Utilization of Solid Waste from Tannery for Production of *Aspergillus niger* Lipase

A PROJECT REPORT

Submitted by

in partial fulfillment for the requirement of award of the degree

of

M.TECH. BIOTECHNOLOGY

FACULTY OF TECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE 641 049

(An Autonomous Institution Affiliated to Anna University, Chennai)

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BONA FIDE CERTIFICATE

Certified that this project work titled **"Utilization of Solid Waste from Tannery for Production of *Aspergillus niger* Lipase"** is the bona fide work of Mr. MANIKANDAN .A (Reg. No. 1120203004), who carried out the research under my supervision. Certified further, that to the best of my knowledge the work reported here in does not form part of any other thesis or dissertation on the basis of which a degree or award was conferred on an earlier occasion on this or any other students.

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ABSTRACT

A novel bi-substrate fermentation (BSF) process was developed for the production of lipase from *Aspergillus niger* using agro-industrial residues, wheat bran (WB), and Beef fleshing of tannery solid waste. The study was carried out to utilization of solid waste substance from the tannery industry for the production of extracellular lipase enzyme from *A. niger*. The lipase activity was 404.8 ± 17 U/g dry substrate (U/gds) at 30 °C and 96 h and growth studies indicated that addition of WB significantly augmented the biomass and lipase production. Lipase enzyme was produced from tannery waste substance by using the solid state fermentation (SSF) and submerged fermentation. The fungal lipase was produced from a slaughterhouse waste, and also agro-industrial residue as substrate. The high level lipase activity was at 30 ºC and 96h and the growth study indicate that addition of tannery waste substance and agro-industrial residue influence over the production of lipase enzyme. Mixed substrate fermentation was done for single substrate fermentation (SSF), bi-substrate fermentation (BSF), tri-substrate fermentation (TSF), among three fermentation systems the BSF was selected as high enzyme yields one. The optimization of different parameters like substrate concentration, inoculums range, and moisture content was done. The maximum activity was obtained using equal ratio of tannery waste and agro-substance (1:1), when fermented substrate was extracted in phosphate buffer (pH 7). The characterization of fleshing was done using different methods (protein, fat, and moisture content). . The inducers plays important role in increasing the production of lipase enzyme like oily solid substrate, instead of using oily substance tannery waste substance was used. Submerged fermentation was also done using conventional medium and replacement of oil inducers by fleshing as inducer for enzyme production. The maximal activity of lipase is about 25.8 U/ml at 30 ºC and 48h by submerged fermentation using fleshing is one of its substrate. Thus, the extra cellular enzyme has potential industrial applications and furthermore, the direct application of fermented substrate for beef fleshing hydrolysis makes the process economical for industrial production of biofuel.

Keywords:Beef fleshing, solid state fermentation, submerged fermentation, beef hydrolysis.

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1. INTRODUCTION

The enzymes are the focal point of biotechnological process since they are involved in all the aspects of biochemical conversion from the simple fermentation into conversion to the complex techniques in genetic engineering and molecular biology. They are used as cost-effective and eco-friendly substitutes for chemical process in several industries. Enzymes have attracted attention from researchers all over the world because of the wide range of physiological, analytical and industrial applications, especially, from microorganisms, because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation. Despite the fact that more than 3000 different enzymes have been identified and many of them have found their way into biotechnological and industrial applications, the present enzyme toolbox is not sufficient to meet most of the industrial demands. In view of these limitations, researchers have diverted their attention for isolation and characterization of enzymes from different environments. Whenever required, due attention is also paid towards development of recombinant enzymes with desired characteristics and for specific applications.

Among microorganisms, molds are known to be more potent lipase producers, because they produce lipase both by solid substrate and by submerged fermentation. Hence, it is worthwhile to optimize the fermentation medium, which affects the product yield and volumetric productivity of these enzymes (Mala *et al*., 2007).

Solid substrate fermentation (SSF) has built up credibility in recent years for the production of microbial products including enzymes through inexpensive media and it is an appropriate process for developing countries (Singhania *et al*., 2009). Although submerged fermentation (SmF) is widely used in the enzyme industry and has advantages in process control and good yields of enzymes, the products in fermentation are relatively dilute resulting in high volumes of effluents (Nagy *et al*., 2006). As an alternative, SSF has been developed and proved to be an economical way to produce various enzymes including lipases and esterases. Interest in lipases has greatly increased in recent years due to their various applications in food, detergent, cosmetic, organic synthesis, and pharmaceutical industries (Treichel *et al*., 2010). Microorganisms are potent lipase producers and moulds are widely recognized for higher enzyme production due to their ability to utilize various substrates with vigorous growth and sporulation on the substrate matrix.

Several reports are available on lipase production by SSF using solid substrates including wheat bran (Uvarani *et al*., 1998), rice bran (Rao *et al*., 1993) coconut oil cake (Benjamin and Pandey, 1997), gingelly oil cake (Kamini *et al*., 1998) and soybean meal (Han, 2003). However, reports on lipase production by SSF using mixed solid substrates (Mala *et al*., 2007) are very much limited and no report is available on the utilization of fleshing in different substrate combinations for the production of lipase from *Aspergillus niger*. Moreover, lipase production by various SSF processes resulted in low enzyme productivities (Mohanasrinivasan *et al*., 2009). It is, therefore, imperative to develop a suitable process for enhanced lipase production.

Selection of appropriate substrate is a key aspect of SSF (Pandey, 2003) and recently, substrates like and almond meal, sugarcane bagasse (Babu and Rao, 2007), pongamia oil cake (Balaji and Ebenezer, 2008), jatropha seed cake (Mahanta *et al*., 2008), castor bean litters (Godoy *et al*., 2009) and biopharmaceutical oil waste (Mohanasrinivasan *et al*., 2009) have been used for the production of lipases. In SSF, the solid material acts as physical support, source of nutrients and also as appropriate inducer for the production of enzymes. It is difficult to get all required features from a single substrate; however, this could be achieved by combination of different substrates. Accordingly, to enhance the growth and production of lipase from *A. niger*, a novel bi-substrate fermentation (BSF) was developed using combination of substrates like wheat bran (WB), wheat rawa (WR) and coconut oil cake (COC), Beef fleshing that act as support matrix, nutrient source and as an inducer for lipase production, respectively. For the overall economics of the SSF process, scale up and extraction studies were carried out for efficient recovery of lipase from BSF.

Corn steep liquor (CSL), a by-product of the corn wet-milling industry, is used as an ingredient in animal feed and as a nutrient supplement for microorganisms in diverse industrial fermentation processes. It is a mixture consisting of water-soluble extracts of corn soaked (steeped) in water, composed entirely of natural amino acids, minerals, vitamins, reducing sugars, organic acids, enzymes, and other elemental nutrients, which are excellent source of nutrients for microorganisms. The most important application of CSL in microbiology was initially discovered by Moyer and Coghill (1946), who noticed that the addition of CSL to the liquid medium greatly increased the yields of *penicillin* from *Penicillium notatum*. Later, Gern *et al*., (2008) reported that supplementation of CSL to the growth medium increased the mycelial growth of *Pleurotus ostreatus, Aspergillus niger,*

Rhizopus oryzae, and *Aspergillus parasiticus*, respectively, because CSL also adjusts the trace metal balance in the fermentation medium.

Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential. Interest in the production of microbial lipases has increased in recent decades, because they find promising applications in the production of pharmaceuticals, detergents, cosmetics, leather, and foods and in other organic syntheses (Hasan *et al*., 2006).

Furthermore, the fermented solid material was directly evaluated for the hydrolysis of fleshing, a low cost feed stock, which is economically and industrially important for the production of fatty acid esters.

1.1 OBJECTIVES

- To utilize tannery waste substance for production of *Aspergillus niger* lipase enzyme and replacement of oily inducers.
- To analyze the composition of solid waste (Beef fleshing).
- To produce the extracellular enzyme in both solid state fermentation (SSF, BSF, TSF) and optimize parameters for the production of lipase enzyme by SSF.
- Also to produce extracellular enzyme in submerged fermentation.
- To hydrolyse beef fleshing using SSF and SmF lipase enzyme.

2.0 REVIEW OF LITERATURE

2.1. Microorganisms as Bio-Factories

The biosphere is occupied by a wide variety of microorganisms that carry out important functions like global primary energy and element cycling and they form the largest part of living organisms in the sense of total biomass cell numbers (6 x 10^{30} bacteria, 1.3 x 10^{28} archaea, 3.1 x 10^{29} eukarya), cell biomass (6 x 10^{18} kg bacteria, 1.3 x 10^{16} kg archaea and 3.1 x 10^{17} kg eukarya) and species diversity. This diversity of microorganisms is the most common source of genes which can be used in several industrial and research applications (Beloqui *et al*., 2008).

Microorganisms are found everywhere. It means that they can be observed in the deepest ocean sediments, at high atmospheric pressure, at unusually high and cold temperatures and also in highly polluted environments. Microbes are able to break down a variety of usual and unusual carbon and energy sources and convert them into amino acids, nucleotides, vitamins, carbohydrates and fatty acids by producing specific enzymes. Enzymes that carry out metabolic processes in microbes also have several practical and industrial usages to perform certain reactions apart from the cell. So, due to their ability to adapt a wide range of conditions and produce specific enzymes, microorganisms have been paid attention as little bio-factories (Sanchez 2005).

In the beginning of enzyme technology, crude preparations from certain animal tissues like pancreas and stomach mucosa, or plant tissues found applications in textile, leather and other industries. However, such preparations had some disadvantages such as high cost and shortage of tissues from animals and plants. It was realized that some microorganisms produce enzymes similar to that of plants and animals in terms of actions. Dr. Jokichi Takamine (1894, 1914) was the first person to introduce microbial enzymes to industry. Although he was mainly interested in fungal enzymes, Boidin and Effront (1917) were pioneers in the production of bacterial enzymes. Since that time, microbial enzymes have taken the place of enzymes from plants and animals (Underkofler *et al*., 1957).

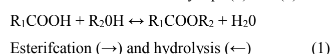
Microbial enzymes have a great number of usages in food, pharmaceutical, textile, paper, leather and other industries (Hasan *et al*., 2006). Their applications have been increasing rapidly. Among industrially important enzymes, hydrolases come in the first place

and include enzymes with wide substrate specificity. Carbohydrases, proteases, pectinases and lipases are classified into hydrolases. They catalyze the hydrolysis of natural organic compounds (Underkofler *et al*., 1957).

2.2 Lipases

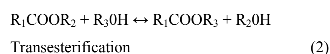
Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. They catalyse the hydrolysis of triglycerides into diglycerides, monoglycerides, glycerol and fatty acids, and under certain conditions, the reverse reaction leads to esterification and formation of glycerides from glycerol and fatty acids. The hydrolysis is a reversible reaction and the potential of lipases for ester synthesis (Cao *et al.,* 1992*;* Chulalaksananukul *et al.,* 1990; Linko *et al.,* 1995a; Malcata *et al.,* 1990*;* and Mustranta *et al.,* 1993) and for interesterification (Bloomer *et al.,* 1990*;* Lamboursain *et al.,* 1996; Safari and Kennasha 1994) in organic media has been well documented. A true lipase will split emulsified esters of glycerine and long chain fatty acids such as triolein and tripalmitin. In contrast to esterases, lipases are activated only when adsorbed to an oil-water interface (Martinelle *et al.,* 1995) and do not hydrolyze dissolved substrates in the bulk fluid. Lipases are serine hydrolases and they display little activity in aqueous solutions containing soluble substrates. In contrast, esterases show normal Michaelis-Menten kinetics in solution (Shanna *et al.,* 200l). Lipases may catalyze three types of reaction: hydrolysis, esterification, and transesterification. It is also well known that some lipases display position specificity (regiospecificity) toward fatty acids in triacylglycerols as well as fatty acid selectivity (Marangoni and Rousseau, 1995).

These reactions can be illustrated by eqs. (1) and (2) as follows:



$R_1COOH + R_20H \leftrightarrow R_1COOR_2 + H_20$

Esterifcation (→) and hydrolysis (←) (1)



$R_1COOR_2 + R_30H \leftrightarrow R_1COOR_3 + R_20H$

Transesterification (2)

2.3 Action of Lipase

Lipases belong to the class of serine hydrolases and therefore do not require any cofactor. The natural substrates of lipases are triacylglycerols, having very low solubility in water. Under natural conditions, they catalyse the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved (Fig. 2.1) (Saxena *et al.,* 1999). Under certain experimental conditions, such as in the absence of water, they are capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol. The occurrence of the lipase reaction at an interface between the substrate and the aqueous phase causes difficulties in the assay and kinetic analysis of the reaction (Macrae and Hammond, 1985). The usual industrial lipases are special classes of esterase enzymes that act on fats and oils, and hydrolyse them initially into the substituted glycerides and fatty acids, and finally on total hydrolysis into glycerol and fatty acids (Bjorkling *et al.,* 1991; Ghosh *et al.,* 1996).

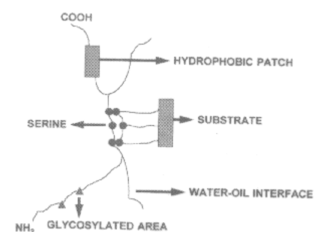


Fig.2. 1 Lipase molecule showing its main feature. Substrate can be any glycerides

In nature, the lipases available from various sources have considerable variation in their reaction specificities: this property is generally referred as enzyme specificity. Thus, from the fatty acid side, some lipases have affinity for short-chain fatty acids (acetic, butyric, capric, caproic, caprylic, etc.), some prefer unsaturated fatty acids (oleic, linoleic, linolenic, etc.), while many others are nonspecific and randomly split the fatty acids from the triglycerides. From the glycerol side of the triglycerides, the lipases often show positional specificity and attack the fatty acids at 1 or 3 carbon position of glycerol or at both the positions, but not the fatty acid at the 2 position of the glycerol molecule (Saxena *et al.,* 1999). However, through random acyl migration, the 2-fatty acid monoglyceride undergoes

rearrangement pushing the fatty acid to the 1 or 3 position of the glycerol molecule. As acyl migration is a slow process and the available lipases do not act on glycerol 2-mono fatty acid esters, the hydrolysis slows down and awaits the acyl migration to complete for enabling the lipase to attack the glyceride at the 1 and l or the 3 position. Interestingly, lipases function at the oil-water interface. The amount of oil available at the interface determines the activity of the lipases (Verger, 1997). This interface area can be increased substantially to its saturation limit by the use of emulsifier as well as by agitation. The saturation limit depends on the ingredients used as well as the physical conditions deployed. Thus, the activities of lipases can be pronouncedly increased by use of emulsifying agents as well as by methods that increase the size of the emulsion micelles (Borgstrom and Brockman, 1984; and Brockerboffand Jenseo, 1974).

2.4 Microbes as Source of Lipase

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.

2.4.1 Bacterial lipases

A relatively smaller number of bacterial lipases have been well studied compared to plant and fungal lipases (Brune and Gotz, 1992; Petersen and Drablos, 1994; Sugiura, 1984; Sztajer *et al.,* 1991). Bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins. 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). Staphylococcal lipases are lipoprotein in nature (Brune and Gotz, 1992).

2.4.2 Fungal lipases

Fungal lipases have been studied since 1950s and comprehensive reviews are available in the literature (Brockerhoff and Jensen, 1974; Lawrence, 1967; Sharma *et al.,*

2001). These lipases are being exploited due to their low cost of extraction, thermal and pH stability, substrate specificity, and activity in organic solvents (Lawson *et al.,* 1994). The chief producers of commercial lipases are *Aspergillus niger, Candida cylindracea, Humicola lanuginosa, Mucor miehei, Rhizopus arrhizus, R. delemar, R.japonicus, R. niveus* and *R. oryzae* (Godfredson, 1990).

Among the different biological sources of the lipases studied, filamentous fungi are thought to be the best source for industrially useful lipases because these lipases are usually extracellular and soluble (Huang *et al.,* 2004). Fungal lipases from the genera *Geotrichum, Penicillium, Aspergillus, Rhizopus,* and *Rhizomucor* (Persson *et al.,* 2000) have been the most widely studied and consequently are the most widely used in industrial applications.

Among Mucorales, the lipolytic enzymes of the moulds *Mucor hiemalis, M. miehei, M lipo/yticus, M pusillus, Rh izop us japonicus, R. arrhizus, R. delemar. R. nigricans, R. nodosus, R. microsporus,* and *R. chinesis* have been studied in great detail (Lazar and Schroder, 1992). The thermophilic *M pusillus* is well known as a producer of thermostable extracellular lipase. From a lipase-producing strain of *M miehei,* two isoenzymes with slightly different isoelectric points, but high degrees of antigenic identity were isolated (Huge-Jensen *et al.,* 1987). Novo Industries have commercialized a lipase of *M miehei,* immobilized on a resin (Lipozyme TM).

Lipase producers within the order Entomophthorales include *Entomophthora apiculata, E. coronata, E. thaxteriana, E. virulenta, Basidiobolus* spp. and *Conidiobolus* spp. The genera *Pichia, Hansenula,* and *Saccharomyces* are also reported to produce lipase (Stead, 1986). Two kinds of cell-bound lipases were purified from *Saccharomyces lipolytica* (Lazar and Schroder, 1992). Lipases are reported from *Candida curvata,* C. *tropicalis,* C. *valida,* and C. *pellioculosa* (Lazar and Schroder, 1992) and are nonspecific towards the different ester bonds. In triglycerides, with the exception of C. *deformans* (Lazar and Schroder, 1992).

The imperfect fungus *Geotrichum candidum* is responsible for acid formation in dairy products by lipolyzing fat. The *G. candidum* lipase features specificity towards fatty acids with a *cis* double bond at *C9,* hence is applied for the structural analysis of triglycerides (Litchfield, 1972).

The intracellular and extracellular lipases of *Aspergillus niger* are 1,3-(regio)-specific (Okumura *et al.,* 1976). *A. oryzae* was reported to be an efficient host for the heterologous expression of the lipase from *Rhizopus miehei* and *Humicola lanuginose* (Lazar and

Schroder, 1992). The lipase of *Penicillium roqueforti* is responsible for the flavour of blue cheese (Eitenmiller *et al.,* 1970). Lipolytic activity has also been detected in *P. camemberti,* the white surface mould of Brie and Camembert cheese. Lipases with specificity for butyric acid have been isolated from strains of the *Penicillium* species such as *P. cyclopium* (Iwai *et al.,* 1975), *P. verrucosum* var. *cyclopium,* and *P. crustosum* (Lazar and Schroder, 1992). The *P. cyclopium* lipase has a much higher activity towards di- and monoglycerides than triglycerides. *H. lanuginosa* lipases show a high degree of hydrolytic activity with coconut oil and oils having a high content of lauric acid. The two lipases differ in their positional specificity (Ibrahim *et al.,* 1987).

2.4.3 Metagenomics and Lipase

Metagenomics, an approach to access global microbial genetic diversity, has been used to discover novel, potentially important enzymes, including lipases (Daniel, 2005; Lorenz and Eck, 2005; Streit and Schmitz, 2004). Several genes encoding metagenomic lipases have been identified in metagenomic libraries prepared from various environmental samples, including soils (Henne *et al., 2000;* Lee *et al.,* 2004), pond and lake water (Ranjan *et al.,* 2005; Rees *et al.,* 2003), and a solfataric field (Rhee *et al.,* 2005). A novel lipase-encoding gene, *lipG,* was isolated from a tidal flat-derived metagenomic library and sequenced (Lee *et al.,* 2006).

2.4.4 Lipase from marine sources

Marine organisms like fishes are found to be rich sources of lipase. But there are not many reports on lipase production by marine microorganisms. Marine *Vibrio* sp. VB-5 produces a lipase that hydrolyzes n-3 polyunsaturated fatty acid (PUFA)-containing fish oil. Saturated and monoenoic fatty acids were liberated easily from fish oil by lipase. It is hoped that the lipase from VB-5 is capable of catalyzing the esterification reaction with n-3 PUFA, since the reversible reaction is known for lipase (Chandrasekaran and Rajeev Kumar, 2002).

2.5 Fermentation Production of Lipase

Numerous papers have been published dealing with suitable fermentation system. A technical method of Solid State Fermentation (SSF) involves the growth and metabolism of microorganism on moist solid without free flowing of water. SSF has many advantages over submerged fermentation (SmF), including an economical use of space that is required for

fermentation, simplification of fermentation media, superior yield and no requirement of complex machinery. However, SSF has some limitation such as poor pool of microorganism capable of growth under restricted condition and the controlling and monitoring of parameters such as temperature, pH, moisture and airflow.

Fungal culture adopts different growth pattern when cultivated in liquid and solid medium substrate. Research studies on SSF are very limited while submerged fermentation is well investigated for number of industrial fermentation. Each form has its own characteristic which greatly affects the process yield and attempts have been made to manipulate the morphology in order to achieve increased productivity.

A number of reports exist on influences of various environmental factors such as temperature, pH, nitrogen, carbon and lipid sources, agitation, and dissolved oxygen concentration on lipase production (Nahas, 1988; Watanabe *et al.,* 1977). Lipase production is generally stimulated by lipids (Omar *et al.,* 1987*;* Suzuki *et al.,* 1988). The lipase activity steadily increases to a peak and declines. Lipase production is usually coordinated with, and dependant on the availability of triglycerides. Besides this, free fatty acids, hydrolysable esters, bile salts, and glycerol also stimulate lipase production. High production of lipase in case of *P. fragi* occurs in peptone-supplemented medium, although different peptones vary in their effectiveness (Lawrence *et al.,* 1967a; Nashif and Nelson, 1953). Though *Pseudomonas* sp. grows in a basal medium with ammonium sulphate, glucose, citrate or pyruvate, it required an organic nitrogen source for lipase production (Alford and Pierce, 1963). A mixture of arginine, lysine and glutamic acid in medium was observed to be effective for lipase production (Alford and Pierce, 1963). A strain of *Penicillium roqueforti* produced maximum amount of lipase when grown in 0.5% casitone and 1% proflobroth (Eitenmiller *et aI.,* 1970). Growth and lipase production by *Micrococcus* sp. were unaffected by peptone of 0.5% to 2%, but lipase production by *Pseudomonas* sp, *A. wentii, M hiemalis, R. nigricans,* and *M. racemosus* were stimulated by peptone (Akhtar *et al.,* 1980*;* Chander *et al.,* 1981; Chander *et al.,* 1980). Soybean meal extract in *Rhizopus oligosporus* culture medium supported good growth and lipase production (Nahas, 1988). Physiological regulation of lipase activity by thermotolerant 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).

Milk is a good medium for growth of psychrotrophic bacteria and for lipase production which was found to be susceptible to catabolite repression by glucose (Akhtar *et*

al., 1980; Chander *et al.,* 1981; Chander *et al.,* 1980; Gilbert *et al.,* 1991). While glucose is essential for production of lipase by *P.fragi* (Alford and Pierce, 1963), *A. wentii* (Chander *et al.,* 1980), *M. hiemalis* (Akhtar *et al.,* 1980*), R. nip'icans,* and *M. racemosus* (Chander *et al.,* 1981). *P. aeruginosa* EF2 (Gilbert *et al.,* 1991) showed no such requirement (Nadkami, 1971). Lipase activity per milligram dry weight of mycelium was much higher on lactose, mannose, xylose, fructose, dextrin, and rhamnose in case of *Talaromyces emersonii* (Oso, 1978). Mannitol, galactose, sucrose (Chander *et al.,* 1980), fructose, lactose, maltose, raffinose or ribose produced less amount of lipase (Chander *et al.,* 1981) and caused decreased growth with corresponding reduction in lipase activity in *M. racemosus* (Chopra *et al.,* 1981). Polysaccharides such as glycogen, hyaluronate, laminarin, gum arabic, and pectin stimulated production of lipase in *Serratia marcescens* (Nishio *et al.,* 1987) and *Saccharomycopsis lipolytica* (Ruschen and Winkler, 1982).

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). Salts of unsaturated fatty acids inhibited lipase production by *P. frag;* (Smith and Alford, 1966), whereas tributyrin and trioctanoin had no effect on lipase production by *P. fragi* and *Mfreudenreichii* (Lawrence *et al.,* 1967). Butter oil, corn oil or olive oil inhibited lipase production by *P. roqueforti* (Eitenmiller *et al.,* 1970), *Saccharomycopsis* sp., *B. licheniformis, M caseolyticus* and *Staphylococcus* sp. (Saxena *et al.,* 1999). Triglycerides such as olive oil, groundnut oil and cotton seed oil, and fatty acids such as oleic acid, linoleic acid and linolenic acid stimulated lipase production by *P. mephitica* (Saxena *et al.,* 1999). Lipids are considered not to be true inducers (Lawrence *et al.,* 1967; Nashif and Nelson, 1953). *A. wentii* showed reduced growth and lipase production when the synthetic and natural lipids were added to the growth medium (Chander *et al.,* 1980). Emulsification of culture media containing oil by gum acacia supported good growth and lipase production in *R. oligosporus* (Nahas, 1988). Triolein, olive oil, tributyrin, and oleic acid butyl ester were able to induce lipase in immobilized protoplasts, whereas Tween 80 enhanced lipase activity (Johri *et al.,* 1990*).*

The initial pH of the growth medium is also important for lipase production. Maximum activity was observed at pH > 7.0 for *P. fragi* (Nashif and Nelson, 1953) and at pH 9.0 for *P. aeruginosa* (Nadkarni, 1971) wherein development of acidity in media reduced lipase activity (Nashif and Nelson, 1953). In contrast, maximum growth at acidic pH (4.0-7.0) was reported for S. *Lipolytica* (Jonsson and Snygg, 1974), *M caseolyticus* (Jonsson and

Snygg, 1974), *B. licheniformis, A. wentiib* (Chander *et al.,* 1980), *M. hiemalis* (Akhtar *et al.,* 1980)*, R. nigricans., Mucor racemosus* (Chander *et al.,* 1981), *R. oligosporus* (Nahas, 1988) and *P. aeruginosa* EF2 (Gilbert *et al.,* 1991).

The best temperature for lipase production by *T. emersonii* was determined to be 45°C (Oso, 1978). 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), and *P. aeruginosa* (Gilbert *et al.,* 1991).

Aeration has variable effect on lipase production by different organisms. The degree of aeration appears to be critical in some cases since shallow layer cultures (moderate aeration) produced much more lipase than shake cultures (high aeration) (Nashif and Nelson, 1953). Vigorous aeration greatly reduced lipase production by *R. oligosporus* (Nahas, 1988), *P. fragi* (Lu and Liska, 1969), *P. aeruginosa* (Nadkami, 1971), and *M. racemosus* resulted in increased lipase production in static culture conditions (Chopra *et aI.,* 1981). However, high aeration was needed for high lipase activity by *A. wentii* (Chander *et al.,* 1980) and *M hiemalis* (Akhtar *et al.,* 1980). Changing the ratio of surface area to volume and hence, aeration of cultures of *P. 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 (Laws on *et al.,* 1994). The stationary conditions favoured maximum lipase production in *T. emersonii* (Oso, 1978). Lipase synthesis by two strains of *P. fluorescens* (psychrotroph), stimulated in milk medium at 7°C, was immediately preceded by a decrease in O_2 tension which resulted in earlier production of lipase (Rowe and Gilmour, 1982). Oxygen is the limiting factor in shake-flask cultures. Low oxygen concentration negatively affects the metabolism of *R delemar,* which explains that low oxygen concentration is a useful tool to scale down fermentation processes in cases where a transient or local oxygen limitation occurs (Giuseppin, 1984).

2.6 Lipase Assay

Lipase hydrolyses triglycerides and gives rise to free fatty acids and glycerol. Numerous methods are available, in literature, for measuring the hydrolytic activity as well as for the detection of lipases (Brockerhoff and Jensen, 1974; Jensen, 1983; Tietz and Shuey, 1993). These methods can be classified as follows: 1. titrimetry, 2. spectroscopy (photometry,

fluorimetry, infrared), 3. chromatography, 4. radioactivity, 5. interfacial tensiometry, 6. turbidimetry, 7. conductimetry, 8. immunochemistry, 9. microscopy (Beisson *et al*., 2000).

Tributyrin plate assay and titrimetry are the most commonly used methods for screening of lipase producers and estimation of lipase activity, respectively (Akhtar *et al.*, 1983; Linfield *et al*., 1984).

A simple and reliable method for detecting lipase activity in microorganisms uses the surfactant Tween 80 in a solid medium to identify a lipolytic activity (Sierra, 1957). The formation of opaque zones around the colonies is an indication of lipase production by the organisms. Modifications of this assay use various Tween surfactants in combination with Nile blue or Neet's foot oil and Cu^{2-} salts. Also, screening of lipase producers on agar plates is frequently done by using tributyrin as a substrate and clear zones around the colonies indicate production of lipase (Cardenas *et al.,* 2001). Screening systems making use of chromogenic substrates have also been described (Yeoh *et al.,* 1986). Plates of a modified Rhodamine B agar are used to screen lipase activity in a large number of microorganisms (Wang *et al*., 1995).

2.7 Lipase Purification

Most of the microbial lipases are extracellular in nature. Usually the fermentation process is followed by the removal of cells from the culture broth, either by centrifugation or by filtration and the cell-free culture broth is then concentrated by ultrafiltration, ammonium sulphate precipitation or extraction with organic solvents (Saxena *et al.,* 2003). About 80 % of the purification schemes attempted thus far have used a precipitation step, with 60 % of these using ammonium sulphate and 35% using ethanol, acetone or an acid (usually hydrochloric) followed by a combination of several chromatographic methods such as gel filtration and affinity chromatography. Precipitation is used as a crude separation step, often during the early stages of a purification procedure, and is followed by chromatographic separation. Increase in lipase activity depends on the concentration of ammonium sulfate solution used (Pabai *et al.,* 1995). In comparison to other techniques, which give lower yields (60-70 %), precipitation methods often have high average yield (87.l%) (Aires-Barros *et al.*, 1994).

This is followed by gel filtration, and ion exchange chromatography. In recent years, affinity chromatographic techniques have come into use as this technique decreases the number of steps necessary for lipase purification as well as increases specificity. Currently,

reversed-micellar (Oekker *et al.,* 1986; Yadav *et al.,* 1998) and two-phase systems (Ounhaupt *et al.,* 1991; Hustedt *et al.,* 1985), membrane processes, and immunopurification (Harlow and Lane, 1988; Hill *et al.,* 1989) are being used for purification of lipases.

2.8 Lipase Properties

Lipases are active in organic solvents, and they catalyze a number of useful reactions including esterification (Chowdary *et al.,* 2001; Hamsaveni *et al.,* 2001; Krishna and Karanth, 2001; Rao and Divakar, 2001); transesterification, regioselective acylation of glycols and menthols; synthesis of peptides (Ducret *et al.,* 1998; Zhang *et al.,* 2001), and other chemicals (Azim *et al.*, 2001; Berglund and Hurt, 2000; Bomscheuer, 2000).

Lipases purified from S. *aureus* and S. *hyicus* show molecular weights ranging between 34 - 46 kDa. They are stimulated by Ca+ and inhibited by EDT A. The optimum pH varies between 7.5 and 9.0. The purified lipase of *P. jragi, P. jluorescens,* and *P. aeruginosa* were monomeric with molecular weight of 33 kDa, 45 kDa, and 29 kDa, respectively (Brune and Gotz, 1992; Nishio *et al.,* 1987*;* Sztajer *et al.,* 1991). The lipase was inhibited by Zn^{+}, FeH, and Ar^{++} and activated by Ca^{++} (Kugimiya *et al.,* 1986). The lipase gene of *P. fragi* has been cloned and sequenced (Lawrence, 1967). Lipases from different species of *Psuedonomas* were purified by acidification of the culture supernatant, ammonium sulphate precipitation (Nishio *et al.,* 1987), Sepharose CL-6B chromatography (Sztajer *et al.,* 1991), and isoelectric focussing using CHAPS (Nishio *et al.,* 1987*).*

2.8.1 pH optima

Extracellular lipase of *A. niger, Chromobacterium viscosum* and *Rhizopus* sp. are active at acidic pH (Fukumoto *et al.,* 1963; Laboureur and Labrousse, 1966). An alkaline lipase active at pH 11.0 has been isolated from *P. Nitroreducens* (Watanabe *et al.,* 1977*).*

2.8.2 Temperature optima and thermal stability

Lipases of *A. niger* (Fukumoto *et al.,* 1963), *R. japonicus* (Aisaka and Terada, 1980), and *C. viscosum* (Yamaguchi *et al.,* 1973) are stable at 50°C, while lipases of then no tolerant *H. lanuginosa* and *P. nitroreducens* are stable at 60°C and 70°C (Liu *et al.,* 1973) respectively. *C. gigantea* lipase had half life for inactivation of 35.7, 46.4 and 22.9 min respectively at 45°C, 50°C and 55°C (Tombs, 1991), similar to lipases of *R. japonicus* (Suzuki *et al.,* 1986). Purified lipase from *A. terreus* (Yadav *et al.,* 1998) retained 100% of its

activity at 60°C after 24 h. But, the maximum activities of C. *gigantea* and other lipases from mesophiles were at 30 - 35°C. Thermophilic bacterial lipases obtained from Icelandic hot spring showed higher lipase activity at 40 to 60°C (Sigurgisladottir *et al.,* 1993).

2.8.3 Activation and inactivation of the enzyme

Cofactors are not required for the expression of lipase activity. Divalent cations, such as calcium, generally stimulate the activity. It has been postulated that this is based on the formulation of calcium salts of long-chain fatty acids (Godfredson, 1990; Macrae and Hammond, 1985). The lipase activity is inhibited drastically by Co^{++}, Ni^{++}, $Hg^{++,}$ and Sn^{++}; and is slightly inhibited by Zn^{--}, Mg^{--}, EDTA, and SDS (Patkar and Bjorkling, 1994). In *H.lanuginosa* S-38, sulphhydryl reducing agents, like dithiothreitol, did not alter the enzyme activity, but did render it more susceptible to heat inactivation. Inactivation is accelerated by the addition of urea. Reducing compounds (cysteine, 2-mercaptoethanol), chelating agents, (EDTA, o-phenanthroline), and thiol group inhibitors (p-chloro mercuric benzoate, monoiodoacetate) did not show a detectable effect on lipase in *M pu.sillus,* suggesting that lipase is not a metallo-enzyme and it does not require either free -SH group or an intact S-S bridge for its activity. Spontaneous and cyclic AMP induced lipase formation is greatly enhanced in *Serratia marcescens* SM-6 on exposure to glycogen, hyaluronate, pectin B, and gum Arabic (Nishio *et al.,* 1987).

2.8.4 Substrate specificity

Specificity of lipases is controlled by the molecular properties of the enzyme, structure of the substrate, and factors affecting binding of the enzyme to the substrate (Jensen, 1983). Substrate specificity of lipases is often crucial to their application for analytical and industrial purposes. Specificity is shown both with respect to either fatty acyl or alcohol parts of their substrates (Jensen, 1983). Many microbes produce two or more extracellular lipases with different fatty acid specificities. Tributyrin is hydrolysed slowly by some microbial lipases (Patkar and Bjorkling, 1994). In contrast, *M. miehei* lipase preferentially releases butyric acid from milk fat especially at low pH (Moskowitz *et al.,* 1977). *Geotrichum candidum* produces a lipase, which shows pronounced specificity for the hydrolysis of esters of a particular type of long-chain fatty acid. Lipases show both regio- and stereospecificity with respect to the alcohol moiety of their substrates (Chapman, 1969).

Lipases can be divided into two groups on the basis of the regiospecificity exhibited acylglycerol substrates (Macrae and Hammond, 1985). Lipases in the first group catalyse the complete breakdown of triacylglycerol to glycerol and free fatty acids together with diacylglycerols and monoacylglycerol as intermediates in the reaction. These intermediates do not accumulate since they are hydrolysed more rapidly than the triacylglycerol. Examples of the first group of lipases include lipase from *C. cylindracea* (Benzonana and Desnuelle, 1965). The second group of lipases release fatty acids regiospecifically from the outer 1 and 3 positions of acylglycerols. These lipases hydrolyse triacylglycerol to give free fatty acids, 1,2- diacylglycerols (Macrae and Hammond, 1985; Priest, 1992), and 2-mono acylglycerol. Many extracellular microbial lipases, such as those from *A. niger* (Okumura *et al.,* 1976) and *R. arrhizus* (Saxena *et al.,* 1999), show 1,3-(regio)specificity. Lipases excreted by *R. japonicus, M. miehei, H. lanuginosa,* C. *viscosum,* and *P. fluorescens* are also 1,3-(regio)-specific (Macrae and Hammond, 1985). Till date, there are no authentic reports of lipases which catalyse the release of fatty acids selectively from the central 2-position of acylglycerols (Macrae and Hammond, 1985), except for a report of Asahara *et al.,* (1993). Partial stereospecificity in the hydrolysis of triacyl glycerols' has been observed in *R. arrhizus, R. delemar,* C. *cylindracea,* and *P. aeruginosa.* Owing to this property, these enzymes can be used to isolate optically pure esters and alcohols (Lavayre *et al.,* 1982).

2.9 Sequencing and Cloning of Lipase Gene

Lipase gene from many microorganism and higher animals has been cloned. The lipase genes from S. *hyicus* and S. *aureus* have been cloned, sequenced, and compared with other lipases. This revealed two conserved domains separated by 100 amino acids, which are likely to form active site (Brune and Gotz, 1992). Putative active site residue around His 269 and Ser 369 of the *S. hyicus* lipase are highly conserved in the two S. *aureus* lipases and in several eukaryotic lipases.

2.10 Applications of Lipase

The industrial applications of lipases have grown rapidly in recent years and are likely to markedly expand further in the coming years. Lipases may be used to produce fatty acids (Linko *et al.,* 1990; Marangoni, 1994), biosurfactants (Chopineau *et al.,* 1988), aroma and flavor compounds (Gandhi *et al.,* 1995), lubricant and solvent esters (Linko *et al.,* 1994), polyesters (Linko *et al*., 1995), and biomodified fats (Marangoni, 1994; West, 1988). Lipases

are also widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals (Kazlauskas and Bomscheuer, 1998). Lipase can be used to accelerate the degradation of fatty waste (Masse *et al.,* 2001; Masse *et al.,* 2001) and polyurethane (Takamoto *et al.,* 2001).

There is a renewed interest in the development of more industrial applications of lipases. For each application, the lipase selection is based on specific characteristics including substrate, positional and stereoisomer specificity as well as temperature and pH stability (Yamaguchi and Mase, 1991). The use of lipases for the modification of the positional distribution of fatty acids in butter fat triacylglycerols was reported (Safari and Kennasha, 1994). Indeed, it was shown that the interchange of palmitic or myristic acid with oleic acid at the sn-2-position of the glycerol backbone can suppress the cholesterol-raising potential of milk fat (Hayes *et al.,* 1991). Lipases from *Pseudomonas jluorescens* (Kalo *et al.,* 1989), *Aspergillus niger* (Kalo *et al.,* 1988), *Candida cylindracea* (Kalo *et al.,* 1988), and *Mucor mielIei* (Kalo *et al.,* 1988: Safari *et al.,* 1993) have been investigated for the interesterification of butter fat. Among several commercial enzymes, lipase from *Rhizopus niveus* showed an interesting potential for the production of interesterified butter fat with an increased proportion of oleic acid at the sn-2 position (Safari and Kennasha, 1994). *R.japonicus* lipase has been used to produce hard butter suita.ble for chocolate manufacture by interesterification of palm oil with methyl stearate (Matsuo *et al.,* 1981).

The importance of thermostable lipases for different applications has been growing rapidly. Most of the studies realized so far have been carried out with mesophilic producers. Many lipases from mesophiles are stable at elevated temperatures (Sugihara *et al.,* 1991). Thermostable lipolytic enzyme has been applied to the synthesis of biopolymers and biodiesel and used for the production of pharmaceuticals, agrochemicals, cosmetics, and flavours (Haki and Rakshit, 2003).

2.10.1 Lipases in fat and oleochemical industry

The lipase catalysed transesterification in organic solvents is an emerging industrial application such as production of cocoa butter equivalent, human milk fat substitute "Betapol", pharmaceutically important polyunsaturated fatty acids (PUFA) rich/low calorie lipids, "designers' fats or structured lipid" and production of biodiesel from vegetable oils (Jaeger and Reetz, 1998; Nakajima *et al.,* 2000). *Mucor miehei* (IM 20) and *Candida*

antarctica (SP 382) lipases were used for esterification of free fatty acids in the absence of organic solvent or transesterification of fatty acid methyl esters in hexane with isopropylidene glycerols (Akoh, 1993).

Immobilized *M miehei* lipase in organic solvent catalysed the reactions of enzymatic interesterification for production of vegetable oils such as; corn oil, sunflower oil, peanut oil, olive oil and soybean oil containing omega-3 polyunsaturated fatty acids (Li and Ward, 1993). The scope for the application of lipases in the oleochemical industry is enormous. Fats and oils are produced worldwide at a level of approximately 60 million tpa and a substantial part of this (more than 2 million tpa) is utilized in high energy consuming processes such as hydrolysis, glycerolysis and alcoholysis. The saving of energy and minimization of thermal degradation are probably the major attractions in replacing the current chemical technologies with biological ones. Miyoshi Oil & Fat Co., Japan, reported the commercial use of *Candida cylindracea* lipase in the production of soaps (McNeill *et al.,* 1991). The company claimed that the enzymatic method yielded a superior product and was cheaper overall than the conventional Colgate-Emery process.

2.10.2 Use of lipase in textile industry

Lipases are used in the textile industry to assist in the removal of size lubricants, in order to provide a fabric with greater absorbency for improved levelness in dyeing. Its use also reduces the frequency of streaks and cracks in the denim abrasion systems. Commercial preparations used for the desizing of denim and other cotton fabrics, contains both alpha amylase and lipase enzymes (Cortez, 2000).

PCT Publication No. WO 97/43014 (Bayer AG) describes the enzymatic degradation of polyesteramide by treatment with an aqueous solution comprising an esterase, lipase or protease. JP 5344897 A (Amano Pharmaceutical KK) describes a commercial lipase composition, which dissolves in solution with aliphatic polyester with the result that the fibre texture is improved without losing strength. Polymers of aliphatic polyethylene are also disclosed which can be degraded by lipase from *Pseudomonas* spp. PCT Publication No. 97/33001 (Genencor International, Inc.) discloses a method for improving the wet ability and absorbance of a polyester fabric by treating with a lipase (http://www.wipo.int).

2.10.3 Lipases in detergent industry

The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry and in household dishwashers. The cleaning power of detergents seems to have peaked; all detergents contain similar ingredients and are based on similar detergency mechanisms. To improve detergency, modem types of heavy duty powder detergents and automatic dishwasher detergents usually contain one or more enzymes, such as protease, amylase, cellulase and lipase (Ito *et al.,* 1998). The lipase of *H. lanuginosa* DSM 3819 is suitable as a detergent additive because of its thermostability, high activity at alkaline pH, and stability towards anionic surfactants (Huge and Gormsen, 1987).

In 1994, Novo Nordisk introduced the first commercial lipase, Lipolase, which originated from the fungus *T. lanuginosus* and was expressed in *A. oryzae.* Lipases used as detergents also include those from *Candida* (Nishioka *et al.,* 1990) and *Chromobacterium* (Minoguchi and Muneyuki, 1989). Laundering is generally carried out in alkaline media, lipases active under such conditions are preferred (Gerhartz, 1990; Satsuki and Watanabe, 1990; Umehara *et al.,* 1990), and for example, the *A. oryzae* derived lipase. Alkaline lipase produced by *Acinetobacter radioresistens* had an optimum pH of 10.0 and was stable over a pH range of 6.0 - 10.0; therefore have great potential for application in the detergent industry (Chen *et al.,* 1998).

2.10.4 Lipases in food processing, flavour development and improving quality

Lipases have also been used for addition in food to modify flavour by synthesis of esters of short chain fatty acids and a1cohols, which are known flavour and fragrance compounds (Macedo *et al.,* 2003). Lipases are used in production of leaner meat such as in fish. The fat is removed during the processing of the fish meat by adding lipases and this procedure is called biolipolysis. The lipases also play an important role in the fermentative steps of sausage manufacture and to determine changes in long-chain fatty acid liberated during ripening. Earlier, lipases of different microbial origin have been used for refining rice flavour, modifying soybean milk and for improving the aroma and accelerating the fermentation of apple wine (Seitz, 1974).

2.10.5 Diagnostic tool

Lipases are also important drug targets or marker enzymes in the medical sector. They can be used as diagnostic tools and their presence or increasing levels can indicate certain infection or disease. Lipases are used in the enzymatic determination of serum triglycerides to generate glycerol, which is subsequently determined by enzyme linked colorimetric reactions. The level of lipases in blood serum can be used as a diagnostic tool for detecting conditions such as acute pancreatitis and pancreatic injury (Lott and Lu, 1991).

2.10.6 Bakery products, confectionery and cheese flavouring

Lipases are extensively used in the dairy industry for the hydrolysis of milk fat. Current applications include the flavour enhancement of cheeses, the acceleration of cheese ripening, the manufacturing of cheese like products, and the lipolysis of butterfat and cream (http://www.au-kbc.orglframeresearch.html).

A whole range of microbial lipase preparations has been developed for the cheese manufacturing industry: *Mucor meihei* (Piccnate, Gist-Brocades; Palatase M, Novo Nordisk), *A. niger* and *A. oryzae* (Palatase A, Novo Nordisk; Lipase AP, Amano; Flavour AGE, Chr. Hansen) and several others (http://www.aukbc. orglframeres(arch.html). Lipases also play a crucial role in the preparation of so called enzyme modified cheeses (EMC). EMC is a cheese that is incubated in the presence of enzymes at elevated temperature in order to produce a concentrated flavour for use as an ingradjent in other products (dips, sauces, dressings. soups, snacks, etc.) (http:lhWlW.au-kbc.org1frameresearch.html).

2.10.7 Cosmetics

Unichem International (Spain) has launched the production of isopropyl myristate, isopropyl palmitate and 2- ethylhexylpalmitate for use as an emolient in personal care products such as skin and sun-tan creams, bath oils etc. Immobilized *Rhizomucor meihei* lipase was used as a biocatalyst. The company claims that the use of the enzyme in place of the conventional acid catalyst gives products of much higher quality, requiring minimum downstream refining (http://www.aukbc. orglbetalbioproj2/uses.htlnl).

Retinoids (Vitamin A and derivatives) are of great commercial potential in cosmetics and pharmaceuticals such as skin care products. Water-soluble retinol derivatives were prepared by catalytic reaction of immobilized lipase (Maugard *et al.*, 2002). Lipases have

been used in hair waving preparation (Saphir, 1967), as a component of topical antiobese creams (August, 1972) or in oral administration (Smythe, 1951).

2.10.8 Lipases in tea processing

The quality of black tea is dependent largely on the dehydration, mechanical breaking, and enzymatic fermentation to which tea shoots are subjected. During manufacture of black tea, enzymatic breakdown of membrane lipids initiate the formation of volatile products with characteristic flavour properties, emphasize the importance of lipid in flavour development. Lipase produced by *Rhizomucor miehei* enhanced the level of polyunsaturated fatty acid observed by reduction in total lipid content (Latha and Ramarethinam, 1999).

2.10.9 Medical applications

Lipases isolated from the wax moth *(Galleria mellonella*) were found to have a bacteriocidal action on *Mycobacterium tuberculosis* (MBT) H37Rv. This preliminary study may be regarded as part of global unselected screening of biological and other materials for detecting new promising sources of drugs (Annenkov *et al.,* 2004).

Lipases may be used as digestive aids (Gerhartz, 1990). Lipases are the activators of Tumor Necrosis Factor and therefore can be used in the treatment of malignant tumors (Kato *et al.,* 1989). Although human gastric lipase (HGL) is the most stable acid lipase and constitutes a good candidate tool for enzyme substitution therapy (Ville *et al.,* 2002). Lipases have earlier been used *as* therapeutics in the treatment of gastrointestinal disturbances, dyspepsias, cutaneous manifestations of digestive allergies, etc. (Mauvemay *et al.,* 1970). Lipase from *Candida rugosa* has been used to synthesize Iovastatin, a drug that lower serum cholesterol level. The asymmetric hydrolysis of 3-phenylglycidic acid ester which is a key intermediate in the synthesis of diltiazem hydrochloride, a widely used coronary vasodilator, was carried out with *S. mmcescens* lipase (Matsumae *et al.,* 1993).

2. 10.10 Lipases as biosensors

A biosensor based on the enzyme-catalysed dissolution of biodegradable polymer films has been developed. The polymer enzyme system; poly (trimethylene) succinate, was investigated for use in the sensor, which is degraded by a lipase. Potential fields of application of such a sensor system are the detection of enzyme concentrations and

construction of disposable enzyme based immunosensors, which employ the polymer degrading enzyme as an enzyme label (Swnner *et al.,* 2001).

Lipases may be immobilized onto pH/oxygen electrodes in combination with glucose oxidase, and these function as lipid biosensors (Karube and Sode, 1988) and may be used in triglycerides (lwai, 1990) and blood cholesterol detenninations (lmamura *et al.,* 1989).

2. 10.11 Degreasing of leather

Lipases represent a more environmentally sound method of removing fat. For bovine hides, lipases allow tensides to be replaced completely. For sheepskins, which contain upto 40 % fat, the use of solvents is very common and these are replaced with surfactants. If surfactants are used for sheepskins, they are usually not as effective and may be harmful to the environment. Maps (India) offers a range of lipases for degreasing which work in different pH conditions; Palkodegrease, lipase for degreasing in neutral to alkaline pH conditions and Palkodegrease AL, Lipase for degreasing m acidic pH conditions (http://www.mapsenzymes.comlEnzymes Leather.asp).

Lipase enzymes can remove fats and grease from skins and hides, particularly those with a moderate fat content. Both alkaline stable and acid active lipases are used in skin and hide degreasing. Deliming and bating are the most suitable processing stages for using lipases. The enzyme loosens and removes the hair on the skins, which can then be filtered off. The end product is of a higher quality when compared to leather manufactured using traditional methods. *Rhizopus nodosus* lipase was used for the degreasing of suede clothing leathers from wooled sheep skins (Muthukumaran and Dhar, 1982). Acid active lipases can be used to treat skins that have been stored m a pickled state (http://www.biowise.org.uk).

2.10.12 Waste/ effluent/ sewage treatment

Lipases are utilized in activated sludge and other aerobic waste processes, where thin layers of fats must be continuously removed from the surface of aerated tanks to permit oxygen transport (to maintain living conditions for the biomass). This skimmed fat-rich liquid is digested with lipases (Bailey and Ollis, 1986) such as that from *C.rugosa.* Effective breakdown of solids and the clearing and prevention of fat blockage or filming in waste systems are important in many industrial operations. Examples include: (i) degradation of organic debris-a commercial mixture of lipase, cellulase, protease, amylase, inorganic nutrients, wheat bran, etc. is employed for this purpose; (ii) sewage treatment, cleaning of

holding tanks, septic tanks, grease traps, etc. Effluent treatment is also necessary in industrial processing units, such as abattoirs, the food processing industry, the leather industry, the poultry waste processing (Godfrey and Reichelt, 1983). Both *P. aeruginosa* LP602 cells and the lipase were shown to be usable for lipid-rich wastewater treatment (Dharmsthiti and Kuhasuntisuk, 1998). A fat in wastewater treatment plants that contains mainly triglycerides is hydrolysed by immobilized lipase (Tschocke, 1990).

Bacterial lipases are involved in solution of such environmental problems as the breakdown of fats in domestic sewage and anaerobic digesters (Godfrey and Reichelt, 1983).
Simple alkyl ester derivatives of restaurant grease were prepared using immobilized lipases as biocatalysts. The lipase from *Pseudomonas cepacia* was found to be the most effective in catalysing the methanolysis and ethanolysis of grease (Hsu *et al.,* 2002). A mixture of industrial cellulase, protease, and lipase, in equal proportion by weight, reduced total suspended solids (TSS) by 30-50% and improved settling of solids in sludge. An increase in solid reduction was observed with increase enzyme concentration.

2. 10.13 Oil biodegradation

Monitoring of soil microbial lipase activity is a valuable indicator of diesel oil biodegradation in freshly contaminated, unfertilized and fertilized soils (Margesin *et al.,* 1999). Fungal species is used to degrade oil spills in the coastal environment, which may enhance ecorestoration as well as in the enzymatic oil processing in industries (Gopinath *et al.,* 1998).

2.10.14 Pulp and paper industry

The pulp and paper industry processes huge quantities of lignocellulosic biomass every year. The technology for pulp manufacture is highly diverse, and numerous opportunities exist for the application of microbial enzymes. Historically, enzymes have found some uses in the paper industry, but these have been mainly confined to areas such as modifications of raw starch. The enzymatic pitch control method using lipases have been in use in a large-scale paper-making process as a routine operation since early 1990s (Bajpai, 1999). Lipase for wastepaper deinking can increase the pulping rate of pulp, increase whiteness and intensity, decrease chemical usage, prolong equipment life, reduce pollution level of waste water, save energy and time and reduce composite cost. The addition of lipase from *Pseudomonas* species (KWI-56) to a deinking composition for ethylene oxide-

propylene oxide adduct stearate improved whiteness of paper and reduced residual ink spots (Fukuda *et al.,* 1990).

2.10.15 Use of lipases in production of biodiesel

The limited (and fast diminishing) resources of fossil fuels, increasing prices of crude oil, and environmental concerns have been the diverse reasons for exploring the use of vegetable oils as alternative fuels (Shah *et al.,* 2004). The biodiesel fuel from vegetable oil does not produce sulphur oxide and minimize the soot particulate one third times in comparison with the existing one from petroleum. Because of these environmental advantages, biodiesel fuel can be expected as a substitute for conventional diesel fuel (Iso *et al.,* 2001). Immobilized *P. cepacia* lipase was used for the transesterification of soybean oil with methanol and ethanol (Noureddini *et al.,* 2005). Fatty acid ethyl esters have also been prepared from castor oil using n-hexane as solvent and two commercial lipases, Novozym 435 and Upozyme IM, as catalysts (Oliveira *et al.,* 2004). Novozyme 435 has also been used to catalyse the transesterification of crude soybean oils for biodiesel production in a solvent-free medium (Du *et al.,* 2004).

Simple alkyl ester derivatives of restaurant grease were prepared using immobilized lipases from *Thermomyces lanuginose* and C. *antarctica,* as biocatalysts (Hsu *et al.,* 2002). Fatty acids esters were produced from two Nigerian lauric oils, palm kernel oil and coconut oil, by transesterification of the oils with different alcohols using PS30 lipase as a catalyst. In the conversion of palm kernel oil to alkyl esters (biodiesel), ethanol gave the highest conversion of 72 %. Some of the fuel properties compared favourably with international biodiesel specifications (Abigor *et al.,* 2000).

2.10 Current status of lipase research in India

Research on microbial lipases in India date back to late seventies when a few reports on screening and production of lipase from a few fungi and bacteria appeared. The initial emphasis on screening exercises was followed by process optimization in maximum lipase production. Physico-chemical conditions of lipase production by *AI. racemosus, A. wenlii,* and *P. chlysogenum* was reported (Akhtar *et al.,* 1980; Chander *et al.,* 1980). Lipolytic activity of thermophilic fungi of paddy straw compost was reported (Satayanarayan and Johri, 1981). *A. niger, A. flavus, A. fumigatus* and *Penicillium glaucum* were reported as the

potential lipase producers isolated from the kernels of chironji and walnut (Saxena *et al.,* 1999).

Large-scale process optimization for lipase production was reported for *A. lerreus, A. carneus* and *B. stearothermophilus* (Yadav *et al.,* 1998). Extracellular microbial lipases was utilized for transesterification reactions for producing valuable transformed edible oils which cannot be obtained by chemical interesterification methods (Chakrabarty *et aI.,* 1987). Lipases from *H. lanuginosa* and *Y. /ipolytica* have also been reported for the synthesis of geranyl esters (Chand and Kaur, 1998). An extracellular lipase isolated from the conidia of *N. crassa,* had an apparent molecular weight of 54 kDa and 27 kDa, suggesting thereby the presence of two identical subunits (Kundu *et al.,* 1987).

Extensive work on various aspects of lipase from production and purification to characterization and industrial applications has been carried out on various fungi and bacteria (Ghosh *et al.,* 1996; Yadav *et al.,* 1998). Novel thermostable and alkaline lipases from *A. terre us* and *A. carneus* are being developed for the production of biosurfactants, glycerides, and pharmaceutically important compounds. These lipases show regio- and chemoselective cleavage of polyphenolic compounds. Lipase from a strain of *B. stearothermophilus* shows remarkable activity even at 100°C. Besides this, a rapid zymogram for lipase activity in polyacrylamide gels was developed (Yadav *et al.,* 1998).

The ability of lipases to show increased stability and selectivity in organic solvents has been exploited (Parrnar *et al.,* 1992). Biotransformations on polyacetoxy arylmethyl ketones, benzylphenylketone peracetates, esters of polyacetoxy aromatic acids, and peracetylated benzopyranones, using commercial lipases are reported (Parmar *et al.,* 1992). The enantioselective behaviour of microbial lipases for the resolution of racemic drugs (Qazi, 1997), lipase-catalysed ester interchanges for the modification of selected Indian vegetable oils into cocoa butter substitutes and high oleic oils (Sridhar *et al.,* 1991), and enhancement of enzyme activity in aqueous-organic solvent mixtures (Gupta, 1992) were reported.

3. MATERIALS AND METHODS

3.2 Materials

All the chemicals used in the present study were of AR grade and purchased from Hi-Media Limited and S.D. Fine Chemicals Limited, Mumbai, India. Wheat bran (WB), coconut oil cake (COC), wheat rawa (WR) was purchased from local market, Chennai, India. Beef fleshing was obtained from the tannery department, Central Leather Research Institute, Chennai, India.

3.2. Methods

3.2.1. Screening of Lipase Producing Fungal Microorganism

3.2.1.1 Tributyrin agar medium

Ingredient	g/100ml
Czapek Dox agar	4.9
Tributyrin	1(v/v)
Agar Agar	2

Tributyrin agar medium prepared in appropriate volume with above ingredients and sterilized at 121°C for 30min.

3.2.1.2 Rhodamine B agar medium

Ingredient	g/100ml
Sodium nitrate	0.2
Potassium chloride	0.05
Magnesium sulphate	0.05
Dipotassium hydrogen phosphate	0.1
Sucrose	2
Agar	1.5
Tributyrin	0.5(v/v)

The Rhodamine agar medium was prepared in distilled water, autoclaved, and cooled to 60°C. The cooled medium was added with 0.5 % of tributyrin previously sterilized at 160°C for 2 h in hot air oven and 1 % filter sterilized Rhodamine B (1 mg/ml). The contents were mixed well to dissolve and the medium was poured into petri dishes.

3.2.2. Beef Fleshing and Their Characterization

Beef fleshing was used in the present study was solid substance, rich in protein with a mild odour and was obtained from slaughterhouse at Chennai, India. The main components of beef fleshing are: oleic, palmitic and stearic acid. Other fatty acids account for <0.1% of the product.

3.2.2.1. Estimation of Protein Content (Kjeldhal method or Digestion, 1883)

Reagents

1) 36N H_2SO_4
2) Catalyst (Potassium sulphate and copper sulphate)
3) 20 % NaOH
4) Mixed indicator (Methylene blue + Methyl red)

Procedure

1g of sample (Beef fleshing)

↓

20ml of con H_2SO_4 + catalyst

↓

Addition of 50ml of H_2O

↓

Solution became dark colour

↓

Addition of 50ml of Baric acid

↓

Back titration

↓

Pink to green colour

↓

Titration with 0.03N H_2SO_4

Formula

$$\text{Nitrogent (\%)} = \frac{(A-B) \times \text{Normality of } H_2SO_4 \times \text{Mw of Nitrogen}}{\text{Weight of the sample}} \times 1000 \quad (3.1)$$

Khjeldhal method is used to estimate the nitrogen content of sample. The conversion factor (6.25) can be used to predict the quantity of protein present in sample.

3.2.2.2. Extraction Lipids from Beef Fleshing using Soxhlet Method

Reagents

1) Sample (Beef fleshing)
2) Petroleum ether

Procedure

12g of sample

↓

Soxhlet apparatus fixed in position

↓

Petroleum ether solvent was taken (150ml)

↓

4-5 hours of reflux

↓

Solvent evaporated in boiling water bath

↓

Weigh the lipids

3.2.2.3. Moisture Analysis of Beef Fleshing

1g of sample

↓

Incubated at 100 °C in hot air oven for 1hour

↓

Sample weighs in moisture analyzer

3.2.2.4 Ash Content of Beef Fleshing

Fleshing maintained in 500 - 600 °C for 1 hour and fleshing became ash, sample weighed in the balance.

3.2.3 Electrophoretic Method

Ammonium sulphate: precipitated sample and crude, dialysis sample were collected. Sample was electrophoresed by SDS-PAGE in a 30 % acrylamide gel according to the method of Laemmlli (1970).

Reagents

1) 30% Acrylamide solution (30:0.8)
2) Stacking gel buffer stock (0.5 M Tris-HCI, pH 6.8)
3) Resolving Gel buffer stock (3M Tris-HCl, pH 8.8)
4) Protein Staining solution
5) Destaining Solution
6) Protein Marker for SDS-PAGE

Procedure

1) The gel plates were cleaned using absolute ethanol and acetone, and assembled.
2) Resolving gel- All the components except APS were added into a beaker, mixed gently and finally added APS. Immediately poured the mixture into the cast and poured a layer of butanol over the gel and allowed to solidify at least for 1 h.
3) Stacking gel- The components of stacking gel except APS was added into a beaker, mixed gently and finally added APS. Poured the contents into the cast above the resolving gel and immediately inserted the comb between the glass plates. Allowed it to solidify at least for 30 min.
4) Gel was placed in the electrophoresis apparatus, and upper and lower reservoir was filled with reservoir buffer for PAGE.
5) The gel was pre run for 1h at 80V.
6) Loaded the gel with the protein sample.
7) The gel was run at 80V till the sample entered the resolving gel.
8) When the dye front entered the resolving gel, increased the current to 100 V.

9) The current was stopped when the dye front reached 1 cm above the lower end of the glass plate.
10) Removed the gel from the cast and stained for at least 1 h in the staining solution.
11) Destained till the bands became clear and observed under UV transilluminator.

3.2.4. Solid State Fermentation

3.2.4.1 Inoculum and Fermentation Conditions

A fungal strain of *A. niger* was isolated in our laboratory was used in the present study and it was maintained on Czapek Dox agar slants at 4°C. The spore suspension for inoculation was prepared by adding 2 ml of sterile distilled water to the culture slant and the spores were dislodged using an inoculation needle under aseptic conditions. A spore suspension containing 4.3×10^8 spores/ml was used as inoculums.

Lipase production was carried out in 250 ml Erlenmeyer flasks, each containing 10 g of different solid substrates either alone or in mixed combinations with two substrates, bi-substrate fermentation (BSF) or three substrates, tri-substrate fermentation (TSF), respectively, with initial moisture content adjusted to 55% with distilled water. The flasks were inoculated with 250 µl of spore suspension and the contents were mixed and incubated at 30°C for 5 days. Samples were taken at 24 h intervals and extraction of the enzyme was carried out according to Kamini *et al*., (1998). All the experiments were carried out in triplicates and the results were expressed as the average of triplicates.

Mixed Substrate Fermentation

3.2.4.2. Lipase Production by Single Substrate Fermentation

Fermentation studies were investigated by the initial moisture content was 55% and inoculum concentration 2.5% (4.3×10^8 spores/g substrate). Using optimized parameters, time course studies on lipase production were carried out for single substrate fermentation.

Substrates are wheat bran, wheat raw, coconut oil cake and beef fleshing

3.2.4.3 Lipase Production by BSF

Consecutive optimization studies were investigated by the initial moisture content was 55% and inoculum concentration was about 2.5% (4.3×10^8 spores/g substrate). Using optimized parameters, time course studies on lipase production were carried out for BSF.

Table 3.1 Lipase Production by BSF

S.No	Substrate (%)
1	WB(7.5)+FL(2.5)
2	WB(5)+FL(5)
3	WB(2.5)+FL(7.5)

3.2.4.4 Lipase Production by TSF

TSF studies were investigated by varying the initial moisture content 60% and inoculum concentration 2.5% (4.3×10^8 spores/g substrate). Using optimized parameters, time course studies on lipase production were carried out for TSF.

Table 3.2 Lipase Production by TSF

S.No	Substrate (%)
1	WB(5)+WR(2.5)+COC(2.5)
2	WB(5)+WR(2.5)+FL(2.5)
3	WB(5)+FL(2.5)+COC(2.5)
4	FL(5)+WR(2.5)+COC(2.5)

3.2.5. Optimization of BSF Parameters

3.2.5.1 Effect of Inoculums Size on Lipase Production for BSF

Various concentrations of inoculums were used for BSF medium. The percentage ranges from 1 % to 10% of inoculums. The production of enzyme various with inoculums percentage. The spore suspension for inoculation was prepared by adding 2 ml of sterile distilled water and the spores were dislodged using an inoculation needle under aseptic conditions. A spore suspension containing 4.3×10^8 spores/ml was used as inoculums.

3.2.5.2 Effect of Moisture Content on Lipase Production by BSF

The moisture content was optimized ranges from 30 % to 60 %. The production of lipase varies with moisture percentage.

Calculation Formula

$$\text{Moisture (\%)} = \frac{\text{Vol. of dis. } H_2O}{\text{Vol. of dis. } H_2O + \text{Weight of the sample}} \times 100 \qquad (3.2)$$

3 .2.5.3 Effect of Extraction of the Enzyme

Extraction of the enzyme from BSF was carried out with tap water, distilled water, buffer, salts (sodium chloride - 1%) and ammonium sulphate - 1%) and surfactants (Triton X-100 – 0.5%).

3.2.5.4 Scale up of Lipase Production

Scale up of lipase production from 10 g flask level to 40 g were carried out. Substrate inoculums grown for 72 h were inoculated into 40g of mixed substrate (moisture content adjusted to 55%) in series of sterilized flasks 96 h and assayed for lipase activity at 24 h intervals. The variation of temperature and humidity during the process was monitored using a portable humidometer.

3.2.5.5 Enzyme Assay

Tritimetric Determination of Lipase Assay (Yamada *et al*., 1962)

Reagents

1) Olive oil
2) 2% PVA
3) 0.05M NaOH
4) 0.05M phosphate buffer
5) 1% Phenolpthalein
6) Olive oil emulsion

   25ml of olive oil with 75ml of 2% PVA solution and sonicated at 60% amplitude for 3min (9 second pulse)

Procedure

5ml of emulsion was added to 4ml of 0.05M phosphate buffer (pH 7) and mixed well.

↓

The reaction mixture was pre-incubated at 37°C for 10minutes.

↓

1ml of enzyme solution was then added & incubated at 37°C for 20minutes.

↓

20ml of acetone was added to stop the reaction

↓

A controlled was performed by adding the enzyme after addition of acetone

↓

The released fatty acids were estimated by titration against 0.05M NaOH using phenolphthalein as indication

Calculation Formula

$$\text{Lipase activity} = \frac{\text{Vol of NaOH} \times \text{Normality of NaOH}}{\text{Time of incubation}} \times 1000 \quad (3.3)$$

At the end of fermentation period, 1 g of the fermented substrate was homogenized with 10 ml of 0.05 M phosphate buffer (pH 7.0) and the supernatant was squeezed through a double layered muslin cloth and centrifuged at 10,000 rpm for 5 min. The supernatant obtained was used as the enzyme source. Lipase activity was determined according to Yamada *et al*. (1962) using olive oil as substrate. One unit of activity was defined as the amount of enzyme releasing 1 μmol of free fatty acid in 1 min at pH 7.0 and 37 °C and the lipase activity was expressed as U/g of dry substrate (U/gds). Moisture content was determined after total drying of the sample in a hot air oven at 100 °C for 6 h.

3.2.6 Submerged Fermentation

3.2.6.1 Inoculum and Culture Conditions

The spore suspension for inoculation was prepared by adding 2 ml of sterile distilled water to the culture slant, and the spores were dislodged using an inoculation needle under aseptic conditions. A spore suspension containing 4.3×10^8 spores/ ml was used as an inoculum. Lipase production was carried out in 250mL Erlenmeyer flasks, each containing 10 ml of the sterile production medium of pH 7.0. The medium was inoculated with 240 µl of spore suspension and grown for 48 h on a rotary shaker at 30 °C and 120 rpm. After growth, the biomass was removed by filtration, and the cell-free supernatant was used as crude enzyme preparation. All of the experiments were carried out in batches, and the results are expressed as the average of triplicates.

3.2.6.2 Medium Optimization with CSL as Nutrition Adjunct

Lipase production was initially carried out in the production medium of the following composition

Ingredient	g/100ml
CSL	2
Urea	0.25
Sucrose	0.5
NaCl	0.5
$NH_4H_2PO_4$	0.05
KH_2PO_4	0.1
Na_2HPO_4	0.75
olive oil	2

3.2.6.3 Replacement of Oil Inducers

Beef fleshing used to replace the various oil as inducers and increase the production of lipase enzyme by submerged fermentation. The beef fleshing about 2% was used in the production medium

3.2.6.4 Scale up of Lipase Production

Scales up of lipase production from 10ml flask level to 200ml were carried out. Substrate inoculums were inoculated into 200ml of CSL medium in series of sterilized flasks

96 h and assayed for lipase activity at 24 h intervals. The variation of temperature during the process was monitored.

3.2.7 Direct Application of Fermented Substrate for Fleshing Hydrolysis

The fermented substrate was directly evaluated for the hydrolysis of fleshing. The hydrolytic reactions were carried out in series of 100 ml screw caped Erlenmeyer flasks containing 10 g of sliced fleshing, 25 ml of 0.1 M phosphate buffer, pH 7.0 and hydrolysis was carried out with fermented substrate (lipolytic activity of 25 U/g of fleshing) for 96 h with shaking at 150 rpm and 30 °C. Samples were taken at 24 h intervals and reaction was stopped by addition of 20 ml of acetone and the free fatty acids liberated were titrated with 0.1 N KOH using phenolphthalein as indicator. A control was carried out similarly, except the fermented substrate was added after the addition of acetone. The control value was subtracted from the experimental value and the acid value was calculated.

3.2.7.1 Estimation of Saponification Value

Reagents

1) Fleshing
2) 0.5N HCl
3) 0.5N standard alcoholic KOH
4) 0.1% Phenolpthalein in ethanol

Procedure

12g of sample 250ml round bottom flask

↓

150ml of 0.5N alcoholic KOH added and mixture

↓

5 times refluxed

↓

After refluxing few drops of phenolphthalein added to hot solution

↓

Excess KOH present in solution titrated against 0.5N HCl

Calculation Formula

$$\text{Saponification} = \frac{(\text{Test - Control}) \times 0.02805}{\text{Weigh of fleshing (g)}} \times 1000 \quad (3.4)$$

3.2.7.2 Hydrolysis of Beef Fleshing Using Lipase Enzyme

Procedure

10g of sliced beef fleshing

↓

25ml of 0.1M phosphate buffer (pH 7)

↓

Mix well

↓

Addition of enzyme (250U)

↓

Incubated at shaker for 96h

↓

24h interval the sample taken

↓

Addition of 20ml of acetone

↓

1% Phenolphthalein indicator

↓

0.1N KOH titrated

The hydrolysis ratio was calculated by the following equation:

Formula

$$\text{Hydrolysis ratio (\%)} = \frac{\text{Acid value}}{\text{Saponification value}} \times 100 \quad (3.5)$$

4. RESULTS

4.1 Lipolytic Activity of *Aspergillus niger*

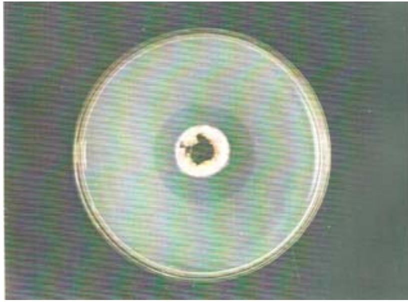


Fig 4.1.1 Tributyrin Agar Plate Medium

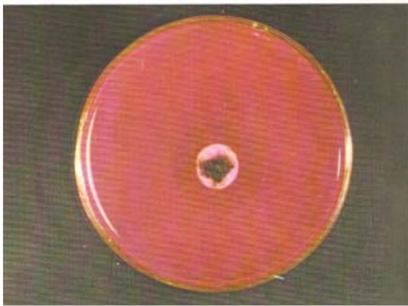
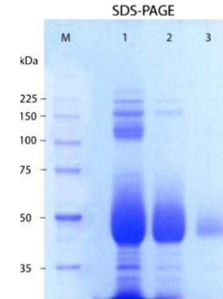


Fig 4.1.2 Rhodamine B Agar Plate medium

Screening of fungal lipolytic activity, using tributyrin and rhodamine B agar plate for lipase production, culture was recognized as potential lipase producers. The zone of clearance in tributyrin agar plate indicate the lipolytic activity of *Aspergillus niger*. The view of the cultures, which showed orange-red fluorescent halos upon UV irradiation in rhodamine B agar plates. Hence, this strain was selected as potential strain for lipase production.

4.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis (SDS-PAGE)

Fig. 4.2 Electrophosis of Protein Sample



Lane 1: crude enzyme

Lane 2: ASP enzyme

Lane 3: Purified enzyme (Dialysis)

The purified lipase of *A.niger* were monomeric with molecular weight of 43 kDa. An extracellular lipase isolated from the *A.niger,* had an apparent molecular weight. Other proteins and contamination also present in the agarose gel. The molecular weight of purified enzyme about 43kda.

4.3 Beef Fleshing Characterization

Proximate analysis of crude protein, fat, ash and moisture were carried out according to the methods of AOAC. All determinations were done in triplicate and the mean value was recorded.

Fig 4.3 Composition of Beef Fleshing

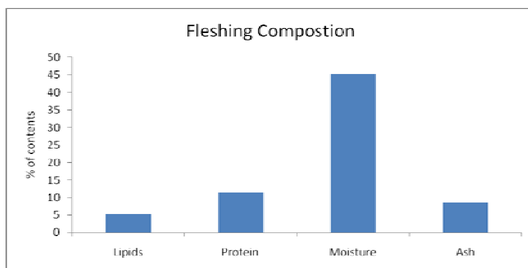


Table 4.1 Composition of Beef Fleshing

S.No	Composition	Percentage
1	Lipids	5.27
2	Protein	11.5
3	Moisture	45.4
4	Ash	8.5

4.4 Solid State Fermentation

The effect of different substrates such as oil cakes, wheat bran, wheat raw, cotton seed oil cake and spent barley on lipase production by *A. niger* was reported earlier by Kamini *et al*. (1998). Mixed solid substrates are viable substrates for the growth of microorganisms on SSF, since they act as support matrix, nutrient source and as inducers for the production of enzymes. Accordingly, to enhance the growth and production of lipase from *A. niger*, a BSF was developed using the substrates WB and beef fleshing.

4.4.1 Mixed Substrate Fermentation

4.4.1.1 Single Substrate Fermentation

Lipase production from *A. niger* was carried out with the substrates WB, COC, WR and beef fleshing and the results are shown in Table 4.2. The lipase activities were 260, 192 and 301 U/gds with WB, FL and COC, respectively, at 96 h, while a higher lipase activity of 310 U/gds was obtained with WR at 72h.

Fig 4.4 Lipase Production by Single Substrate Fermentation

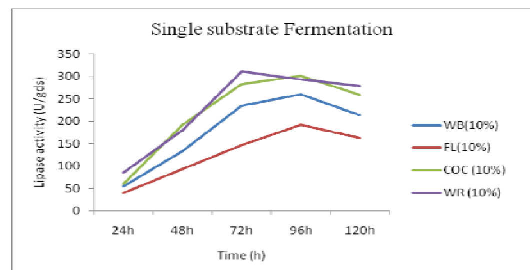


Table 4.2 Lipase Production by Single Substrate Fermentation

Substrate	24h	48h	72h	96h	120h
WB(10%)	55.3 ± 12	134 ± 19	235.8 ± 15	260.5 ±12	214.1 ± 11
FL(10%)	40.5 ± 17	93.8 ± 13	147.4 ± 18	192.9 ± 17	163 ±14
COC (10%)	60.1 ± 19	192.3 ± 18	281.7 ± 11	301.4 ± 18	258.6 ± 18
WR (10%)	85.5 ± 12	179 ± 17	310.1 ± 16	293.1 ± 18	278.5 ± 16

4.4.1.2 Bi-substrate Fermentation

Accordingly, BSF was carried out by addition of fleshing to WB in different ratio (Table 4.3). Maximal lipase activities of 404 and 345 U/gds were obtained with combinations of FL: WB (1:1) and WB: FL (1:3), respectively, at 96 h. The enzyme yields obtained with other combinations were also higher than individual substrates.

Fig 4.5 Lipase Production by BSF

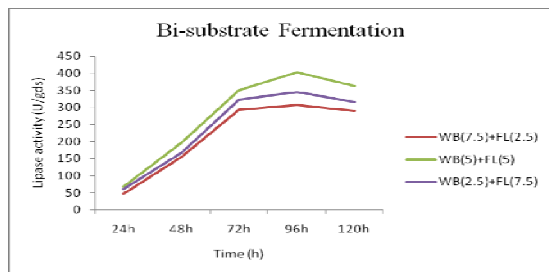


Table 4.3 Lipase Production by BSF

Substrate Combination	24h	48h	72h	96h	120h
WB(7.5)+FL(2.5)	48.2 ± 12	156.5 ± 16	294.8 ± 12	308.7 ± 18	291.8 ±12
WB(5)+FL(5)	68.1 ± 15	198.7 ± 10	352.3 ± 18	404.8 ± 17	363.5 ±11
WB(2.5)+FL(7.5)	62.4 ± 19	168.6 ± 18	323.5 ± 15	345.3 ± 15	315.2 ±18

4.4.1.3 Tri-substrate Fermentation

In order to enhance the lipase production, TSF experiments were carried out using FL, COC, WB and WR (Table 4.4), since maximum lipase activity was obtained with a combination of 5 g of FL and 5 g of WB in BSF (Table 4.3). In TSF, an optimal lipase activity of 422 U/gds was obtained using a mixture of WB, 5 g; COC, 2.5 g and WR, 2.5 g (Table 4.4), which was comparatively higher than other combinations of TSF.

The initial moisture content and inoculum concentration were found to be the critical factors for lipase production. Accordingly, a maximum yield of 422 U/gds was obtained at 96 h with an initial moisture content of 60% and an inoculum concentration of 2.5%.

Fig 4.6 Lipase Production by TSF

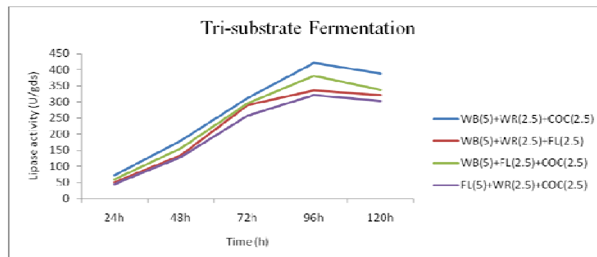


Table 4.4 Lipase Production by TSF

Substrate Combination	24h	48h	72h	96h	120h
WB(5)+WR(2.5)+COC(2.5)	72.1 ±11	180.8 ±16	312.2 ±14	422.7 ±11	389.4 ±18
WB(5)+WR(2.5)+FL(2.5)	51.3 ±15	135.1 ±13	289.7 ±13	335.6 ±17	320.2 ±11
WB(5)+FL(2.5)+COC(2.5)	60.8 ±10	156.7 ±12	295.3 ±11	381.4 ±15	338.1 ±15
FL(5)+WR(2.5)+COC(2.5)	44.5 ±18	128.6±12	258.4 ±17	321.9 ±13	302.1 ±19

4.4.2 Effect of Inoculums Size and Moisture Content on Lipase Production

Fig 4.6 Effect of Inoculums on Lipase Production

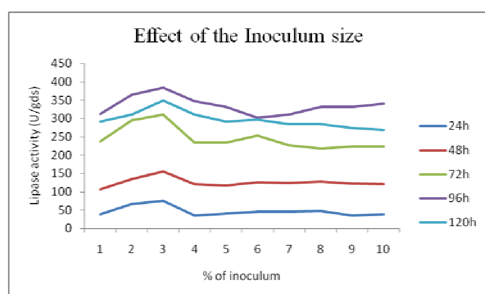


Table 4.5 Effect of Inoculum on Lipase Production

Percentage of Inoculum	24h	48h	72h	96h	120h
1	38.1 ± 11	106.7 ± 16	238.8 ± 13	313.2 ± 17	292.1 ± 13
2	67.2 ± 16	134.1 ± 18	295.9 ± 16	365.3 ± 12	310.2 ± 13
3	75.7 ± 18	156.5 ± 16	310.4 ± 17	384.5 ± 14	350.6 ± 12
4	36.7 ± 19	121.8 ± 12	234.2 ± 18	347.6 ± 13	310.9 ± 17
5	41.2 ± 10	118.1 ± 19	234.4 ± 13	332.5 ± 11	292.8 ± 18
6	46.5 ± 14	126.6 ± 12	253.8 ± 15	302.1 ± 12	296.6 ± 18
7	46.7 ± 19	125.6 ± 16	227.5 ± 18	310.2 ± 14	284.5 ± 15
8	47.1 ± 15	128.6 ± 16	219.1 ± 10	332.5 ± 19	284.4 ± 12
9	36.7 ± 18	122.4 ± 17	223.6 ± 14	3326 ± 15	275.6 ± 11
10	38.3 ± 16	121.5 ± 18	223.3 ± 16	341.9 ± 16	269.8 ± 17

Fig 4.7 Effect of Moisture Content on Lipase Production

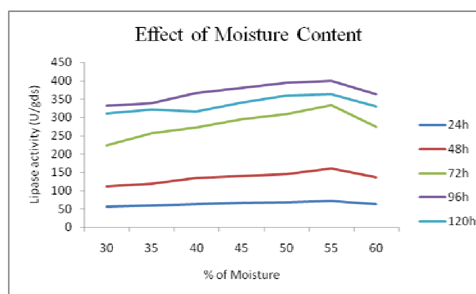


Table 4.6 Effect of Moisture Content on Lipase Production

Percentage of Moisture	24h	48h	72h	96h	120h
30	56.1 ± 17	112.6 ± 14	224.2 ± 12	332.6 ± 12	310.4 ± 19
35	58.8 ± 13	120.4 ± 15	257.3 ± 16	340.6 ± 14	321.2 ± 11
40	62.3 ± 18	135.1 ± 18	273.6 ± 13	367.1 ± 12	315.1 ± 12
45	66.4 ± 17	140.2 ± 18	296.8 ± 13	380.4 ± 18	340.4 ± 12
50	69.6 ± 18	146.3 ± 19	309.3 ± 15	394.6 ± 19	360.3 ± 17
55	72.7 ± 16	161.4 ± 16	334.5 ± 14	399.4 ± 11	363.2 ± 14
60	63.9 ± 16	136.6 ± 17	275.1 ± 12	363.2 ± 18	330.2 ± 11

There was a decline in enzyme production an increase or decrease in moisture content from 60% to 30%, respectively, at 96 h (Table 4.6). This could be due to the suboptimal growth of the fungus and lower degree of substrate swelling at lower moisture levels as reported by Zadrazil and Brunnert (1981), while at increased moisture levels, the porosity of the substrate was decreased and thereby, limiting the gas exchange and increasing the chances of contamination (Mahadik *et al*., 2002; Adinarayana *et al*., 2003b). There was no

significant increase in lipase activity upon increasing the inoculum concentration up to 4.3 × 10^8 spores/g substrate. Supplementation of additives such as carbon and nitrogen sources and inducers to the TSF did not show any effect on production of lipase (data not shown). Moreover, these additives could possibly lead to contamination problems and also increase the cost of production as reported by Mala *et al*. (2007). Hence, the control system without any additives was used for further studies.

4.4.5 Extraction of the Enzyme

Fig 4.8 Effect of Salts and Surfactants on Lipase Extraction

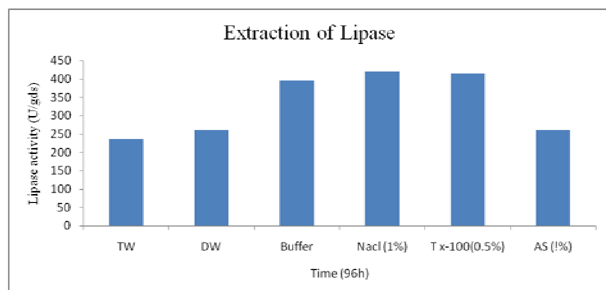


Table 4.7 Effect of Salts and Surfactants on Lipase Extraction

Salts and Surfactants	Activity (U/gds)
Tap Water	235.5 ± 12
Distilled Water	260.2 ± 18
Buffer	396.1 ± 15
Nacl (1%)	420.2 ± 15
Triton x-100(0.5%)	415.6 ± 14
Ammonium Sulphate (1%)	260.7 ± 16

Extraction of the enzyme from TSF was carried out with tap water, distilled water, buffer, salts (sodium chloride or ammonium sulphate) and surfactants (Tween 80 or Triton X-100). The recovery of the enzyme was optimal with sodium chloride (1%), however, supplementation of sodium chloride with Triton X-100 (0.5%) helped in increasing the recovery of enzyme from 420 and 415 U/gds (Table 4.7). This could be due to the use of surfactant, Triton X-100, which might increase the permeability of cells resulting in higher recovery of enzyme from TSF. Similar results were reported for the extraction of lipase from *A. niger* with sodium chloride (1%) and Triton X-100 (0.5%) (Mahadik *et al*., 2002),

4.5 Submerged Fermentation

The production of lipase from *A. niger* in submerged fermentation was reported earlier by Kamini *et al*. 1998. Economic analysis of the above medium revealed that addition of meat extract alone to the medium contributes about 66.8% of medium cost. Therefore, attempts were made to make the process economically viable by using an inexpensive nutrition adjunct, CSL, because it is an excellent source of nitrogen and carbon for most of the microorganisms. Addition of beef fleshing for lipase production in submerged fermentation show comparable enzyme production (25.8 U/ml).

Fig 4.9 Lipase Production by Conventional Medium

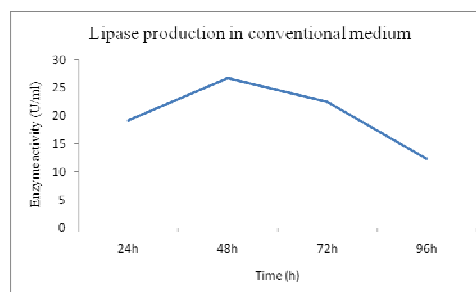


Table 4.8 Lipase Production using CSL and Oil as Inducers

Time (h)	Activity (U/ml)
24h	19.2
48h	26.7
72h	22.5
96h	12.3

Fig 4.10 Lipase Production using Beef Fleshing and CSL

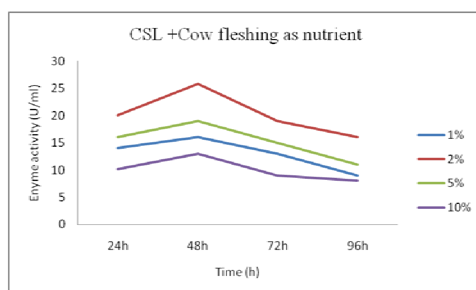


Table 4.9 Lipase Production using Beef Fleshing and CSL

Percentage of Fleshing	24h	48h	72h	96h
1%	14	16	13	9
2%	20	25.8	19	16
5%	16	19	15	11
10%	10.2	13	9	8

4.6 Hydrolysis of Beef Fleshing using Lipase from *Aspergillus niger*

The fleshing was hydrolyzed efficiently to 71.3% at 24 h initially and optimization studies showed an increase in the hydrolytic ratio by 15.6% at 24 h, when the reaction mixture contained 10 g of fleshing, 25 ml of 0.1 M phosphate buffer (pH 7.0), and 250 U of lipase. Accordingly, the fermented substrate could be directly used for the hydrolysis of tallow and in the production of fatty acid esters, since the fuel produced from tallow has the advantage of a higher calorific value and cetane number than the fuels obtained from vegetable oils as reported by Lebedevas *et al*. (2006). Moreover, the reported hydrolytic ratio was comparatively higher with our lipase, than the hydrolytic ratios of beef fleshing obtained with lipases from Rhizomucor miehei (73%) and Yarrowia lipolytica (65%) (Adamczak and Bednarski, 2004), while a higher hydrolytic ratio of 93.86% was reported by Gao *et al*. (2009) using surfactant coated commercial lipase of C. Rugosa (Novoenzymes, China). However, the direct application of the fermented substrate for fleshing hydrolysis by *A. niger* makes the process economical and avoids the need for expensive enzyme recuperation and immobilization processes. The potential of this system in transesterification reaction deserves to be explored further.

Fig 4.11 Hydrolysis of Fleshing by *A. niger* Lipase

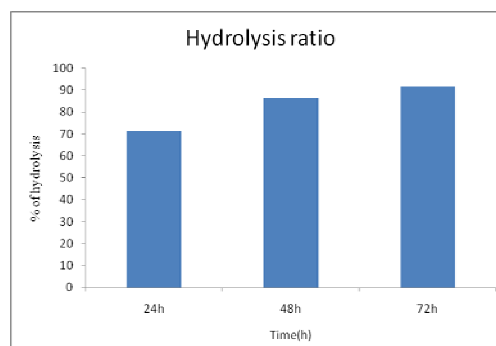


Table 4.10 Percentage of Fleshing Hydrolysis by Lipase

S.No	Incubation	Hydrolysis Percentage
1	24h	71.30
2	48h	86.58
3	72h	91.67

5. DISCUSSION

The purified lipase of *P. jragi, P. jluorescens,* and *P. aeruginosa* were monomeric with molecular weight of 33 kDa, 45 kDa, and 29 kDa, respectively (Brune and Gotz, 1992; Nishio *et al.,* 1987*;* Sztajer *et al.,* 1991). An extracellular lipase isolated from the conidia of *N. crassa,* had an apparent molecular weight of 54 kDa and 27 kDa, suggesting thereby the presence of two identical subunits (Kundu *et al.,* 1987).The molecular weight of purified enzyme about 43kda.

The effect of different substrates such as gingelly oil cake, cottonseed oil cake, groundnut oil cake, rice bran, wheat bran, gingelly seed, castor bean seed, groundnut seed, sugarcane bagasse, groundnut kernel, coffee husk and spent barley on lipase production by *A. niger* was reported earlier by Kamini *et al*., (1998). Among the substrates, gingelly oil cake was found to be the best substrate with a lipase activity of 363.6 U/dgs at 72 h. Later, *Mala et al*. (2007) reported a 36.0% increase in lipase activity by adding GOC to WB in a mixed solid substrate fermentation (GOC: WB, 1:3, w/w), which was comparatively higher than the activity obtained with WB alone. Mixed solid substrates are viable substrates for the growth of microorganisms on SSF, since they act as support matrix, nutrient source and as inducers for the production of enzymes. Accordingly, to enhance the growth and production of lipase from *A. niger*, a TSF was developed using the substrates WB, WR and COC.

Lipase production by mixed substrate fermentation lipase production from *A. niger* was carried out with the substrates WB, COC, WR and beef fleshing. The lipase activities were 260, 192 and 301 U/gds with WB, FL and COC, respectively, at 96 h, while a higher lipase activity of 310 U/gds was obtained with WR at 72 h. The reported activities with our strain were higher, when compared to the activities of various microorganisms including *A. niger* and *Penicillium candidum* with WB (Rivera-Munoz *et al*., 1991) and *Candida rugosa* with COC (Benjamin and Pandey, 1997) as substrates. In general, mixed solid substrates are viable substrates for SSF processes.

Accordingly, BSF was carried out by addition of COC and WR to WB independently. Maximal lipase activities of 459.1 ± 16 and 362.2 ± 18 U/gds were obtained with combinations of COC: WB (1:3) and WR: WB (1:1), respectively, at 96 h. The enzyme yields obtained with other combinations were also higher than individual substrates, since the

nutrients and trace elements present in the BSF enables the organisms to yield more protein favorably by influencing its biochemical pathways for lipase production (Cordova *et al*., 1998). WR has been used as a substrate for the production of antibiotics like neomycin (Adinarayana *et al*., 2003a), cephalosporin C (Adinarayana *et al*., 2003b) and cephamycin C (Kota and Sridhar, 1999) and the present work will serve as a baseline for the production of enzymes using WR as substrate either alone or in mixed combinations with other substrates.

In addition, all the three substrates of TSF, synergistically and also individually exhibited a vital role in bringing higher titre values. WB being a universal substrate provides sufficient nutrients and is able to remain loose even in moist conditions, thereby providing a large surface area for the growth of *A. niger* (Mahadik *et al*., 2002). The high lipid and protein content of COC (Ramachandran *et al*., 2004), made the fungus to yield more lipase in mixed substrate combinations. The addition of WR in TSF significantly augmented the biomass and lipase production by 24% and 35%, respectively, at 96 h, which were significantly higher than that of the biomass (15.48 mg/gds) and activity (459.1 U/gds) obtained in BSF using 7.5 g of WB and 2.5 g of COC.

Lipase production initially started by 24 h followed by an exponential curve up to a maximum of 459.1 ± 16 U/gds in BSF and 628.5 ± 13 U/gds in TSF, respectively, at 96 h. The increase in lipase activity was linearly proportional to that of the biomass concentration in BSF and TSF, indicating that the lipase production is a growth associated process. The glucosamine contents were 15.48 and 19.96 mg/gds at 96 h in BSF and TSF, respectively, and the increase in biomass could be due to the addition of WR in TSF, thereby increasing the lipase yield.

Extraction of lipase was inhibited by ammonium sulphate (1%) and this could be due to the increased hydrophobic interactions between lipase and solid support, thereby preventing the enzyme release as reported by Rodriguez *et al*. (2006). However, supplementation of sodium chloride with Triton X-100 (0.5%) helped in increasing the recovery of enzyme from 662.4 ± 16 to 745.7 ± 11 U/gds. This could be due to the use of surfactant, Triton X-100, which might increase the permeability of cells resulting in higher recovery of enzyme from TSF. Similar results were reported for the extraction of lipase from *A. niger* with sodium chloride (1%) and Triton X-100 (0.5%) (Mahadik *et al*., 2002), while Balaji and Ebenezer (2008) reported a maximum recovery of lipase from *Colletotrichum gloeosporioides* using Triton X-100 alone. Furthermore, the reported lipase activity (745.7 ±

11 U/gds) and productivity (7.76 U/g/h) obtained with our strain are comparatively higher than that of Mahadik *et al*. (2002), 4.37 U/g/h, ul-Haq *et al*. (2002), 2.16 U/g/h, Babu and Rao (2007), 0.05 U/g/h, Mahanta *et al*. (2008), 5.21 U/g/h and Godoy *et al*. (2009), 0.62 U/g/h.

The lipase activity was comparatively higher than the lipase activity of *Fusarium oxysporum*, which showed 5 U/ml of lipase activity after 72 h of fermentation in a medium containing 8% CSL (Quadros, *et al*., 2007), whereas a higher lipase activity (20 U/ml) was reported for *Geotrichum* sp. in a medium with 13% CSL (Burkert *et al*., 2004).

The direct application of fermented substrate on fleshing hydrolysis was evaluated, since the use of fleshing is declined due to the changing feeding habits of human beings and all the excess tallow produced are not used in soap industry (Bhatti *et al*., 2008). Hence, it is economical to consider fleshing, a low cost feedstock, in oleochemical industries for the production of fatty acids and their corresponding esters using enzymes, because they make the process energy efficient than the conventional thermal fat splitting process, which requires operations at elevated temperature and pressure (Edwinoliver *et al*., 2009; Bajaj *et al*., 2010).

6. CONCLUSIONS

By modification of various agro-products, a new BSF was developed, which could enhance the growth and production of lipase from *A. niger*. The lipase production has been scaled up from 10g to 40 kg level and the production of lipase from *A. niger* is a growth associated process. Having established that *A. niger* lipase could be effectively used as an additive in detergent formulation, it is of utmost relevance to produce the enzyme in an economically feasible medium. CSL, a cost-effective nutrition adjunct as well as an agro-industrial residue. Furthermore, the lipase productivity obtained with our strain was comparatively higher than the values already reported and an efficient extraction of the enzyme from BSF made this process economically viable for industrial applications. The direct use of the fermented substrate for fleshing hydrolysis potentially reduces the cost of biocatalysts with lipases, which will be of great importance in the production of relatively low-value products, such as fatty acid esters.

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