation (Abada *et al*., 2008).

There are various documents available on the production of bacterial lipases particularly from *Pseudomonas* and There are various documents available

on the production of bacterial lipases particularly from *Pseudomonas* and *Bacillus sp.*,*Pseudomonas fluorescens* (Yang *et al*., 2009), *B. cereus* (Dutta and Ray, 2009), *P. aeruginosa* (Madan and Mishra, 2010), *B. pumilus* (Sangeetha *et al*., 2010 ), *B. subtilis* (Ahmed *et al*., 2010) and *B. licheniformis* (Sangeetha *et al*., 2010b). Other genera like A*chromobacter, Arthrobacter, Alcaligenes and Chromobacterium* (Riaz *et al*., 2010) too have been studied. Lipase-producing *P. aeruginosaKM110* were isolated from wastewater of an oil processing plant by Qamsari *et al*. (2011). Boonmahome and Mongkolthanaruk (2013) screened lipase-producing bacteria from soil sample contaminated with cooking oil in areas of Khon Kaen region, Thailand. The isolate NA37 was identified to be *P. aeruginosa*. The lipase production was maximum at pH7, temperature 37°C and incubation time 48 hours by the lipase producing bacteria *BLP2 P. gessardii* isolated from soils collected from different oil mills located at Dharmapuri and Salem district (Veerapagu *et al*., 2013).

**Fungal lipases**

A list of lipase producing fungi mentioned in table 1 Peter and Nelson (1948) reported on lipase production by *Mycotorula lipolytica*. Growth at 30°C was optimum for the production of lipase by *M. lipolytica*. Ramakrishanan and Banerjee (1951) investigated lipolytic activity of different strains isolated from moulds grown in sesamum. It was found that *Penicillium chrysogenum* showed appreciable lipolytic activity. Zones of clearance were formed in tributyrin agar layer around the lipase producing colonies, which could be isolated. Kundu and Pal

(1970) reported a new method for the isolation of lipolytic fungi from the soil on oil mineral medium spread on silica gel plates.

Arima *et al*. (1972) purified an extracellular lipase from *Humicola lanuginose* strain Y-38, isolated from compost in Japan. Maximum lipase production at acidic pH (4.0-7.0) was reported for *Saccharomycopsis lipolytica* (Jonsson and Snygg, 1974). The best temperature for lipase production by *Talaromyces emersonii* was determined to be 45°C in stationary condition (Oso, 1978). High aeration was needed for high lipase activity by *Aspergillus* wentii (Chander *et al*., 1980) and *Mucor hiemalis* (Akhtar *et al*., 1980). Triglyceride is important for lipase production as it can act both as an inducer and as inhibitor. Among the triglycerides, olive oil was observed to be effective in inducing lipase (Akhtar *et al*., 1980). *A. wenti*i showed reduced growth and lipase production when the synthetic and natural lipids were added to the growth medium (Chander *et al*., 1980).

*M. racemosus* demonstrated increased lipase production in static culture conditions (Chopra *et al*., 1981). Polysaccharides such as glycogen, hyaluronate, laminarin, gum arabic, and pectin stimulated production of lipase in *S. lipolytica* (Ruschen and Winkler, 1982).Temperatures in the range of 22-35°C were, however observed to be optimum for maximum lipase production for *A. wentii* (Chander *et al*., 1980), *M. heimalis* (Akhtar *et al*., 1980), *R. nigricans* (Chander *et al*., 1981*), M. racemosus* (Chopra *et al*., 1981) *R. oligosporus* (Nahas, 1988).

Roberts *et al*. (1987) studied extracellular lipase production by fungi from sunflower seed. Vigorous aeration greatly reduced lipase production by *R. oligosporus* (Nahas, 1988). Novotny *et al*. (1988) examined the activities of the free and the cell-bound lipases of *Candida rugosa, C. guilliermondii, C. curvata, C. deformans and Yarrowia lipolytica* that had grown on a complex medium with various carbon and nitrogen sources. The cells grown in the presence of the organic nitrogen sources (peptone, urea, soy flour) exhibited highest lipase levels than those cultivated in the medium containing ammonium as the nitrogen source. The extracellular enzyme was detected only in cultures grown with urea, peptone or soy flour as the nitrogen source.

Emulsification of culture media containing oil by gum acacia supported good growth and lipase production in *Rhizopus oligosporus* (Nahas, 1988). He also reported that soybean meal extract in *R. oligosporus* culture medium supported good growth and lipase production. Hegedus and Khachatourians (1988) reported the production of extracellular lipase by *Beauveria bassiana*. The time course of lipase production in the presence of olive oil was studied and which was shown to induce lipase. The addition of fatty acids, such as myristic, palmitic, stearic, oleic, linoleic and arachidic acids inhibited both growth and lipase production.

Roblain *et al*. (1989) studied the improvement of *Mucor* lipase production by mutant selection. This study deals with the characterization of lipase from *Mucor javanicus* and selection of *M. javanicus* mutants, lipase hyper producers. Mutants were induced from *M. javanicus* with NTG (N-methyl-N'-nitro-N-nitrosoguanidine). After growth on petridishes for3-4 days at 28°C,

colonies with distinct clear zones larger than parents were tested for lipase production in liquid medium. Toshihiko *et al*. (1989) reported that among 556 microorganisms isolated from soil on potato dextrose agar plates, a fungus identified as *Rhizopus oligosporus* showed highest lipase production. A medium containing 2% olive oil and 0.2% lecithin was optimal for lipase production. Jacobsen *et al*. (1989) also reported that cell bound lipase was produced using peptone as the nitrogen source and using olive oil supplemented with Tween 80 as the carbon source.

Triolein, olive oil, tributyrin, and oleic acid butylester were able to induce lipase in immobilized protoplasts, whereas Tween 80 enhanced lipase activity (Johri *et al*., 1990). Lipase producing fungal strains were isolated from various habitats such as water, soil, air, milk, rotten fruits/vegetables, bread roasted in oil, pickle and oil mill (Akano and Atanda, 1990). The chief producers of commercial lipases are *Aspergillus niger, Candida cylindracea, Humicola lanuginosa, M. miehei, R. arrhizus, R. delemar, R. japonicus, R. niveus and R. oryzae* (Godfredson, 1990).

Nadj *et al*. (1992) investigated the ability for biosynthesis of extracellular lipase by *Geotrichum candidum* during cultivation in different liquid media. Sztajar *et al*. (1992) studied the purification and properties of lipase from *Penicillium simplicissimum. P. simplicissimum* strain has been found to produce an inducible extracellular lipase. Asahara *et al*. (1993) studied the production of two types of lipases with opposite positional specificity by *Geotrichum sp*. Olama and El-Sabaney (1993) demonstrated the maximum lipase production by *Aspergillus*

*niger* on 8th day of incubation. Venkateshwarlu and Reddy (1993) investigated the lipaseproduction by five thermophilic fungi. The fungi named *Emericella rugulosam, Humicola sp., Thermomyces lanuginosus, P. purpurogenum and Chrysosporium sulfureum* were used for lipase production in five synthetic media. A temperature of 45°C was optimum for secretion of lipase by all the fungi. The optimum condition for lipase activity was pH 8.0, temperature 45°C, a substrate enzyme ratio of 0.9:1.1 and 3 hours of incubation time for all the fungi under study.

Ohnishi *et al*. (1994) reported that *Aspergillus oryzae* produced maximal lipase in a medium that contained yeast extract (1%), polypeptone (2%) and soybean meal (3%) as nitrogen sources. The enzyme produced had an activity optimum at pH 7.5 and 10.0 respectively, with olive oil and tributyrin as substrates.

Chen *et al*., (1995) demonstrated that Oils were good carbon sources for biomass and lipase production of *Aspergillus sp.* Saad (1995) isolated a strain of *A. tamari*. Optimal conditions for lipase production were 6 days incubation period at temperature 30°C, pH 7.0 and 0.15% of olive oil. The crude enzyme showed a high level of pH stability but was not thermostable. The enzyme remained more than 90% of its activity in the presence of some compound detergents.

Peter (1995) reported that lipase activity produced during cultivation of *Fusarium oxysporium* in shake flasks was predominantly extracellular. Only a small amount was found in or on the mycelium. The fungus required peptone for

significant lipase production. The lipase activity remained constant during 1-hour incubation at pH 4.0 to 5.0 and 30°C. It was stable in25% acetone, ethanol and n-propanol and was stimulated remarkably in n-hexane.

Gao and Breuil (1995) reported that the extracellular lipase production of sapwood containing fungus *Ophiostoma piceae*, grown in liquid medium was optically active at pH 5.5 and 37°C.Lui *et al*. (1995) reported the production of thermostable lipase and esterase by *Monascus fulginosus* strain M-101. The optimal conditions for lipase production were incubation temperature of 30°C, pH 3.0 to 5.0 and incubation period of 4-5 days. Huang *et al*. (1995) investigated the conditions for the production of alkaline lipase with *Penicillium expansum* PF868 was studied. The optimum conditions were as follows: corn meal as carbon source and soybean cake meal as nitrogen source, with initial pH 7.0-7.5 and fermentation time 60 hours. The average lipase activity was up to 1200 U/mL.

Ren (1996) selected a lipase producing strain of *Aspergillus oryzae* from 520 fungi through solid plate assay. Conditions were optimized for lipase production. Olive oil was found best carbon source and yeast extract as best nitrogen source at pH 5.8 and culture period of 96 hours. Toide *et al*. (1996) reported the stable production of triglyceride lipase (130 U/mL) by *Aspergillus oryzae* after the optimization of cultural medium and cultural conditions. Berto *et al*. (1997) reported the production and purification of a novel extracellular lipase from *Alternaria brassicicola*. Yadav *et al*. (1998) reported the lipase production by *Aspergillus sp*. and*Penicillium sp*. Forty each of *Aspergilli and Penicillia* were screened for extracellular lipase production on agar plates and in

liquid medium containing olive oil as substrate. Twenty-nine *Aspergilli* and 26 *Penicillia* produced lipase.

Ahmed and Dahot (1998) studied lipase production by *A. niger* using 0.3and 0.6 N sulfuric acid and ammonium hydroxide pretreated sugarcane bagasse (collected from city and industry) as carbon source. Aqueous olive oil emulsion was used as a substrate for the denaturation of lipase activity in the culture broth of *A. niger*. Results showed that maximum production of lipase (30.25 Units/mL) was achieved at 72 hours with initial pH 6.0, when culture was grown in 0.6N NH4OH pretreated sugarcane bagasse (collected from city) in comparison to other pretreated sugarcane waste.

Benjamin and Pandey (1997) reported maximum production of extracellular lipase from *Candida rugosa* on coconut cake as substrate. To optimize the condition, the substrate was enriched by supplying mineral solution, carbon source and several organic and inorganic nitrogen sources such as urea (1%), peptone (3%), maltose (5%) and olive oil (10%). The raw cake was fermented for 96 hours and gave 87.76 U/g.

Kamini *et al*. (1998) investigated the lipase production from *A. niger* using gingelly oil cake. Cultural conditions were standardized for the process. The strain used was MTCC 2594. A lipase activity of 363.6 U/g was obtained at 72 hour under optimum conditions. Addition of various nitrogen sources, carbohydrates and inducers to the substrate was found to be ineffective. The enzyme was optimally active at pH 7.0 and 37°C and was found to be stable between pH4.0-

10.0 and 4-50°C. The enzyme also showed remarkable stability in the presence of detergents and it could be effectively used for the removal of triglyceride soils in laundry.

Maia *et al*. (1999) reported the production of extracellular lipase by phytopathogenic fungus *Fusarium solani* FS1. A Brazilian strain of *Fusarium solani* was tested for extracellular lipase production in peptone-olive oil medium. The optimum lipase activity was achieved at pH8.6 and 30°C and good enzyme stability (80% activity retention) was observed in the pH ranging from 7.6 to 8.6 and the activity rapidly dropped at temperatures above 50°C. Namboodiri and Chattopadhyaya (2000) reported the purification and biochemical characterization of novel thermostable lipase from *Aspergillus niger*. Minoshima and Yoriko (2000) reported the preparation of thermostable lipase of Alcaligenes.

Mala *et al*. (2001) investigated the enhanced lipase production from *A. niger* by ultraviolet (UV) and nitrous acid mutagenesis, and the mutants were selected on media containing bile salts. Nitrous acid mutants exhibited increased efficiency for lipase production as compared to UV mutants in submerged fermentation. Their investigation indicates a possible role for the *A. niger* mutant strains in the biodegradation of oil-polluted environments for the development of ecofriendly technologies.

Abdel-Fattah and Hammad (2002) reported that filamentous fungi isolated from soil were screened for their ability to produce extracellular lipase. Among ten filamentous fungi tested, two strains identified as *Aspergillus niger* and *A. terreus*

were selected as the highest lipase producer. Maximum lipase production was obtained in five days cultures utilized 0.4% (w/v) corn oil as a carbon source. Optimum pH for crude lipase production by the tested fugal strains was 6.0, while L-glutamic acid as a nitrogen source gave the highest lipase production. Among the various fungal strains screened for lipase production, *Rhizopus arrhizus* NCIM 877, 878, 879 and *A. niger NCIM 1207* produced significant quantities of enzyme when grown in synthetic oil based (SOB) medium under submerged conditions (Mahadik, *et al*., 2002). They observed pH and temperature optima were at pH 2.5 and 45°C.

Rotticci (2003) reported that Candida lipase has high thermostability, broad substrate specificity and high selectivity. Recently it has been cloned and expressed in lower eukaryotes as well as in *Escherichia coli* (Liu *et al*. 2006). Pastore *et al*. 2003 verified that the lipase from *Rhizopus sp.* showed a better stability between pH values of 5.0 and 8.5 Lima *et al*. (2003) reported a wild fungal strain isolated from soybean oil and identified as *Penicillium aurantiogriseum* initially presented a volumetric lipase activity of 0.4 U/mL in submerged culture in a medium containing 0.5 % yeast extract and 1 % olive oil. They succeeded in increasing the lipolytic activity 62-fold over the initial values obtained with the non-optimized medium. Lipases from *Aspergillus* source, usually show pH optima in the acidic to neutral range except *Aspergillus carneus* with alkaline optimum pH (9.0) (Saxena *et al*., 2003).

Adinarayana *et al*. (2004) employed different incubation periods (24, 48, 72, 96, 120, 144 and 168 hrs) to study their effect on lipase production by

*Aspergillus* sp. (AU15). The maximum enzyme yield (1934 U/g) was obtained at 96 hrs incubation. Mahadik *et al*. (2004) investigated lipase producing mutants of *A. niger*NCIM 1207, isolated by subjecting *conidia* to UV- irradiation. Mutants UV-10 and ANCR-1 showed seven and five-fold enhanced productivity of enzyme, respectively, over the wild strain in shake flask culture when grown in SOB medium containing 1% olive oil. Cihangir and Sarikaya (2004) studied that fungi isolated from soil were screened for exogenous lipolytic activity. The highest lipase activity was found in a new soil isolate of *Aspergillus sp*. Some optimal cultural parameters influencing the growth and production of extracellular lipase from this *Aspergillus sp*. were investigated. The lipase yield was maximum on day 4 of incubation at pH 5.5 and 30$^{o}$C.

Gopinath *et al*. (2005) reported about 34 wild fungal species associated with edible oil mill wastes which were isolated by the serial dilution technique methods for rapid screening of fungal species against production of extracellular enzymes such as amylase, protease, cellulase, and lipase. Among all the species, fungal species *A. fumigatus, A. japonicus, A. nidulans, A. terreus, Curvularia pallescens, Fusarium oxysporum, Geotrichum candidum, Mucor racemosus, Penicillium citrinum, P. frequentans, Rhizopus stolonifer,* and *Trichoderma viride* exhibited maximum lipase activity, between 7.0 and 12.0 pH and at temperatures up to 80$^{o}$C. Carbon and nitrogen sources, the presence of activators and inhibitors, incubation temperature, pH, inoculum amount and oxygen tension can influence lipase production (Gupta, *et al*. 2004). Savitha *et al*. (2007) reported that fungal strains of different genera were isolated from various sources

of which 4 (3 *Aspergillus sp*. and 1 *Mucor sp*.) were found to be positive for lipase production.

Damaso *et al*. (2008) evaluated *A. niger* mutant 11T53A14 for lipase production using wheat bran and corn cob, supplemented with olive oil as substrates. The best results were obtained with wheat bran. Additionally, three industrial byproducts from corn oil refining (soapstock, stearin and fatty acids) were evaluated as substitutes to the olive oil in the function of lipases production inducer. Among them, soapstock and stearin were the best inducers, whereas fatty acids presented an inhibitor effect. The highest lipase activities using soapstock, stearin and fatty acids were 62.7 U/gds, 37.7 U/gds and 4.1 U/gds, respectively. Mehtras *et al*. (2009) observed maximum *A. niger* lipase activity at pH 2.5 and 50°C.

Different agricultural byproducts such as brassica meal, almond meal, coconut meal, rice husk and wheat bran were used by Iftikhar *et al*. (2011) for extracellular lipase production by *A. niger*, *Penicillium chrysogenum, Rhizopus microsporus, Mucor mucedo, Alternaria alternata,Trichophyton sp., Fusarium semitectum, E (un-identified), Curvularia sp., and Aspergillus flavus.* Brassica meal gave significantly highest enzyme activity (46.5 ± 1.13a U/g), as compared to other substrates. Twelve fungal species from oil-mill effluent composts at Nsukka have been studied and it was found that *Aspergillus* sp. are more common; however, the higher lipase producers are *Trichoderma sp.* followed by *Aspergillus sp*. (Nwuche and Ogbonna 2011).

Lipolytic activity has been observed in *Pencillium sp*. by Salihu *et al*., (2011). Scientific literature cites the maximum amount of lipase activity is obtained on the day 4 with recoverable enzyme activity gradually decreasing thereafter. The medium containing olive oil as carbon source showed both the highest lipase production and also the highest biomass when compared with other media. Extracellular lipase of *Rhizopus sp*. isolated from oil-contaminated soil was recently characterized (Thota, *et. al.,* 2012). Optimization of lipase production by *A. niger* in solid state fermentation from rice bran as solid substrate was investigated by Hosseinpur *et al*. (2012).

Extracellular secretion has been well studied for *Aspergillus sp*. (Abrunhosa *et al*., 2013). Sundar *et al*. (2013) demonstrated that tween 80, soybean meal and ammonium sulfate was found to be the best source for carbon, organic nitrogen and inorganic nitrogen source respectively for the production of lipase by *A. niger* isolated from fungal infected coconut, coconut oil cake, soil from coconut oil mill, spoiled bread and castor oil soaked cotton. Sethi *et al*. (2014) used agro-industrial substrates such as, pearl millet (PM), finger millet (FM), orange peels (OP), mustard oil cake (MoC) and chikling vetch peels (CVP) were appraised for enhanced production of lipase at liquid static surface (LSSF) and liquid shaking fermentation (LShF). MoC was found to be most suitable among these above substrates taken for optimal biosynthesis of lipase by *A. terreus* NCFT 4269.10. Sumathy and Vijayalakshmi (2014) investigated the lipase production by SSF of agro-industrial wastes like coconut oil cake, wheat rava, wheat bran, gingelly oil cake etc., by *Aspergillus niger* MTCC 2594. Their study

revealed that highest enzyme production was obtained by combination of wheat bran, coconut oil cake, wheat rava.

**Detection of lipase production**

Various methods have been developed to detect and determine lipase activity. They are based on either the disappearance of the substrate, the production of free fatty acids (FFAs), or the clarification of emulsions (Hasan *et al*. 2009). There must be three factors to detect a lipase- positive bacterium by culturing it. These factors include

- growth of the organism,
- production of lipase by that organism under suitable growth conditions and the presence of a sensitive method to detect lipase activity (Shelley *et al*., 1987).

Tributyrin is considered as a gold standard for screening of lipase producing microorganisms. It was used for the screening purpose. The method was suggested by Sarkar *et al*. (1998). Microorganisms are often tested for lipase production on solid media. Plate methods are usually used for the preliminary screening of lipase-producing bacteria for the convenience and rapidness. All methods containing agar can be grouped into two categories;

1. Methods based on substrate changes in appearance as a result of lipolysis and
2. Methods, including the usage of an indicator dye to detect lipolysis (Thomson *et al*., 1999).

In all methods, it is important that there must be a contact between substrate and enzyme. Thus the agar content in screening media can be reduced for increased diffusibility of extracellular lipase (Hou and Johnston 1992).

Lipase activity is identified by using triacylglycerols composed of long-chain fatty acids. Triolein is the most ideal substrate due to its liquid form at common assay temperatures. This feature facilitates emulsification of it into growth media. Alternatively, olive oil can be used instead of triolein. Olive oil has the advantage of including high concentration of oleic acid and being more economical (Jensen 1983). Substrates like tweens and tributyrin can also be used for the detection of lipases. However, since they can also be hydrolyzed by esterases, they don't give lipase specific results (Jensen 1983, Shelley, *et al*. 1987). In conclusion, tweens and tributyrin can be used for only primary screening procedures.

Researchers also developed another plate assay for bacterial lipases with fluorescent dye Rhodamine B (Kouker & Jaeger 1987). Yadav *et al*. 1998 demonstrated Zymography for the identification of a lipase by electrophoresis. The dyes phenol red and Victoria blue have been used to stain the active lipase by the overlay technique. Fluorogenic substrate, 4- methylumbelliferone (MUF) derivatives, also used in detection of lipase activity (Diaz *et al*.1999). The native PAGE gel was incubated with overlay containing Victoria blue and olive oil for 12 h, and blue zones developed in the overlay showing the presence of active lipases. Gilham & Lehner, (2005) employed a Chromogenic lipase assay method

which utilized a special substrate that gives a colored end product, or that can be easily converted to a colored product, such as p-nitrophenyl or naphthyl esters.

Singh *et al*. 2006 incorporated the substrate along with phenol red in growth media. Isolates producing esterase gave positive results only on tributyrin plates, while those producing lipase were positive on both tributyrin and triolein plates. Titration methods for the determination of the lipase activity are wide spread by using its tributyrin or olive oil as a substrate (Starodub, 2006). Methods make use of pH indicator dyes, such as victoria blue and phenol red, to indicate the lipolytic activity by the change in dye color as pH drops due to released FFAs (Hasan *et al*., 2009). These methods can differentiate esterase and lipase by using tributyrin and triolein respectively, as substrates.

Kumar *et al*. (2011) screened fungi with bromophenol blue dye supplemented agar plates with olive oil as the substrate. Using tributyrin formation of the clear zone around the fungal colony showed different mutant strains that produced extracellular lipases (Toscano *et al*., 2011). Rhodamine method with olive mill wastewater was used to determine the production of lipases by *Aspergillus ibericus* (Abrunhosa *et al*., 2013).

**Purification of Lipases**

Different workers have followed diverse purification strategies for purification of lipase. *A. niger* lipase was purified by precipitation with ammonium sulfate, hydrophobic interaction (butyl Toyopearl 650 M), gel filtration (Sephadex G-75), anion exchange chromatography (DEAE-Sepharose CL-6B) and adsorption

on hydroxyapatite by Sugihara *et al*. (1988). Various purification strategies have been reviewed for the lipase enzyme (Palekar *et al*., 2000; Sharma *et al*., 2001 and Saxena *et al*., 2003). In the case of extracellular lipases, it is primarily important to remove other contaminants from the compound mixture containing lipase by suitable strategy. The conventional purification strategies give a low yield due to a large hydrophobic surface near the active site. Novel purification steps are mandatory to increase overall enzyme yields and it could be achieved by opting an appropriate chromatography system.

An extracellular lipase from *Acinetobacter calcoaceticus BD 413* was purified to homogeneity using hydrophobic interaction Fast Performance Liquid Chromatography (FPLC) (Kok *et al*., 1995). Lipase of *Aspergillus oryzae* was purified by ammonium sulfate fractionation, anion exchange chromatography (Toida *et al*., 1995). Similarly, Toida *et al*. (1998) purified *A. oryzae* lipase by ammonium sulfate fractionation, acetone precipitation, anion exchange chromatography and gel filtration with 11% yield.

Also, a lipase from *Penicillium roqueforti* IAM 7268 was purified to homogeneity by a procedure involving ethanol precipitation, ammonium sulfate precipitation, and three chromatographic steps on different matrices (DEAE-Toyopearl 650 M, Phenyl Toyopearl 650 M, Toyopearl HW-60). *Pichia burtonii* lipase was purified to homogeneity by a combination of DEAE-Sephadex A-50 ion exchange chromatography, Sephadex G-100 gel filtration, and isoelectric focusing (Sugihara *et al*., 1995).

The molecular mass of purified lipase was 25 kDa by electrophoresis (Mase *et al*., 1995). Kim *et al*. (1996) purified a highly alkaline extracellular lipase of Proteus vulgaris by ion exchange chromatography. An extracellular lipase from *P. aeruginosa KKA-5* was purified using ammonium sulfate precipitation and successive chromatographic separations on hydroxyl appetite (Sharon *et al*., 1998).

Lipase from *Aspergillus carneus* has also been purified by us using a simple two-step procedure involving ammonium sulfate precipitation and hydrophobic interaction chromatography on octyl-Sepharose (Davidson, 1998). Lipase produced by *Staphylococcus epidermidis* RP 62A was purified to homogeneity by a combination of precipitation techniques, metal affinity chromatography, and gel filtration (Simons *et al*., 1998). A recombinant lipase (rROL) produced by *S. cerevisiae* was purified by ethanol precipitation, butyl-Toyopearl 650 M chromatography, and Sephacryl S-100 HR gel filtration, to a single band by native PAGE (Takahashi *et al*., 1998). *A. repens* lipase were purified using a DEAE-Sephadex A-50 column and preparative electrophoresis (Kaminishi *et al*., 1999). Hiol *et al*. (2000) purified an extracellular lipase produced by *Rhizopus oryzae* by ammonium sulfate precipitation, sulfopropyl Sepharose chromatography, Sephadex G-75 gel filtration, and a second sulfopropyl Sepharose chromatography step. A lipase was purified to homogeneity from extracellular culture of *Aspergillus nidulans* WG312 by phenyl- Sepharose chromatography and affinity binding on linolenic acid–agarose (Mayordomo *et al*., 2000).A thermostable lipase produced by a thermophilic *Bacillus sp. J 33* was

purified to 175-fold by ammonium sulfate and phenyl Sepharose column chromatography (Nawani and Kaur, 2000).

Lipase from *Candida rugosa* was purified by ammonium sulfate precipitation, dialysis, ultrafiltration and gel filtration using Sephadex-200 to a 43-fold purification and 64.35 mg/ml specific activity (Benjamin and Pandey, 2001). Ion exchange and gel filtration chromatography are commonly preferred methods (Abdou, 2003). Saxena *et al*. (2003) have purified a novel lipase from *Aspergillus terreus* to electrophoretic purity by means of methods including ammonium sulfate and acetone precipitation, gel filtration (G-100) and ion exchange chromatography. A reversed micellar system, membrane processes, immunopurification, hydrophobic interaction chromatography with an epoxy-activated spacer arm (ligand), column chromatography using polyethylene glycol (PEG)/sepharose gel, and aqueous two -phase systems are also recommended (Gupta *et al*., 2004).

Kumarevel *et al*. (2005) reported a stepwise purification strategy for fungal lipases to remove other components released from the fungus *Cunninghamella verticillata* extracellularly, using acetone precipitation as the important step. To avoid many steps in this study and to minimize the impurities as much as possible the experiment was repeated with 50% acetone saturation with a gradual increments of 5% acetone. An extra cellular lipase was isolated and purified from the culture broth of *Pseudomonas aeruginosa* SRT9 to apparent homogeneity using ammonium sulfate precipitation followed by chromatographic techniques on phenyl Sepharose CL- 4B and Mono Q HR 5/5 column by Borkar *et al*., (2006).

Extracellular lipase from *Antrodia cinnamomea* BCRC 35396 was first purified by ammonium sulfate precipitation and DEAE-Sepharose Chromatography (Shu *et al*., 2006). One of the choices is hydrophobic interaction chromatography and it is considered as a common strategy (Fucinos, *et al*., 2011). Shah and Bhatt (2011) purified the extracellular lipase produced by *B. subtilis* Pa2 acetone precipitation and ion exchange chromatography. Different strategies for lipase purification with the varied sources were recently described in detail by Singh and Mukhopadhyay (2012), and it seems that the production of lipases from fungal species results in different molecular sizes, due to variations in the number of amino acid residues.

Overall, traditional purification strategies are considered time consuming with lower yields and the trends are moving towards aqueous two-phase extraction, and purification in ionic liquids and purification based on lipase-lipase interaction (Nagarajan, 2012). Extracellular lipase obtained from *Pseudomonas fluorescens* Lp1 was purified by ammonium sulfate precipitation followed by dialysis against 0.05 M Sodium phosphate buffer solution (pH 7.2). Dialyzed lipase was further purified by Sephadex G -100 previously equilibrated with 0.1M Tris- HCl (pH 7.0) (Kanimozhi and Perinbam, 2013). *Streptomyces bambergiensis OC 25-4* strain was partially purified with ammonium sulfate precipitation, dialysis, and gel filtration chromatography 2.73- fold and with 92.12 IU/mg specific activity (Ugur *et al*., 2014).

## Characterization of Lipases

The first microbial lipase structure studied was that of the *Rhizomucor miehei* lipase by Brady *et al*. (1990) from X-ray crystallographic analysis. Recently, especially in this millennium, other approaches also have come into practice for structural analyses, including the use of bioinformatics tools for structure predictions up to the tertiary levels of protein organization (Mala and Takeuchi, 2008).

## FTIR (Fourier-transform infrared)

FTIR (Fourier-transform infrared) spectroscopy is emerging as a powerful technique for the determination of the secondary structure of proteins in solution, with no restriction on their molecular mass (Surewicz *et al*., 1993). Fourier transform infrared (FTIR) spectroscopy is being increasingly used for investigating protein structure and stability (Haris and Severcan, 1999). Vecchio *et al*. (1999) employed Fourier-transform infrared (FT-IR) spectroscopy was employed to investigate structure of lipases from *Candida antarctica* B and *Pseudomonas cepacia*. Different conformational types result in different absorption bands in an FTIR spectrum, which are usually broad and overlapping. To overcome these, Severcan *et al*. (2004) have successfully reported the use of artificially generated spectral data to improve protein secondary structure prediction from FTIR spectroscopy. Indeed, conformational changes and structural stability have been successfully studied by FTIR spectroscopy for a large number of proteins,

including lipases (Auria *et al*., 2000; Noinville *et al*., 2002; Severcan *et al*., 2003 and Scheirlinckx *et al*., 2004).

FTIR study of the enzyme CRL1 (*C. rugosa* lipase 1) with the purpose of assessing the usefulness of this technique for the analysis of conformation and post-translational modifications of this enzyme, in both basic and applied research was conducted by Natalello *et al*., 2005. Foresti *et al*. (2009) have withdrawn samples from the supernatant immobilization medium and utilized FTIR spectroscopy in the 1700–1600 cm−1 range in order to obtain quantitative information on the structure elements of the lipase B produced by *C. antarctica*. A simple and fast infrared spectroscopy method were used by Rajan *et al*., (2011) for the characterization of 1, 3-specific and non-specific lipases produced by *Aspergillus fumigatus MTCC 9657*.

**Nuclear magnetic resonance (NMR)**

Nuclear magnetic resonance (NMR) is another important method in the study of lipase structure analysis. Jonas (2002) has reviewed the studies of proteins using high resolution NMR. It is stated that the combination of advanced high resolution NMR technique with high pressure capability is a powerful experimental tool in studies of protein folding. The main advantages of using high resolution and high pressure NMR are the uses of 1-D NMR for determination of structural and dynamic changes in different regions of the protein and the allowance of distance specific information to be obtained between amino acid residues in different regions of the protein. NMR analyses of *Pseudomonas mendocina* lipase and its F180P/S205G mutant indicated virtually identical

structures with notable differences in local dynamics (Sibille *et al*., 2006). The structure of a thermostable lipase '6B' isolated from *Bacillus subtilis* was analysed using NMR by Kamal *et al*. (2011). This NMR study suggested a very rigid structure of 6B lipase. Silva *et al*. (2012) used 1H NMR spectroscopy as a practical tool to aid the selection of two microbial lipases from *Burkholderia cepacia* and *P. fluorescens*, as the most suitable lipase source to mediate the synthesis of biodiesel.

**Mass spectroscopy**

Mass spectroscopy is a versatile tool for protein analysis and has contributed much to the field of proteomics, in conjunction with two-dimensional electrophoresis. Proteomics helps to define the functions and interrelationships of proteins in an organism. As genome sequence information has accumulated, the paradigm has shifted from sequencing to identification of proteins, which has been facilitated by advances in ionization and mass analysis techniques for mass spectrometry. Electrospray ionization (ESI) and Matrix-assisted laser desorption/ionization (MALDI) methods are currently the principal methods for peptide/protein ionization and have been linked to high-throughput sample preparation techniques. Large-scale protein identification has been made possible using mass spectrometry as reviewed by Lin *et al*. (2003). MALDI-TOF mass spectroscopy is widely used for identification of proteins.

Meunier *et al*. (2005) have investigated data analysis methods for detection of differential protein expression using 2DGE (Two dimensional gel electrophoresis) in order to minimize false positives, at the same time, without

losing information with false negatives. Chu *et al*., (2008) characterized the lipase from *Antrodia cinnamomea* by GCMS analysis. *A. cinnamomea* lipase represents the first enzyme of the lipase family from a basidiomycetous fungus.

**SDS-PAGE**

Conventional characterization of lipase includes determination of molecular weight. Molecular weight of the protein is estimated by SDS-PAGE, according to Laemmli (1970). Two lipase isoforms from *O. piliferlipase* were identified on SDS-PAGE having molecular masses 60 and 5 kDa respectively (Brush *et al*., 1999). Ferrer *et al*. (2000) purified the *Penicillium chrysogenum* lipase isolated from wastes of industrial cultures and the molecular mass of the purified lipase was estimated to be 40 kDa on SDS-PAGE. Hoil *et al*. (2000) demonstrated the purified lipase run on SDS-PAGE for molecular weight determination, was found to be 32 kDa. The purified lipase of *Serratia marcescens* was estimated by SDS-PAGE with a MW of 52 kDa (Abdou 2003). Molecular weight of lipases generally ranges between 22-69 kDa (Pandey *et al*.2005).

The molecular mass of Lipase-A and Lipase-B produced by *Geotrichum sp.* were determined to be approximately 41.1 and 35.8 kDa, respectively by SDS-PAGE (Caia *et al*., 2009). Liu *et al*. (2009) reported *Fusarium solani* lipase with the molecular mass 31.6 kDa as determined by SDS-PAGE after purification using acetone fractionation and Q-sepharose ion exchange technique. Mehtras *et al*. (2009) purified *Aspergillus niger* NCIM 1207 lipase to homogeneity using ammonium sulfate precipitation followed by phenyl sepharose and sephacryl-100

gel chromatography. The purified enzyme showed a prominent single band of32.2 kDa molecular weight on SDS–PAGE. The lipase from *Bacillus subtilis* Pa2 was characterised by SDS-PAGE by Shah and Bhatt (2011) and the molecular weight of the pure protein was estimated to be 19.4 and 19.2 kDa. Jayaprakash and Ebenezer (2012) determined the molecular weight of the purified lipase from *A. japonicas* by SDS-PAGE on 12% polyacrylamide gels.

**Application of lipases in industries**

Major applications of lipases are summarized in Table 3. And List of available commercial enzymes are given Table 4.

**Table 3**

**Industrial application of microbial lipases (Vulfson, 1994)**

| Industry | Action | Product of application |
|---|---|---|
| Detergents | Hydrolysis of fats | Removal of oil stains from fabrics |
| Dairy foods | Hydrolysis of milk fat, cheese ripening, modification of butter fat | Development of flavoring agents in milk, cheese and butter |
| Bakery foods | Flavor improvement | Shelf – life prolongation |
| Beverages | Improved aroma | Beverages |
| Food dressings | Quality improvement | Mayonnaise, dressings, and whippings |
| Health foods | Transesterification | Health foods |
| Meat and fish | Flavor development | Meat and fish products; fat removal |
| Fats and oils | Transesterification; hydrolysis | Cocoa butter, margarine, fatty acids, glyercol, mono and diglycerides |
| Chemicals | Enantioselectivity, synthesis | Chiral building blocks, chemicals |
| Pharmaceuticals | Transesterication, hydrolysis | Speciality lipids, digestive aids |
| Cosmetics | Synthesis | Emulsifiers, moisturizers |
| Leather | Hydrolysis | Leather products |
| Paper | Hydrolysis | Paper with improved quality |
| Cleaning | Hydrolysis | Removal of fats |

**Table 4**

**Commercially available microbial lipases (Jaeger and Reetz, 1998)**

| Type | Source | Application | Producing company |
|---|---|---|---|
| Fungi | *Candida rugosa* | Organic synthesis | Amano, Biocatalysts, Boehringer, Mannheim, Fluka, Genyzme, Sigma |
| | *Candida Antarctica* | Organic synthesis | Boehringer mannhein, Novo Nordisk |
| | *Thermomyces anuginosus* | Detergent additive | Boehringer Mannhein, Novo Nordisk |
| | *Rhizopus miehei* | Food processing | Novo Nordisk, Biocatalysts, Amano |
| Bacteria | *Burkholderia cepacia* | Organic synthesis | Amano, Fluka, Boerhinger Mannheim |
| | *Pseudomonas alcaligenes* | Detergent additive | Genencor |
| | *Pseudomonas mendocina* | Detergent additive | Genencor |
| | *Chromobacterium viscosum* | Organic synthesis | Asahi, Biocatalysts |

### 2.1 Lipases in the detergent industry

The major commercial application for hydrolytic lipases is their use in laundry detergents. Lipases for use in detergents needs to be thermostable and remain active in the alkaline environment of a typical machine wash.

An estimated 1000 tons of lipases are added to approximately 13 billion tons of detergents produced each year (Jaeger and Reetz, 1998).

Detergent lipases are especially selected to meet the following requirements: (1) a low substrate specificity i.e., an ability to hydrolyze fats of various compositions (2) ability to withstand relatively harsh washing conditions (pH 10-11, 30-60℃) (3) ability to withstand damaging surfactants and enzymes (e.g., linear alkyl benzene sulfonates (LAS) and proteases), which are important ingredients of many detergent formulations. Lipases with the desired properties are obtained through a combination of continuous screening (Yeoh *et al.,* 1986; Cardenas *et al.,* 2001) and protein engineering (Kazlauskas and Bornscheuer, 1998).

In 1995 two bacterial lipases were introduced – 'Lumafast' from *Pseudomonas mendocina* and 'Lipomax' from *Pseudomonas alcalingenes* – by Genencor International (Jaeger and Reetz., 1998). Gerritse *et al.,* (1998) reported an alkaline lipase, produced by *P. alcaligenes* M – 1, which was well suited to removing fatty stains under conditions of a modern machine wash. The patent literature contains examples fo many microbial lipases that are said to be suitable for use in detergents (Bycroft and Byng, 1992).

## 2.2 Lipases in food industry

Fats and oils are important constituents of foods. The nutritional and sensory value and the physical properties of a triglyceride are greatly influenced by factors such as the position of the fatty acid in the glycerol backbone, the chain length of the fatty acid, and its degree of unsaturation. Lipases allow us to modify the properties of

lipids by altering the location of fatty acid chains in the glyceride and replacing one or more of the fatty acids with new ones. This way, a relatively inexpensive and less desirable lipid can be modified to a higher value fat (Colman and Macrae, 1980; pabai *et al.*, 1995a, b; Undurraga *et al.*, 2001).

Cocoa butter, a high – value fat, contains palmitic and stearic acids and has a melting point of approximately 37℃. Melting of cocoa butter in the mouth produces a desirable cooling sensation in products such as chocolate. Lipase based technology involving mixed hydrolysis and synthesis reactions is used commercially to upgrade some of the less desirable fats to coca butter substitutes (Colman and Macrae, 1980; Undurraga *et al.*, 2001).

Pabai *et al.,* (1995a) described a lipase-catalyzed intersterification of butter fat that resulted in a considerable decrease in the long – chain saturated fatty acids and a corresponding increase in C18:0 and C18:1 acid at position 2 of the selected triacylglycerol.

## 2.3 Lipases in pulp and paper industry

'Pitch' or the hydrophobic components of wood (mainly triglycerides and waxes), causes severe problems in pulp and paper manufacture (Jaeger and Reetz., 1998). Lipases are used to remove the pitch from the pulp produced for paper making. Nippon Paper Industries, Japan, have developed a pitch control method that uses the *Candida rugosa* fungal lipase to hydrolyze up to 90% of the wood triglycerides.

## 2.4 Lipases in organic synthesis

Use of lipases in organic chemical synthesis is becoming increasingly important. Lipases are used to catalyze a wide variety of chemo-, region-, and stereo selective transformations (Rubin and Dennis, 1997b; Kaziauskas and Bornscheuer, 1998; Berglund and Hutt, 2000). Majority of lipases used as catalysts in organic chemistry are of microbial origin. These enzymes work at hydrophilic-lipophilic interface and tolerate organic solvents in the reaction mixtures. Use of lipases in the synthesis of enantiopure compounds has been discussed by Berglund and Hutt (2000).

The enzymes catalyze the hydrolysis of water-immiscible triglycerides at water-liquid interface. Under given conditions, the amount of water in the reaction mixture will determine the direction of lipase-catalyzed reaction. When there is little or no water, only esterification and transesterification are favoured (Klibanov, 1997). Hydrolysis is the favoured reaction when there is excess water (Klibanov, 1997). Lipases – catalyzed reaction in supercritical solvents have been described by Rantakyla *et al.,* (1996), Turner *et al.,* (2001) and King *et al.,* (2001).

## 2.5 Lipases in bioconversion in aqueous media

Hydrolysis of esters is commonly carried out using lipase in two-phase aqueous media (Vaysse *et al.,* 1997; Chatterjee *et al.,* 2001). Penreach and Baratti (1996) reported on the hydrolysis of p-nitro phenyl palmitate (pNPP) in n-heptane by a lipase preparation of *Pseudomonas cepacia.*

Mutagenesis has been used to greatly enhance the enantioselectivity of lipase (Bornscheuer, 2000; Gaskin *et al.,* 2001). For example, in one case, the enantioselectivity of lipase catalyzed hydrolysis of a chiral ester (*Pseudomonas aeruginosa* lipase) was increased from e.e 2% to e.e 81% in just four mutagenesis cycles. The lipase acyl transferase from *Candida parapsilosis* has been shown to catalyze fatty hydroxamic acid biosynthesis ina biphasic liquid/aqueous medium. The substrates of the reaction were acyl donors (fatty acid or fatty acid methyl ester) and a hydroxylamine. The transfer of acyl group from a donor ester to hydroxylamine (aminolysis) was catalyzed preferentially compared to the reaction of free fatty acids. This feature made the *C.parapsilosis* enzyme the catalyst of choice for the direct bioconversion of oils in aqueous medium (Vaysse *et al.,* 1997). Yeo *et al.,* (1998) reported a novel lipase produced by *Burkholderia sp.,* which could preferentially hydrolyze a bulky ester, t-butyl octanoate (TBO). This lipase was confirmed to be 100-fold superior to commercial lipases in terms of its TBO-hydrolyzing activity.

## 2.6 Lipases in ester synthesis

Lipases have been successfully used as catalyst for synthesis of esters. The esters produced from short-chain fatty acids have applications as flavoring agents in food industry (Vulfson, 1994). Methyl and ethyl esters of long-chain acids have been used to enrich diesel fuels (Vulfson, 1994). From *et al.,* (1997) studied the esterification of lactic acid and alcohols using a lipase of *Candida Antarctica* in hexane. Esterification of five positional isomers of acetylenic fatty acids (different chain lengths) with n-butanol was studied by Lie *et al.,* (1998), using eight different lipases. Arroyo *et al.,* (1999) noted that an optimum pre-equilibrium water activity

value was necessary for obtaining a high rate of esterification of (R, S) – ibuprofen. Janssen *et al.,* (1999) reported on the esterification of sulcatol and fatty acids in toluene, catalyzed by *C. rugosa* lipase (CRL). Krishnakant and Madamwar (2001) reported using lipase immobilized on silica and microemulsion-based organogels, for ester synthesis.

## 2.7 Lipases in oleochemical Industry

Use of lipases in oleochemiccal processing saves energy and minimizes thermal degradation during alcoholysis, acidolysis, hydrolysis and glycerolysis (Vulfson, 1994; Bornscheuer, 2000). Although lipases are designed by nature for the hydrolytic cleavage of the ester bonds of triacylglycerol, lipases can catalyze the reverse reaction (ester synthesis) in a low-water environment. Hydrolysis and esterification can occur simultaneously in a process known as interesterification. Depending on the substrates, lipases can catalyze acidolysis (where an acyl moiety is displaced between an acyl glycerol and a carboxylic acid), alcoholysis (where an acyl moiety is displaced between an acyl glycerol and an alcohol) and transesterification (where two acyl moieties are exchanged between two acylglycerols) (Balcao *et al.,* 1996).

### Biodiesel

Biodiesel is primarily produced by transesterification of edible oils. Increasing concern about using food supplies for fuel has generated interest in alternative raw materials. Biodiesel production process is sensitive to the quality of the feedstock requiring vegetable oil or animal fats with very low amount of FFA. The most

common feedstock for biodiesel is edible oil such as soybean, rapeseed, canola, sunflower, palm, coconut and even corn oil (Mondala, 2008). However, high cost of vegetable oils had attracted researcher to produce biodiesel from waste cooking oil because it is available with relatively cheap price. Although, the use of waste oils can lower the feedstock cost significantly, complicated procedures are needed to remove impurities, resulting in high operating costs (Nisworo, 2006; Zhang *et al*., 2003).

Knothe and Steidley (2005), reported that biodiesel has a better lubricity than petro- diesel hydrocarbons, because of the polarity that is introduced with the presence of oxygen atoms which is lacking in petro-diesel. Lubricity it was reported improves with the chain length and the presence of double bonds. One major technical advantages of biodiesel over petro-diesel is lubricity (Knothe *et al*., 2005). Christopher *et al*. (2010) also showed that blending biodiesel with petro-diesel decreases the brake thermal efficiency (BTE). Research conducted by Reddy *et al*. (2010), showed that BTE of diesel fuel and cotton seed methyl ester (biodiesel) blends; it was found that the BTE is always found to be lower with biodiesel blends as compared with petro-diesel.

Cumali *et al*. (2011) experimented on a diesel engine using biodiesel fuel produced from sunflower oil with petro-diesel. They found that using biodiesel in comparison to using petro- diesel increased the brake specific consumption, but a decrease in pollutants such as particulate matter and carbon monoxide. Fallahipanah *et al*. (2011), investigated the performance of biodiesel in an engine which completes a specific cycle. Their results showed that when biodiesel is applied as a fuel in the

engine, similar results was obtained and in some cases even better results was obtained over petro-diesel fuel.

Rashid (2011), reviewed that in an assessment of the environmental hazards caused by the use of fossil fuels, biodiesel is being considered to be the best fuel for diesel engines since burning biodiesel and its blends has the lowest Green House Emissions on a life cycle basis. The emission of carbon monoxide gas is reduced by using biodiesel as a fuel. His review showed that biodiesel is a fuel that is clean and environment friendly, which can supplement or replace petro-diesel as a fuel in the future.

Indirect injection diesel engine was studied with petrodiesel and 100% biodiesel at various fuel injection pressures by Kumar *et al*. (2012). It was observed that at 100% load, brake thermal efficiency of biodiesel increases as the fuel injection pressure is increased keeping advance angle of fuel injection constant, whereas for petro-diesel, the brake thermal efficiency decreases under the same conditions. Rubianto *et al*. (2013) found that biodiesel has a contribution to reduce particulate emission from combustion on a boiler burner with reduction of 29.796%. This percentage of reduction is significant to decrease air pollution, creating a better and healthier environment. Similarly Innocent *et al*. (2013) demonstrated that the combustion of biodiesel emits particulate matter and gases which is lower than petrodiesel, combustion of biodiesel and biodiesel blends have shown a significant reduction in particulate matter and exhaust emissions.

## Vegetable oil as feed stock for biodiesel production

India has rich and abundant forest resources with a wide range of plants and oilseeds. Economics of the biodiesel production process can be improved if non-edible oils are used. Table 5 gives various non-edible oil sources available in India.

**Table 5**

**Non-edible oil potential in India by National Oilseeds and Vegetables Oil Development (NOVOD) Board, India**

| Oil | Botanical name | Oil potential |
|---|---|---|
| Neem | *Azadirachta indica* | 100 000 |
| Karanja | *Pongamia pinnata* | 55 000 |
| Kusum | *Schleichera oleosa* | 25 000 |
| Ratanjyot | *Jatropha curcus* | 15 000 |
| Pilu | *Salvadora oleoides* | 17 000 |
| Tumba | *Citrullus collocynthis* | 21 000 |
| Sal | *Shorea robusta* | 180 000 |
| Mahua | *Madhuca indica* | 180 000 |
| Mango | *Mangifera indica* | 45 000 |
| Phulware | *Cheura* | 3 000 |
| Kokum | *Garcinia indica* | 500 |
| Simarouba | *Simaruba glauca* | - |
| Jojoba | *Simmondsia chinesis* | - |
| Chullu | *Prunus armeniaca* | 110 |
| Rice bran | *Oryza sativa* | 474 000 |

Amongst the various non edible oil seed crops, *Jatropha* (Plate No.1) and *Pongamia* plant (Plate No.2) is considered as the sole resources that can meet the growing demand of biodiesel in India due to their high productivity and less maturity cycle.

***Jatropha* oil**

*Jatropha* oil is suitable raw material for biodiesel production. *Jatropha* has primarily gained so much attention because it is claimed to avoid the food-versus-fuel debate and thus negative food security implications (BP, 2007; Tattersall, 2007). Yaakob *et al*. (2009) investigated that one way of reducing the biodiesel production costs is to use the less expensive feedstock containing. The fact that *Jatropha* oil cannot be used for nutritional purposes without detoxification makes its use as energy/fuel source very attractive. It has been reported that in 2008, 900, 000 hectares *J. curcas* was planted globally with majority of it located in Asia, and the rest in Africa and Latin America. The report also made a projection of 12.8 million hectares of *J. curcas* plantation by 2015.

*Jatropha* oil has been investigated as a fuel source by many researchers. Banerji *et al*. (1985) compared the fatty acid profile of four different species of *Jatropha*, namely *J. curcas, J. glandulifera, J. gossypifolia and J. multifida,* all of which were found to be suitable for methyl ester production. *J. curcas* was found to have the highest oil content at 48.5% and *J. multifida* had the highest energy value. *J. curcas* is the most widespread species among the *Jatropha* species, particularly because of its high oil content. It has been used directly in engines (Senthil Kumar *et al*., 2003) and transesterified to methyl ester (Oliveria *et al*., 2008) as well as blended with alcohol.

# PLATE 1

***Jatropha curcas* – Habit, Inflorescence, Seeds, Oil**

**Habit**

**Inflorescence**

**Seeds**

**Seed oil**

Muniyappa *et al*. (1996), investigated the correlation between the density, viscosity and cloud point of biodiesels from soybean and tallow oil. It was found that the high cloud point obtained for methyl ester from beef tallow oil was due to its high concentration of saturated fatty esters. Three of the inedible oils considered (*J. curcas, Pongamia pinnata* and *Azadirachta indica*) also contain high percentages of unsaturated fatty acids, and so they are unlikely to suffer from this problem. *J. curcas* contains the highest percentage of unsaturated fatty acid at 72.9 %, while *P. pinnata* and *A. indica* each contain 68.4 %. Therefore biodiesel from *S. oleidis* is likely to have poor cloud point properties.

*A. indica* is more renowned for its medicinal properties than its capability as a new raw material in biodiesel production. Out of the four inedible oils *J. curcas* remains the best option as a raw material for biodiesel production. The CN of the oil may be the lowest of the four, but it is still within the EN 14214 minimum limit. The oil fatty acid composition in *J. curcas* is dominated by oleic (18:1) and linoleic (18:2) acids, both of which are unsaturated fatty acids and thus the high cloud points of oils with high percentages of saturated fatty acids will be avoided. The longest fatty acid chain in *J.curcas* is arachidic acid (20:0), which contributes to 0.4% of its overall composition. The lack of long fatty acids in *J. curcas* will help to avoid the CFPP problem. This is very important in order to ensure that the biodiesel would be accepted at higher latitudes.

A two-step process consisting of pre-esterification and transesterification was developed to produce biodiesel from crude *Jatropha curcas* L oil by Lu *et al*. (2009) and they demonstrated an yield of higher than 98%. Study conducted by Rahman

*et al*., 2010, have recommended the usage of *Jatropha* oil in biodiesel production and adopting bio-fuel as an alternative to petroleum fuels.

*Jatropha* vegetable oil is one of the prime non edible sources available in India. The vegetable oil used for biodiesel production might contain free fatty acids which will enhance saponification reaction as side reaction during the transesterification process (Raja *et al*., 2011). Soumanou *et al*., (2012) demonstrated that enzymatic biodiesel production can be made commercially more viable, especially using a non-edible plant oil, *Jatropha*. Lee *et al*., (2013) compared the biodiesel production by enzymatic process using *Jatropha* oil and waste soybean oil. Their study showed that conversion of about 98% at 4 h could be achieved for biodiesel production using *Jatropha* oil, while a conversion of about 97% at 4 h was achieved from waste soybean oil.

### ***Pongamia* oil**

*Pongamia pinnata* (Leguminosae, subfamily Papilionoideae) is a medium sized tree that about 7 - 8 meters high with a trunk diameter of more than 50 cm. The trunk is generally short with thick branches spreading into a dense hemispherical crown of dark green leaves. The alternate, compound pinnate leaves consist of 5 or 7 leaflets arranged in 2 or 3 pairs, and a single terminal leaflet. Leaflets are 5 - 10 cm long, 4 - 6 cm wide, and pointed at the tip. Flowers are pink, light purple, or white. Pods are elliptical; (3 - 6 cm long and 2 - 3 cm wide) thick walled, and usually contain a single seed.

**Botanical Description of *Pongamia Pinnata* (Gaurav et al., 2011)**

- Fast growing, medium sized legume tree
- Height: 7 - 10 m, stem diameter: 50 - 80 cm
- Smooth grey-brown bark with vertical fissuring
- Leaves compound, pinnate and alternate
- Mature leaves glossy dark green above, pale below
- Flowers white, pink or lavender pea-like blossoms, which bloom late spring/early summer
- Seeds are 1.5 cm long, light brown, oval and contained in clusters of brown, eye-shaped pods
- Reproduces via seeds but can be cultivated from root suckers or stem cuttings
- Yields from 9 to 90 kg seed/tree/annum
- Can produce up to 40% solvent extractable oil per seed
- Approximately 50% of oil is C18:1, which is suitable for biodiesel production (low cloud point)

**Chemical Composition of *Pongamia* Oil**

The seeds of *Pongamia pinnata* contain 30 to 40% oil which is thick, reddish brown in colour oil known as Pongam/Pongamol/Hongay oil which can be converted to biodiesel by transesterification with methanol in the presence of KOH. FFA composition of *Pongamia* seed oil which shows that the oil has 20.5% saturated and 79.4% unsaturated long chain fatty acids the major monounsaturated fatty acid is oleic

# PLATE 2

## *Pongamia pinnata* – Habit, Inflorescence, Seeds, Oil

**Habit**

**Inflorescence**

***Seeds***

***Seed oil***

acid (46%) linoleic acid (27.1%) and linolenic acid (6.3%) which constitutes the total polyunsaturated fatty acids. Low molecular weight fatty acids such as lauric and capric acids occur in very small amount of about 0.1% each.

**Catalysts for Biodiesel production from Vegetable oil**

A very large number of potentially useful catalysts have been investigated as a mean to enhance the reaction rate in biodiesel synthesis. Without catalysts, some degree of rearrangement can be obtained but only under extreme conditions of temperature, pressure and time leading to undesirable effects such as isomerisation, polymerization and decomposition. Catalysts are classified as homogeneous (alkali -- NaOH, KOH; acid – $H_2SO_4$ and alkali-alcoholic - MeOH) or heterogeneous (CaO, MgO, BaO and $Z_nO/Al_2O3$; alkaline metal oxides supported on zeolite; ion exchange resin).

Potassium hydroxide (KOH) was commonly used as catalyst for the transesterification of oils such as *Jatrophacurcas*; used frying oil and soybean used frying oil (Foidl, *et al*., 1996). When rice bran oil was used as feedstock, sulphuric acid was used as catalyst (Zullaikah, *et al*.,2005). Sodium hydroxide was also chosen to catalyse the transesterification of rubber seed oil because it is cheaper (Ramadhas, *et al* 2005). Different homogeneous catalysts were used to transesterify sunflower oil. Vicente, *et al* (2005) pointed out that near by100% biodiesel yields were only obtained with sodium methoxide catalyst.

Pryde *et al*. (1986) showed that the methanolysis of soybean oil, in the presence of1 mol% of $H_2SO_4$, with an alcohol/oil molar ratio of 30:1 at 65°C, takes 50 hour to reach complete conversion of the vegetable oil (> 99%). Attempts

have been made to use alkaline- earth metal compounds in the transesterification of rapeseed oil for the production of FAME (Gryglewicz, 1999). The catalytic activity of magnesium oxide, calcium hydroxide, calcium oxide, calcium methoxide, barium hydroxide, and for comparison, sodium hydroxide during the transesterification of rapeseed oil was investigated by the author. Sodium hydroxide exhibited the highest catalytic activity in this process. Barium hydroxide was slightly less active with a conversion of 75% after 30 min. Calcium methoxide was medially active. Magnesium oxide and calcium hydroxide showed no catalytic activity in rapeseed oil methanolysis.

Zang *et al*. (2003) obtained a 97% oil conversion to FAME within 240 min, using a 50:1 molar ratio of methanol to oil and a 0.14:1 weight ratio of sulphuric acid to oil at $80^0$C. However, this process gives rise to problems linked with the corrosive action of the liquid acid catalyst and to the high quantity of obtained by-products (Loreto *et al*., 2005). According to Lotero *et al*., (2006), the homogeneous alkaline catalysts enable a faster reaction at moderately low temperature and pressure, high conversion rate at low catalyst concentration and are relatively cheap, though, their performance is adversely affected by moisture and soap formation in the presence of high free fatty acids in the seed oil feedstock reducing yield. The homogenous acid catalyst on the other hand can be used even if the seed oil feedstock has higher free fatty acid contents. However, it requires higher temperatures (373–473K) and higher methanol/oil molar ratio (20–35:1) and also poses environmental problem due to its corrosive and polluting nature.

Investigation into the use and the development of the heterogeneous catalyst process which is expected to be an effective biodiesel production process with low cost and minimal environmental impact is still on-going because of the possibility of simplifying the production and purification processes under mild conditions. For example, the 20 transesterification reaction of soybean oil with ETS-10 zeolite has been studied; conversion in excess of 90% was achieved at a temperature of 10000C (Suppes *et al*., 2004). It has also been reported that the conversion to methyl ester reaches 87% with the potassium- loaded alumina catalyst, when a mixture with a molar ratio of methanol to oil of 15:1 is refluxed for a reaction time 7 h (Xie *et al*., 2006).

Besides these, there have been several other reports on heterogeneous catalysts(Gryglewicz, 1999; Suppes *et al*., 2001; Kim *et al*., 2004; Cantrell *et al*., 2005; Jitputti *et al*.,2006). Kouzu *et al* (2007), using CaO as a solid base catalyst for transesterification of soybean oil with refluxing methanol, found that after 1 hour of reaction time, the yield of fatty acid methyl ester (FAME) was 93% for CaO, 12% for $Ca(OH)_2$ and 0% for $CaCO_3$. Under the same conditions, sodium hydroxide brought about the almost complete conversion (99%) into FAME. Jacobson *et al* (2008) synthesized and utilized various solid acid catalysts such as MoO3/SiO2, MoO3/ZrO2, WO3/SiO2, WO3/SiO2–$Al_2O_3$, zinc stearate supported on silica, zinc ethanoate supported on silica and 12-tungstophosphoric acid (TPA) supported on zirconia.

Patil *et al*. (2009) performed a two-step process for production of BDF from *Jatrophacurcas* oil with a maximum yield of 95% attained according to the reaction conditions: at the first acid esterification, i.e., methanol to oil molar ratio of 6:1, sulfuric acid of 0.5 wt.%, and reaction temperature of 40 ± 5 °C; followed by alkaline transesterification with methanol to oil molar ratio of 9:1, KOH of 2 wt.%, and reaction temperature of 60 °C. Wen *et al*. (2010) used mixed oxides of TiO2–MgO produced by the sol–gel method to convert waste cooking oil into biodiesel.

Biodiesel production from *Jatropha* oil by catalytic and non-catalytic means was studied by Juan *et al*. (2011). Sivakumar *et al*. (2011) produced biodiesel from raw material dairy waste scum and the FAME yield reached 96.7% under the optimal conditions, the reaction being catalyzed by KOH 1.2 wt.%; molar ratio of methanol to oil 6:1; reaction temperature 75 °C; reaction time 30 min at 350 rpm. Folaranmi, 2012 studied the production of biodiesel (B100) from *Jatropha* oil using sodium hydroxide as a catalyst. His result showed that the product met the set standard for biodiesel.

Farouk *et al*. (2014) subjected two oil sample from *Jatropha* to alkaline base step using base-catalyst process parameters of 1.2 w/w potassium hydroxide (KOH), 4.5:1 w/w methanol to oil mole ratio, reaction temperature of 60°C, and 120 min of reaction time. The final biodiesel yield obtained was 82% and 90% from the first and the second *Jatropha* oil sample respectively. The basic physiochemical properties of the *Jatropha* methyl ester produced from both *Jatropha* oil samples were found to be within the ASTM D6751 specified limits.

**Lipases as catalyst in Biodiesel production**

Chemical catalysts give a high conversion of triglycerides to methyl esters in a short reaction time. However, chemical catalyst used in biodiesel production has several drawbacks including the difficulty of recycling glycerol, the need to eliminate the catalyst and salt leading to development of alternative pathways (Zhang, 2003). To overcome these problems an enzymatic process using extracellular or intracellular lipase catalyst has been developed (Kaieda, 1999).

Lipases are widely used in industry and capable of catalyzing a variety of reactions such as hydrolysis, alcoholysis, esterification, and transesterification. The study of Du *et al*, 2005, showed that higher yield (90%) was achieved for biodiesel production by using a sn-1, 3-specific lipase, *Thermomyces lanuginosa* immobilized on silica gel. The enzymatic process can utilize low quality feedstocks with high levels of FFA because FFAs can be directly converted to biodiesel via lipase-catalyzed esterification; it also requires less energy input and the glycerol byproduct is easier to separate (Hasan, *et al*., 2006). Many microbial lipases have been identified as potential enzyme catalysts for biodiesel synthesis with a wide range of stabilities and catalytic efficiency (Joshi and Vinay, 2007)

Substrate specificity of lipases is also a crucial factor towards the biodiesel production which acts on the choice of the proper enzyme based on the composition of raw materials by consisting in the capability of distinguishing structural features of acyl chains (Robeiro, 2007). The use of sn-1, 3- specific lipases can give rise to biodiesel yield of above 90% under appropriate conditions (Antczak, *et al*.,

2009). Lipases from *Pseudomonas fluorescens, P. cepacia, Candida rugosa, C. antarctica* and *C. cylindracea* are suitable for transesterification reaction by displaying both wide substrate specificity and regiospecificity. Using lipases as catalysts can overcome many drawbacks of the chemical-catalyzed process (Bisen, *et al*., 2010). Even with many desirable properties, the enzymatic process has a very limited commercial success mainly due to the high cost of lipases; one of the more cost effective approaches often adopted to reduce the cost is to recycle the enzyme through immobilization.

Till date, immobilized lipases and whole cell lipase catalysts have been the most studied biocatalysts for production of biodiesel. As recombinant yeast, whole cell catalysts in the form of intracellular enzymes, *Saccharomyces cerevisiae* yeast cells with intracellular expression of *Rhizopus oryzae* lipase were engineered to generate biodiesel (Matsumoto *et al*., 2001). Pichia pastoris yeast whole cell catalysts for biodiesel synthesis with intracellular expression of *Thermomyces lanuginosus* lipase (Yan *et al*., 2007). A number of commercial immobilized lipase preparations such as Novozyme 435 and Lipozyme TLIM have been evaluated for this process (Martin and Otero, 2008) and recombinant *Aspergillus oryzae* expressing heterologous lipases have been used as whole cell catalysts for biodiesel production (Takaya *et al*., 2011).

Immobilized enzymes have flexible performance on activity and stability, but suffer high preparation cost. Wild-type filamentous fungus *R. oryzae* containing homogenous cell-bound lipases (Balasubramanian *et al*., 2012).

Recombinant *Escherichia coli* cells producing intracellular lipase were employed to catalyze biodiesel formation (Yan *et al*., 2012). *Saccharomyces cerevisiae* and *Pichia pastoris* yeast cells with surface displayed lipases were also tested for the same purpose (Matsumoto *et al*., 2002 and Jin *et al*., 2013). You *et al*. (2013) studied the Biodiesel production from *Jatropha* oil catalyzed by immobilized *Burkholderia cepacia* lipase on modified attapulgite. Their results indicated that the best conditions for biodiesel preparation were: 10g *Jatropha* oil, 2.4g methanol (molar ratio of oil to methanol is 1:6.6) being added at 3h intervals, 7 wt% water, 10 wt% immobilized lipase, temperature 35°C, and time 24h. Under these conditions, the maximum biodiesel yield reached 94% and suggested that the immobilized lipase catalyzed process has potential industrial use for biodiesel production to replace chemical-catalyzed method.

**Lipase catalyzed *Jatropha* oil biodiesel production**

Zarei *et al*. (2013) investigated the production of biodiesel from non-edible crude *Jatropha* oil in the presence of an immobilized lipase catalyst. Their experimental results revealed that the highest biodiesel yield was 87.10% at 40 °C reaction temperature, 5:1 methanol/oil molar ratio, 70 wt% water content, and 17 h reaction time. The immobilized lipase catalyst beads exhibited good activity for biodiesel production. Cesarini *et al*., (2014) studied the one-step biodiesel production by combining phospholipase and a liquid lipase using crude oils and achieved 95% transformation into FAME and a good reduction of phosphorous (< 5ppm) was achieved.

**Lipase catalyzed *Pongamia* oil biodiesel production**

Transesterification of *Pongamia* and *Jatropha* oil was carried out using a novel isolate *Rhizopus* strain JK 1 as extracellular lipase producing whole cell biocatalyst by Jayshree and Asmita (2015). Ethanol gave maximum conversions when compared with the methanol as acyl acceptor. Ethanol gave up to 95% ethyl ester production from *Pongamia* oil after 36h of transesterification reaction. Different parameters like temperature, time and oil to alcohol ratio were standardized for optimum conversion. Maximum conversion of oil into ethyl ester was found at $30^0$C. Ethyl ester production increased with time and maximum production (95% from *Pongamia* oil and 88% from *Jatropha* oil) was achieved after completion of 36h of reaction at $30^0$C. Oil to ethanol concentration also affected the conversion rate in both the oils. Oil to ethanol concentration which gave best conversion was 1:4.

**Biodiesel Scenario and Land Requirement in India**

As India is deficient in edible oils, non-edible oils become the main choice for biodiesel. Generally, a blend of 5% to 20% is used in India (B5-B20). Indian oil corporation (IOC) has taken up Research and Development work to establish the parameters for the production and use of biodiesel in its R&D center at Faridabad Table 6 gives the requirements for different blends up to B20 which gives the engine performance similar to diesel in terms of power output & other parameters.

**Table 6**

**The biodiesel and land requirement in India for future**

| Year | Diesel demand | Biodiesel requirement (MT) | Area requirement (Mha) |
|---|---|---|---|
| | | $BD_{20}$ | $BD_{20}$ |
| 2007-08 | 60.18 | 12.0 | 21.0 |
| 2008-09 | 90.27 | 18.0 | 30.0 |
| 2009-10 | 95.23 | 19.0 | 31.7 |
| 2010-11 | 100.47 | 21.1 | 33.5 |
| 2011-12 | 106.00 | 21.2 | 35.3 |
| 2012-13 | 111.83 | 22.3 | 37.2 |
| 2013-14 | 117.98 | 23.6 | 39.3 |
| 2014-15 | 124.47 | 24.9 | 41.5 |
| 2015-16 | 131.31 | 26.2 | 43.7 |
| 2016-17 | 138.54 | 27.7 | 46.1 |
| 2017-18 | 146.16 | 29.2 | 48.7 |
| 2018-19 | 154.19 | 30.8 | 51.4 |
| 2019-20 | 162.67 | 32.5 | 54.2 |

**Conclusion**

Demand for transport fuel is increasing unabatedly in India. On the other hand there are frequent hikes of prices of fossil fuel and uncertain supply in international market. To minimize the import of crude oil we must go for Bio fuels which are renewable and eco-friendly. *Pongamia* oil and *Jatropha* oil may stand as feed stock for Bio-diesel which is renewable, safe and non-polluting. It holds great promise to the rural sectors of India to meet the energy and organic fertilizer requirements.