"ISOLATION OF LIPASE PRODUCTION FROM *ASPERGILLUS NIGER* BY USING COCONUT OIL CAKE"

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"ISOLATION OF LIPASE PRODUCTION FROM *ASPERGILLUS NIGER* BY COCONUT OIL CAKE"

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DECLARATION

I, Aswathi P.R. (VM20FPT005) hereby declare that this project entitled “ISOLATION OF LIPASE PRODUCTION FROM *ASPERGILLUS NIGER* BY USING COCONUT OIL CAKE” is a bonafide record of the project work done by us during the course of study and that the report has not previously formed the basis for the award to us for any master degree, diploma, fellowship or other title of any other university or society.

Place: ASWATHI P R

Date:

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ABSTRACT

Cultural conditions for the production of lipase by *Aspevgillus niger* strain by solid-state fermentation using coconut oil cake were standardized. Coconut oil cake was collected from coconut oil processing mill. Addition of various nitrogen sources, carbohydrates and inducers to the substrate was found to be ineffective. It is used as a substrate. The coconut oil cake is the cheap raw material for lipase production. *Aspergillus niger* was identified to be the best producer of lipase. When *Aspergillus niger* was incubated for 5 days at 30°C, it shows surrender of lipase in production.

Keywords: Aspergillus niger, lipase, production, characterization, solid-state fermentation, coconut oil cake.

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ABBREVATIONS AND SYMBOLS

Min	- Minute
Hr	- Hour
Wt	- Weight
L	- Liter
%	- Percentage
SSF	- Solid State Fermentation
COC	- Coconut Oil Cake
Ppm	- Parts per million
g	- Gram
µl	- Microliter
ml	- Milli liter
°C	- degree Celsius
Cm	- Centi meter

INTRODUCTION

CHAPTER I

INTRODUCTION

1.1 AIM

The aim of the project is to isolation of lipase from *Aspergillus niger* by using solid state fermentation using coconut oil cake.

1.2 OBJECTIVES

i. Production of lipase under solid state fermentation using coconut oil cake *Aspergillus niger*.
ii. Utilization of agro wastes for lipase production
iii. Effect of substrate in the production of lipase and its activity

Food production is a top priority issue, as the lack of food for the continuously growing population is becoming an increasing problem in the world (Zvonko.B.Njezic et al...). On the other hand large amount of food is wasted all around the world every year. So there is an urgent need to reuse them and convert it to useful products. (1). Microbial enzymes play a major role in food industries because they are more stable than plant and animal enzymes. E management of solid waste in an eco-friendly way. Solid state fermentation (SSF) has more advantages than submerged fermentation (SMF) due to low capital investment, simplification of the fermentation media, and absence of complex machinery, reduced energy requirement and improved product recovery, more thermos table (Lonsane et al., 1985; Pandey et al., 1999).

Lipases (triacylglycerol acyl hydrolases, E.C.3.1.1.3) catalyze the hydrolysis of triglycerides to glycerol and free fatty acids at an oil-water interface. Microorganisms are potent lipase producers and moulds are widely recognized as producing the highest amounts of enzymes. It has been reported that solid-state fermentation (SSF) with fungal strains results in much greater productivity than submerged fermentation and it also offers many other advantages. Hence, the aim of this study was to devise a SSF process using a novel substrate with a new *Aspergillus niger* strain MTCC 2594 isolated in our laboratory and identified for its capacity to grow rapidly on a solid support and elaborate large quantities of extracellular lipase.

Lipases or triacylglycerol acyl hydrolases are a class of hydrolase enzymes, which helps in the hydrolysis of triglycerides and acts on carboxylic ester bonds. Lipases normally occur in

humans and animals with monogastric stomach. Lipase enzymes are usually produced in the pancreas and stomach where they help in the digestion of fats and lipids.

Lipases are water-soluble, ester hydrolases that are marked preference for apolar, water-insoluble ester substrates. This group of enzymes also includes species referred to as cholesterol esterases. Lipases and cholesterol esterases are distinguished from phospholipases that catalyze the hydrolysis of acyl ester bonds of highly amphipathic phospholipids having a *sn*-glycero-3-phospho-X moiety and from carboxylesterases that hydrolyse polar, water-soluble esters. These distinctions are relative, however, because some lipases exhibit activity toward phospholipids or soluble esters. Typical natural lipase substrates include, in order of amphipathicity, long aliphatic chain acyl esters of cholesterol (cholesteryl esters), triacyl esters of glycerol (triacylglycerols), acyl esters of long chain alcohols (wax esters), diacyl esters of glycerol (diacylglycerols), and monoacyl esters of glycerol. Because lipase substrates tend to be oily and only weakly amphipathic, they reside primarily in a bulk oil phase in preference to the aqueous phase or to the interface, that is, monomolecular surface phase that separates the bulk oil and aqueous phases. It follows, because lipases are water-soluble enzymes, that the site of lipolysis is the quasi-two-dimensional interface.

Enzyme is a substance that acts as a catalyst in living organisms, regulate rate at which chemical reactions proceed without itself being altered in the process. Almost all metabolic process in the cell need enzyme catalysis in order to occur s fast enough to sustain life (Stryr.L.Berg J M and Tymoczko J L, 2002). Metabolic pathways depend upon enzymes to catalyse individual steps. Enzymes are known to catalyse more than 5,000 biochemical reaction types (Schomburg 1 et al,) .These catalytic/enzymatic activities are essential to the information and energy management requirements of a cell. Specific enzymatic activities are found within all cellular organelles (D.C. DeLuca, J. Lynda York, in Encyclopedia of Genetics, 2001). Enzymes can be classified in different ways, including by the location of the enzyme, the kind of substrate (difference between substrate specificity and reaction singularity) and the enzyme reaction (Tsugikazu Komoda, Toshiyuki Matsunaga, in Biochemistry for Medical Professionals, 2015), Among enzymes lipases, esterases, cellulases, xylanapectinases, amylases and proteases are some of the most important (Cherry and Fidantsef, 2003; Gupta et al., 2004). The most significantly used enzyme is Lipase.

Lipases are the most pliable biocatalyst and bring about a wide range of bioconversion reactions, such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and

aminolysis. Lipases can act on a variety of substrates including natural oils, synthetic triglycerides and esters of fatty acids. They are resistant to solvents and are exploited in a broad spectrum of biotechnological applications. Lipase catalyzed transesterification, hydrolysis and esterification are the important class of reactions for food technology applications in fats and oil industry, dairy industry, pharmaceuticals and bakery industry. Lipases are very peculiar as they hydrolyse fats into fatty acids and glycerol at the water-lipid interface and can reverse the reaction in non-aqueous media. Novel biotechnological applications, like biopolymer synthesis, biodiesel production, treatment of fat-containing waste effluents, enantiopure synthesis of pharmaceuticals and nutraceutical agents etc...

Lipase have wide range of uses and they can be extracted from various sources (Abhijith Ray 2012). Best plant lipase sources include raw, unheated, unrefined oil (egg yolk, cream, whole coconut, whole avocado etc.) Neutralize by heat (pasteurization) or acids (vinegar, stomach acid etc.). Lipases are also important in the fermentation and yoghurt, freeing fatty acid and breaking down fat. Some of the industrially important chemicals which are manufactured from fats and oils, by chemical process, could be produced by lipase with great rapidity and better specificity under mild conditions (Sih and Wu 1989; Vulfson 1994). Lipase isolated from different sources have a wide range of properties depending on their source with respect to positional specificity, fatty acid specificity, thermo stability, pH optimum etc. (Huang 1984). Lipases have been intensively investigated for their multiplicity of catalysis with unique specificities, which have multifold applications in oleo chemistry, organic synthesis, detergent formulations and nutrition. They also display useful properties related to their stability as organic solvent-tolerant and thermo stable enzyme. Therefore, microbial lipases have been of recent research interests and a number of lipases have been identified, purified and characterised to date.

Microbial lipases are produced by bacteria, fungi and yeast. This enzyme finds application in various industries including food, biofuel, detergents and animal feed, dairy. It is also used in leather, textile and paper processing applications. In the food and beverage industry, lipases find major application in dairy, baking, fruit juice, beer and wine industries. Microbial enzymes are often more useful than enzymes derive from plants or animals because of the great variety of catalytic activities, their high yielding, ease of genetic manipulation, regular supply due to absence of seasonal fluctuation and rapid growth of microorganism on inexpensive media. Microbial enzymes are also more stable than corresponding plant and animal enzyme and their production is more convenient and Safer (wiseman 1995). Microbial lipases are commercially

Significant because of low production cost, greater stability and wide availability that plant and animal lipases. They may originate from fungi, mold or bacteria and most of them are formed extracellularly. The most preferable source is fungi because fungal enzymes extraction from fermentation media and mostly are extracellular Ahmed et al, 2007). Lipases are widely present in nature, but due to greater stability, wide availability and low product cost, microbial lipase are more significant than lipases extracted from plant and animal sources (Beisson et al, 2000). Being more stable than animal or plant lipases, microbial lipases have earned special industrial attention due to their stability, selectivity and broad substrate specificity. The energy consumption required to conduct reactions at elevated temperature and pressure is eliminated as lipases are active under room temperature, reducing the denaturation of labile reactants and products (Dutra et. al, 2008, Griebeler et al., 2011). Microbial lipase used in industry, is extracted from yeast, fungi and bacteria.

Fungal lipases are widely diversified in their enzymatic properties and substrate specificity, which makes them very attractive for industrial applications. They constitute an important group of biotechnologically important enzymes because of the versatility of their properties and case of mass production. The industrial applications of fungal lipases have been reviewed by many researchers [Ray S. 2015, 1Thakur S. 2012, Hasan et al., Horchani H et al, Kapoor M, Gupta MN. 2012]. Development of lipase-based technologies for the synthesis of novel compounds is rapidly expanding the uses of these enzymes [Azim A et al., 2001). The increase the productivity of lipase enzyme, different physiological parameters are studied like pH, temperature, incubation time, inoculation, etc... The effect of temperature is mostly used for the selection of optimum temperature for the production of lipase enzyme.

Lipase enzymes produced by the microbes are generally extracellular and are determined by the factors such as temperature, pH, carbon source, nitrogen source, inducers, and metal ions. Therefore, optimization of the medium composition plays an important role in the production of enzymes for industrial applications. A prominent number of reports have dealt with the isolation, characterization and purification of solvent-tolerant thermophilic lipases (Masomian et al., 2010; Yoo et al.2011; Shariff et al., 2011). However, finding lipases that can tolerate high temperatures and the presence of organic solvents remains a challenge. It is also important that the lipase-producing species better withstand changes in growing conditions and produce more enzymes compared to other microorganism.

Lipases are used to enrich PUFAs from animal and plant lipids, and their mono and diacylglycerides are used to produce a variety of pharmaceuticals. PUFAs are increasingly used as food additives, pharmaceuticals and neutraceuticals because of their metabolic benefits. Liposomes are used in the medical field to optimize the action of drug by transporting them to target areas, thus circumventing drug waste inactivation and anatomical barriers. Profens, a class of no steroidal anti-inflammatory drugs are active in the S-enantiomeric form. Nutriceuticals are food components that have health benefits beyond nutritional value. Lipases are also used in the synthesis of artificial sweetener sucralose by regioselective hydrolysis of octaacetylsucrose.

Lipases possess the unique feature of acting at the interface between an aqueous and a non-aqueous phase. They synthesize esters from glycerol and long chain fatty acids when the water activity is low. A “true lipase” will split emulsified esters of glycerine and long chain fatty acids, such as triolein and tripalmitin. The growth of application of commercial enzymes is very significant and promising, particularly in food industry.

Agro-industrial residues (Oil cakes) are used as potential raw materials in the production of enzymes and supply essential nutrients which reduce the production time, provide high calorific value and enhance the growth of microbes. In the present work coconut oil cake (COC) was used for the production of lipases.

Coconut oil cake is the by-product of the Mechanical expelling / Solvent Extraction process of Copra, wherein the main product is Crude Coconut oil. Coconut oil cake is a high energy, high fiber, feed formulation for the animal feed industry, particularly in order to increase weight of fully grown animals. It can provide significant protein and energy in livestock diets and may be used to reduce feed costs. Organic coconut meal is a delicious, healthy alternative to wheat and other grain flours. Oil cake can also be directly used as cattle feed. It contain starch, soluble sugars, soluble protein, lipids and trace amount of nitrogen. SSF has been tried using coconut oil cake for different applications in bioconversion processes.

Sl.No.	Nutrients	Percentage
1	Crude Fiber	10.8%
2	Crude Protein	25.2%
3	Ash Content	0.67%

4	Calcium	0.8%
5	Saturated fat	60%

Table 1: Nutrient composition of coconut oil cake

Lipases have been successfully used as a catalyst for the synthesis of esters. The esters produced from short-chain fatty acids are used as flavouring agents in the food industry. Lipase immobilized on silica and micro emulsion based organelles were widely applied for ester synthesis. Accurate control of lipase concentration, pH, temperature and emulsion content are required to maximize the production of flavour and fragrance. Uhling has explained the preparation of lipase modified butter fat which found wide application in various food processes. Chocolates with coco butter substitutes, bread, and structured lipids like human milk fat replacers, low calorie health oils, nutraceuticals, EMC, etc. are few examples for lipase mediated food products. Since it is a new technology, more research on usage of lipase to develop new commercial food products must be promoted. Oil from soybean is hydrolysed by lipase in making Koji, a traditional Asian food. Another soybean fermented food Tempeh utilized lipase from R. oligosporous. This Tempeh forms a base material for many delicious, easily digestible and nutritious food preparations, providing a good number of human populations with a valuable and affordable source of protein.

1.3 APPLICATION OF LIPASE IN FOOD INSUTRY

The majority of enzymes used in industry are for food processing, mainly for the modification and breakdown of biomaterials. A large number of fat clearing enzymatic lipases are produced on an industrial scale. Most of the commercial lipases produced are utilized for flavour development in dairy products and processing of other foods, such as meat, vegetables, fruit, baked foods, milk product and beer.

Commercial lipases are mainly used for flavour development in dairy products and processing of other foods containing fat. They can improve the characteristic flavour of cheese by acting on the milk fats to produce free fatty acids after hydrolysis. Different types of cheese can be made by using lipases from various sources, e.g. Romano cheese using kid/lamb pre-gastric lipase, Camembert cheese using lipase from Penicillium camemberti and cheddar cheese using Aspergillus niger or A. oryzae. Lipase catalysis could improve the texture and softness of cheese. Lipases are also used as flavour development agents in butter and margarine, also to prolong the shelf life of various baking products. In alcoholic beverages such as wine, the aroma can be modified using lipase. They are used to improve the quality of cocoa butter,

which has a melting point of 37 °C due to the presence of palmitic and stearic acids and can easily melt at 37 °C. A patent has been filed by Unilever using immobilized Rhizopus miehei lipase, which can replace palmitic with stearic acid to give desired stearic-oleic-stearic triglyceride. Functionalized phenols were esterified for the synthesis of lipophilic antioxidants for the application in sunflower oil using immobilized lipase from Candida antarctica (CALB), Candida cylindracea Ay30, Helvina lanuginosa, Pseudomonas sp. and Geotrichum candidum. Lipases also find application as a biosensor in food industry. Immobilized lipase was successfully used for the determination of organophosphorous pesticides with a surface acoustic wave impedance sensor by lipase hydrolysis. It may also be used in the determination of triglycerides and blood cholesterol if the lipase is immobilized onto pH/oxygen electrodes in combination with glucose oxidase. Microbial lipases such as lipase from Candida rugosa have many applications which cannot be met by chemical synthesis. This lipase finds application in the production of ice cream, single-cell protein, and carbohydrate esters and amino acid derivatives. In addition to this, lipase could also be used in the processing of different waste streams that are released from food industries.

Phospholipases have found industrial applications in egg yolk treatment for the production of mayonnaise and other emulsifiers, in lecithin modification, and for the oil-degumming step in the refining of vegetable oils. Introduction of a microbial phospholipase (Lecitase Nova) has significantly improved the economy of enzymatic degumming of vegetable oils. In this process, the phospholipids are hydrolyzed and rendered more water soluble, hence facilitating their washout. The function of phospholipase in egg yolk treatment is to hydrolyze egg lecithin, iso-lecithin, which improves the emulsifying capacity and heat stability. The egg yolk thus produced can be useful in the processing of custard, mayonnaise, baby foods, and dressings and in dough preparation. It can also be applied in the processing of sauces, like hallandise, bernaise and cafe de Paris.

Lipases are extensively used in the dairy industry for the hydrolysis of milk fat. The dairy industry uses lipases to modify the fatty acid chain lengths, to enhance the flavours of various cheeses. Current applications also include the acceleration of cheese ripening and the lipolysis of butter, fat and cream.

The production of low molecular weight esters as flavour compounds by biotechnological processes has potential interest for the food industry. The use of naturally available substrates and enzymes is an essential part of the process design. Laboret and Perrand91 performed direct

esterification of citronellol and geraniol with short-chain fatty acids, catalyzed by free lipase from M. miehei, which gave high yields in n-hexane. The initiative to scale-up applications was also attempted, using a reactor that allowed the production of ester in quantities up to 100 cm.

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 flavour content of bakery products by liberating short-chain fatty acids through esterification. Along with flavour enhancement, it also prolonged the shelf-life of most of the bakery products. Texture and softness could be improved by lipase catalyzation. An artificially expressed lipase in A. oryzae was used as processing aid in the baking industry.

REVIEW OF LITREATURE

CHAPTER II

REVIEW OF LITREATURE

Johanna Blom et al. (2013) reported that Food lost or wasted constitutes a substantial environmental, economic and social problem; today roughly one-third of edible parts of food that is produced for human consumption globally gets lost or wasted. Thus a reduction of waste would contribute to a sustainable use of resources.

As of today the possibilities of using enzymes are very widespread, starting from scientific studies, cosmetic studies medical diagnostics and chemical analysis, therapeutic uses, up to an industrial catalysis of such synthesis as e.g. the production of wine and beer, dairy products, in the sugar or pulp and paper industry, sewage treatment and many others. All this contributes to the constant increases in demand for these valuable products. Approximately 4000 enzymes are known, from which 200 have practical applications. These are especially enzymes of bacterial origin.

2.1 ENZYME

Berg and Tymoczko et al. (2007) reported that Enzymes, the biological catalysts are remarkable molecular devices that determine the patterns of chemical transformations. These catalysts also mediate the transformation of one form of energy into another. The most striking characteristics of enzymes are their catalytic power and specificity. They are produced by living organisms to catalyse the biochemical reactions required for life. Thus, enzymes mainly formed within living cells, can continue to function in vitro (in the test-tube). Their ability to perform highly specific chemical transformations making them increasingly useful in industrial processes. Enzyme-catalysed process are gradually replacing chemical in many areas of industries.

Cherry and Fidantsef, (2003), Krishna, (2002) and Schulze and Wubbolts, (1999) reported that Enzymes offer substantial and significant advantages over chemical catalysts. It can be derived from renewable resources and are biodegradable. The enzymes can operate at ambient temperature and pH, and tend to offer exquisite selectivity in both reactant and product stereochemistry unlike their chemical counterparts. Growing environmental concern due to population explosion and global industrialization advocate the need to find more benign methods of processing. This leads to the escalating interest in enzymes. Of all the enzymes, the group hydrolases are most often employed for industrial biotransformations. It is estimated that

approximately 80% of all industrial enzymes belong to the group hydrolases. Within the hydrolases, lipases are most explicitly used for food applications as well as in the area of fine chemicals manufacture.

Kenji Takahashi et al. (2013) investigated that A group of enzymes, mostly hydrolases or certain transferees, utilize one or a few side chain carboxyl groups of Asp and/or Glu as part of the catalytic machinery at their active sites. The author and his colleagues have been working for over fifty years mainly on the structure and function of proteins and enzymes, especially certain groups of enzymes such as ribonucleases (RNases), peptidases and glutathione S-transferases. The studies on peptidases involved numbers of extra- and intracellular peptidases F. Sanger's group first succeeded in the determination of the amino acid sequence of bovine insulin (51 residues). After that, various proteins of molecular weight over 10,000 became the major targets of sequencing. Among these sequence studies, that of RNase at (124 residues) was most advanced and its amino acid sequence was determined. In 1982, Heinemann and Saenger first reported the crystal structure of RNase Tlcomplexed with 2B-GMP, in which Glu58, His40, His92 and Arg77 were shown to reside in close proximity with each other in the active site38) as previously predicted mainly from chemical modification studies. The crystal structure was also consistent with the results obtained previously using gel filtration regarding the interaction of guanine base with the enzyme. In addition, it was revealed that the region from Tyr42 to Tyr45 interacts with the guanine base in the substrate through multiple hydrogen bonds and that Tyr45 is stacked with the guanine base.

L.Carvalho et al., (2012) studied that enzymatic hydrolysis of three cellulosic substrates: filter paper (FP), used as a low recalcitrance substrate model; steam exploded sugarcane bagasse (SB), and weak acid pre-treated SB (1:20 dry bagasse: H2SO4 solution 1% w/w), the last two delignified with 4% NaOH (w/w). The influence of substrate concentration was assessed in hydrolysis experiments in a shaker, using Accellerase 1500, at pH 4.8, in 50 mM sodium citrate buffer. Cellulose loads (weight substrate/weight total) were changed between 0.5% -13% (for FP) and 0.99% -9.09% (for SB). For FP and low loads of steam exploded SB, it was possible to fit pseudo-homogeneous Michaelis-Menten models (with inhibition). For FP and higher loads of steam exploded SB, modified Michaelis-Menten models were fitted. Besides, it was observed that, after retuning of the model parameters, it is possible to apply a model fitted for one situation to a different case.

Sharma.R et al., (2001) and Kojima et al., (2003) investigated that Lipase is a physiologically necessary enzyme. It occurs in many plant and animal organisms, as well as in microorganisms. However, its richest source is bacteria, fungi and yeast. At least 75% of enzymes with industrial significance (including lipases) have hydrolytic properties.

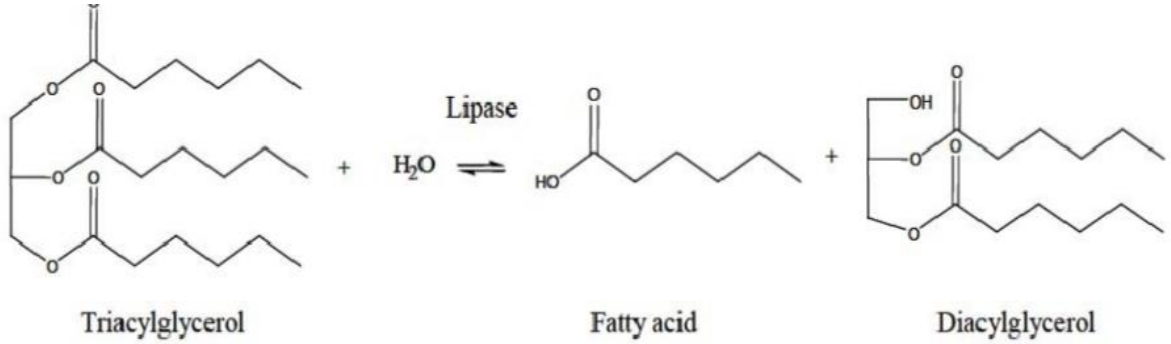
Sumita Thakur, (2012) and B. Joseph et al., (2008) researched that Lipase have emerged as one of the leading biocatalyst with proven potential. The number of available lipases has increased since the 1980s. Lipases are produced by animals, plants and microorganism.

2.2 LIPASE

Reis et al., (2009) and Mala and Takeuchi, (2008) investigated that Lipases are glycerol ester hydrolases (E.C. 3.1.1.3), which hydrolyse ester linkages of glycerides at water-oil interface. The increasing demand of lipases has been attributed to their efficiency in catalysing hydrolysis along with various reverse reactions, such as esterification, Trans esterification and aminolysis in organic solvents. Moreover, the endowed substrate specificity, regioselectivity and enantioselectivity of microbial lipases contribute to their catalytic activity under mild temperatures with reduced side products at low waste treatment costs.

(Hasan et al., 2006; Kumari et al., 2009a & 2009b; Jegannathan et al., 2008; Mahapatra et al., 2009a & 2009b) reported that The activity retainment in organic solvents extend the range of applications of lipases as commercial biocatalysts in industry.

Svendsen, et al. (2000) and Schmid and Verger et al. (1998) investigated that Lipases catalyze particularly the hydrolysis of the ester bond of tri-, di- and monoacylglycerols (glycerides). Generally, lipases have a preference for tri- and diglycerides rather than monoglycerides. Lipases are often 1, 3 regioselective, acting on the positions sn-1 and sn-3 (end positions) rather than on sn-2 (mid position). Lipases also catalyse the hydrolysis or synthesis of a rather broad range of substrates containing ester linkages, such as aliphatic, alicyclic, bicyclic and aromatic esters along with naturally occurring triglycerides, the often preferred substrates.



Triacylglycerol Fatty acid Diacylglycerol

Jaeger et al., (1994) studied that lipases are also effective biocatalysts for the acylation and. deacylation of a wide range of synthetic substrates, while still showing high regioselectivity and chiral recognition. The catalytic actions of lipases are affected by the water content of the reaction mixture. In aqueous media, lipases catalyse the hydrolysis of triacylglycerol's into diglycerides, monogylcerides, glycerol and fatty acids. In non-aqueous environment, lipases catalyse ester synthesis.

The study investigated the screening, production and optimization of an extracellular lipase from a fungus isolated from the contaminated soil of a palm oil processing shed. This was with a view to obtaining a strain that can secrete lipase with biochemical properties exploitable for biotechnological applications such as bioremediation of oil contaminated sites. Soil samples were collected from palm oil contaminated sites in Gbogan, Osun State, Nigeria (Latitude N 7°29.1481 and Longitude E 4°20.7587'). The isolated fungal strains were screened on tributyrin agar for exogenous lipolytic activity. Molecular identification was carried out by amplifications of ITS-1, 5.88 and ITS-2 regions. The effects of incubation time, inducers, and pH, temperature, carbon and nitrogen sources were varied for optimal lipase production using one factor at a time approach.

Rhizopus oryzae et al. and Zainab Adenike Ayinla et al, (2017) ZAC3 (NCBI accession No: KX035094) was identified that the highest lipase-producing strain. Maximum lipase production was observed on the fourth day, pH 5.0 and a temperature of 45°C. Olive oil, xylose and yeast extract were the best inducer, carbon and nitrogen sources respectively for lipase production. Lipases were found as lipolytic enzyme with numerous industrial applications. The culture and desired growth conditions for production of extracellular lipase from fungus *Aspergillus niger* strain NCIM 584 was extensively investigated. Enzyme production was carried out in a submerge culture using major nutrients from soya flour as main constituent of the media. The optimum weight percentage of soya flour, glucose and olive oil concentrations on lipase production were defined as 7.5 wt%, 12.5 and 12 g/l, respectively. Combination of nitrogen sources such as yeast extract and peptone were suitable nitrogen sources. The activity of lipase was maximized at pH value of 7. In addition, the optimum growth temperature was observed at 30°C. Also, maximum enzyme activities were observed in presence calcium ions with concentration of 7.5Mm (Maryam N. Hosseinpour et al., 2011).

Hasan et al., (2006); Bornscheuer et al., (2002); Menoncin et al., (2009) and Abada et al., (2008) investigated that the first lipase was discovered in pancreatic juice in the year 1856 by Claude Bernard. Animal pancreatic extracts were traditionally used as the source of lipase for

commercial applications. However, microbial sources of lipase were explored when industrial potential of lipases enhanced and their demand could not be met by the supply from animal sources. The number of available lipases has increased mainly as a R'-OH 19 result of achievements made in the cloning and expression of enzymes from microorganisms, as well as of an increasing demand for these biocatalysts with novel and specific properties such as specificity, stability, pH, and temperature. Lipases are widely distributed in animal, plants and microorganisms however, microbial lipases are commercially most important mainly because of the ease of their cultivation and genetic manipulation to obtain higher yield. Commercially important microbial lipases are produced from bacteria, fungi and yeast.

Sharma et al, (2001) reported that the industrial demand for new sources of lipases with different catalytic characteristics stimulated the isolation and selection of new strains. 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.

2.3 SOURCES

Hasan F et al. (2006) studied that lipases are ubiquitous in nature and are found in multiple unicellular and multicellular organisms. However, yeast and fungi are one of the most important sources of lipases for industrial applications. Most commercially important lipase-producing yeasts belong to the class of ascomycetous yeast, like Candida sp. Most of the lipases are extracellular and can be obtained either by submerged fermentation (SmF) or by solid state fermentation (SSF). Lipolytic yeasts are found in a variety of oil contaminated habitats including soil contaminated with oil, wastes of vegetable oils, dairy waste and deteriorated food. There are a number of lipase producing yeast sources compiled by several authors, however only a few have been commercially exploited for the bulk production. Some important sources are: C. antarctica, C. rugosa, Candida tropicalis, Candida curvata, Candida cylindracea, Candida deformans. Candida parapsilosis, Candida utilis, Candida valida, Candida viswanathii, Galactomyces geotrichum, Arxula adeninivorans, Saccharomyces cerevisiae, Yarrowia lipolytica. Trichosporon fermantans, Trichosporon asahii, Rhodotorula mucilaginosa, and Aureobasidium pullulans.

Villeneuve et al., (2000) reported that in general lipases do not require cofactors, operate in a wide pH range, are stable at high temperatures, have high specificity and exhibit regio-, chemo- and enantioselectivity.

Verma et al., (2012) reported that owing to their unique properties, lipases are widely used in various industrial sectors, such as food, pharmaceutics, biofuels, oleochemical, textile, agro-chemical, paper manufacturing, cosmetics and many others. In the food industry, lipases can be used as flavor modifiers by synthesis of short chain fatty acids esters and alcohols, and to obtain products of increased nutritional value by modifying the triacylglycerol structure for inter- or trans esterification.

Barros et al., (2010) reported that Lipases are ubiquitous in nature and are produced by plants, animals and microorganisms. However, microbial lipases, native or recombinant, are mostly used in different biotechnological applications.

Borrelli and Trono, (2015); Bonugli-Santos et al., (2015) investigated that nature provides a wide biodiversity of microbial resources. Microorganisms have great adaptive capabilities, even in inhospitable environments, such as the Dead Sea, Antarctica, alkaline lakes, hot springs, volcanic vents and contaminated soils, which offer remarkable potential for production of enzymes with specific characteristics.

Basheer et al., (2011); Dewapriya and Kim, (2014) studied that the proportion of enzymes used in the food industry is constantly growing, with prospects of growing more in the coming years, due to the demand for new applications in the fields of dairy and baking, among others. Studies report that marine microorganism capacity in the production of active compounds, including proteins and enzymes reveal new sources of industrial enzymes, some producing microorganism of lipases and their application in the food industry.

Kumar.R et al., Dandavate. V et al. (2002) reported that most lipases are inducible enzymes and addition of oils proved to enhance lipase production Bacterial cultures were isolated from industrial and oil spilled areas and was screened for Lipase production and activity. Seven bacterial strains were found to have lipolytic ability. These bacterial strains were grown in production media and the lipase enzyme produced was estimated.

Devaraj Bharathi et al., (2017) studied that lipase enzyme from bacterial strains were produced and eight bacterial strains were isolated from petrol spilled soil by serial dilution technique. Olive oil was used as the substrate in tributyrin agar medium for screening and showed the zone of activity in five of those bacterial strains.

2.4 MICROBIAL LIPASE

Gutarra et al., (2005) and Illanes, (2008) are investigated that most microbial lipases are produced extracellularly in large quantities and are quite stable under non-natural conditions

such as high temperature and in non-aqueous organic solvents. Stability, inexpensive manufacturing, as well as broad synthetic potential of microbial lipases make them ideal biocatalysts for various industrial applications.

Archer et al., (2008) and Peberdy et al., (2001) reported that Filamentous fungi are widely distributed metabolically versatile organisms. Many fungi are regarded as nature's primary degraders because they secrete a wide variety of hydrolytic enzymes that degrade waste organic materials Fungi serve as the source of many commercial enzymes acted as hosts for production of heterologous enzymes which accounts for multifold industrial applications.

Holker et al., (2004) studied that the utilization of agricultural residues as the carbon/energy source or the solid support/substrate offers the advantage of combining the utilization of low-valued problematic residues for the production of costly enzymes and chemicals. For their ability to secrete a large amount of hydrolytic enzymes, filamentous fungi are capable of utilizing the cellulosic materials, starchy products, and pentose sugars present in agricultural residues and plant biomass. Therefore, direct fermentation by filamentous fungi is a promising process, leading to many applications.

Pandey et al. (1999) investigated that traditionally, enzymes are produced using the submerged fermentation (SmF), in which the cultivation of microorganisms occurs in an aqueous solution containing nutrients. In recent years, a significant interest has been evolved in using solid-state fermentation (SSF) technique to produce a wide variety of enzymes, mainly from mold origin.

Viniegra-González, (1998) and Viniegra-González et al., (2003) investigated that the SSF has led to a wide range of laboratory scale applications and comparative studies between SmF and SSF claim higher yields for products made by SSF.

2.5 General aspects and advantages of SSF

Couto and Sanorman, (2005) and Pandey et al., (2000) studied that SSF is the growth of microorganisms (often fungi) on solid substrates in systems with a continuous gas phase and no free-flowing water by employing either a natural support or an inert support as a solid material.

Durand et al., (1993) and Ralph, (1976). Rodriguez et al. (2006) reported that the non-inert materials such as wheat bran, bagasse and oil cakes used in SSF serve as an attachment as well as supplier of some nutrients to the microorganism utilized sugarcane bagasse with liquid

medium as the substrate for lipase production by Rhizopus homothallicus through solid state fermentation. Rice and wheat bran supplemented with rice bran oil were utilized as the substrates for lipase production by SSF from Candida sp.

Cordova et al., (1998) reported that enhanced lipase activity in the case of lipase production by R. pusillus and R. rhizopodiformis with the mixture of olive oil cake and sugarcane baggase as the substrate for the solid state fermentation Several researchers utilized wheat bran as the substrate for lipase production by Rhizopus oligosporus through solid state fermentation.

The advantages include:

- High volumetric productivity.
- Low capital investment and energy requirements.
- Ease of product recovery.
- Less waste-water output.
- Fermentation conditions well adapted to the requirements of fungal cultures. Possibility of using new substrates (e.g. solid by-products, plants) without the need for pre-treatments.

The drawbacks associated with the SSF include:

- Difficulties in fermentation control and automation.
- Limited mass and heat transfer.
- Necessity for adequate aeration to remove excess heat;
- Sterilization and contamination problems:
- Problems with downstream processing, for some applications and difficulties with scale-up of the process.

Lotti et al., (1998) investigated that the production of lipases through SSF is influenced by various fermentation conditions such as pH, temperature, liquid to solid ratio and incubation time, presence of lipids. Lipases are mostly induced in presence of fats and oils in the culture medium.

Sztajer et al. (1993) reported that the maximal lipase activity by Penicillium expansum upon addition of 0.1% olive oil in the culture medium. The extracellular lipase production by Candida rugosa was induced by addition of fatty acids to the culture medium resulted in different isoforms.

Burkert et al., (2004) studied that the optimal temperatures of 30 °C and 37 °C were reported for lipase production by Geotrichum sp. and Aspergillus carneus respectively through SSF.

Sugihara et al., (1999) and Tenga and Xu, (2008) reported that the alkaline and acidic pH was preferred for lipase production by Bacillus sp and Rhizopus chinensis.

2.6 ASSAY FOR LIPASE ACTIVITY

Winkler and Stuckmann, (1979) and M.Prasanna Rajesh kumar et al., (2013) reported that the activity of lipase was demonstrated spectrophotometrically at 30°C by using p nitrophenol palmitate (PNPP) as a substrate 20 isolates were subjected for lipase production along with the standard lipolytic bacteria Enterococcus faecium MTCC5659 in lipase production medium. This showed a maximum lipase activity of 4.280U/ml. Among these 6 isolates were capable producing lipase above 3U/ml in 24 hrs of incubation.

Sharma et al. (2002) investigated that the lipase activity after 24 h of inoculation the fermentation broth was taken in falcon tubes. These falcon tubes were centrifuged for 10 minutes at 10000 rpm and the supernatant was then used as the crude enzyme. This crude enzyme was then tested for its enzyme activity by titrating it against 0.05 M NaOH. The amount of NaOH used the amount of acid present in the solution which was directly proportional to the amount of lipase produced.

C.Schmidt-Dannert (1999) reported that acid value was calculated by the formula: (mi NaOH for sample mi NaOH umol fat for blan ty k) acid x N ml U Mx1000-Where: U-umol of fatty acid released/ml N-the normality of the NaOH titrant used (0.05 in this case) M-Total volume of reaction mixture used One lipase unit has been defined as the amount of the enzyme that releases one mol fatty acid per ml under standard assay conditions (U-umol of fatty acid released/ml) (Production and Optimization of Lipase Enzyme from Mesophiles and Thermophiles.

H.Park et al. (2006) reported that the samples collected from the culture broth were centrifuged for 15 min at the speed of 5000 RPM and the supernatant was assayed for extracellular lipase activity. Lipase was determined titrimetrically on the basis of olive oil hydrolysis by modified method of Jensen, 1983 (Jensen R.G et al., 1983). One ml of culture supernatant was added to assay substrate containing 10 ml of 10% (w/v) homogenized olive oil in 10 % (w/v) gum acacia, 2ml of 0.6% (w/v) CaC12 solution 5 ml of phosphate buffer (pH 7). The enzyme substrate was incubated on rotator shaker with 150 C for 1 hour. To stop the reaction 20ml of acetone: alcohol

(1:1) mixture was added to reaction RPM at 30 mixture. The liberated fatty acid were titrated with 0.1 NaOH using phenolphthalein as an indicator. One unit of lipase activity was defined as the amount of enzyme that liberated Imilli mol fatty acid per min at 37°C and at pH 7 under the assay conditions. I unit of Lipase activity- Volume of NaOH consumed (ml) x molarity of NaOH / volume of lipase x reaction time.

V.K. Winkler, M. Stuckmann, J. Bacteriol., (1979) investigated that the lipase activity was assayed quantitatively by using para-nitro phenyl palmitate (PNPP) as the substrate Ten millilitre of isopropanol containing 30 mg pNPP (Sigma) was mixed with 90 ml 0.05 M sodium phosphate buffer (pH 8) containing 207 mg sodium deoxycholate and 100 mg gum arabic. A total volume of 2.4 ml freshly prepared substrate solution was prewarmed at 37°C and mixed with 0.1 ml enzyme solution. After 15 min incubation at 37°C, absorbance at 410 nm was measured against the blank. One enzyme unit was defined as amount of enzyme required to release lumol of p nitrophenol from the substrate in milliliters per minute.

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1 COLLECTION OF SAMPLES

Coconut oil cake was collected from coconut oil processing mill (Kollam) in a sterile plastic bag and brought to laboratory for further analysis.

3.2 ORGANISM

Aspergillus niger, strain was provided by the institution.

3.3 SUBSTRATE

Different substrates viz., coconut oil cake is tested for lipase production by SSF. Oil cakes were ground to a coarse powder before use. They collect from coconut oil mill. It is rich source of lipase production.

3.4 PRELIMINARY ANALYSIS OF COCONUT OIL CAKE

To determine the moisture content, 5 g of the ground substrate was dried at 100°C for 6 h, cooled and weighed until constant weight was achieved.

Figure 1: Sample taken

3.5 GROWTH MEDIA

Growth media used for the cultivation of production of a strain and subsequent production of either microbial culture or a biochemical product.

Potato dextrose agar is a common microbiological growth media made from potato infusion and dextrose. Potato dextrose agar (PDA) is the most widely used medium for growing fungi and bacteria which attack living plants or decaying dead plant matter. Potato Dextrose Agar is composed of dehydrated potato infusion and dextrose that encourage luxuriant fungal growth. Agar is added as the solidifying agent. Many standard procedures use a specified amount of sterile tartaric acid (10%) to lower the pH of this medium from 3.5 to 0.1, inhibiting bacterial growth. Do not reheat the acidified medium, Heating in the acid state will hydrolyse the agar.

Composition	
Potato infusion	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled water	1 L

Table 2: Composition for PDA

3.6 PREPARATION OF POTATO DEXTROSE AGAR

- To prepare potato infusion, boil 200 g sliced, unpeeled potatoes in 1 litre distilled water for 30 min.
- Filter through cheesecloth, saving effluent, which is potato infusion (or use commercial dehydrated form).
- Mix with Dextrose, Agar and Water and boil to dissolve.
- Autoclave 15 min at 121°C.
- Dispense 20-25 ml portions into sterile 15 × 100 mm petri dishes.
- Final pH, 5.6 ± 0.2.

Figure 2. Preparation PDA

3.7 INOCULATION PROCEDURE

- Take 4 test tubes.
- Prepare the potatoes dextrose agar medium using standard composition
- Sterilize the conical flask containing medium and test tubes by autoclaving.
- At laminar airflow cabin (LAF), transfer the medium to test tubes (switch on the LAF with UV for 20 minutes and swap the LAF cabin with alcohol before using LAF)
- Allow the media in the test tube to solidify
- At LAF, after solidification inoculate this spore of *Aspergillus niger* into the medium
- Plugged the test tubes and wrapped with paper.

3.8 SOLID STATE FERMENTATION

Solid state fermentation was carried out for the present study, to study the effect of various incubation periods taken as parameters required for the optimum production of lipase by *Aspergillus niger*. The incubation period is taken as a parameter with an interval pf 24 hours (24, 48, 72, 96, 120), a total of five days. Requirements and procedures for solid state fermentation are as follows:

3.8.1 Mineral salt media

Mineral salt media is used as nutrient solution for *Aspergillus niger* for its growth and lipase production. Mineral salt medium is prepared by using the composition shown in table no.3 with a final volume of 50 mg/50 ml. mineral salt medium used as a nutritive media for *A.niger* on the substrates.

Sl. no:	Name of constituents	g/ml (g in 100 ml)
1	Ammonium nitrate	1.65

2	Potassium nitrate	1.90
3	Magnesium sulphate	0.37
4	Potassium dihydro phosphate	0.17
5	Ammonium ferrous sulphate	0.005
6	Calcium chloride	0.004
7	Distilled water	100

Table 3: Composition for mineral salt solution- macro nutrients

Sl.no.	Name of constituents	g/ml (g in 100ml)
1	Boric acid	0.3125
2	Manganese sulphate	1.115
3	Zinc sulphate	0.43
4	Sodium molybdate	0.0125
5	Copper sulphate	0.01
6	Distilled water	50

Table 4: Micro nutrients

3.8.2 Preparation of spore suspension

- Take 4 test tubes
- Add 2 ml of distilled water to each test tube
- Each test tube was plugged with cotton and autoclaved for 10 minutes
- Allow them to cool at room temperature
- At LAF, dissolve 2 full loop (before inoculation make the loop red hot in flame) of *Aspergillus niger* colony taken from the culture into the test tube to produce spore suspension. Repeat the step for rest of the 3 test tube.
- Switch on the LAF with UV for 20 mins and swap the LAF cabin with alcohol before using LAF.

Figure 3: Spore formation

3.9 FERMENTATION CONDITION

A sample (10 g) of substrate was taken in a series of 250ml Erlenmeyer flasks, moistened with 10ml of water (1:1, substrate: water, w/v) and sterilized at15 lbs/in² for 20 rain. After cooling, the flasks were inoculated with 250µl of spore suspension (4.3x 108spores/ml) and the contents, after mixing, were incubated at 30°C for 5 days.

Figure 4: Addition of mineral salt medium

Figure 5: Mixing

Figure 6: Incubation

Figure 7: Sample after fermentation

3.10 EXTRACTION OF ENZYME

At the end of the fermentation period, 2g of the mouldy substrate was homogenized with 20 ml of distilled water using a pestle and mortar and filtered. The filtrate was centrifuged at 10000 rpm for 5 rain and the supernatant was used as the enzyme source.

Figure 8: Crude enzyme

3.11 LIPASE ASSAY

The crude enzyme is obtained by centrifugation at 10,000 rpm, 4°C for 20 minutes. Lipase activity was determined by spectrophotometric method using p-NPP (p-nitrophenyl palmitate) at pH 9. To carry out the study following reagents were required such as, Reaction Buffer (1 ml) (It contains 1M Tris HCl (pH 9.0) and Triton X-100) and 2-Propanol p-nitrophenyl palmitate (pNPP). 1ml crude enzyme or blank solution was taken in test tube, to this 1ml of reaction buffer was added and this content was incubated for 5 minutes at room temperature and to this 10mM PNPP in 2-propanol (0.04 ml) was added to the enzyme buffer solution and shaken well. The mixture was emulsified for 2 minutes at 50°C and absorbance was measured at 400 nm (UV-3600 Shimadzu UV-VIS-NIR spectrophotometer). The molar extinction coefficient of p-nitrophenol was estimated from the absorbance measured at 400nm of standard PNP. One unit of the enzyme activity was defined as the amount of enzyme which releases 1 µmol of fatty acid per minute under the assay conditions.

Enzyme activity (U) =Ax.V/tx.ex.v

A = Absorbance

V=Total volume of reaction mixture (ml)

v = Enzyme volume (ml)

t = Incubation time (minute)

e = Extinction coefficient

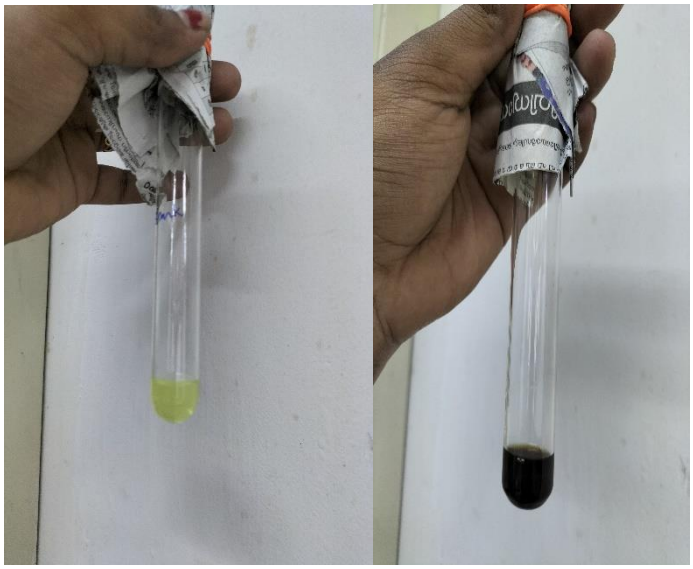


Figure 9: Blank and test sample

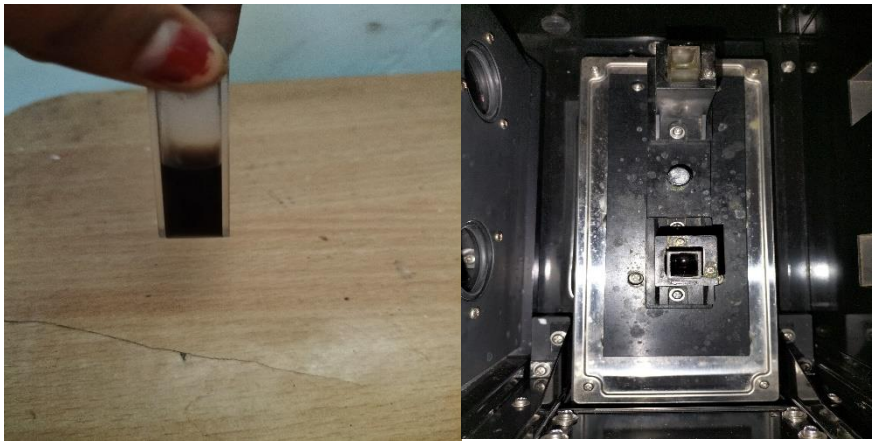


Figure10: Samples at spectrophotometer

RESULT AND DISCUSSION

CHAPTER IV

RESULT AND DISCUSSION

4.1 Collection of sample

Coconut oil cake was collected from coconut oil processing mill at Kollam Market in a sterile plastic bag and brought to laboratory for further analysis.

4.2 Screening and identification of lipase

Solid state fermentation (SSF) technique was used to isolate fungal strain. The present study, the fungal strain *Aspergillus niger* was used to produce using coconut oil cake as a solid substrates in solid state fermentation the *Aspergillus niger* was cultured in potato dextrose agar (PDA). In solid state fermentation with coconut oil cake substrate, lipase enzyme was produced under incubation period of 5 days (120hrs) at an optimum pH 9 and temperature 30 **°C that** we provided. In this study, pNPP is used as stock solution in different concentration. pNPP is used for enzyme assay. Maximum optical density was recorded for the standard at 400nm.

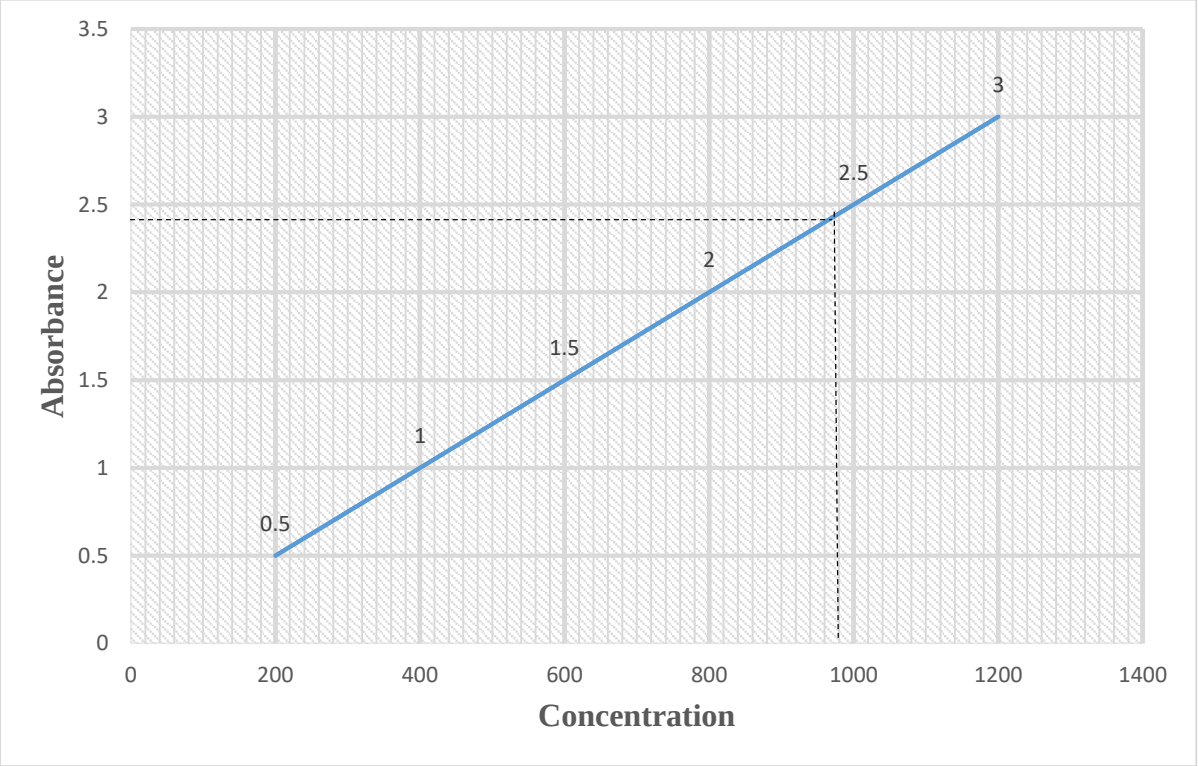


Figure 11: Graph

The absorbance value for the test sample obtained was 2.469.The result was found graphically. In the graph X axis stands for standard concentration and Y axis for absorbance values. And the result was a linear line. And on that the absorbance value of the test sample 2.469 was marked.

The lipase activity obtained from the graph was 960µ/ml.

So a 10g Coconut oil cake sample used as the substrate for SSF with the help of Aspergillus niger produces 960µ/ml lipase activity.

SUMMARY AND CONCLUSION

CHAPTER V

SUMMARY AND CONCLUSION

Lipases are the enzymes that breakdown dietary fats into smaller molecules called fatty acids. They belongs to the class serine hydrolases and subclass esterases. Plants, animals, and microorganisms produce lipases. Lipases normally occur in humans and animals with monogastric stomach. In this study the lipase was produced from the coconut oil cake. The organisms used for solid state fermentation was *Aspergillus niger*. The lipase enzyme was produced by incubating the coconut oil cake of optimum pH 9, about 5 days at a temperature around 30°C. The stock solution was made from pNPP. The pNpp was used to find enzyme activity. The recorded maximum optical density for the standard was 400nm. The result of the test sample's absorbance value was 4.69. The activity of the enzyme was found by graphically. So 10g Coconut oil cake sample used as the substrate for SSF with the help of *Aspergillus niger* produces 960u/ml lipase activity.

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CHAPTER VI

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APPENDIX

APPENDIX I

SPECIFICATION

Sl.no.	Equipment
1.	Centrifuge ❖ A centrifuge is a laboratory device that is used for the separation of fluids, gas or liquid, based on density. ❖ Separation is achieved by spinning a vessel containing material at high speed; the centrifugal force pushes heavier materials to the outside of the vessel. ❖ There are various types of centrifuges, depending on the size and the sample capacity. ❖ A centrifuge works by using the principle of sedimentation: Under the influence of gravitational force (g-force), substances separate according to their density
2.	Spectrophotometer ❖ It is an instrument that measures the amount of photons (the intensity of light) absorbed after it passes through sample solution. ❖ With the spectrophotometer, the amount of a known chemical substance (concentrations) can also be determined by measuring the intensity of light detected. ❖ Consists of three primary components: a light source, optics to deliver and collect the light, and a detector.

APPENDIX II

❖ Preparation of PDA

Composition	
Potato infusion	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled water	1 L

❖ Composition of mineral salt macro nutrients

Sl. no:	Name of constituents	g/ml (g in 100 ml)
1	Ammonium nitrate	1.65
2	Potassium nitrate	1.90
3	Magnesium sulphate	0.37
4	Potassium dihydro phosphate	0.17
5	Ammonium ferrous sulphate	0.005
6	Calcium chloride	0.004
7	Distilled water	100

Micro nutrients

Sl.no.	Name of constituents	g/ml (g in 100ml)
1	Boric acid	0.3125
2	Manganese sulphate	1.115
3	Zinc sulphate	0.43
4	Sodium molybdate	0.0125
5	Copper sulphate	0.01
6	Distilled water	50