Thesis report

On

Fungal Lipase Production from Lab to Semi-pilot Scale by Solid State Fermentation

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Under the guidance of

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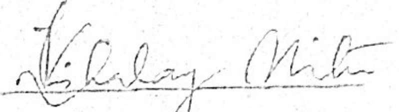

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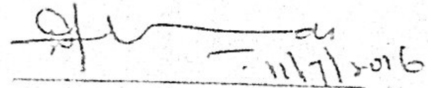

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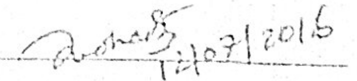

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Nomenclature

EC	Enzyme Commission
MTCC	Microbial Type Culture Collection
NCIM	National Collection of Industrial Microorganisms
SmF	Submerged Fermentation
SSF	Solid State Fermentation

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1. Abstract

Lipases (triacylglycerol acyl hydrolases (EC 3.1.1.3) which account for up to 10% of the enzyme market are widely used in different processes of industrial importance based on their ability to catalyze both synthetic and hydrolytic reactions. Some of the industrial applications that employed the use of lipases include biofuel production, detergent formulation, fine chemical synthesis, perfumery and cosmetics, leather processing, pharmaceuticals and medical diagnostics, food and feed processing. Lipases of microbial origin have greater industrial attraction because they are available in large quantities and can be produced with high yields. Moreover, advances in industrial biotechnology offer potential opportunities for economic utilization of agro industrial residues for the production of lipase.

In this study, Solid State Fermentation (SSF) is used for the production of lipase using a fungal strain, *Aspergillus Niger* (MTCC 872). A total of five substrate ratios, using three agricultural residues, rice husk, cotton seed cake and red gram were considered for experiments of lipase production by SSF. Out of these, 2:1:1 ratio of the respective substrates yielded maximum lipase activity of 27.2U/gds after 48h of fermentation. The optimum incubation, temperature, moisture content and pH for 5g of this substrate were found to be 48h at 40°C, 70% and 6.0 respectively. The lipase produced using these optimal process parameters when scaled up to 100g and semipilot 1kg level yielded 24.38U/gds and 21.62U/gds respectively. This is the first report on *Aspergillus Niger* MTCC 872 strain that can give maximum activity of 27.2U/gds, a potential strain for industrial application.

2. Objectives

I. To develop a novel tri-substrate using agricultural residues viz., rice husk, cotton seed cake and red gram for production of maximum lipase activity.
II. To study the effect of process parameters of temperature, moisture and pH on lipase activity in a flask level.
III. To study the scale up of lipase in large scale fermentation for substrate of 100g and 1000g.
IV. To compare the lipase activity by SSF from flask level to tray fermenter level.

3. Introduction

3.1 Fungal Lipases

Lipase belongs to the class of enzymes called hydrolases that catalyze the breakdown of fats i.e., it catalyses triglycerol to yield diglycerol and fatty acid anion.The mechanism is seen in figure 1. Lipase has a molecular formula of $C_{11}H_9N_3NaO_2$and a molecular weight of 238.197829 g/mol. There are two types of lipases: Acidic and Alkaline lipases. Among lipases from plant, animal and microbial sources, it is the microbial lipase which are used for commercial applications as they can be easily cultivated, relatively stable and their lipase can act as a catalyst for wide range of hydrolytic and synthetic reactions. Lipase is an enzyme that can be secreted by a wide range of micro-organisms. Fungi and bacteria may secrete lipase to facilitate nutrient absorption from

the external medium. The production of lipase requires carbon and nitrogen sources as required by any fermentation process. The effect of carbon and nitrogen source plays an important role in efficient production of lipase in microbes. The physical parameters like pH, temperature etc., also influences the production rate.

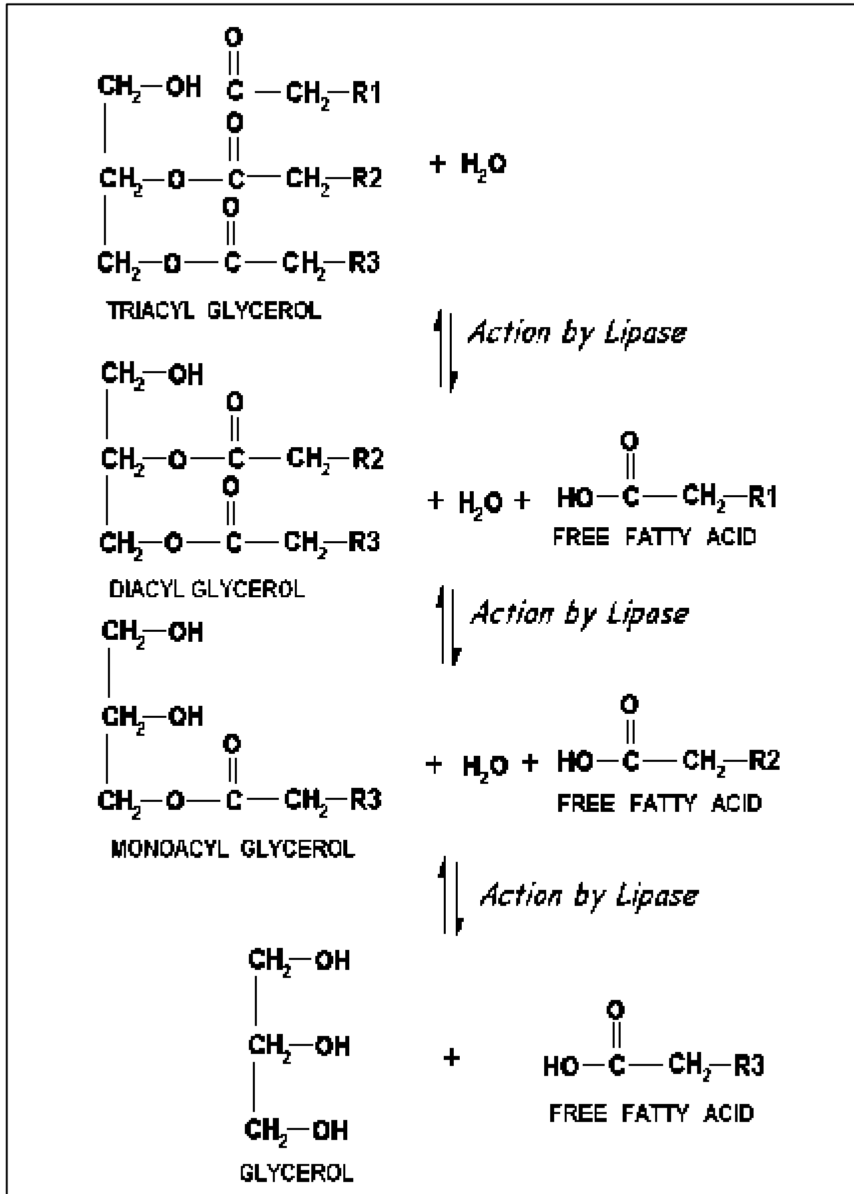

Fig 1: Action of lipase as catalyst in transesterification

Fungal lipases are mostly extracellular. Most commercially important lipase-producing fungi are recognized as belonging to the genera *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Geotrichum* sp., *Mucor* sp., and *Rhizomucor* sp. Lipase produced by different fungi and their optimum production conditions are mentioned in table 1. Lipase producing microorganisms have been found in different habitats such as industrial wastes, vegetable oil processing factories, dairy plants, and soil contaminated with oil and oilseeds among others. Recently, some works reporting the use of immobilized whole biomass of filamentous fungi have also been published.

The immobilization is advantageous since it can avoid biomass washout at high dilution rates. Also, high cell concentration in the reactor could be achieved and the separation of biomass from the medium is favored.

Various techniques have been used to determine the lipase enzyme activity. The commonly used techniques are turbidimetry, interfacial tensiometry, atomic force microscopy, infrared spectroscopy, titrimetry, colorimetry, flourimetry, chromatography, electron microscopy and immunodetection.

Lipases are one of the important groups of biocatalysts used in biotechnological applications. Lipases extracted from microorganisms are used in various industries such as dairy, food, detergents, and textile, pharmaceutical, cosmetic and biodiesel industries. It is also used for synthesis of fine chemicals, agrochemicals and new polymeric materials. Lipases are added to detergents such as household and industrial laundry and also in household dishwashers, where their function is removal of fatty residues and cleaning clogged drains.

Table 1: Comparison of lipase production by various fungi.

Microorganisms	**Solid Substrate**	**Inducers**	**Fermentation conditions(hours)**	**Lipase Activity Units**
Fusarium oxysporum	Wheat bran	Cetyl trimethylammonium bromide	96 h, pH-8.5, 40°C, high MC	111.48 U/ml
Aspergillus flavus	Wheat bran and Castor oil cake	-	96 h, pH-7.0, 30°C, MC 64% w/w	121.35 U/gds
Aspergillus niger	Rice bran	-	pH-6.87, 30.3°C	121.53 (U/g dss)
Colletotrichum gloeosporioides	Sunflower oil	Triton X-100	pH-6.5, 25°C	2560 U/g DM
Yarrowia lipolytica NCIM 3589	Palm Kernal cake	-	MC 70% v/w, 96 h	18.58 U/ gds
Aspergillus niger J-1	Olive oil and Glucose	-	pH-7.0, 30°C for 7 days	9.14 IU/gdss
Aspergillus niger MTCC 2594	Wheat bran, Coconut oil cake and Wheat rawa	Olive oil, Sesame oil and Sunflower oil	72h, 30°C, pH-7.0, MC 60% v/w	745.7 ± 11 U/gds
Rhizomucar rhizopodiformis	Sugar cane bagasse and Olive oil	-	20 h-24 h	79.60 U/gds

Aspergillus niger MTCC 2594	Gingelly oil cake	-	250 ml EF, 30°C,120h, pH-7.0, MC 60%	363.6 /gds
Penicillium restrictum	Babassu oil cake	Peptone, Oliveoil and Starch	EF, 30°C, 15-65 h,	30.3 U/g
Rhizopus oligosporous NCIM	Wheat bran and Olive oil	Nacl, Triton X-100	500ml EF, 45°C, pH-2.5, 24 h, MC 71.4%	630 IU/gds
Aspergillus.sps	\Wheat rawa	-	250 ml EF, 30°C, pH-7.0, 96 h	1934 U/g
Penicillium restrictum	ssu oil cake and Olive oil	-	Tray reactor, 37°C, 24 h, pH-7.0	5.8 U/ml
Rhizopus oryzae PTCC 5176	Bagasse and Urea	Olive oil and Canola oil	Tray-bioreactor, pH8.0, 35°C, MC80%	229.355 U/gds
Candida rugosa	Coconut oil cake	Urea, Peptone and Maltose	96 h	87.76 U/g ds
Candida rugosa DSM-2031 *Yeast Specie*	Coconut oil cake and Wheat bran Deoiled rice bran, Rice bran oil and Mineral salts	- -	28°C 96h, 30°C	48.61 U/ml 58 LU/gm dry bran
Aspergillu s Terreus	Mustard oil cake	-	30°C, 96 h, pH-6.0	1566.67 ± 133.33 U/ml
Aspergillus niger IIT 53A14	Wheat bran and Olive oil	- Soap stock	32°C, 48 h, pH 6.3-6.6 32°C, 48 h, pH 6.3-6.6	48.6 U/gds 62.5 U/gds
Aspergillus niger	Shea butter cake	Tween 20	30°C, 7 days, pH 7.0	3.35 U/g
Aspergillus. sp	Wheat bran	-	30°C, 96 h, pH 7.0	13.1 U/ml
Aspergillus niger	Soya bean and Rice husk	-	37°C, pH 7.7, 96 h	4.23 U/ml

3.2 Solid State Fermentation

SSF is defined as the fermentation involving solids in the absence (or near absence) of free water. However, the substrate must possess enough moisture to support growth and metabolism of microorganism. Therefore, it can be used either as support or as a carbon-energy source. Solid state fermentation (SSF) is an interesting process for lignocellulose-degrading enzymes

production at low cost, due to the possibility of using agricultural and agroindustrial residues as substrate for microbial growth. Filamentous fungi are considered to be the most adapted microorganisms for SSF, since their hyphae may grow on the particles surface and penetrate the intra-particle spaces, and thus colonize solid substrates. The selection of an appropriate substrate is a key factor for the success of SSF. Besides having the adequate composition to induce the desired product, the particles size should also be considered, since this is a factor that greatly influences SSF. Small particles offer more contact surface, allowing more access of the microorganism to the nutrients; however, depending on the type of substrate and on the moisture level, it can get compacted, impairing aeration and oxygen availability, as well as heat dissipation, limiting microbial growth. Big particles, on the other hand, facilitate aeration, however may hinder microbial access, limiting substrate contact surface and making heat transfer difficult.

Other parameters should also be evaluated and optimized for higher process efficiency, such as initial moisture and pH, incubation temperature, aeration, inoculum size, nutrient supplementation, extraction and purification of the final product. Also the differences between SSF and SmF are given in table 2.

Interestingly, many workers have reported exploitation of agricultural by-products in SSF for enzyme production and several of these reports deal on lipase production by SSF. Extraction of products from the fermented solids is one of the most critical unit operations in a SSF process and an efficient downstream processing is essential for the overall economics of the SSF process. Also the traditional static SSF has difficulties in mass and heat transfer. Scale up, purification of end products and biomass estimation are the major challenges that led the researchers to thrive hard to find the solutions. Scale up in SSF has been a limiting factor since long but recently with advent of biochemical engineering a number of bioreactors have been designed which could overcome the problems of scale up and to an extent also the on-line monitoring of several parameters, as well as heat and mass transfer.

Table 2: Basic Differences in Solid state Fermentation and Submerged Fermentation

Solid State Fermentation	Submerged Fermentation
Medium is not free-flowing	Medium free-flowing
Shallow depth	Greater
Single food substrate provides C, N_2, minerals and energy	Employed
Medium absorbs water, uptakes nutrients	Dissolved in water
Gradients of T, pH	Uniform
Minimum water, (less volume)	More water, more volume
3-phase system	2-phase system
T, O2, water control (water critical)	T, O2 control
Inoculums ratio large	Low
Intra particle resistances	No such resistances
Highly concentrated product	Low concentrated product

3.3 Design of Fermenter for SSF

Design of fermenter for SSF processes is an important aspect**.** There are different types of bioreactors for SSF used as fermenters. Most of the designs are based on two models: tray type or drum type with or without mixing devices and modifications. Koji process for soya sauce is considered as a representative of SSF processes. Attempts were also made to operate koji process in drum types of bioreactors. Some researchers proposed some modifications such as dividing the drum by baffles to achieve mixing and better product formation. Drum type of bioreactors have often been used for the bioprocesses where mixing of substrate during fermentation was recommended useful, which could be achieved by rotation. One disadvantage, in particular, with high rotation is damage of fungal mycelia. Tray type of bioreactors represent the simplest type of bioreactors that are used for SSF. The chamber consists of a large number of trays one above the other with a gap in between for aeration. The tray may be constructed using various materials such as wood, bamboo, metal or plastic. The top of the trays is typically opened in every category, the bottom and sides of the tray may be perforated for following aeration of the undersurface. The temperature of the fermented substrate is controlled by circulating warm or cool air as necessary; also the relative humidity can be controlled by passing saturated or dry air through the chamber.

The transfer of heat into or out of the SSF system is closely related with the aeration of fermentation system. The temperature of the substrate is also very critical in SSF as it ultimately affects the growth of the micro-organism, spore formation, germination, and product formation. High moistures results in decreased substrate porosity, which in turn prevents oxygen penetration. This may help to avoid bacterial contamination. On the other hand, low moisture content may lead to poor accessibility of nutrients resulting in poor microbial growth. Aeration is achieved in the reactors by blowing air through the substrate or flowing air around a static bed. Aeration rate depends on the rate of heat removal. Water transfer, air supply and heat removal are achieved simultaneously through proper aeration. In the same way, maintenance of uniformity within the substrate bed could be as effective as possible. The effect of the shear forces generated by mixing of both the substrate and the microorganism should be also considered. In this way, it has been observed that these forces can damage the penetrative hyphae and affect the gel substrate.

4. Literature Review

Lipases have come into prominence because of new and novel applications in oleo-chemistry, detergent formulation, organic synthesis and nutrition. Consequently, the sourcing of enzymes is also being expanded from the conventional animals and plants to microbial sources. The search for newer and rugged products is continuing to find materials having specific application potential. The development of technology for lipase production and synthesis of novel compounds using lipase-mediated reactions will result in their expansion into new areas and will have a major impact on a range of industries.

Lipases act on ester bonds. The usual industrial lipases are special classes of esterase enzymes that act on fats and oils, and hydrolyze them in steps into the substituted glycerides and fatty acids, and finally on total hydrolysis into glycerol and fatty acids. In nature, the available lipases

from various sources have considerable variations in their reaction specificities, this property being referred to the enzyme specificity. Thus from the fatty acid side, some lipases have an affinity for short chain fatty acids, some have a preference for unsaturated fatty acids.

Since these enzymes act at the oil-water interface, they can be used as catalysts for the preparation of industrially important compounds. As lipases act on ester bonds, they have been used in fat-splitting, transesterification, development of different flavors in cheese, improvement of pallatibility of beef fat for making dog food, etc. Lipases which are stable and alkaline at pH which are usually the suitable wash conditions for enzyme containing detergent powders and liquids, have also been found, and these hold potential for use in the detergent industry.

Many workers have exploited fungi as valuable sources of lipase due to their following properties: thermal stability, pH stability, substrate specificity and activity in organic solvents. Fungal lipases have benefits over bacterial ones due to the fact that present day technology favors the use of batch fermentation and low cost extraction methods. The chief producers of commercial lipase are *Aspergillus Niger, Candida cylindrecia, Rhizopus oryzae* etc.

It can be said that lipase producers are widespread in the fungal kingdom and are of much biotechnological interest in both research and applications.

4.1 Applications of Lipases

Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly due to versatility of their applied properties and ease of mass production.

Fat and Oil Industry:

Fats and oils are important constituents of foods. Lipases allow us to modify the properties of lipids by altering the location of fatty acid chains in the glyceride and replacing one or more of these with new ones. In this way, a relatively inexpensive and less desirable lipid can be modified to a higher value fat. Lipases catalyze the hydrolysis, esterification and inter-esterification of oils and fats. Among the lipolytic conversion of oils and fats, esterification and interesterification are used to obtain value added products, such as specialty fats and partial glycerides by using positional and fatty acid specific lipases, and have greater industrial potential than fatty acid production in bulk through hydrolysis.

Bakery Industry:

In baking industry, there is an increasing focus on lipolytic enzymes. Recent findings suggest that (phospho) lipases can be used to substitute or supplement traditional emulsifiers since the enzymes degrade polar wheat lipids to produce emulsifying lipids in situ. Lipase was primarily used to enhance the flavor content of bakery products by liberating short-chain fatty acids through esterification. Along with flavor enhancement, it also prolonged the shelf-life of most of the bakery products. Texture and softness could be improved by lipase catalyzation.

Lipases as Biosensors for Food Industry:

Immobilized lipases are fast, efficient, accurate and cost effective as sensors for the quantitative determination of triacylglycerol. This application is important in the food industry, especially in fats and oils, beverages, soft drinks, pharmaceutical industries and also in clinical diagnosis. The basic concept of using lipase as biosensors is to generate glycerol from the triacylglycerol in the analytical sample and to quantify the released glycerol by a chemical or enzymatic method.

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Lipases for Pharmaceutical Application:

Profens, a class of non-steroidal anti-inflammatory drugs, are active in the(s)-enantiomer form. Lee et al (1995) and Xieet al (1988) synthesized pure (s)-ibuprofen using lipase catalyzed kinetic resolution via hydrolysis and esterification, respectively. In addition to racemization in situ, lipases are also capable of catalyzing synthetic reactions, which has led to the production of life saving drugs. Efficient kinetic resolution processes are available for the preparation of optically active homochiral intermediates for the synthesis of nikkomycin-B, nonsteroid anti-inflammatory drugs (naproxen, ibuprofen, suprofen and ketoprox), the potential anti-viral agent lamivudine, and for the enantiospecific synthesis of alkaloids, antibiotics, vitamins, and anti- arteriosclerotic, anti-tumor and anti-allergic compound.

Lipases in pulp and paper industry

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

Table 3: Uses of lipases

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

Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality
Cleaning	Hydrolysis	Removal of fats

4.2 Lipase production conditions

Most of the microbial lipases are extracellular, being extracted through the cell membrane into the culture medium. The amount of lipase produced is dependent on several environmental factors, such as temperature, pH, carbon and nitrogen sources, agitation and dissolved oxygen concentration. Certain inducers have a profound effect on the stimulation of lipase production. These include: triglycerides, free fatty acids, bile salts and glycerol.

Agro industrial wastes are valuable sources of lignocellulosic materials. The lignocellulose is the main structural constituent of plants and represents the primary source of renewable organic matter on earth. It can be found at the cellular wall, and is composed of cellulose, hemicellulose and lignin, plus organic acids, salts and minerals. Therefore, such residues are superior substrates for the growth of filamentous fungi, which produce cellulolytic, hemicellulolytic and ligninolytic enzymes by solid state fermentation (SSF). These fungi are considered the better adapted organisms for SSF, since their hyphae can grow on the surface of particles and are also able to penetrate through the inter particle spaces, and then, to colonize it. Filamentous fungi are the most distinguished producers of enzymes involved in the degradation of lignocellulosic material, and the search for new strains displaying high potential of enzyme production is of great biological importance.

Ashok Pandey reported that cassava can serve as an ideal substrate for microbial processes for the production of value added products. This is because cassava bagasse is a fibrous material and it contains 30-50% starch on dry weight basis. It is also having rich organic nature and low ash content. Attempts have been made to produce several products such as organic acids, flavor and aroma compounds, and mushrooms from cassava bagasse.

Vijay Gunasekaran reported the lipase production by *A.Niger* using wheat bran as substrate. The enzyme is characterized with regard to thermostability, pH stability, optimum temperature and pH for enzymatic reaction. Effects of oils such as olive oil, mustard, sesame, castor, sunflower and coconut oils in the growth medium have been studied. Several researchers have already reported the use of immobilized whole cells system for lipase production. Batch and repeated batch experiments have been carried out for lipase production using immobilized *C.Rugosa* cells in expanded bed reactors, having various gel supports with enriched medium. *R.Arrizhus* cells have been immobilized on polyurethane foams and effect of parameters like glucose concentration, pH, inoculums size and agitator speed has been studied on lipase production.

There were reports of effect of soap stock acting as an inducer in lipase activity. Soapstock is composed of soap, neutral oil and water. The neutral oil is available for fungus growth and lipase production. The presence of cations (soap content and/or ash content of respectively 27.10% and 4.04%) is a beneficial factor for fungus growth and enzyme production. Lipase activity is dependent upon the type and not the amount of the lipid used as inducer. The high concentration of free fatty acids in the fatty acid residue seems to have inhibited lipase production. Stearin was

a slightly better inducer than olive oil, under the test conditions whereas soapstock was the best for lipase production. The effect of lipase activities using different inducers is given in table 3.

Table 4: Maximal lipase activities obtained in SSF using different substrates and inducers

Organism	Substrate	Inducer	Activity(U/gds)
Rhizomucor Pusillus	Sugarcane bagasse	-	4.99
R.rhizopodiformis	Sugarcane bagasse	-	2.67
Rhizomucor Pusillus	Sugarcane bagasse	Olive oil cake	20.24
R.rhizopodiformis	Sugarcane bagasse	Olive oil cake	79.6
Aspergillus Niger	Gingelly oil cake	-	363.6
Aspergillus Niger	Wheat bran	-	303.2
P.restrictum	Babassu oil cake	Olive oil	30.3
R. oligosporous	Almond meal	-	48

As reported by Bala, Plackett-Burman design was used to efficiently select important medium components affecting the lipase production by *Aspergillus niger* using shea butter cake as the main substrate. Out of the eleven medium components screened, six comprising of sucrose, $(NH4)_2SO_4$, Na_2HPO_4, $MgSO_4$, Tween-80, and olive oil were found to contribute positively to the overall lipase production with a maximum production of 3.35U/g. Influence of tween-80 on lipase production was investigated, and1.0%(v/w) of tween-80 resulted in maximum lipase production of 6.10U/g.

A report on the lipase production by *Aspergillus Niger* and *Pencillium* species suggests sugarcane bagasse as a good co-substrate along with wheat bran and soy bran. Strains studied achieved maximum lipase activities with 25% sugarcane bagasse combined with 75% wheat bran or soyabran at 40% moisture content. The highest lipase activities were observed for WB and SB, and for SB combined with CB using *Aspergillus* sp. Fermentation of 96 h was the optimum period for enzyme production.

For an extracellular lipase of *Pe. citrinum*, Sztajer and Maliszewska obtained maximal production in a medium that contained 5% (w/v) peptone (pH 7.2). Nitrogen sources such as corn steep liquor and soybean meal stimulated lipase production but to a lesser extent than peptone. Urea and ammonium sulfate inhibited lipase synthesis. Lipolytic activity (1120 U/L) was determined by titration of the free fatty acids released from olive oil incubated with the cell-free broth.

Aspergillus oryzae produced maximal alkaline lipase in a medium that contained yeast extract (1%), polypeptone (2%), and soybean meal (3%) as nitrogen sources. The enzyme produced had an activity optimum at pH 7.5 and 10.0, respectively, with olive oil and tributyrin as substrates. A Brazilian strain of *Pencillium citrinum* produced a maximal lipase activity of 409 IU/mL in a medium that contained yeast extract (0.5%) as the nitrogen source. A decrease in yeast extract concentration reduced the attainable lipase activity. Replacement of yeast extract with ammonium sulfate diminished lipase production.

Pokorny et al reported that lipase production by *A. niger* was enhanced in the presence of Mg^{2+}. Maximal lipase production by *P. pseudoalcaligenes* KKA-5 occurred at Mg^{2+} concentration of 0.8 M. Exclusion of the magnesium ions from the medium caused approximately 50% reduction in lipase production , but supplementing the medium with calcium ions did not affect lipase production.

There are few commercial lipases mainly from fungi and bacteria and are used in various applications.

Table 5: Commercially available lipases, their sources and industrial applications.

Type	Source	Application	Marketing Company
Fungal Lipases	*Candida Rugosa*	Organic Synthesis	Amano(Japan), Biocatalysts(UK), Genzyme, Sigma
	Candida Antarctica A/B	Organic Synthesis	Novonordisk, Boehringer Mannheim
	Thermomycis Lanuginosus	Detergent additive	Novonordisk, Boehringer Mannheim
	Rhizomucor Miehei	Food processing	Novonordisk, Biocatalysts, Amano
Bacterial Lipases	*Burkholderia cepacia*	Organic synthesis	Amano, Fluka, Boehringer Mannheim
	Pseudomonas Alkaligenes	Detergent additive	Genencor International (USA)
	P. Mendocina	Detergent additive	Genencor International (USA)
	Chromobacterium Viscosum	Organic synthesis	Asahi, Toyo Jozo(Japan)

4.3 Lipase Assays

Various techniques have been used to determine the lipase enzyme activity. The commonly used techniques are turbidimetry, interfacial tensiometry, atomic force microscopy, infrared spectroscopy, titrimetry, colorimetry, flourimetry, chromatography, electron microscopy and immunodetection.

Table 6: Different techniques for lipase assays

Assay	Substrate used	Product analyzed	Principle	Remarks
Turbidimetry assay	Tween20 in the presence of CaCl2	Released fatty acids	Optical density increase at 500 nm due to precipitation in the form of calcium salts	Simple and quantitative but Tweens are not specific substrates for lipases
Interfacial tensiometry	Lipid monolayer spread on surface of aqueous phase	Fatty acids	Monitoring of surface pressure change due to dissolution of lipids using electro microbalance and Teflon barrier	Highly sensitive reliable measurements,
	Oil water interface	Fatty acids	Tensiometers for oil drop method	Low amounts of lipids used, but requires very sophisticated equipments
Atomic force microscopy	Lipid bilayer supported on Mica	Fatty acids	Lipid dissolution forms holes in the bilayer an the increase in area of holes with time is monitored using real time images	Nano scale assay and hence requires very sophisticated instruments
Infrared spectroscopy	Triacyl glycerols (TAG)	Free fatty acids and Fatty acid esters	In the Fourier transform IR spectrum Fatty acid esters peak at 1751 cm-1 and FFAs at 1715 cm-1 and hence can be quantitated on the basis of molar extinction coefficients	Expensive and sophisticated equipments required
Titrimetry	Stirred emulsion of TAG, tributyrin, Olive oil emulsified with gum Arabic	Fatty acids	pH stat method – Neutralization of released FFAs using titrated NaOH	Most common procedure, sensitive to within 1 μmol fatty acid released per min, disadvantage if FFAs are not fully ionized

Spectrophotometry (Colorimetry)	Lipid at lipid water interface Olive oil emulsion Olive oil emulsion in presence of copper Reagent	Safranin Free fatty acids converted to copper soaps. Rhodamine G-FFA complex	Absorbance change of Safranin due to change in net negative charge at the lipid water interface Formation of a copper soap. The copper complex is estimated spectrophotometrically at 440 nm The complex develops a pink color. Absorbance read at 513 nm	Lipase activities as low as 50 mU can be detected Sensitivity and efficacy improved for specific purposes by many researchers Reproducibility is difficult
	Para-nitrophenly esters	Para nitro phenol	Yellow coloured product which is measured at 410 nm	Convenient and quick method, used commonly. These esters are liable to spontaneous hydrolysis and also by non-specific estetrases
Flourimetry	TAG with alkyl groups substituted with fluorescent group (Pyrenic acylglycerol derivatives)	Free fatty acids	Shift in fluorescence wavelength after hydrolysis	Rapid assay but expensive substrate, Chemically modified Tag is poorly hydrolyzed
	Triaclyglycerol in the presence of fatty acid binding protein conjugated to an acrylodan fluorophore	Free fatty acids	Fluorescence emission wavelength changes from 432 nm to 505 nm upon binding	Detection of concentration as low as 1 nM. Kit commercially available
	Phosphatidylch online containing naturally fluorescent parinaric acid	Parinaric acid	Detection of parinaric acid. Excitation and emission wavelengths of parinaric acid -324 nm and 420 nm	Low quantities can be detected
Chromatography	Lipids, TAGs	Fatty acids	Thin layer	Detection of as

			chromatography and quantititative analysis of FFAs by densitometric or auto radiographic methods when TAGs are labeled	small as a few pmoles of fatty acids. Time consuming and not continuous
Electron microscopy	Lipids	Fatty acids	Electron microscopy detection of fatty acids	----------------
Immunodetection	-----------------	Lipase	ELISA using monoclonal antibodies specific to antigens on Lipase	Detection of both active and inactive form

5. Materials and Methods

5.1 Lipase production by Solid State Fermentation

There are several recent publications describing the solid state fermentation of agro-industrial residues such as rice bran, rice husk, potato wastes, cassava husk, wheat bran, sugar cane bagasse, sugar beet pulp, palm kernel cake, rice straw, cocoa pod, fruit wastes etc. into bulk chemicals and value added fine products such as ethanol, enzymes, antibiotics, biofuel, mushrooms, organic acids, amino acids, biologically active secondary metabolites. In principle SSF refers to the microbial growth on moist solid substrates or within the pores without free flow of water. The required moisture for SSF exists in the solid as absorbed or complex form is more helpful for oxygen availability to the microbial population. In SSF the microbe is in contact with atmospheric oxygen unlike in submerged fermentation (SmF). SSF is simpler and requires less processing energy.

SSF Process Methodology

In any SSF process the basic steps carried out are:
1. The preparation of a solid substrate .
2. Sterilization of the substrate.
3. Rising of suitable inoculum. Traditional or pure culture technique.
4. The inoculation of the moist substrate
5. The incubation in appropriate culture of vessels or reactors.
6. Maintenance of optimal conditions. (pH, T, Hm, mixing, aeration, flow pattern, Q, NA)
7. Harvesting of solids
8. Drying / Extraction of product
9. Further downstream processing if necessary.

5.2 Developing a novel substrate

Experiments were done to produce lipase by SSF. The different types of substrates tried were rice husk, wheat bran, cotton seed cake and red gram husk. Different mixtures of substrates rice husk, cotton seed cake and red gram husk were prepared by taking different proportions of them. Using these substrates for SSF, the lipase activity in each case is determined and the maximal lipase activity obtained is noted of all these. That particular substrate which gave maximal lipase

activity is considered as a novel substrate. The ratios of rice husk, cotton seed cake and red gram taken are 2:2:1, 1:2:1, 2:1:1, 1:2:2, 1:1:1.

Preparation of SSF medium

To 100mL of distilled water, the following are added in g/gdss

1. Glucose-3%
2. Olive oil-3%
3. NaH_2PO_4-1.8%
4. KH_2PO_4-0.3%
5. $MgSO_4.7H_2O$-0.045%
6. $CaCl_2$-0.0375%
7. Yeast Extract-1.5%

Methodology

5g of substrate is taken in a 250 mL Erlenmeyer flask. After adding saline medium such that the moisture content is 60%, the contents are stirred properly. The inoculum is added by taking a loop of the fungal strain *Aspergillus Niger* (MTCC 872). Before adding the inoculums, both the medium and substrate are autoclaved or sterilized in an autoclave at 121° C for 15 min. The flask is incubated at a temperature of 30° C for 96h.

Sample Collection

After every 24h of fermentation, the sample from the flask is collected and is processed for enzyme extraction and assay.

Enzyme Extraction

The sample collected is taken in a mortar. To this, a 10mL of solution containing 1% (w/v) NaCl and 1% triton(w/v) X-100 is added and the mixture is ground with a pistel. The resulting solution is then filtered and centrifuged at 10000 rpm and the supernatant obtained is used as an enzyme source.

Lipase Assay

Enzyme assays are performed to serve two different purposes: (i) to identify a special enzyme, to prove its presence or absence in a distinct specimen, like an organism or a tissue and (ii) to determine the amount of the enzyme in the sample. While for the first, the qualitative approach, a clear positive or negative result is sufficient, the second, the quantitative approach must deliver data as exact as possible. During the enzyme reaction product accumulates in amounts exceeding by far the intrinsic enzyme concentration. However, the conclusion from the product formed back to the amount of enzyme in the sample comprises various difficulties and pitfalls.

Basically, lipases act on triglycerides and the released products are fatty acids. These acids are titrated with a known base and using the burette reading, the lipase activity is calculated.

Materials Required

(1) 2% (w/v) Polyvinyl alcohol (PVA): 2g of PVA was added to 100ml of distilled water and dissolved by boiling in a waterbath with continuous stirring.
(2) Commercial grade olive oil
(3) Preparation of substrate emulsion: 75ml of 25 (w/v) PVA was sonicated with 25ml of olive oil
(4) 0.1 M potassium phosphate buffer, pH 7.0
(5) Acetone-alcohol mixture: 1 vol of acetone mixed with 1 vol of 95% ethanol.
(6) Standardized NaOH : 0.05N
(7) Indicator : 0.1g of phenolpthalein dissolved in 100ml of 95% ethanol.

Methodology

5ml of olive oil emulsion substrate is taken in an Erlenmeyer flask. To that, 20ml of 0.1M potassium phosphate buffer and 1ml of the cultural enzyme is added. The contents are well stirred and the flask is incubated in a rotary shaker at a temperature of 30°C at 130 rpm for 30 minutes of time. After that, the reaction is quenched with a 15ml acetone-ethanol mixture each containing in the ratio 1:1. The fatty acids are released in the flask after this and then it is titrated against NaOH of 0.05N by adding a phenolphthalein indicator. The solution turns to pale pink. The burette reading is noted. Similarly, a blank assay is done by adding the enzyme after adding acetone-ethanol mixture and it is used as a control.

Lipase Activity Calculation

Lipase Activity (U/l) = $(A*c*10^{6})/V$
A = mL of NaOH per minute (burette reading)
U= µmoles of fatty acids released per minute
C= concentration of NaOH in mmoles/lit
V=sample volume in µl
10^{6} converts volume sample to liters

One lipase unit is defined as the amount of enzyme that liberated 1 µmol fatty acid per minute under the assay conditions described.

5.3 Optimization of process parameters

After determining the novel substrate**,** time course studies of lipase activity is studied. By this, optimum time for fermentation is determined. Then the remaining parameters such as temperature, moisture content and pH are also optimized. The temperature is optimized by

carrying out SSF at different temperatures viz., 30°C, 35°C, 40°C and 45°C. Similarly the moisture content is optimized by varying the volume of saline medium in different percentages 55, 60,65,70,75. The optimum pH is determined by using phosphate buffers of different pH, used during lipase assay. The phosphate buffers used were of pH 6, 6.5, 7, 7.5.

5.4 Scale up of lipase production by SSF

Scale-up has been defined technically in a variety of ways by different workers. Scale-up is not just a one-way procedure involving smaller to larger size systems but also includes the reverse, commonly referred to as scale-down. The latter is also valuable for obtaining additional information in less-expensive ways of carrying out procedures and is characterized by simplicity and efficiency. Scale-up is therefore the crucial link in transferring a laboratory scale process to commercial production scale. It also provides a large quantity of the product which might be required for product evaluation and toxicological studies. Unsuccessful scale-up results in wasted time spent on cost-intensive laboratory scale work and also forces the withdrawal of prospects thought earlier to be potentially profitable. The stages of scale-up are (a) Flask level (b) Laboratory fermenter level (c) Pilot fermenter level (d) Production fermenter level.

There are few problems associated with scale-up:

1. Variations in the biomass formed
2. Large scale inoculum development
3. Medium sterilization
4. Aeration
5. Agitation
6. Heat removal
7. Moisture content of the solids
8. Contamination control
9. pH control
10. Heterogeneity
11. Downstream processing
12. Waste management

Using the novel tri-substrate and the optimized parameters: time, moisture content, temperature, pH the lipase production by SSF is carried out at a level of 100g and 1000g. 100g scale up is done in a 1 litre Erlenmeyer flask whereas scale up to 1 kg is done in a tray fermenter and the lipase activities are determined. Thereby effect of scale up by SSF is studied.

5.4.1 Scale-up to 100g capacity

Methodology

The novel tri-substrate of quantity 100g which contains rice husk, cotton seed cake and red gram pulse in the ratio 2:1:1 i.e., 50g rice husk, 25g cotton seed cake and 25g of red gram pulse are taken in a 1 liter Erlenmeyer flask. The contents are sterilized in an autoclave at 121°C for 15 minutes. Inoculum is then added to the flask and incubated at optimum temperature i.e., at 40° C for 48hrs. The lipase activity is then determined.

5.4.2 Scale-up in a tray fermenter

Methodology

A steel tray of dimensions length: 45.2cm, breadth: 42.7cm and height: 2.2cm is taken. In that 1000g of sterilized tri-substrate, containing rice husk, cotton seed cake and red gram pulse in the ratio 2:1:1 is taken. The inoculum is then added and the tray is kept in an incubator at a temperature of 40°C for 48hrs. After fermentation, a sample is collected for determining lipase activity.

6. Results and Discussions

6.1 Effect of substrates for maximum lipase production

Experiments were done to determine the optimal lipase activity by using different ratios of the components of substrate mixture. The maximum lipase activity is obtained for the substrate ratio 2:1:1 after 24h of fermentation. All substrate ratios except 1:2:2 showed maximum activity only after 24 h of fermentation. Substrate containing wheat bran alone showed an enzyme activity of 4.17U/ml while substrate of 2:1:1 proportion showed an enzyme activity of 6.74U/ml. After determining the optimum substrate proportion, experiments on optimization of physico-chemical parameters is carried out. The lipase activities for different ratios of substrates are given in table below.

Table 7: Effect of substrate composition on lipase activity

Substrate ratio (R:C:P)	Maximum Lipase Activity (U/gdss)			
	24h	48h	72h	96h
2:2:1	5.19	2.11	1.60	4.23
1:2:1	4.90	1.93	1.90	1.79
2:1:1	6.74	1.95	1.69	2.33
1:2:2	2.16	1.77	1.71	4.37
1:1:1	3.11	1.86	1.77	1.54

R: Rice husk, C: Cotton seed cake, P: Red gram pulse

6.2 Effect of parameters for maximum lipase production

6.2.1 Effect of Temperature

Experiments were done to optimize the temperature at which the novel tri-substrate gives maximum lipase activity. Four different temperatures were maintained by keeping the flasks in incubator. The maximum lipase activity is obtained at a temperature of 40°C, after 48h of fermentation and the value is 12.08 U/gds. The results are illustrated by table 8 and figure 2.

Table 8: Effect of Temperature on lipase activity

Temperature (^{o} C)	Maximum Lipase activity (U/gds)			
	24h	48h	72h	96h
30	11.47	11.33	11.33	8.44
35	8.46	9.21	8.32	8.97
40	11.75	12.08	10.41	7.63
45	10.23	10.74	9.17	8.74

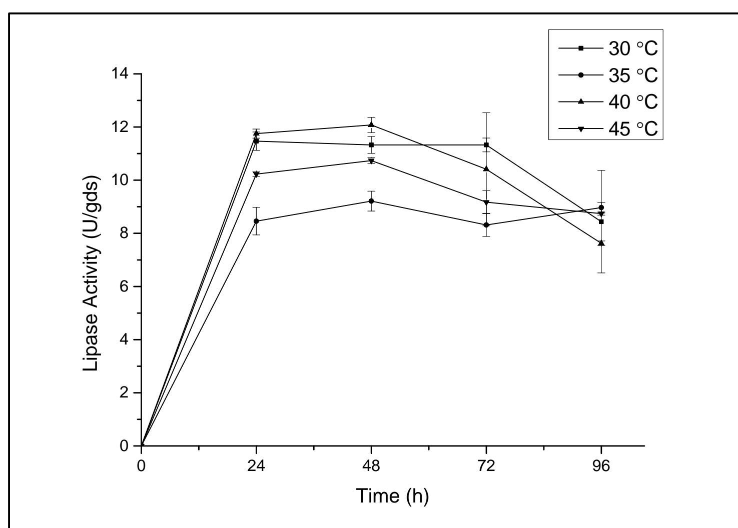

Fig 2: Effect of different temperatures (30°C-45°C) for maximum lipase activity

6.2.2 Effect of Moisture

By varying the volume of saline medium, different percentages of moisture contents were attained. For the different moisture contents used, maximal lipase activity was determined in a time interval of 24h for duration of 96h fermentation. After 48 h of fermentation at 40°C, the maximum lipase activity is found for the moisture content of 70 %(w/v). After doing this experiment, optimum time, temperature and moisture content are obtained. The results are illustrated by table 9 and figure 3.

Table 9: Effect of moisture on lipase activity

Moisture Content (%)	Maximum Lipase activity (U/gds)			
	24h	48h	72h	96h
55	9.65	9.32	9.73	8.88
60	10.94	11.43	11.00	11.45
65	16.62	16.59	15.94	12.55
70	22.76	25.75	17.72	16.02
75	28.19	25.52	18.83	17.54

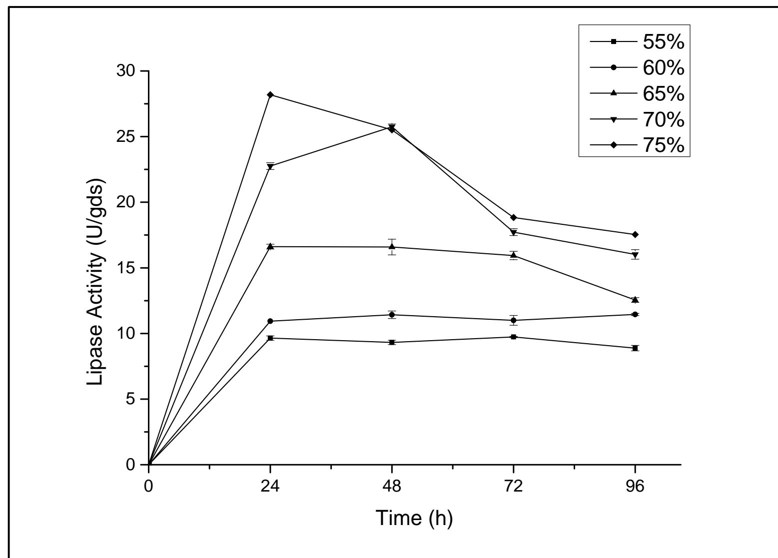

Fig 3: Effect of different percentages of moisture (55-75) for maximum lipase activity

6.2.3 Effect of pH

It can be noticed that the lipase exhibits maximum activity when assayed with buffer pH 6.0. This shows that the lipase is acidic in nature. The maximum activity obtained is 25.12U/gds.The potassium buffers of different pH i.e., in the range 6 to 8 are taken. The results are illustrated by table 10 and figure 4.

Table 10: Effect of pH on lipase activity

pH	Maximum Lipase activity (U/gds)			
	24h	48h	72h	96h
6.0	23.5	25.12	21.98	21.02
6.5	17.72	18.61	17.58	17.32
7.0	12.47	12.84	12.32	11.62
7.5	6.25	6.59	6.33	6.14
8.0	3.44	3.66	3.40	3.22

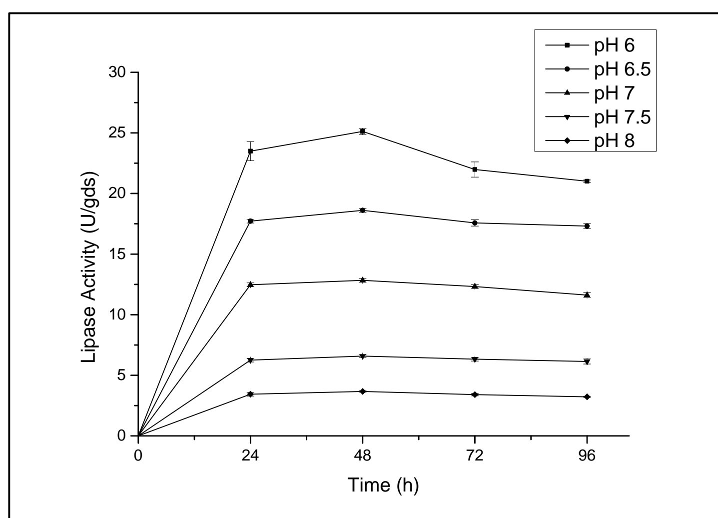

Fig 4: Effect of different pH (6-8) for maximum lipase activity

6.3 Effect of scale up in maximum lipase production to 100g

By taking the optimized ratio of tri-substrate i.e., 2:1:1 (rice husk: cotton seed cake: red gram) of 100g in an Erlenmeyer flask and maintaining the optimized parameters, the lipase activity is determined after 48h of fermentation. The lipase activity is found to be 24.38U/gds which is less than the activity found using 5g of substrate 27.2U/gds. This shows that during scale up the activity has decreased due to the problems associated with scale up.

6.4 Effect of Scale up in maximum lipase production to 1kg in a semi-pilot level fermenter

This experiment was carried out in a tray in static condition in which 1 kg of substrate is taken. The substrate is inoculated and incubated at optimum temperature. Also optimum moisture content and pH are maintained. After 48h of fermentation, the lipase activity is found to be 21.62U/gds. The lipase activity declined when scaled up to 1 kg. It may be because of the problems like aeration and agitation when scaled up in a tray.

Table 11: Lipase activity for different amount of tri-substrates-scale up

Substrate (g)	Lipase Activity(U/gds) at 48h
5	27.2
100	24.38
1000	21.62

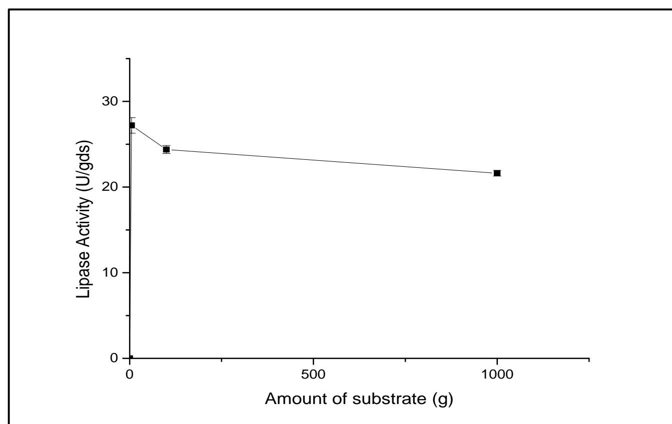

Fig 5: Effect of Lipase activity for different amount of tri-substrates-scale-up.

7. Conclusions

The lipase produced by SSF from the fungal strain *Aspergillus Niger* (MTCC 872) is acidic in nature because the optimum pH found is 6.0. The lipase production supports Solid State Fermentation as the optimum moisture content is 70%. After 48h of fermentation at a temperature of 40° C, the lipase activity is found to be maximum for 5g of substrate. But as the lipase production has been scaled up from flask level to tray level, the lipase activity has been declined.

8. Future Perspectives

In future, downstreaming processes for purification of lipases and estimation of protein should be done. There can be studies to be done on effect of salts, surfactants and metal ions for maximum lipase activity. Optimization of process parameters for production of lipase by SSF in semi-pilot

scale have to be done. Flask experiments for biomass estimation by glucosamine method and to perform biomass studies from semi pilot plant can be done.

9. References

1. Arzoglou, P., Goudoula, C., Tsantili, P., Lessinger, J. M., Ferard, G., Samyn, N., & Avranas, A. (1994). Transferability of lipase titrimetric assays: deductions from an interlaboratory study. *Clinical Chemistry and Laboratory Medicine*, *32*(10), 773-778.
2. Castilho, L. R., Polato, C. M., Baruque, E. A., Sant'Anna, G. L., & Freire, D. M. (2000). Economic analysis of lipase production by *Penicillium restrictum* in solid-state and submerged fermentations. *Biochemical Engineering Journal*, *4*(3), 239-247
3. Chen, H. Z., & He, Q. (2012). Value-added bioconversion of biomass by solid-state fermentation. *Journal of Chemical Technology and Biotechnology*, *87*(12), 1619-1625.
4. Colla, L. M., Ficanha, A. M., Rizzardi, J., Bertolin, T. E., Reinehr, C. O., & Costa, J. A. V. (2015). Production and characterization of lipases by two new isolates of *Aspergillus* through solid-state and submerged fermentation. *Biomed research international*, *2015*.
5. Cordova, J., Nemmaoui, M., Ismaıli-Alaoui, M., Morin, A., Roussos, S., Raimbault, M., & Benjilali, B. (1998). Lipase production by solid state fermentation of olive cake and sugar cane bagasse. *Journal of Molecular Catalysis B: Enzymatic*, *5*(1), 75-78.
6. Damaso, M. C. T., Passianoto, M. A., Freitas, S. C. D., Freire, D. M. G., Lago, R. C. A., & Couri, S. (2008). Utilization of agroindustrial residues for lipase production by solid-state fermentation. *Brazilian Journal of Microbiology*, *39*(4), 676-681.
7. Dos Santos, R. R., Muruci, L. N. M., Damaso, M. C. T., Da Silva, J. P. L., & Santos, L. O. (2014). Lipase Production by *Aspergillus niger* 11T53A14 in Wheat Bran Using Experimental Design Methodology. *Journal of Food and Nutrition Research*, *2*(10), 659-663.
8. Dos Santos, R. R., Muruci, L. N. M., Santos, L. O., Antoniassi, R., Da Silva, J. P. L., & Damaso, M. C. T. (2014). Characterization of different oil soapstocks and their application in the lipase production by *Aspergillus niger* under solid state fermentation. *Journal of Food and Nutrition Research*, *2*(9), 561-566.
9. Edwinoliver, N. G., Thirunavukarasu, K., Naidu, R. B., Gowthaman, M. K., Kambe, T. N., & Kamini, N. R. (2010). Scale up of a novel tri-substrate fermentation for enhanced production of *Aspergillus niger* lipase for tallow hydrolysis. *Bioresource Technology*, *101*(17), 6791-6796.
10. Falony, G., Armas, J. C., Mendoza, J. C. D., & Hernández, J. L. M. (2006). Production of extracellular lipase from *Aspergillus niger* by solid-state fermentation. *Food Technology and Biotechnology*, *44*(2), 235-240.
11. Fleuri, L. F., de Oliveira, M. C., Arcuri, M. D. L. C., Capoville, B. L., Pereira, M. S., Delgado, C. H. O., & Novelli, P. K. (2014). Production of fungal lipases using wheat bran and soybean bran and incorporation of sugarcane bagasse as a co-substrate in solid-state fermentation. *Food science and biotechnology*, *23*(4), 1199-1205.
12. Ghosh, P. K., Saxena, R. K., Gupta, R., Yadav, R. P., & Davidson, S. (1996). Microbial lipases: production and applications. *Science Progress (1933-)*, 119-157.
13. Gunasekaran, V., & Das, D. (2005). Lipase fermentation: progress and prospects. *Indian journal of Biotechnology*, *4*(4), 437-445.

14. Hasan, S., Ahmad, A., Purwar, A., Khan, N., Kundan, R., & Gupta, G. (2013). Production of extracellular enzymes in the entomopathogenic fungus *Verticillium lecanii. Bioinformation*, *9*(5), 238-242.
15. Hosseinpour, M. N., Najafpour, G. D., Younesi, H., Khorrami, M., & Vaseghi, Z. (2012). Lipase production in solid state fermentation using *Aspergillus niger*: Response surface methodology. *International Journal of Engineering*,*25*(3), 151-159.
16. JSMP, T. Mathematical Modeling of Macroscale Phenomena: Oxygen Transfer for Solid-state Fermentation in Static Tray Bioreactor.
17. Kotogán, A., Németh, B., Vágvölgyi, C., Papp, T., & Takó, M. (2014). Screening for extracellular lipase enzymes with transesterification capacity in *Mucoromycotina* strains. *Food Technology and Biotechnology*, *52*(1), 73-82.
18. Kumar, D. S., & Ray, S. (2015). Fungal lipase production by solid state fermentation-an overview. *Journal of Analytical & Bioanalytical Techniques*,*2015*.
19. Lonsane, B. K., Saucedo-Castaneda, G., Raimbault, M., Roussos, S., Viniegra-Gonzalez, G., Ghildyal, N. P., & Krishnaiah, M. M. (1992). Scale-up strategies for solid state fermentation systems. *Process Biochemistry*,*27*(5), 259-273.
20. Mahadik, N. D., Puntambekar, U. S., Bastawde, K. B., Khire, J. M., & Gokhale, D. V. (2002). Production of acidic lipase by *Aspergillus niger* in solid state fermentation. *Process Biochemistry*, *38*(5), 715-721.
21. Martins, D. A. B., Gomes, E., Do Prado, H. F. A., Ferreira, H., De Souza Moretti, M. M., da Silva, R., & Leite, R. S. R. (2011). *Agroindustrial wastes as substrates for microbial enzymes production and source of sugar for bioethanol production*. INTECH Open Access Publisher.
22. Mohanasrinivasan, V., Dhrisya, P., Dipinsha, K. P., Unnithan, C. M., Viswanath, K. M., & Devi, C. S. (2009). A comparative study of the lipase yield by solid state and submerged fermentations using fungal species from biopharmaceutical oil waste. *African Journal of Biotechnology*, *8*(1).
23. Pandey, A., Soccol, C. R., Nigam, P., Soccol, V. T., Vandenberghe, L. P., & Mohan, R. (2000). Biotechnological potential of agro-industrial residues. II: cassava bagasse. *Bioresource Technology*, *74*(1), 81-87.
24. Prabhakar, A., Krishnaiah, K., Janaun, J., & Bono, A. (2005). An overview of engineering aspects of solid state fermentation. *Malaysian Journal of Microbiology*, *1*(2), 10-16.
25. Prabhakar, T., Bhogavalli, P. K., Vallem, P. R., & Venkateswar, S. (2012). Studies on optimization of extracellular lipase from potential fungal strain (s) isolated from oil contaminated soil. *J. Microbiol. Biotech. Res*, *2*(3), 418-425.
26. Prabhakar, T., Kumar, K. A., & Ellaiah, P. (2002). The effect of cultural conditions on the production of lipase by fungi. *Journal of Scientific and Industrial Research*, *61*(2), 123-127.
27. Raimbault, M. (1998). General and microbiological aspects of solid substrate fermentation. *Electronic Journal of Biotechnology*, *1*(3), 26-27.
28. Ray, A. (2012). Application of lipase in industry. *Asian Journal of Pharmacy and Technology*, *2*(2), 33-37.
29. Rodriguez, J. A., Mateos, J. C., Nungaray, J., González, V., Bhagnagar, T., Roussos, S., ... & Baratti, J. (2006). Improving lipase production by nutrient source modification using *Rhizopus homothallicus* cultured in solid state fermentation. *Process Biochemistry*, *41*(11), 2264-2269.

30. Salihu, A., Bala, M., & Alam, M. Z. (2015). Lipase production by *Aspergillus niger* using sheanut cake: an optimization study. *Journal of Taibah University for Science*.
31. Salihu, A., Bala, M., & Bala, S. M. (2013). Application of Plackett-Burman experimental design for lipase production by *Aspergillus niger* using shea butter cake. *ISRN biotechnology*, *2013*.
32. Savitha, J., Srividya, S., Jagat, R., Payal, P., Priyanki, S., Rashmi, G. W., & Shantala, Y. M. (2007). Identification of potential fungal strain (s) for the production of inducible, extracellular and alkalophilic lipase. *African journal of biotechnology*, *6*(5), 564.
33. Sharma, R., Chisti, Y., & Banerjee, U. C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnology advances*, *19*(8), 627-662.
34. Silva, W. O. B., Mitidieri, S., Schrank, A., & Vainstein, M. H. (2005). Production and extraction of an extracellular lipase from the entomopathogenic fungus *Metarhizium anisopliae*. *Process Biochemistry*,*40*(1), 321-326.
35. Singhania, R. R., Patel, A. K., Soccol, C. R., & Pandey, A. (2009). Recent advances in solid-state fermentation. *Biochemical Engineering Journal*,*44*(1), 13-18.
36. Subramaniyam, R., & Vimala, R. (2012). Solid state and submerged fermentation for the production of bioactive substances: a comparative study. *Int J Sci Nat*, *3*, 480-486.
37. Sumathy, R., Vijayalakshmi, M., & Deecaraman, M. (2012). Studies on Lipase production from fungal strains by different inducers at varied concentrations-A comparative study. *International Journal of Environmental Sciences*, *3*(3), 1072.
38. Thakur, S. (2012). Lipases, its sources, properties and applications: a Review. *Int J Sci Eng Res*, *3*, 1-29.
39. Toscano, L., Montero, G., Stoytcheva, M., Gochev, V., Cervantes, L., Campbell, H., & Gil-Samaniego, M. (2013). Lipase production through solid-state fermentation using agro-industrial residues as substrates and newly isolated fungal strains. *Biotechnology & Biotechnological Equipment*,*27*(5), 4074-4077.