Production and Characterization of Lipase from *Pseudomonas* sp.

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(2014- 2016)

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"In the name of ALLAH The Most Gracious and The Most Beneficial"

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FINAL APPROVAL

It is certified that we have read the final thesis entitled "**Production and Characterization of Lipase from *Pseudomonas* sp.**" submitted by **Mr. Hanif Ullah** and it is our Judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the MS Degree in Biotechnology.

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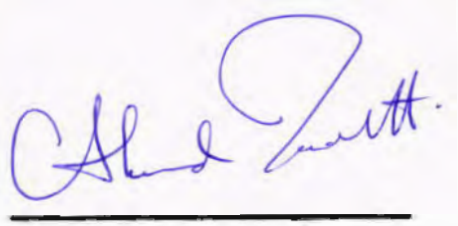

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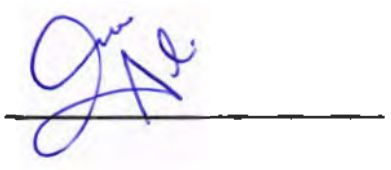

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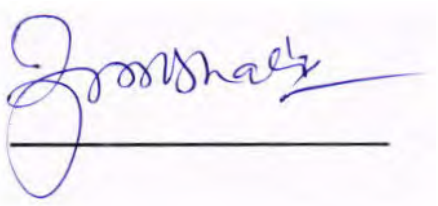

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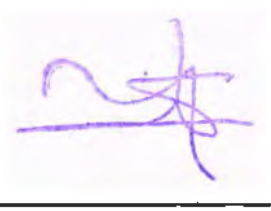

A thesis submitted to Department of Bioinformatics and Biotechnology,
International Islamic University, Islamabad as a partial Fulfillment of requirement of the award of the
Master in Sciences of Biotechnology
(MSBT)

This humble effort is

Dedicated

To

My beloved

Parents,

Brothers, Sisters

And

Teachers

Who inspired me for higher ideals of Life

DECLARATION

I hereby solemnly declare that the work **"Production and Characterization of Lipase from *Pseudomonas* sp."** presented in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated: 28/10/2016

Hanif Ullah

164-FBAS/MSBT/F-14

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All praises and thanks to **ALLAH Almighty**, The Lord of all creation, The Beneficent, The most Merciful and the most Compassionate, Who created man and taught him manner and is The source of entire knowledge and wisdom endowed to mankind. All respect and reverence for The Holy Prophet (PBUH) whose teaching are complete guidance for humanity.

My family is one of the greatest blessings of ALMIGHTY ALLAH that gave me strength to persevere during difficult times. Without endless love of my father and mother, I have no doubt that success in any capacity would be unattainable for me. I am very grateful to my parents, elder brothers and sisters.

Sincere thanks from the depth of my heart to my supervisor Dr. Syed Ali Imran Bokhari for his valuable suggestion, consistent help in research, experimental work and compilation of this thesis. I express my deep gratitude and special thanks to Prof. Dr. Zafar Mahmood Khalid for his valuable guidance and enduring support during my research work. A special thanks to Dr. Bashir Ahmad for providing the help in getting the microbial strain and Dr. Islam Ud Din for providing space in lab during my research work.

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Hanif Ullah

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is that to replace glycerol with ethyl or methyl alcohol by transesterification reaction and reduce the oil viscosity. Transesterification reactions are of two types chemical and enzymatic. Chemical transesterification reactions are catalyzed by acids or bases. The yield of 93 % of ethyl esters of fatty acids was observed after one hour when ethanol and oil mixture was mixed together at temperature of 70 °C (Shah *et al.*, 2004).

Modern world have many challenges to face due to increasing population on daily bases like food, shelter and some of other resources which are not directly related to their daily life but have great impact over their life and such limitation includes the increasing demand of biodiesel which compelled scientist to design and though about such fruitful ideas which may overcome the limitations of these diesel using certain other organism like bacteria and fungi for their production. Some of the approaches were very much useful such as the production of biodiesel in industries in which fats and oils are used as a precursor and these are then converted by certain enzymes into biologically important diesels although some other drawback are also their which involves the contamination during extracting of catalyst and it also need an extra dosage of energy because of the need of high temperatures and pressures. Many researchers admitted that lipase have prime uses in catalysis process of many other products which have industrial importance as well (Soumanou and Bornscheuer, 2003). Luo *et al.*, (2006) also worked on some of lipases production from *Pseudomonas fluorescens* (strain B68) and this type of particular lipase was made stationary for the process of transesterification activity for biodiesel preparation.

APPENDICES

ABBREVIATIONS

AA	Amino Acids
Ala	Alanine
ATPase	Adenosine triphosphatase
bp	Basepair
C-terminal	Carboxyle-terminal
DAP	Diammonium phosphate
DEAE	diethyl aminoethyl
Dsb	Disulfide bond
EDTA	Ethylenediaminetetra acetic acid
FAMEs	fatty acid methyl esters
Gly	Glycine
h	Hours
kDa	Kilodalton
LFH	Laminar flow hood
MFP	Membrane fusion protein
min.	Minutes
mM	Millimolar
N-terminal	Amino-terminal
PAGE	Polyacrylamide Gel Electrophoresis
PMSF	Phenylmethylsulfonyl fluoride
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
Ser	Serine
U/mg	Unit/ milligram
U/ml	Unit/ milliliter
V/V	Volume/ volume
W/V	Weight/ volume
W/W	Weight/ weight

ABSTRACT

Lipases chemically named as (triacylglycerol acylhydrolases, EC 3.1.1.3) are industrially important enzymes which has the ability of biotransformation and act upon triglycerides and convert it into their residual compounds free fatty acid and glycerol over an oil-water interface. Lipases are used in detergent, cosmetics, pharmaceutical and other industries. The proposed study was based on isolation, production and characterization of lipase enzyme from *Pseudomonas* sp. For optimum lipase production, growth parameters like time course, inoculum size, carbon sources, selected carbon source concentration optimization, nitrogen sources and fermentation conditions like pH and temperature were evaluated. The incubation period of 24 h using 10 % of inoculum, 3 % of olive oil cake, ammonium nitrate, pH of 7 and 35°C were reported the most favourable for lipase production. Crude extract was subjected to ammonium sulphate precipitation using 20 to 40 % saturation. Ammonium sulphate saturation of 20 % was reported best for lipase purification. The purified lipase enzyme characterization was done for various kinetic parameters. The thermo stability and effect of pH range of (5.6 to 7.2), temperature ranges from 25 °C to 80 °C, organic solvent and different concentration of olive oil substrate on purified extracellular lipase enzyme activity were evaluated. Thermo stability of the enzyme was optimized in temperatures range from (65 to 90 °C) and reported 65.6 kJ/mol activation energy (E_a) for lipase enzyme denaturation was calculated. The highest activity (68 U/ml/min) at pH (7.0) was reported while enzyme activity was obtained in pH range of (5.6 to 7.2). The highest enzyme activity (72 U/ml/min) was obtained at 55 °C and decreased above 60 °C. The K_m and V_{max} of lipase measured by Lineweaver Burk plot was K_m of 0.28 ml and V_{max} of 87.71 U/ml/min. Organic solvents effect was checked on enzyme activity. Maximum lipase activity of (40 U/ml/min) was obtained with methanol, while the minimum activity of lipase (20 U/ml/min) was obtained with n-hexane.

INTRODUCTION

1. INTRODUCTION

Lipases are industrially important enzymes which are chemically named as (triacylglycerol acylhydrolases, EC 3.1.1.3) and these enzymes are again grouped into a sub group of those enzyme which requires H^+ and OH^- for the reaction process to carry out its enzymatic reaction. It has the ability to act upon triglycerides and convert it to their residual compound fatty acid and glycerol over an oil-water interface. Lipases are also having some special properties like selectivity of enantiomers and also help in the esterification of some ester. Lipases are also able to carry out the process like biotransformation. All such properties make lipases an important candidate for industrial use. Some of the other special properties of lipases are its uses in industries like surface active agents to remove the spots and dirt from clothes. Lipases are also used in medicines and pharmaceuticals industries. (Grbaveic *et al.*, 2007). After a lot of study in the late 1990 enormous increase in numbers of lipases and also its groups were achieved. All these achievements have become possible due to biotechnological expertise like insertion of gene into bacterial cell which will then express and increasing the chances for production of lipases and all these increases the market value of lipases. This also increase the demand for lipases which having narrative and specific properties like stability, pH, specificity, and temperature (Bornscheuer *et al.*, 2002)

Discovery of bioelements are always been of a prime importance and always adds to both its industrial use and commercialization. Lipases were discovered by Claude Bernard in 1856 which was studied during fat digestion in pancreases (Peterson and Drablos, 1994). His discovery makes it possible to identify some other lipases and which have different origin of their extraction like plants, animals, bacteria and fungi. The historical background of lipases shows that it was first time reported as a proper group of enzyme by Claude Bernard in 1856 after studied fat digestion in pancreases. Some lipases were investigated early in 1901 from some group of bacteria like *Bacillus pyocyaneus*, *Bacillus prodigiosus*, and *Bacillus fluorescens* today known as *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Pseudomonas fluorescens* respectively (Hasan *et al.*, 2006). There are some group of lipases which are isolated from some microorganism like bacteria and specially from those bacteria which are having an extra peptidoglycan layer and these bacteria are called *Pseudomonas* which contributes to large number of lipases and these genera from which lipases are isolated are *Pseudomonas aeruginosa*.

1999). The hydrolase folding of these enzymes having active site which contains many residues which are positive charge loving and they are intended to positive charge and these residues includes serine, glutamate or aspartate and histidine. Those portions which are having catalytic activity contain residues of glutamate or aspartate residue. Serine active site is highly conserved structurally which is present at most conserved region (Joseph *et al.*, 2008). Those lipases which are isolated from the strain of bacteria that is *Bacillus* the Gly amino acid is substituted by Ala residue in those most conserved region which remain same throughout the species (Jaeger *et al.*, 1994).

Most lipases having a lid composed of α-helices and present on those site of enzymes which are exposed to co factor or co enzyme to which it may act easily and it moves down to make enzyme available for substrate interaction (Angkawidjaja and Kanaya, 2006).These properties are not same in all lipases because lipases from *Candida Antarctica* and *Pseudomonas glumae* having a lid but not able to perform interfacial activation (Schmid and Verger, 1998). Some lipases from *Pseudomonas* having calcium binding site (Figure 1.2) present near the active site having no role in catalytic activity of the enzyme but stabilize the general structural of the enzyme.

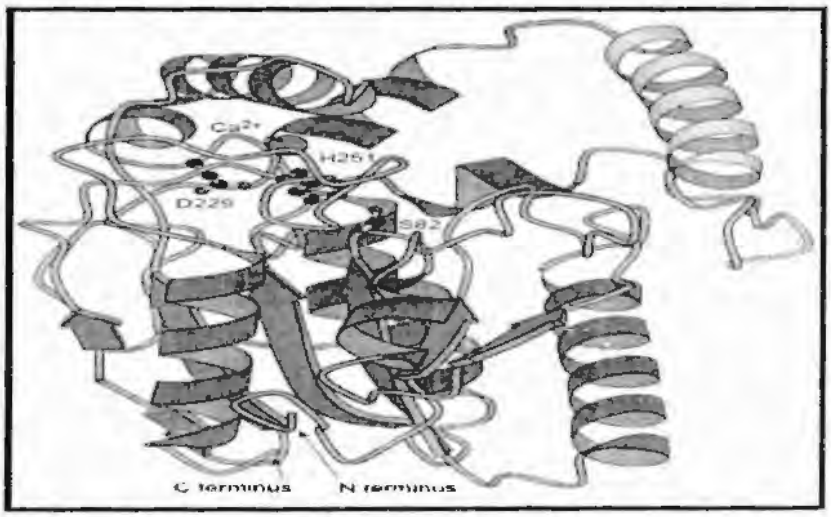

Figure 1.2 3D structure of *Pseudomonas aerogenosa* lipase (Jaeger and Reetz., 1998)

Pseudomonas lipases are grouped on the bases of amino acid homology and other properties into three groups (Zhang *et al.*, 2009). Group I constitute the group which contain lipases from *Pseudomonas aeruginosa, Pseudomonas fragi* and *Pseudomonas alcaligenes*. The

lipases of this group consist of 285 amino acids with 30,000 dalton molecular weight. The structural analysis of lipases showed that lipases of group I having disulfide bond formed by two cysteine residues. The lipases of this group having an N-terminal sequence and required lipase specific foldase enzyme for their secretion and proper folding. Group II lipases consist of 320 amino acids with 33,000 dalton molecular weight and structurally it having N-terminal sequence and one disulfide bond. This means the core structure remain the same in all lipases and these lipases are isolated from *Pseudomonas glumae* and *Pseudomonas cepacia* and are grouped as a group II. Group III constitute a larger group of *Pseudomonas* lipases which contain 475 amino acids with 50,000 dalton molecular weight. *Pseudomonas fluorescens* lipases are placed in this group (Arpigny and Jaeger, 1999).

Group III lipases are structurally different from other two groups because they do not require any enzyme for their folding neither they contain an N-terminal signal sequence nor they contain cysteine residues and on the bases of these structural differences these lipases are thought to be having different secretion pathways. Lipases from Group I and II use secreton-mediated secretion pathway also called type II secretion pathway while group III lipases uses ABC exporters also called type I secretion system for their secretion (Rosenau and Jaeger, 2000). As mentioned above, although Group III lipases contain C-terminal sequence instead of N-terminal sequence and this is responsible to secrete these lipases through ABC exporter (Figure 1.3) (Amada *et al.*, 2000).Three different proteins an inner membrane ATPase, membrane fusion protein (MFP), and outer membrane protein are required for type I secretion pathway. MFP are connected with inner and outer membrane and act like a bridge (Rosenau and Jaeger, 2000). Through this system lipases are directly secreted into the extracellular area and not required an extra step like type II secretion pathway.

Both the group I and II lipases have an N- terminal sequences that help in the secretion of lipases though the transmembrane channel though special mechanism called Sec dependent mechanism. This contains protein known as Sec translocase having many subunits. Sec translocase recognised the amino terminal of lipases sequences. Signal sequence is removed, lipase interact with its specific foldase and secreted through the inner membrane (Rosenau *et al.*, 2004). Before interacting with foldase these lipases are inactive and when they interact so lipases get activated in cell periplasm. Furthermore, this process is based on Dsb (disulfide bond formation)-proteins and after this lipases are then transported through outer membrane and

are esterification and transesterification and both the processes are catalyzed by lipases. Some other carbohydrates like oil can also be changed from one form to another form by using enzymatic activities of lipases. Some of the products may be enhanced by its quality and its nutritional value can be increased or enhanced through use of the industrial process like glycerol are modified into triacylglycerols which is particularly having a low energy but high nutritional values such as cocoa butter (Hasan *et al.*, 2006).

The constituents of Cocoa butter varies like some of its key constituents which are palmitic and stearic acids and the physical properties of these like its melting points is approximately normal room temperature that is 37 °C which impart it the property of melting in human mouth. Unilever tested number of experiments in 1976 to prepare and produce cocoa butter at industrial level using lipases in static conditions which was mainly based on immohilization of lipase. The lipase which was used for such function was isolated from bacteria. In some of the transesterification processes lipases carried out function by substituting palmitic acid with stearic acid which form the stearic- oleic- stearic triglyceride which having suitable melting point use for cholotae (Sharma *et al.*, 2001). Some other uses of lipases are to impart special flavour and also taste in some of the cases to different industrial products which involved the process of addition of fatty acid and alcohol due to which the products have different flavour and fatty acid and alcohol are the two important flavours compounds (Hasan *et al.*, 2006).

Although lipase has wide range of usage but it is also used in dairy industries. In dairy industry lipases carried out milk fat hydrolysis. There are some other important industrial foods products in which lipases are used in several ways as an enzyme and also as flavour such is ripening of cheese and production of other cheese like products are carried out in the presence of lipases, it is also used in butter formation at industrial level. The function of lipases to such product have the function of releasing a short chain fatty acid that is (C4 and C6) which is popular for sharp flavour while delicious and smooth are produce with the release of medium chain (C12 and C14) fatty acids (Saxena *et al.*, 1999). Lipase enzyme is also used in the processing of fish meat in which it remove the access of fat from the fish meat through the process of biolipolyses in whieh degradation of lipids take place (Sharma *et al.*, 2001). In 2004 the global industrial enzyme market was estimated $ 2 billion and in 2009 reaches nearly $ 2.4 billion (Hasan *et al.*, 2006).

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1.1 AIMS AND OBJECTIVES

- Effect of various carbon sources on lipase production.
- Effect of various nitrogen sources on lipase production.
- Effect of pH and temperature on lipase production.
- Purification of lipase.
- Effect of pH on lipase activity and stability.
- Calculation of various kinetics parameters (*Km* and *Vmax*) to find enzymatic reaction rate.
- Effect of various organic solvents on lipase activity.

2. REVIEW OF LITERATURE

Saeed *et al.*, (2005) evaluated the purification of two different lipase enzymes and reported *Pseudomonas aeruginosa* Ps-x was able to produce these two lipases. The purification of these enzymes was performed using ammonium sulphate precipitation. Both the lipases were monomeric in nature and one having (15.5 KDa) and other having (54.97 KDa) molecular weight. The most favourable activity by lipase I was found at 45 °C and lipase II at 50 °C. Similarly both the lipases I and II gave maximum yield at pH 10.0 and 9.0 correspondingly. Calcium ions having great effect upon these two enzymes and increased their thermostability. The purified lipases are reported that they are not dependent on metal ion for their activity. While using EDTA solution upto 10 mM slightly inhibit the activity of these lipases. Protease inhibitor based on serine and PMSF are reported that they also inhibit the activity of these two purified lipases.

Fujii *et al.*, (2005) reported the directed molecular evolution of *Pseudomonas aeruginosa* lipase to increase their amide hydrolyzing (amidase) activity. Mutants were derived by random mutagenesis and all the mutants were screened for their hydrolytic activity using oleoyl 2-naphthylamide and oleoyl 2-naphthyl ester. Five mutants were identified with 1.7–2.0 fold increase in relative amidase activity using oleoyl 2-naphthyl ester. There were different location of mutations (A213D, F207S and F265L) that were having effect upon amidase and esterase activity ratio. The study indicated that the activity was higher in those mutants which were double mutant A213D/F207S and reported the 1.1 min^{-1} most favourable molecular activity using amide as a substrate. The increase in mutant was double as that to the normal types of wild type of lipases. The stercochemistry of lipases shows that alteration occurred in those sites which were near to the catalytic triad and closer to the sites to which calcium binds. The reported study open a gate way to understand that the serine proteases and lipases are similar in their active site structure and the reaction mechanism then why lipases don't hydrolysed amide. The study also provide base to perform amides biotransformation by preparing acyl group transfer catalyst.

Singh *et al.*, (2006) reported the isolation of a bacteria from soil capable of lipase production and their lipase was subjected to hydrolyze the (±)-methyl trans-3(4-methoxyphenyl) glycidate. This is used as intermediate in the drugs to cure

Kumar *et al.*, (2016) stated that lipases are industrial biocatalysts and involved in several novel reactions occurring in aqueous medium as well as non-aqueous medium. Lipases are well-known for their remarkable ability to carry out a wide variety of chemo-, regio- and enantio-selective transformations. Lipases are preferred to use at large level because of its property that it can be easily handled in the field of organic chemistry. Lipase has good performance in those solvent which are organic in nature and are non-polar. It is very important to have knowledge about lipases to use and to increase its uses range. When lipase was studied properly scientist came to know that the interfacial action help researchers in getting knowledge how the structure of these lipases have a relationship with the function which is always been an interesting topic for researchers, enzymologist etc.

Cai *et al.*, (2016) conducted experiment on bacterial strain which were having abilities of producing lipase and was confirmed as *Pseudomonas synxantha* PS1 by 16 S rRNA sequence and study. The further study on the strain reported that it gave a maximum enzymatic activity 10.8 U/mL after culturing for 48 h at 30 °C, with lactose (4 g/L) as carbon source, tryptone (8 g/L) as nitrogen source, olive oil (0.5 %, v/v) as inductor, and the initial pH 8. The lipase gene from the selected strain was cloned and expressed in *Escherichia coli* BL21 with the vector pET28a. The novel gene (*lipPS1*) has an open reading frame of 1425 bp and encodes a 474 AA lipase (LipPS1) sharing the most identity (87 %) with the lipase in *Pseudomonas fluorescens*. LipPS1 preferably acted on substrates with a long chain (C_{10}–C_{18}) of fatty acids. The optimum pH and temperature of the recombinant enzyme were 8.0 and 40 °C respectively. The lipase was activated by 15 % (*v/v*) methanol (112 %), 15 % ethanol (127 %), and 15 % *n*-butyl alcohol (116 %). LipPS1 presented strong biodegradability of waste grease 93 % of waste grease was hydrolyzed into fatty acid after 12 h at 30 °C. This preliminary study of the biodegradability of waste greases shows the potential value in industry applications.

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cardiovascular disease. The selected lipase performed this specific hydrolysis as a result 99 % enantiomeric excess required (±) methyl trans-3(4-methoxyphenyl) glycidate in 44 % yield. 16S rRNA direct sequencing of the isolated bacteria was performed and was confirmed as *Pseudomonas aeruginosa*. The production of lipases is related with some of the other parameters in physiochemical condition which was studied using flasks for lipase production. Some of the best nitrogen sources like beef extract was used to produce lipase. The best conditions that were considered top for favourable cultivation was including pH 8 and 30 °C. The lipases production is directly related with the age and concentration of inoculum. Both the parameters for inoculum have gained positive effect on lipase production and 3 mM of (±)-methyl trans-3(4-methoxyphenyl) glycidate was the best inducer for lipase production.

Devanesan *et al.*, (2007) conducted experiment for the production of biodiesels and he investigated that by the process of transesterification esters are produced which may lead as precursors for the production of biodiesel. This process is also used as an alternative process for biodiesel production. A commercial immobilized *Pseudomonas fluorescens* lipase was used in the biodiesel production from Jatropha oil through the process of transesterification. Batch process is used to make *Pseudomonas fluorescens* stationary through sodium alginate. Maximum production yield of biodiesel was obtained at 40 °C temperature, pH of 7.0, and 3 g beads amount after 48 h reaction time. Some of the physical parameters were compared with that petroleum which is produced through conventional methods and it was concluded that these biodiesel are very much affective as compared to that of conventional.

Shah and Gupta, (2007) reported that monoethyle esters of the long chain fatty acids (biodiesel) were prepared by alcoholysis of Jatropha oil. In this study different process were optimized like commercially valuable lipases preparation, immobilization, pH tuning, water content of the media, enzyme amount that used for reaction and reaction temperature. The most excellent yield 98 % (w/w) was reported in the study using *Pseudomonas cepacia* lipase which were immobilized using celite at 4 to 5 % (w/w) water concentration, 50 °C temperature, and 8 h reaction time. It was reported that both the analytical grade alcohol and commercial grade alcohol give same yield. The activity of the enzymes remains unchanged even using it after four times.

Salis *et al.*, (2008) experimented that a group of commercially important lipases were characterized and immobilized on macro porous polypropylene via physical adsorption. These lipases were made stationary using a solid state medium which was macro porous in which physical absorption at the surface of the stationary phase took place. The efficiency of lipases was studied and study showed that there was mark adaptation to the support when its catalytic characteristics were observed. These lipases were used in the process of methane breakdown to produce biodiesel from it in the stationary phase. Different lipases of different origin means from the organism which produce it and it was indicated that the performance of *Pseudomonas fluorescens* lipase were having the best performance (ester yield = 58 mol % after 22 h). The second good performance was shown by *Pseudomonas cepacia* lipase (ester yield = 37 mol % after 51.5 h) under the same condition in case of medium and it were also studied in stationary phase and lipases from different fungal strains were found with zero percent activity. In the study effect of triglyceride, water content, reaction temperature and enzyme loading was also investigated. Each reaction have proceed in specific condition of temperature, pressure pH and water content and for this reaction the optimal condition were 30 °C temperature, 0.5 mg water/mg, and 600 mg lipase for loading. The above optimize condition yielded an ester of 98 mol % after 70 h reaction period when soybean oil was used as a substrate.

Ruchi *et al.*, (2008) reported that solvent tolerant *Pseudomonas aeruginosa* strain has heen studied for lipase activity. This strain has earlier been reported to be secreting alkaline and solvent stable protease. It produced an extra cellular lipase with suitable properties for detergent applications viz. (i) alkaline in nature, (ii) stability and compatibility towards bleach oxidants, surfactants and detergent formulations and (iii) resistant to proteolysis. Since the culture supernatant contains both protease and lipase which are together required in detergent formulations. Enzymes from *Pseudomoas aeruginosa* seem ideal for use as detergent additive. *Pseudomoas aeruginosa* lipase exhibited remarkable stability in wide range of organic solvents at 25 % (v/v) concentration. This property can be useful for solvent bioremediation and biotransformation in non-aqueous media. Media optimization for cost effective

production of lipase was carried out by response surface methodology which led to 5.58 fold increase in lipase production (4580 IU/ml) over un-optimized media.

Borkar *et al.*, (2009) isolated and purified an extra cellular lipase from the culture broth of *Pseudomonas aeruginosa* SRT 9 to apparent homogeneity using ammonium sulphate precipitation followed by column chromatographic techniques resulting in a purification factor of 98 fold with specific activity of 12307.8 U/mg. Molecular study of lipase were identified by using sodium dodicyle sulphate polyacrylamide gel electrophoreses to know it molecular weight which was estimated to be of 4.5 isoelectric point with 29 kDa molecular weight. The activity of lipase was highest at standard condition of temperature and pH was 55 °C and pH 6.9. It is very easy process for lipases to act and convert relatively large fatty acid chain to its smaller subunit ranges in carbon size of 14-16. EDTA has the ability to lower down the enzyme activities suggesting the enzyme might be metalloprotein. Metal ions like Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, and SDS down regulates the enzymatic activity of lipase. The properties like stability in organic solvents make this lipase an excellent biotechnological tool which having uses in preparation of enantiomeric medicinal products and in organo synthetic reactions. The value K_m (1.11 mmol/L) and V_{max} (0.05 mmol/L/min) of the selected lipase enzyme for triolein substrate hydrolysis were calculated.

Salis *et al.*, (2009) investigated the influence of the support surface on the loading and the enzymatic activity of the immobilized *Pseudomonas fluorescens* lipase. Different porous materials like polypropylene, polymethacrylate, silica and an organosilicate (MSE), were used as supports. The immobilized biocatalysts were compared towards sunflower oil ethanolysis for the sustainable production of biodiesel. Since the supports have very different structural and textural features. In order to consider only the effect of the support surface the experiments were performed at low surface coverage. The different functional groups occurring on the support surface allowed either physical or chemical adsorption. The lipase immobilized on the MSE was the most active biocatalyst. However in terms of catalytic efficiency (activity/loading) the lipase immobilized on the silica allowed the lowest loading which was the most efficient.

Jayaraman and Ilyas, (2010) conducted a research on lipase from *Pseudomonas* sp. which was isolated from soil fields like rhizosphere soil. Some mutants were

produced through ultra violet radiations and some other chemical like Sodium Azide and EMS. The study indicated that the lipase activity of the mutant strain using chemical mutagen was 2-fold higher than the wild strain.

Li and Yan, (2010) evaluated an unidentified techniques which were helpful in the process of biological diesels production through Sapium sebiferum oil and this catalytic reaction was carried out by lipase extracted from *Pseudomonas cepacia* G63 which was made in the laboratory. Different factors were studied and their independency and significance were confirmed. The effects of significant factors on biodiesel production were evaluated by using box–behnken design. The optimal conditions like methano/oil molar ratio (4:1), lipase of 2.7 % (w/w) and 41 °C temperature for biodiesel production were evaluated. The maximum yield of biodiesel 98.19 % were obtained with above optimal conditions. It was also found that there will be no loss in immobilized lipase activity while using repeatedly for 20 cycles at the above optimal reaction conditions.

Narasimha *et al.*, (2011) explored different uses of lipases specially their large scale use that of industrial role which includes different activity as a catalyst and all those reaction which are carried out by lipases are breakdown of triglycerides, inter conversion of esters and the important one is chiral synthesis of ester under standard condition of temperature and pH. In this study the lipase enzyme producing bacterial strain was isolated from soil contaminated with groundnut oil and identified as *Pseudomonas* sp. based on its morphological, physiological and biochemical characteristics. The optimal parameters for lipase enzyme production were carried out by using different carbon and nitrogen sources in the medium maintained at pH-7 through submerged fermentation. Various chemical and physical conditions carried out in the present study, Olive oil, Glucose and Ammonium sulphate were served as best carbon and nitrogen sources for lipase production by *Pseudomonas* sp. grown at 30 °C after 24 h incubation period.

Mobarak-Qamsari *et al.*, (2011) reported that lipases are important because of their wide range of properties and uses in each and every industrial level. Catalysis of long chain fatty acids, acylglycerol and oil can be easily carried out by lipases. The selected strain *Pseudomonas aeruginosa* KM110 was confirmed by 16S rRNA

sequencing and their lipase were studied. An overall 3-fold enhanced lipase production (0.76 U mL^{-1}) was achieved after improving conditions of production medium. The olive oil and peptone was found to be the most suitable substrate for maximum enzyme production. Also the enzyme exhibited maximum lipolytic activity at 45 °C where it was also stably maintained. At pH 8.0 the lipase had the highest stability but no activity. It was active over a pH range of 7.0 to 10.0. The lipase activity was inhibited by Zn^{2+} and Cu^{2+} (32 and 27 % respectively) at 1 mM. The enzyme lost 29 % of its initial activity in 1.0 % SDS concentration. Triton X-100 and tween-80 did not significantly inhibit lipase activity.

Tembhurkar *et al.*, (2012) studied an extracellular lipase production by *Pseudomonas* sp. through submerged fermentation. The optimum time for lipase production was found to be 72 h. Lipase production was enhanced when media was supplemented with mustard oil as carbon source and ammonium di-hydrogen phosphate as nitrogen source which was better supported lipase production than organic nitrogen sources. The lipase produced worked optimally at 50 °C, pH 8 and 15 % Olive oil as substrate. Continuous pH assay were applied and mathematical calculations were performed to determine enzyme units.

Zouaoui and Bouziane, (2012) identified six different isolates which were named as (Ps1, Ps2, Ps3, Ps4, Ps5 and Ps6) and these isolates were screened from wastewater on a selective medium agar containing tween 80 or olive oil as the only source of carbon. Ps5 isolate reported highest lipase activity and this isolate were later acknowledged as *Pseudomonas aeruginosa*. The effect of media composition was analysed to maximize the production of lipase. The maximum extracellular lipase present in the broth was purified 4 folds with an overall yield of 19.4 % through the purification procedure of ammonium sulphate precipitation and diethyl aminoethyl (DEAE) cellulose chromatography. The purified lipase had the maximal activity with optimum pH of 7 and optimum temperature at 30 °C for the hydrolysis of olive oil. The enzyme activity of *Pseudomonas aeruginosa* lipase was enhanced by Ca^{2+} and Mg^{2+} but strongly inhibited by heavy metals such as Zn^{2+}, Cu^{2+} and Mn^{2+}.

Gokbulut and Arslanoglu, (2013) isolated an extracellular lipase producing bacterium which was confirmed as *Pseudomonas fluorescens* KE38 by 16S rRNA

sequencing. The selected strain showed psychrotolerant properties with an optimum growth temperature of 25 °C. The lipase enzyme secreted by the selected strain was purified 41.13 fold with an overall yield of 54.99 % and a specific activity of 337.3 U/mg. The molecular mass of purified lipase was estimated to be approximately 43 kDa by SDS-PAGE. The lipase exhibited maximum activity at 45 °C and pH 8.0. The enzyme also showed a broad substrate specificity acting on p-nitrophenyl esters with C8-C18 acyl groups as substrates and was activated by Ca^{2+} and Ni^{2+} at 1 mM. While the enzyme retained its activity levels in the presence of a variety of organic solvents like DMSO and dimethylformamide. High stability, broad substrate specificity and activity at cold temperatures in the presence of organic solvents and metal ions make the extracellular lipase of the selected strain a strong candidate for industrial applications.

Pagu *et al.*, (2013) reported that lipases are able to act over triglycerides and break it to the glycerol and free fatty acids. Twenty lipase producing Bacteria were isolated from oil contaminated soil. One strain in these having ability to produce greater zone of clearance than others which is the indication for superior lipase activity. This strain was then identified on their physicochemical and morphological characteristics. 16s rRNA sequencing was performed and the strain was named as *Pseudomonas gessardii* BLP2. Incubation time, inoculums concentration, medium pH, temperature, carbon and nitrogen sources were optimize for the lipase production. The selected strain reported maximum lipase production at pH 7 and temperature 37 °C after 48 h incubation time. Increased production of enzyme (168.7 Uml-1) was obtained when the cultured medium was supplemented with protease peptone at 1 % concentration. The present study reveals that the selected strain is supreme for the production of extracellular lipase at industrial level.

Cesarini *et al.*, (2014) stated that applications of lipases at industrial level are an interesting topic because of their mild conditions use and versatility. Many lipases have been isolated, studied, improved and are used to catalyzed different chemical reactions. Transesterification of triglycerides with methanol to produce fatty acid methyl esters (FAMEs) is recent application of lipase and the aim was to produce biodiesel. Immobilized and soluble lipases act as biocatalyst for biodiesel production. In present study both the immobilized and soluble preparations of two cold-adapted lipases were evaluated. LipC and its thermo stable variant LipCmut from *Pseudomonas aeruginosa*

42A2 were produced at low cost protocol. Transesterification of soybean oil with such lipases were tested to assess the production process of FAMEs.

Fatima *et al.*, (2015) optimized the production of lipase from *Pseudomonas putida* 922 by modifying various physical parameters such as carbon source, nitrogen source, pH, salt concentration and biochemical parameters of the production medium such as temperature and incubation time of the growth medium. Oil cakes were also used as carbon source to check for an increased production of the enzyme. The bacterium was found to have a maximal growth at pH 10 with the enzyme production being highest (24 U/ml) after 48 hours at 30 °C. The optimum composition of the medium was mustard oil cake as carbon source, yeast extract or peptone as nitrogen source and 1 % sodium chloride concentration. Partial characterization of the enzyme was carried out where the optimum working pH and temperature was found to be 10 and 40°C respectively. Enzyme stability was found to lie in the pH and temperature ranges of 5 to11 and 30 to 40 °C respectively. Partial purification of the enzyme was carried out at 80 % ammonium sulphate saturation. Molecular mass of lipase was determined by SDS PAGE and found to be 45 kDa.

Bharti and Sharma, (2015) isolated a bacterial strain from the diesel oil contaminated soil and screened on tributyrin agar plate for their lipolytic activity which reported outstanding zone of clearance. The selected strain was acknowledged as gram positive rod shaped bacteria and biochemically it were catalase, citrate utilization, methyl red and urease positive. Maximum extracellular lipase activity 28.5 U/ml in the culture supernatant and the maximum intracellular lipase activity 5.6 U/ml from pellet were obtained. Ammonium sulphate precipitation was performed to purify the lipase and the 30-60 % saturation fraction yielded maximum lipase activity. The purified lipase showed stability up to 55 to 60 °C and their activity was decreased above this temperature. In the pH range of 2.0 to 10.0 the lipase activity was stable. Tween-80 and Tween-20 not decreased the lipase activity while SDS caused loss in enzymatic activity. After 12 h incubation of enzyme with organic solvent the enzyme remains 90 % of their activity which indicated that the enzyme was stable in organic solvent.

Kumar *et al.*, (2016) stated that lipases are industrial biocatalysts and involved in several novel reactions occurring in aqueous medium as well as non-aqueous medium. Lipases are well-known for their remarkable ability to carry out a wide variety of chemo-, regio- and enantio-selective transformations. Lipases are preferred to use at large level because of its property that it can be easily handled in the field of organic chemistry. Lipase has good performance in those solvent which are organic in nature and are non-polar. It is very important to have knowledge about lipases to use and to increase its uses range. When lipase was studied properly scientist came to know that the interfacial action help researchers in getting knowledge how the structure of these lipases have a relationship with the function which is always been an interesting topic for researchers. enzymologist etc.

Cai *et al.*, (2016) conducted experiment on bacterial strain which were having abilities of producing lipase and was confirmed as *Pseudomonas synxantha* PS1 by 16 S rRNA sequence and study. The further study on the strain reported that it gave a maximum enzymatic activity 10.8 U/mL after culturing for 48 h at 30 °C, with lactose (4 g/L) as carbon source, tryptone (8 g/L) as nitrogen source, olive oil (0.5 %, *v/v*) as inductor, and the initial pH 8. The lipase gene from the selected strain was cloned and expressed in *Escherichia coli* BL21 with the vector pET28a. The novel gene (*lipPS1*) has an open reading frame of 1425 bp and encodes a 474 AA lipase (LipPS1) sharing the most identity (87 %) with the lipase in *Pseudomonas fluorescens*. LipPS1 preferably acted on substrates with a long chain (C_{10}–C_{18}) of fatty acids. The optimum pH and temperature of the recombinant enzyme were 8.0 and 40 °C respectively. The lipase was activated by 15 % (*v/v*) methanol (112 %), 15 % ethanol (127 %), and 15 % *n*-butyl alcohol (116 %). LipPS1 presented strong biodegradability of waste grease 93 % of waste grease was hydrolyzed into fatty acid after 12 h at 30 °C. This preliminary study of the biodegradability of waste greases shows the potential value in industry applications.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Materials

Materials and equipments like glass slide, cover slip, spirit lamp, dropper, microscope, glass slides, autoclave, wire loop, inoculating loop, shaking and static incubators, Erlenmeyer flasks and petri plates were used in the study.

3.1.1 Chemicals

Chemicals like nutrient agar, phenopthalien, ethanol, acetone, ammonium sulfate, ammonium nitrate, crystal violet dye, gram iodine, decolorizer, safranin, hydrogen peroxide, plasma, urea, DAP (di-potassium hydrogen phosphate), olive oil, citric acid, NaCl, peptone and yeast extract were used in the study. Chemicals of analytical grade were used for study.

3.1.2 Microbial strains

Three bacterial strains were used in the study. *Pseudomonas aeruginosa* and *Pseudomonas florescene* were isolated in Infectious disease lab Room no 127 Department of Bioinformatics and Biotechnology. One strain of *Pseudomonas aeruginosa* was obtained from Dr. Bashir Ahmad (Department of Bioinformatics and Biotechnology).

3.1.3 Sample collection

In present study, to isolate *Pseudomonas* sp. the waste water samples were collected from oil and fats contaminated water of IIUI cafes drainages. The waste water samples were brought to the laboratory in sealed bottles.

3.2 Serial dilution method

All the water samples were subjected to serial dilution upto 10^{-6} concentrion by serial dilution method. Seven test tubes were taken and label with 10, 10^{-1} upto 10^{-6}. Add 9 ml distil water to all of these test tubes. 1ml of waste water sample was taken and transfers to the first test tube label with 10 and mixed well. 1 ml solution was then transfer to next test tube label with 10^{-1}. By repeating the same procedure of transferring 1ml solution to remaining labeled test tubes and dilutions of concentrations (10^{-1} upto 10^{6}) were prepared.

3.2.1 Nutrient agar plates preparation

Nutrient agar plates were used for isolation and purification of *Pseudomonas* sp. Nutrient agar plates were prepared by adding 2 % nutrient agar (20 g/L) in distilled water. Nutrient agar media and petri plates were autoclaved at 121 °C temperature and 15 psi pressure for 15 min. After sterilization the petri plates and media were taken to LFH (Laminar flow hood) for pouring media. About 20 ml nutrient agar media were poured in each petri plate.

3.2.2 Isolation of *Pseudomonas* sp.

After serial dilution of waste water samples, 0.1 ml of dilutions (10^{-3} upto 10^{-6}) were aseptically spread on freshly prepared nutrient agar plates by sterilized spreader. These petri plates were then labeled and transfer to a static incubator settled at 30 °C temperature for 24 h. After 24 h the plates were checked for bacterial growth appeared with characteristic of *Pseudomonas* morphology was isolated and selected for further studies.

3.2.3 Purification of bacterial culture

The purification step (streaking) was carried out to isolate pure culture of *Pseudomonas* sp. The sterile inoculation loop was inserted in bacterial growth and then streak on nutrient agar plates. The streaked plates were than incubated at 30 °C for 24 h. Further single colony was picked up that was grown on nutrient agar plates and streaked on another nutrient agar plate resulted in isolation of pure culture. In this way, the pure culture of *Pseudomonas* sp. was maintained on nutrient agar slants.

3.2.4 Identification of *Pseudomonas* sp.

To identify *Pseudomonas* sp. microscopic observation, Gram staining and biochemical analysis were performed.

3.2.4.1 Gram staining

Small drop of distill water was put on the glass slide. A single colony was picked up with sterile wire loop from purified culture of *Pseudomonas aeruginosa* and *Pseudomonas florescene* and smear on separated glass slides. The glass slide was heat fixed by moving slowly over spirit lamp. A drop of crystal violet dye was added to smear and waited for 1 min. The glass slide was then washed with distilled water to remove

crystal violet dye. Gram iodine was added and wait for 1 min, than rinsed with distilled water. Afterwards, Decolorizer was added for 5 sec and rinsed with gentle stream of distill water. Atlast, Safranin drop was added to cover the smear and wait for 1 min. The stained glass slide was washed with distill water and observed under microscope.

3.2.4.2 Biochemical analysis

Isolated pure culture was then analyzed for identification by biochemical tests including catalase and coagulase tests.

Catalase test

Catalase test identifies bacteria which can produce catalase enzyme. Single drop of hydrogen peroxide solution was poured on a clean glass slide. Single colony of *Pseudomonas aeruginosa* and *Pseudomonas florescene* was removed using sterile wire loop and was immersed in hydrogen peroxide. Bubbling was noted on slide which indicates catalase positive bacteria.

Coagulase test

Bacteria produce coagulase enzyme was identified through this test. Single drop of plasma and distill water were separately placed on each ends of glass slide. Wire loop full of organism was added to plasma and distill water, mixed gently and Clumping of microbe were observed with in 10 sec. Plasma was not added to distill water drop to differentiate any granular appearance of organism from true coagulase clumping.

3.2.5 Selection of *Pseudomonas* sp.

The two isolated strain of *Pseudomonas aeruginosa* and *Pseudomonas florescene* were compared with *Pseudomonas aeruginosa* strain obtained from Dr. Bashir Ahmad. After comparison the obtained *Pseudomonas aeruginosa* strain were selected for further study and lipase enzyme from the selected strain were subjected for optimization, purification and characterization of lipase enzyme.

3.3 Inoculum preparation

0.9 % saline solution was used for inoculum preparation. 10 g of NaCl were added to 100 ml distill water to prepare 10 % stock solution. 0.9 ml from stock solution were taken in a measuring cylinder and add distill water upto 100 ml volume to obtain 0.9 % saline solution. Saline solution was sterilized by autoclaving at 121 ^{o}C temperature

and 15 psi pressure for 15 minutes. 5 ml of sterilized saline solution were added into nutrient agar slant and the selected *Pseudomonas aeruginosa* strain were scrap slowly with a sterile wire loop in a saline solution. The resulting bacterial suspension was used as inoculum.

3.3.1 Citrate buffers preparation

Citrate buffers were prepared by mixing the following two solutions in different proportion given in (table 3.1) to get the required pH. After mixing the 50 ml solution were dilute to 100 ml distill water to prepare 100 ml of each buffer.

(1) 0.1 M solution of citric acid

(2) 0.2 M solution of Di-potassium hydrogen phosphate

Table 3.1 Proportions for required pH

pH	Volume of 0.1 M Citric acid (ml)	Volume of 0.2 M K_2HPO_4 (ml)
4.0	30.7	19.3
4.4	27.8	22.2
4.8	25.2	24.8
5.0	24.3	25.7
5.2	23.3	26.7
5.4	22.2	27.8
5.6	21.0	29.0
6.0	19.9	32.1
7.0	6.5	43.5
7.2	6.4	43.6
7.4	4.5	45.5
7.6	3.2	46.8

3.3.2 Shake flask fermentation technique

The selected *Pseudomonas aeruginosa* strain was analyzed for their lipase production by submerged fermentation using the fermentation media shown in (table 3.2).

Table 3.2 Composition of fermentation medium

Ingredients	Quantity
Peptone	5 gm/l
Yeast extract	10 gm/l
NaCl	5 gm/l
Olive oil	10 ml/l

For lipase enzyme production, 50 ml of fermentation media was transfer to 250 ml Erlenmeyer flasks. Sterilization of flasks were carried out by autoclaving at 121 °C temperature and 15 psi pressure for 15 min. Inoculum concentration of 5 ml was transfer to each test flasks. 5 ml distill water was added to a flask which was marked as a control flask. The test and control flasks were incubated in shaking incubator having 170 rpm speed at 30 °C for specific incubation period. After incubation period the fermented media were analyzed for lipase enzyme activity.

3.4 Extracellular lipase assay

For extracellular lipase assay activity, the titrimetric assay were performed.

3.4.1 Titrimetric assay of lipase.

After incubation time, the content of the fermented flask were aseptically filtered through a filter cloth. The filtrate known as crude extract and were analyzed for extracellular lipase activity. Lipase activity was measured titrimetrically based on olive oil hydrolysis method used by Fatima *et al.* (2015). This method was slightly modified in proposed study. Crude extract of 0.5 ml was added to the test flask, containing 1 ml olive

oil as a substrate, 0.5 ml of citrate buffer (pH 6.0). Distill water were added to the control flask instead of 0.5 ml of crude extract. Both the test and blank flasks were incubated on rotatory shaker with a speed of 170 rpm at 30 °C for 10 minutes. After reaction time, inhibition of the enzyme was carried out by the addition of 1 ml ethanol: acetone solution in 1:1 ratio. Three drops of 0.9 % phenolphthalein indicator was added to the test and control flasks. Titration was carried out against 0.1 N solution of NaOH until the end point (light pink color) appeared. Lipase unit is defined as "The amount of enzyme which releases one micromole fatty acid per minute under specified assay condition".

3.4.2 Lipase unit calculation

Lipase enzyme unit was estimated by the following formula.

$$\text{Enzyme units (U/ml/min)} = \frac{\Delta V \times N \times 1000 \times \text{Dilution factor}}{V\ (\text{sample}) \times T\ (\text{min})}$$

Where;

$\Delta V = V2 - V1$

V1= NaOH volume used for control flask
V2= NaOH volume used for experimental flask
N= NaOH Normality

Dilution factor is obtained due to usage of buffer for extraction.

V (sample) =Amount of enzyme extract taken for the reaction mixture.

T (min) = Time of incubation in minutes.

3.5 Optimization of lipase enzyme activity

3.5.1 Time course production of lipase

Time course of lipase production was studied with the fermentation medium in shake flasks for 48 h. Erlenmeyer flask contain 50 ml medium were inoculated with 5 % (v/v) inoculum and incubated at 30 °C temperature in a shaker incubator at 170 rpm for 48 h. Samples was removed periodically at 12 h interval and lipase activity was determine . The highest lipase production period was selected and the enzyme activity was determined with different interval in that optimized period.

3.5.2 Effect of inoculum size

Different inoculum concentrations were added to the 50 ml fermentation media in 250 ml Erlenmeyer flask to study inoculum effect on lipase production. The flasks

containing 50 ml fermentation media were sterilized by autoclaving and keeping for 121 °C temperature and 15 psi pressure for 15 minutes. Inoculum concentration (5 %, 10 %, and 15 %) was aseptically added to sterilize fermentation media in a test flask. Distill water of above concentration were added to control flask. The test and control flasks were incubated at 30 °C temperature in a rotatory shaker with a speed of 170 rpm for selected time period. Enzyme assay were performed with different time interval.

3.5.3 Effect of carbon source

To optimize best carbon source for lipase production, olive oil in the fermentation media was replace by natural substrates like olive oil cake, mustard oil cake, apricot oil cake and taramira oil cake. The concentrations of (1 % w/v) of each substrate were added into the 250 ml Erlenmeyer flasks for both test and control containing 50 ml fermentation liquid medium. The flasks containing media and carbon sources were sterilized by autoclaving at 121° C temperature and 15 psi pressure for 15 minutes. After sterilization the optimize inoculum concentration were added to test flasks and distilled water was added in place of inoculum into the control flask. Both test and control flasks were incubated at 30 °C on a rotatory shaker at a speed of 170 rpm for selected time period. The enzyme assay was performed with different time intervals. The carbon source supporting highest lipase production was selected for further study.

3.5.4 Effect of carbon source concentration

The selected carbon source was used at various concentrations for upper limit lipase production. The concentrations used were 1-4 %. The concentration yielded maximum lipase production was selected for further study.

3.5.5 Effect of nitrogen source

Different nitrogen sources Ammonium Sulphate (0.71 % w/v), Ammonium Nitrate (0.43 % w/v), Urea (0.32 % w/v) and DAP (0.7 % w/v) were added into the 50 ml fermentation media (table 1.1) with optimize carbon source in 250 ml Erlenmeyer flask. All the flasks were sterilized by autoclaving and keeping for 121 °C temperature and 15 psi pressures for 15 minutes. The flasks were then taken into LFH and optimize inoculum was aseptically added to test flasks. Distill water were added to control flask instead of inoculum. Both the test and control flasks were incubated at 30 °C temperature in a rotatory shaker with a 170 rpm speed for selected time period. The lipase activity was

assayed with different time interval. The nitrogen source supporting maximum lipase production was selected.

3.5.6 Effect of the medium pH and incubation temperature

The effect of pH for lipase production was performed by varying the initial PH of the fermentation media ranging from pH 4 to pH 7. The pH showing maximum lipase production by *Pseudomonas aeruginosa* strain was selected. To optimize most favorable temperature for lipase production various temperature ranges from 30 °C to 45 °C were selected by keep the other optimize parameters same and the optimum temperature for lipase production was selected.

3.5.7 Protein estimation

Total Protein from the crude extract was estimated by using Bradford method (Kruger, 1994). Test tube containing appropriate amount of crude extract i.e. 100 micro liters, 1 ml of Bradford reagent were incubated for 10 minutes at 30 °C temperature. The test tube solution was transferred into cuvette and absorbance was recorded at 595 nm on spectrophotometer (See appendix I). Control blank was prepared in similar way like test sample for analysis except equal volume of distilled water in place of crude extract.

3.6 Partial purification of lipase

After the optimization study the 50 ml fermentation media in a flask containing optimum carbon source, nitrogen source and pH were incubated at optimum temperature in a rotatory shaker for optimize time period. The content of the flask were filter through filter cloth. The filtrate obtain was known as crude extract. The crude extract obtain was assayed for lipase activity and were subjected to ammonium sulfate precipitation (See appendix II).

3.6.1 Ammonium sulphate precipitation

In this solid ammonium sulphate was added to the crude extract at 20 % saturation (w/v). The crude extract were then keep overnight at 4 °C and then centrifuge at 4000 rpm for 5 minutes. The supernatant and pellet were assayed for lipase activity. The supernatant were treated with further 30 % and 40 % concentrations of ammonium sulphate. All the fractions were collected in 10 mM citrate buffer of pH 6.0 and titrimetric lipase assay were applied to all fraction to find optimum lipase activity.

Fraction contain optimum lipase activity were selected and used further for enzyme characterization.

3.7 Characterization of lipase

The enzyme purified from crude extract through ammonium sulphate precipitation was then characterized.

3.7.1 Effect of pH on lipase activity

Citrate buffer of different pH ranging 4.0 to 7.8 were used to study the pH effect on lipase activity. The reaction mixture constaining 1ml olive oil, citrate buffer of 0.5 ml and 0.5 ml enzyme. The control was prepared in similar way by adding distill water to the control flask instead of 0.5 ml enzyme present in test flask. Both the test and control flask for each pH were incubated at 30 °C temperature in a shaker with 170 rpm speed for 10 minutes. The reaction was terminated by 1 ml of ethanol: acetone mixture (1:1). The activity of the lipase was determined under standard assay condition.

3.7.2 Effect of temperature on lipase activity

To optimize this parameter the reaction mixture was incubated with different temperature range from 25 to 80 °C. Standard assay condition was applied to measure enzyme activity.

3.7.3 Effect of different concentration of olive oil on lipase activity

Various concentrations of substrate olive oil range from 0.1 ml to 0.9 ml were used to study olive oil concentration effect on lipase activity. The reaction mixture in a test flask contain different concentrations of olive oil, 0.5 ml citrate buffer (pH 6.0), and 0.5 ml enzyme solution. Distill water were added to the control flask instead of 0.5 ml enzyme present in test flask. Both the test and control flask for each concentration of olive oil were incubated at 30 °C temperature in a rotatory shaker with 170 rpm speed for 10 minutes. The reaction was terminated by 1 ml of ethanol: acetone mixture (1:1). The activity of the lipase was determined under standard assay condition.

3.7.4 Effect of various organic solvent on lipase activity

Ethanol, n-hexane, acetone and methanol with a concentration of 30 % (v/v) were used to study their effect on lipase activity. Reaction mixture in a test flask contains 1 ml olive oil, 0.5 ml citrate buffer (pH 6.0), 0.5 ml enzyme solution and 1 ml of each 30 % (v/v) solution of organic solvent. Distill water were added to the control flask instead of

0.5 ml enzyme present in test flask. Both the test and control flask for each organic solvent were incubated at 30 °C temperature in a shaker with 170 rpm speed for 10 minutes. The reaction was terminated by 1 ml of ethanol: acetone mixture (1:1). Standard assay condition was applied to measure enzyme activity.

3.7.5 Effect of different substrate on lipase activity

Olive oil substrate in a reaction mixture was replaced by other substrate like mustard oil, Apricot oil and tween 80 to study their effect on lipase activity. The reaction mixture in a test flask contain 1 ml of the above substrate, 0.5 ml citrate buffer (pH 6.0), and 0.5 ml enzyme. Distill water were added to the control flask instead of 0.5 ml enzyme present in test flask. Both the test and control flask were incubated at 30 °C temperature in a rotatory shaker with a speed of 170 rpm for 10 minutes. The reaction was terminated by 1 ml of ethanol: acetone mixture (1:1). Titrimetric assay were performed to estimate the liberated fatty acids.

3.7.6 Thermal denaturation of lipase enzyme

The thermal denaturation of the lipase enzyme was performed by incubating the purified lipase at different temperature range from 65 to 90 °C. 3 ml enzyme solution in test tube were incubated in a water bath for 30 minutes at specific temperature and the enzyme assay was performed with the interval of 5 minute periodically. Standard assay condition was applied to measure enzyme activity. The ln (% Residual Activity) and k_d value for the lipase enzyme were evaluated.

RESULTS

4. RESULTS

4.1 Isolation of *Pseudomonas* species

The *Pseudomonas aeruginosa* and *Pseudomonas florescene* were isolated on nutrient agar plates. According to their cultural characteristic *Pseudomonas aeruginosa* produce a greenish blue soluble pigment (Pyocyanin) diffused into the medium. When the plates were observed under fluorescent light, fluorescent color greenish blue was appeared around the dense culture of *Pseudomonas aeruginosa* (figure 4.1). Similarly *Pseudomonas florescene* produce a greenish yellow soluble pigment (Pyoverdin) diffused into the medium. When the plates was observed under fluorescent light, fluorescent color a greenish yellow was appeared around the dense culture of *Pseudomonas florescene* (figure 4.2)

Figure 4.1 *Pseudomonas aeruginosa* greenish blue appearances on nutrient agar

Figure 4.2 *Pseudomonas florescene* greenish yellow appearance on nutrient agar

4.1.1 Purification of *Pseudomonas* species

The *Pseudomonas aeruginosa* and *Pseudomonas florescene* were purified from mixed culture by streaking method. Purified single colonies were obtained after fourth step of purification (figure 4.3 and 4.4).

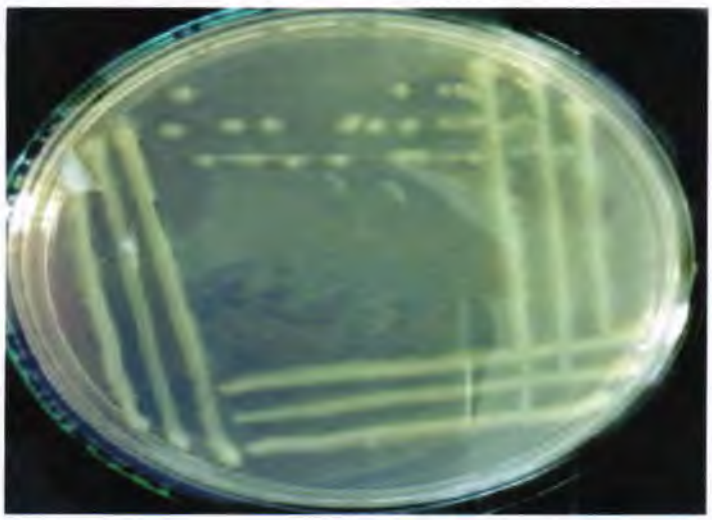


Figure 4.3 *Pseudomonas aeruginosa* purification

Figure 4.4 *Pseudomonas florescene* purification

4.1.2 Identification of *Pseudomonas* species

The *Pseudomonas aeruginosa* and *Pseudomonas florescene* was morphologically identified under microscope (Watanabe, 2002) and further gram staining test, biochemical analysis such as catalase test and coagulase test were performed.

4.1.2.1 Gram staining

The Gram staining results showed that both the *Pseudomonas aeruginosa* and *Pseudomonas florescene* was gram negative rod shaped.

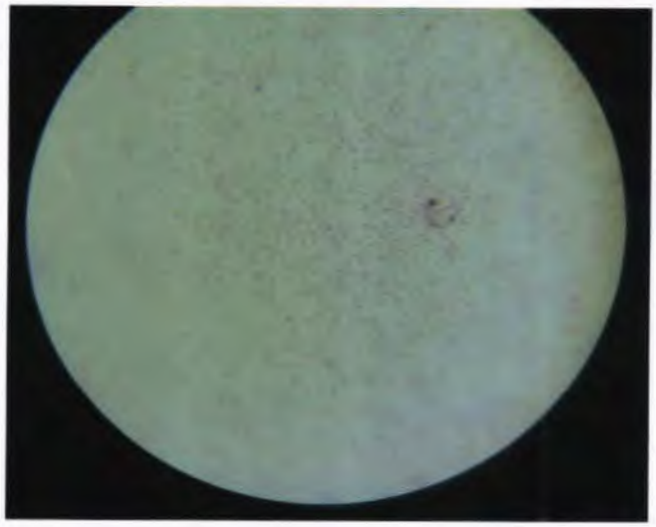


Figure 4.5 *Pseudomonas aeruginosa* gram negative rod shape

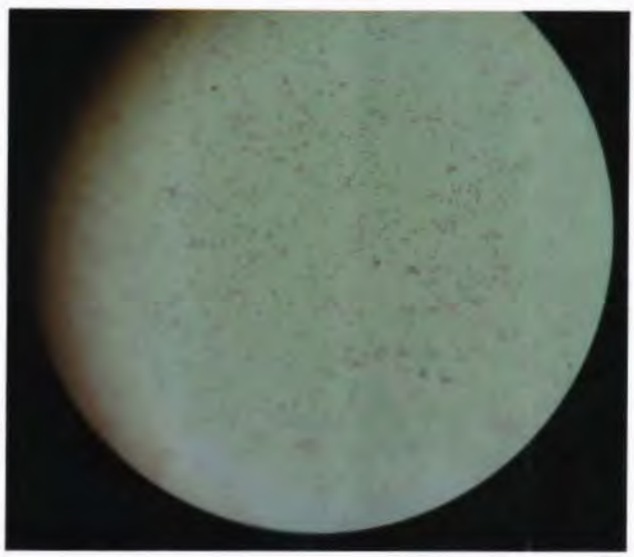


Figure 4.6 *Pseudomonas florescene* gram negative rod shape

4.1.2.2 Catalase test

The catalase test results showed that both the *Pseudomonas aeruginosa* and *Pseudomonas florescene* were catalase positive.

Figure 4.7 *Pseudomonas aeruginosa* catalase positive

Figure 4.8 *Pseudomonas florescene* catalase positive

4.1.2.3 Coagulase test

The coagulase test results showed that both the *Pseudomonas aeruginosa* and *Pseudomonas florescene* was coagulase test negative.

Figure 4.11 Effect of incubation time on a) Extracellular lipase production b) Specific activity

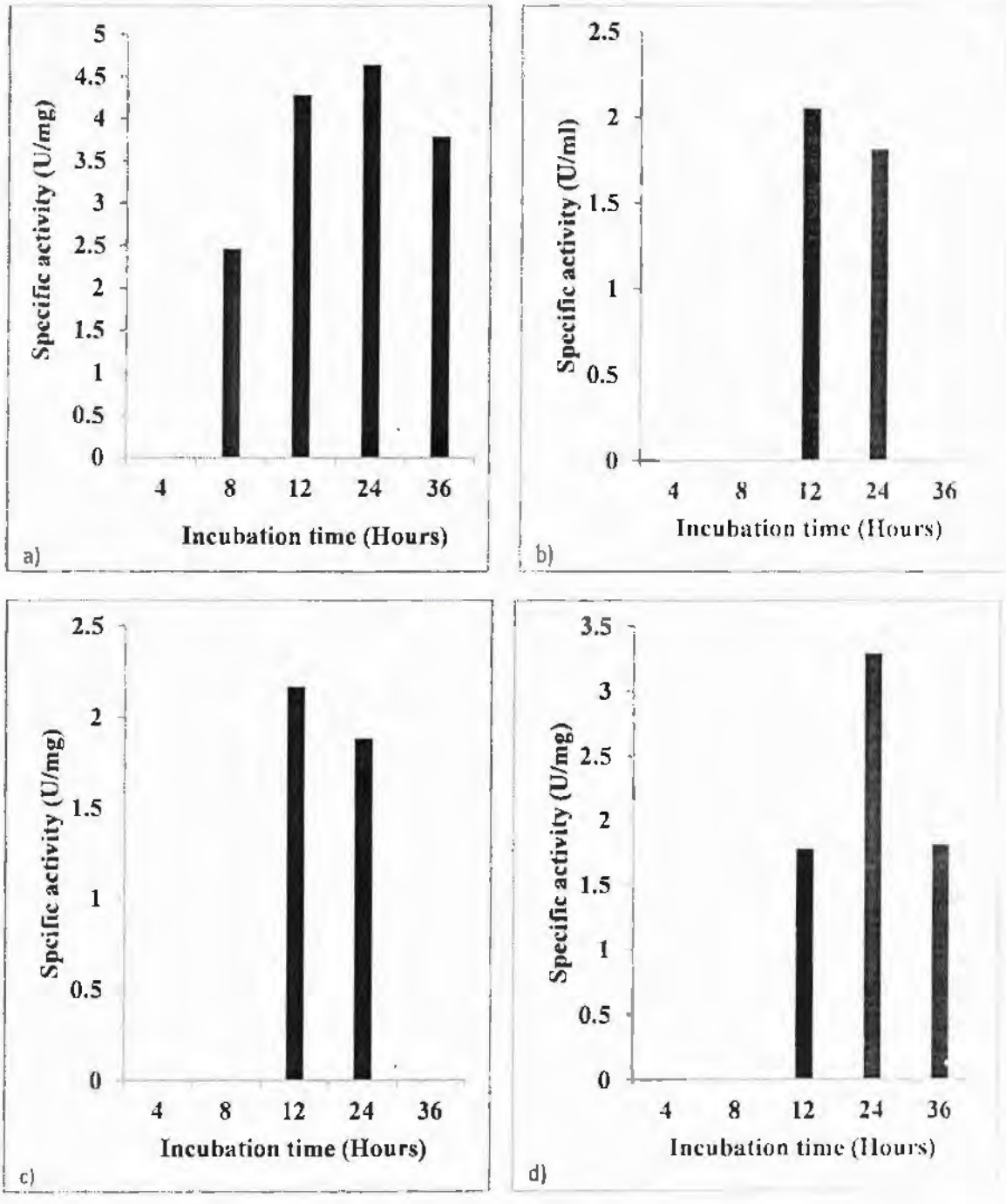


Figure 4.15 Effect of carbon source on specific activity a) olive oil cake b) mustard oil cake c) apricot oil cake d) taramira oil cake

Figure 4.7 *Pseudomonas aeruginosa* catalase positive

Figure 4.8 *Pseudomonas florescene* catalase positive

4.1.2.3 Coagulase test

The coagulase test results showed that both the *Pseudomonas aeruginosa* and *Pseudomonas florescene* was coagulase test negative.

Figure 4.9 *Pseudomonas aeruginosa* (P.A) negative coagulase test

Figure 4.10 *Pseudomonas florescene* (P.F) negative coagulase test

4.2 Optimization of different parameter for lipase production

Various conditions were optimized to acquire the highest production of lipase enzyme from isolated and purified *Psuedomonas* sp.

4.2.1 Time course for lipase production

The effect of incubation period on lipase enzyme activity by *Pseudomonas aeruginosa* was optimized. Olive oil in the fermentation media was used as inducer for lipase production. The whole incubation period was 48 h and lipase enzyme activity was calculated periodically after 12 h interval. The result for 48 h fermentation are shown in (figure 4.11a, table 4.1), while result for their effect on specific activity are shown in (figure 4.11 b). There was a positive correlation of lipase activity with time till 24 h of incubation (4 U/ml). However, when the incubation time increase from 24 h the lipase enzyme activity decrease. The 24 h time period was selected for optimum lipase and lipase enzyme activity was calculated with the interval of 4, 8, 12, and 24 h.

Table 4.1 Effect of incubation time on lipase activity

Incubation time (h)	Enzyme activity (U/ml/min)	Protein assay (mg/ml)	Specific activity (U/mg)
12	2	1.111	1.800
24	**4**	**1.123**	**3.561**
36	2	1.113	1.796
48	0	1.101	0

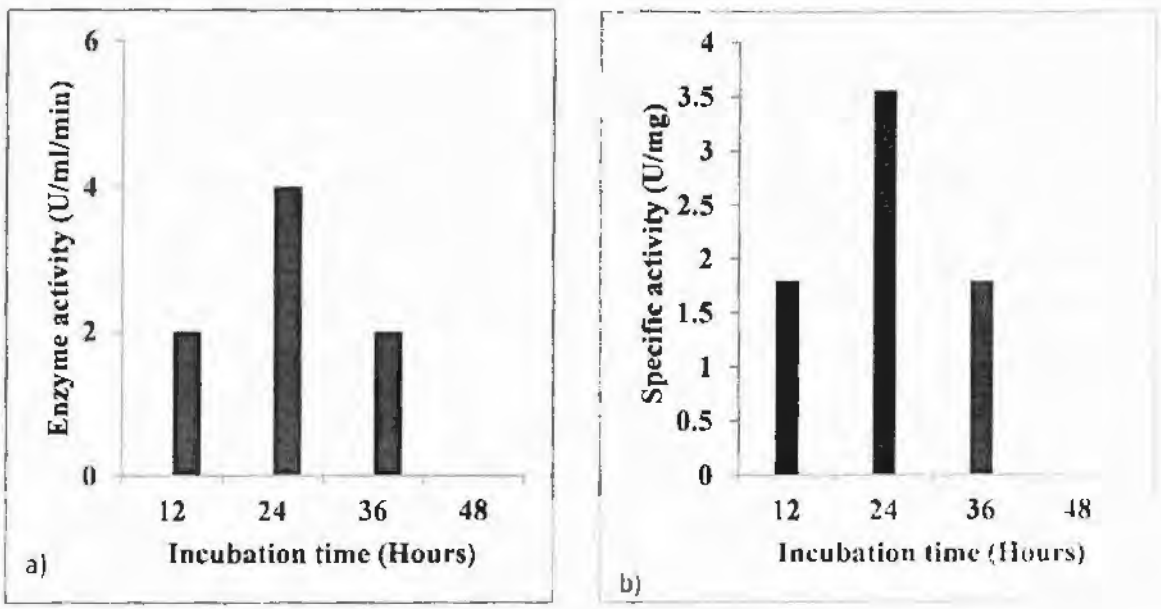


Figure 4.11 Effect of incubation time on a) Extracellular lipase production b) Specific activity

4.2.2 Effect of inoculum size

Effect of different inoculum size was studied for extracellular lipase activity by *Pseudomonas aeruginosa* strain in fermentation media. Result for the effect of 5 %, 10 % and 15 % inoculum on extracellular lipase activity are shown in (table 4.2 and figure 4.12), while result for their effect on specific activity are shown in (figure 4.13). The inoculum size of 10 % yielded maximum extracellular lipase activity (6 U/ml). As the dose of inoculum increase from 10 % to 15 % inoculum there was a gradually decrease of extracellular lipase activity (4 U/ml). The inoculum size of 5 % also yielded low extracellular lipase activity (4 U/ml). Hence 10 % of inoculum was optimize for maximum extracellular lipase activity and were used throughout the study.

Table 4.2 Effect of inoculum size on extracellular lipase activity

Inoculum concentration (%)	Incubation time (h)	Enzyme activity (U/ml/min)	Protein assay (mg/ml)	Specific activity (U/mg)
5	4	0	0.773	0
	8	0	0.901	0
	12	2	1.123	1.78
	24	4	1.228	3.257
	36	2	1.101	1.816
10	4	0	1.091	0
	8	2	1.101	1.814
	12	4	1.250	3.2
	24	**6**	**1.390**	**4.316**
	36	4	1.031	3.879
15	4	0	0.779	0
	8	0	0.801	0
	12	2	0.912	2.192
	24	4	1.123	3.561
	36	0	0.949	0

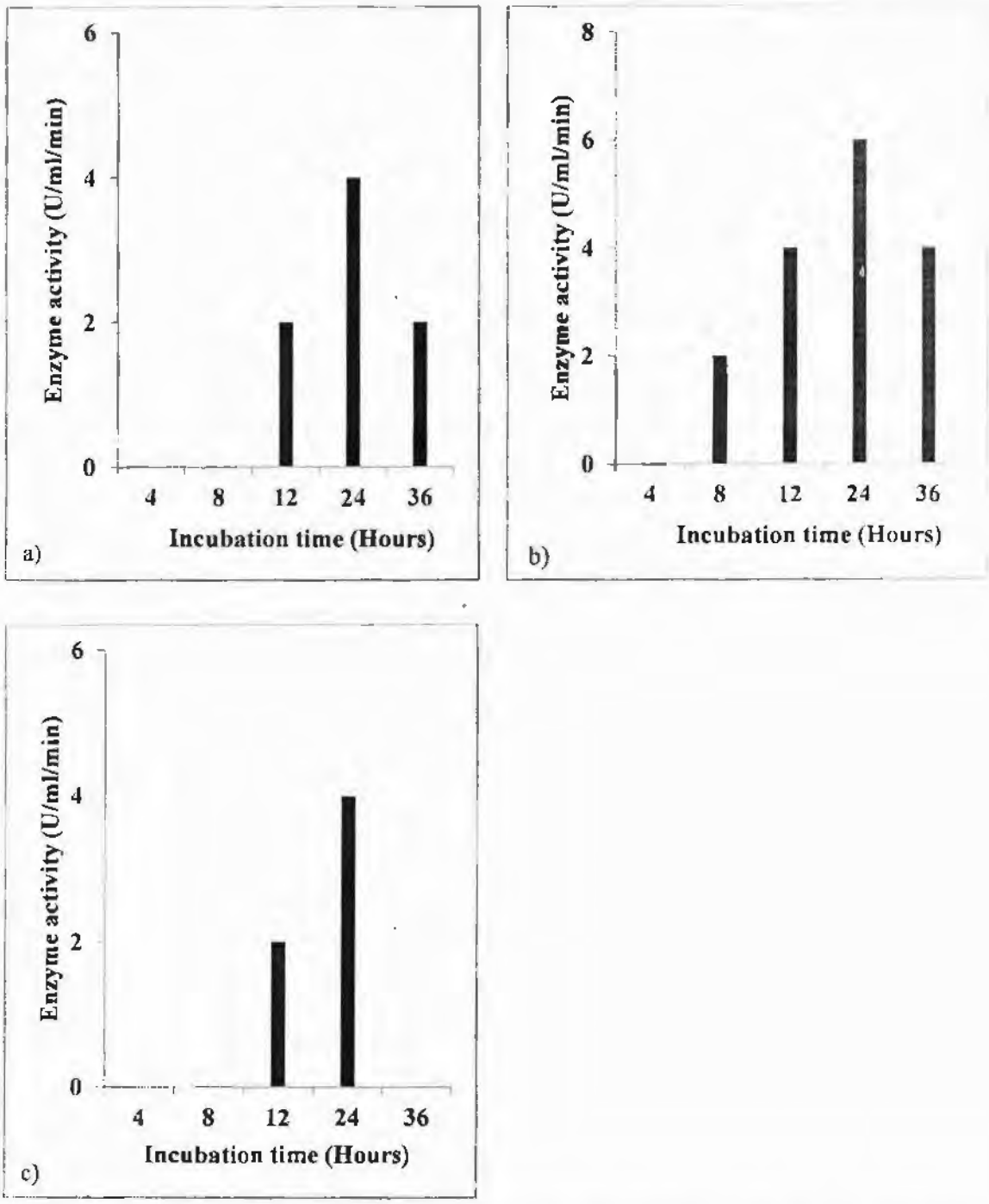
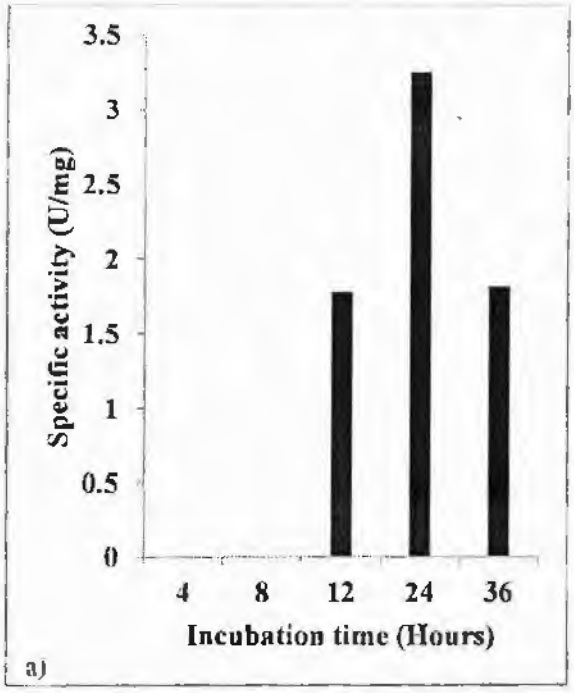
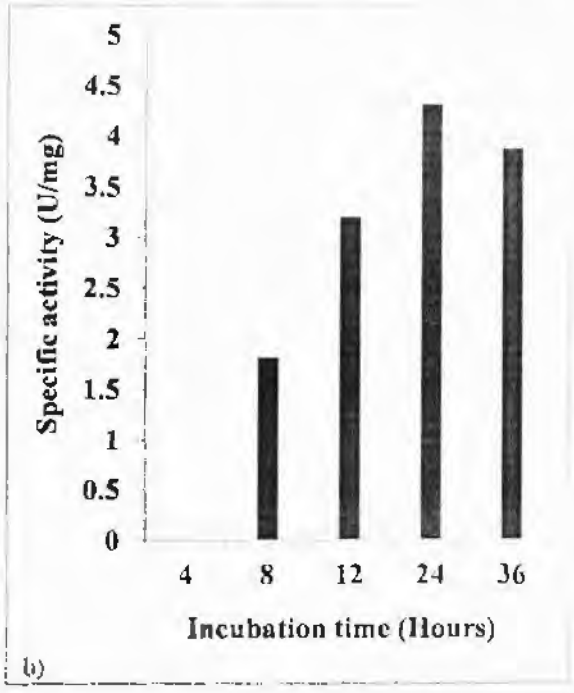


Figure 4.12 Effect of inoculum size on extracellular lipase activity a) 5 % inoculum b) 10 % inoculum c) 15 % inoculum





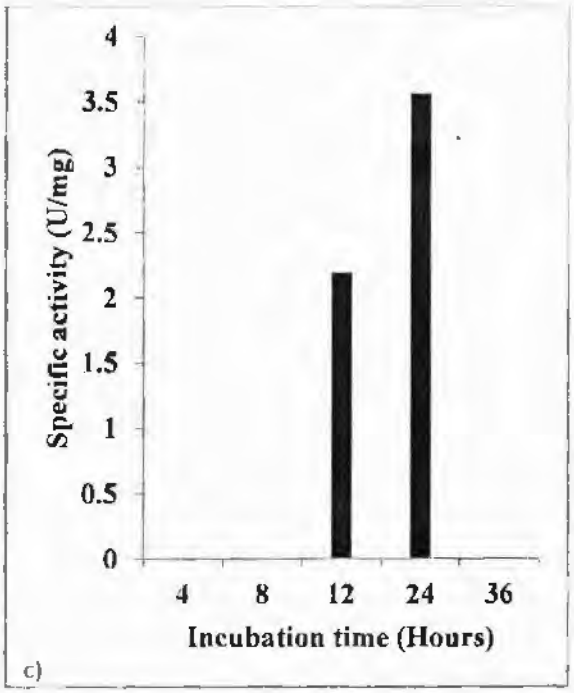


Figure 4.13 Effect of inoculum size on specific activity a) 5 % inoculum b) 10 % inoculum c) 15 % inoculum

4.2.3 Effect of carbon source

To optimize best carbon source for lipase production olive oil in the fermentation media was replace by natural substrates like olive oil cake, mustard oil cake, apricot oil cake and taramira oil cake. The concentrations of (3 % w/v) of each substrate were added into the 250 ml Erlenmeyer flasks for both test and control containing 50 ml fermentation liquid medium. The result for the above substrates on extracellular lipase enzyme activity are shown in (table 4.3 and figure 4.14), while their effect on specific activity are represented in (figure 4.15). Among these four carbon sources olive oil cake showed considerable increase in lipase enzyme activity by *Pseudomonas aeruginosa* strain (6 U/ml/min). The selected *Pseudomonas aeruginosa* strain is reported for maximum protein production (1.291 mg/ml) at olive oil cake after 24 h of incubation period with maximum specific activity (4.647 U/mg). While the other carbon sources showed considerable decrease in lipase activity by *Pseudomonas aeruginosa* strain. Hence olive oil cake was optimized for maximum extracellular lipase activity and was used throughout the study.

Table 4.13 Effect of carbon source on extracellular lipase activity

Carbon sources	Incubation time (h)	Enzyme activity (U/ml/min)	Protein assay (mg/ml)	Specific activity (U/mg)
Olive oil cake	4	0	0.729	0
	8	2	0.811	2.466
	12	4	0.933	4.287
	24	**6**	**1.291**	**4.647**
	36	4	1.054	3.795
Mustard oil cake	4	0	0.846	0
	8	0	0.927	0
	12	2	0.972	2.057
	24	2	1.101	1.816
	36	0	0.782	0
Apricot	4	0	0.611	0
	8	0	0.733	0
	12	2	0.922	2.169
	24	2	1.061	1.885
	36	0	0.813	0
Taramira oil cake	4	0	0.821	0
	8	0	0.933	0
	12	2	1.122	1.782
	24	4	1.213	3.297
	36	2	1.102	1.814

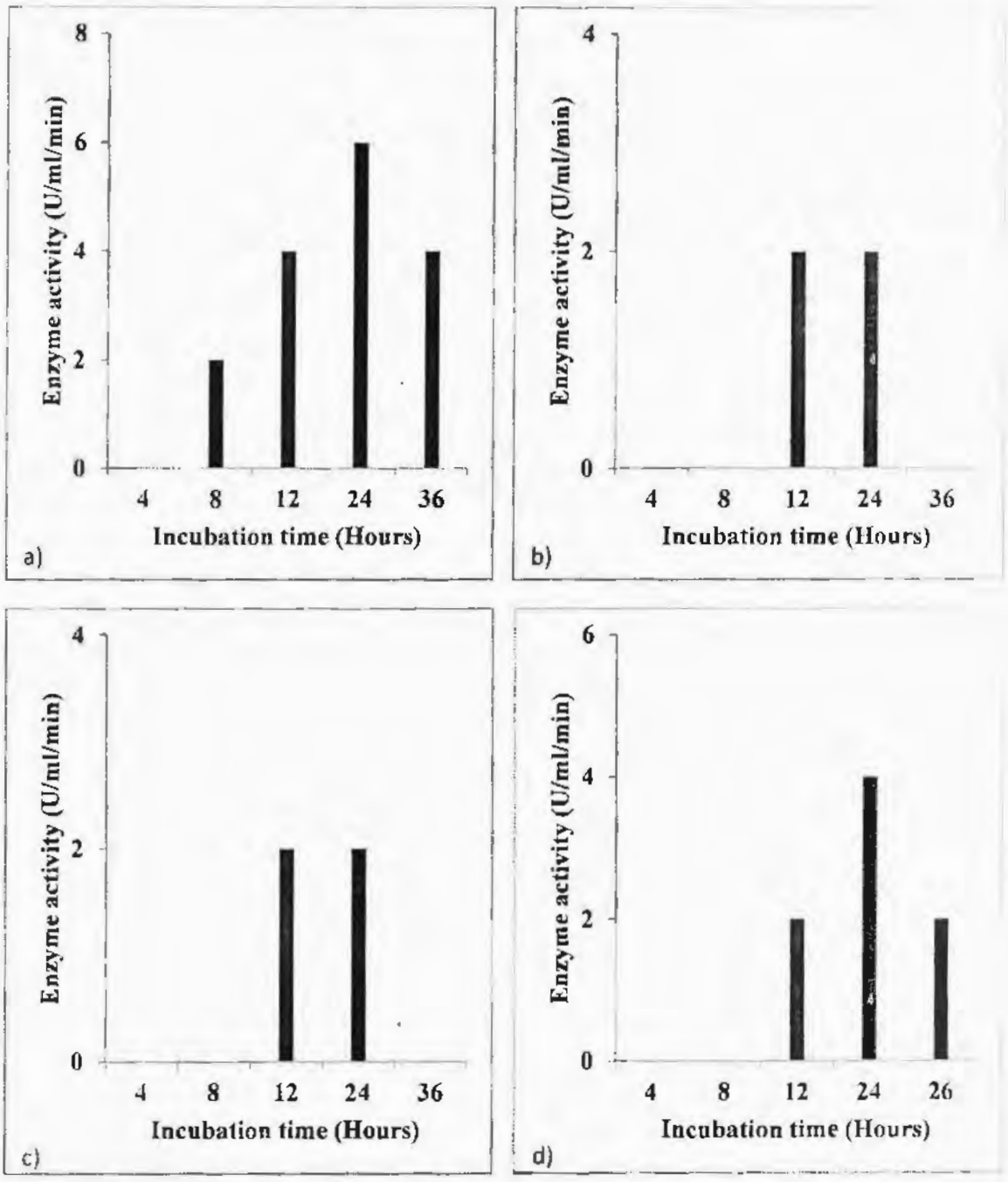


Figure 4.14 Effect of carbon source on extracellular lipase activity a) olive oil cake b) mustard oil cake c) apricot oil cake d) taramira oil cake

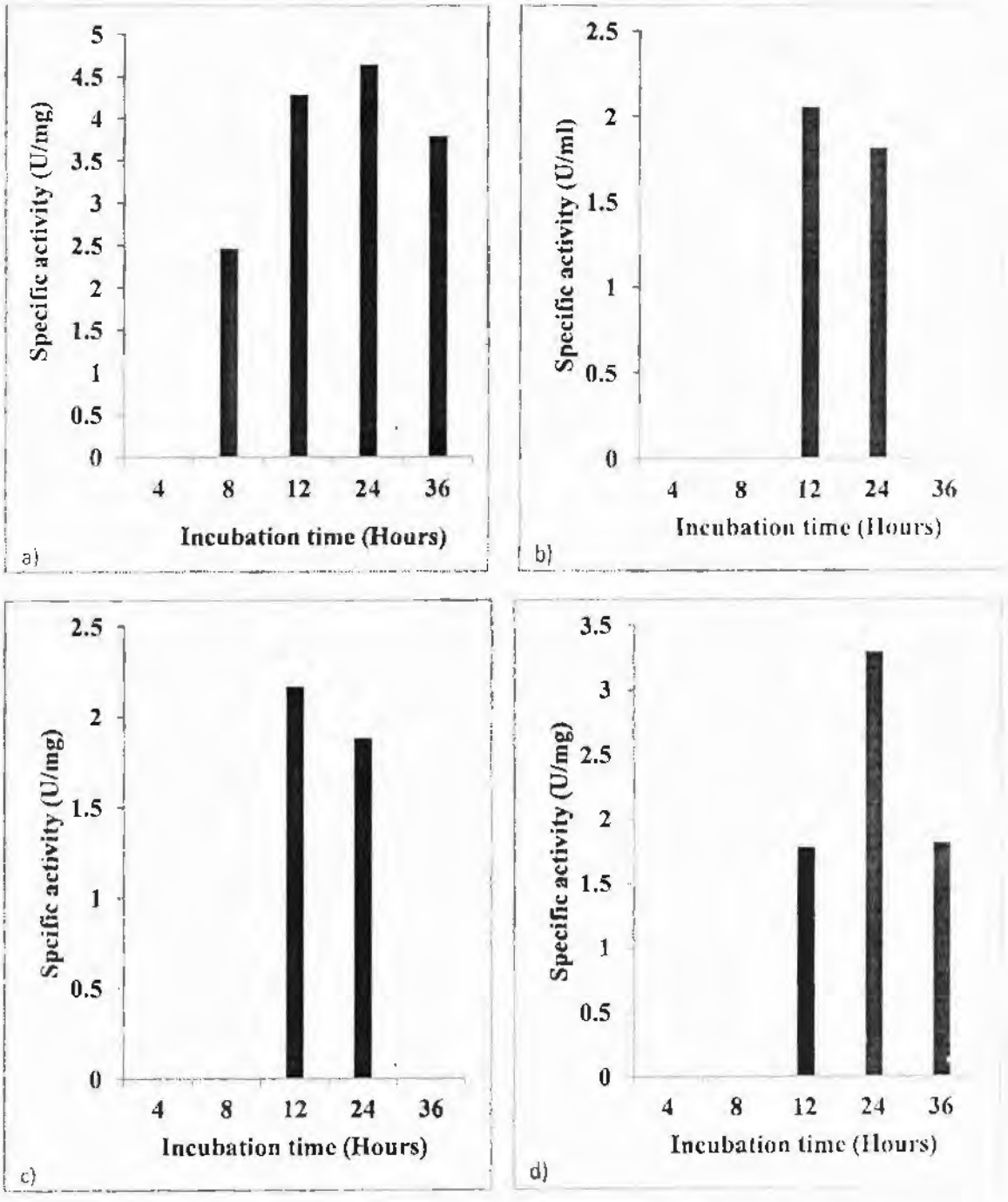


Figure 4.15 Effect of carbon source on specific activity a) olive oil cake b) mustard oil cake c) apricot oil cake d) taramira oil cake

4.2.4 Effect of carbon source concentration

The effect of olive oil cake concentration was evaluated for the production of extra cellular lipase from *Pseudomonas aeruginosa* strain. The result for the effect of 1 %, 2 %, 3 % and 4 % olive oil cake concentration on lipase enzyme activity are shown in (figure 4.16 and table 4.4), while their effect on specific activity are shown in (figure 4.17). Maximum lipase enzyme activity by *Pseudomonas aeruginosa* strain (6 U/ml/min) was obtained at 3 % concentration of olive oil cake. By increasing the concentration of olive oil cake upto 4 % the lipase enzyme activity decreased (4 U/ml/min). The selected *Pseudomonas aeruginosa* strain is reported for maximum protein production (1.218 mg/ml) at 3 % carbon source concentration after 24 h of incubation period with maximum specific activity (4.926 U/mg). The 3 % concentration of olive oil cake were selected and used for further study

Table 4.4 Effect olive oil cake concentration on lipase activity

Olive oil cake (%)	Incubation time (h)	Enzyme activity (U/ml/)	Protein assay (mg/ml)	Specific activity (U/mg)
1	4	0	0.65	0
	8	0	0.733	0
	12	2	0.776	2.577
	24	2	0.911	2.195
	36	0	0.771	0
2	4	0	0.729	0
	8	0	0.811	0
	12	2	0.933	2.143
	24	4	0.991	4.036
	36	2	0.821	2.436
3	4	0	0.621	0
	8	2	0.74	2.702
	12	4	0.96	4.166
	24	**6**	**1.218**	**4.926**
	36	4	1.021	3.917
4	4	0	0.74	0
	8	0	0.810	0
	12	2	0.912	2.192
	24	4	1.237	3.233
	36	2	0.837	2.389

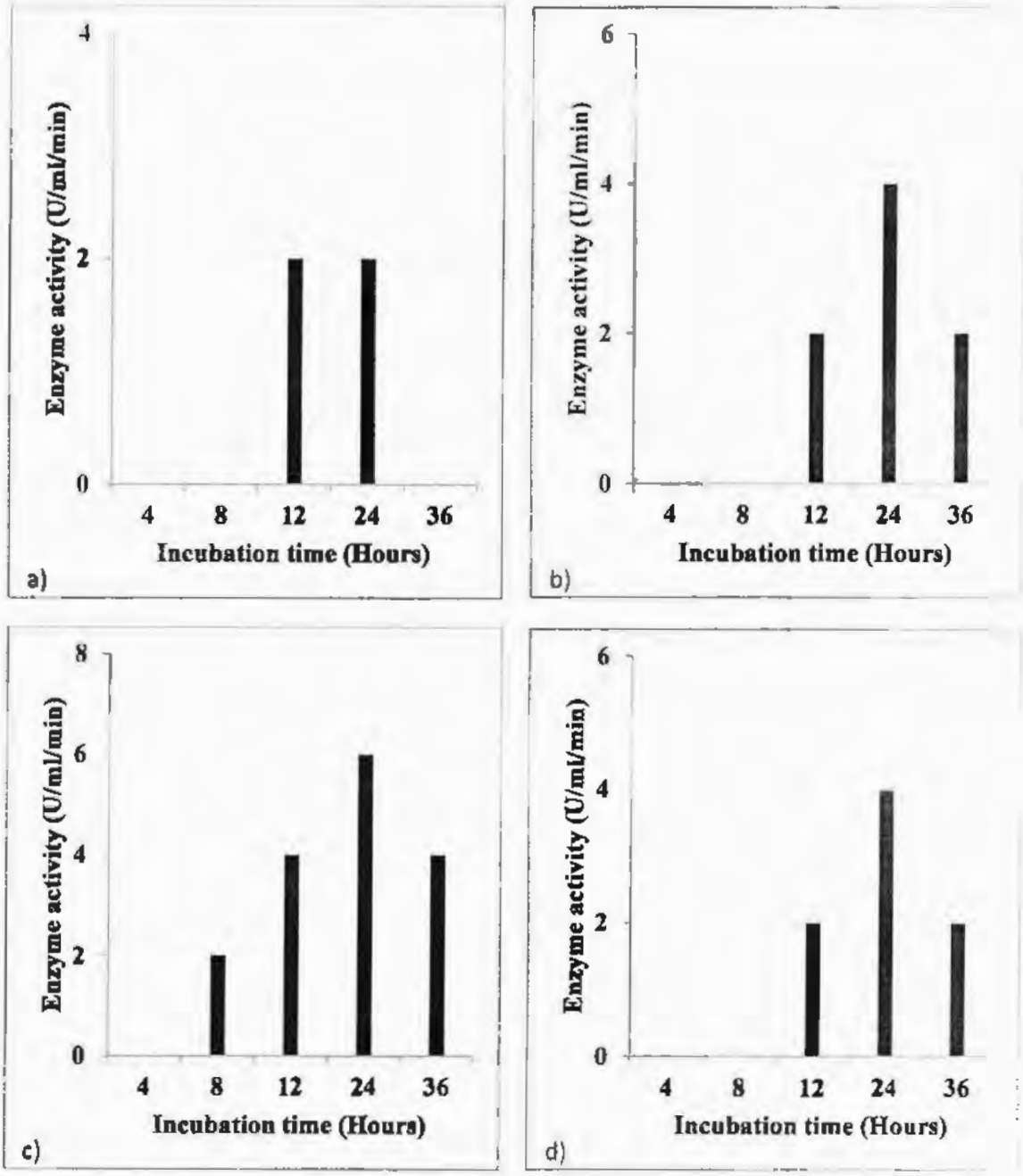


Figure 4.16 Effect of olive oil cake concentration on extracellular lipase activity a) 1 % olive oil cake b) 2 % olive oil cake c) 3 % olive oil cake d) 4 % olive oil cake

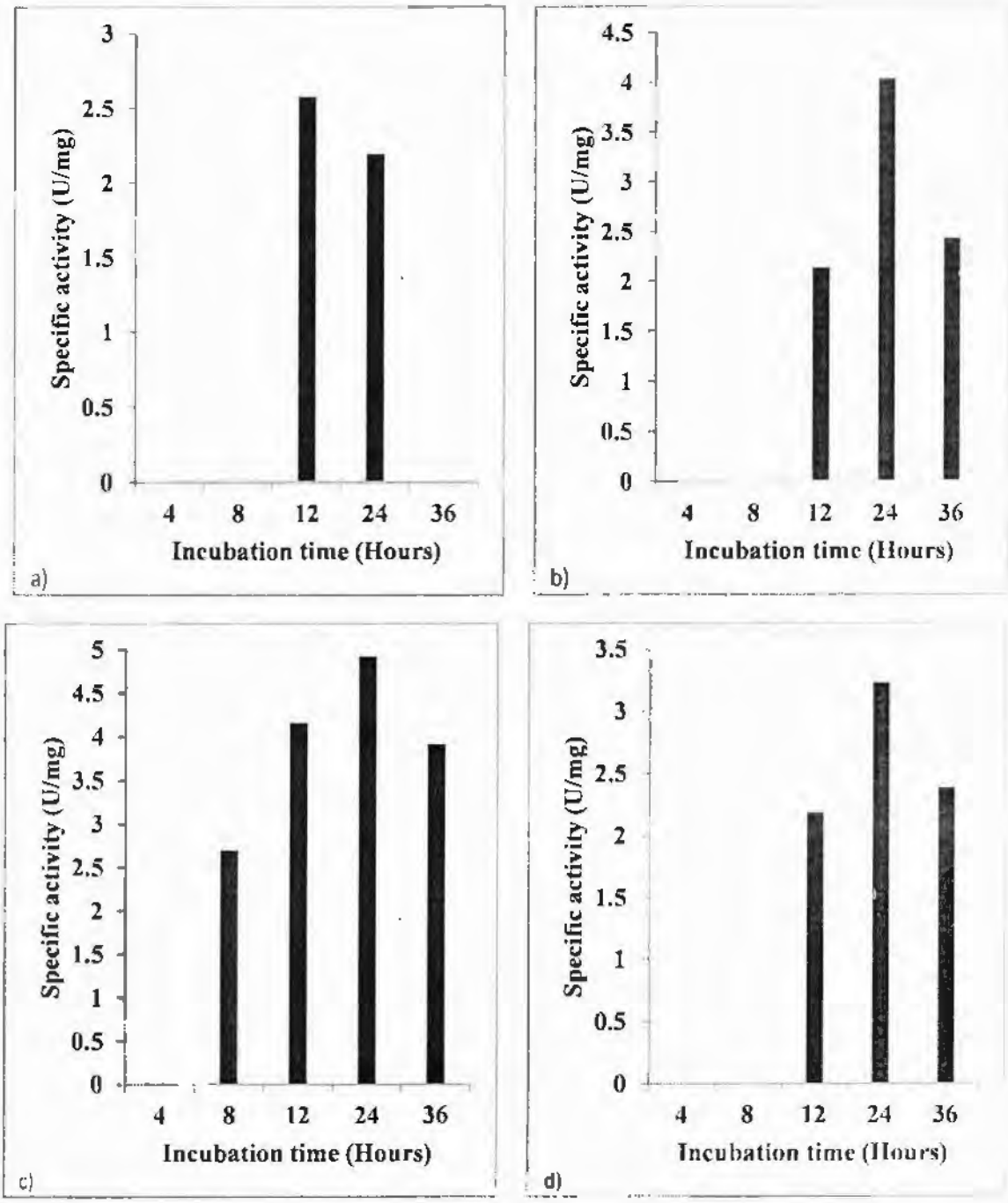


Figure 4.17 Effect of olive oil cake concentration on specific activity a) 1 % olive oil cake b) 2 % olive oil cake c) 3 % olive oil cake d) 4 % olive oil cake

4.2.5 Effect of nitrogen source

The effect of four different nitrogen sources on lipase production from *Pseudomonas aeruginosa* strain was studied. Ammonium Sulphate (0.71 % w/v), Ammonium Nitrate (0.43% w/v), Urea (0.32 % w/v) and DAP (0.7 % w/v) were added to the fermentation media which contain 3 % olive oil cake as a optimum carbon source. The result for the effect of above nitrogen sources on lipase enzyme activity are shown in (figure 4.18 and table 4.5), while their effect on specific activity are shown in (figure 4.19). The maximum extracellular lipase activity (8 U/ml/min) were obtain when ammonium nitrate were added to the fermentation media. The selected *Pseudomonas aeruginosa* strain was reported for maximum protein production (1.202 mg/ml) at ammonium nitrate after 24 h of incubation period with maximum specific activity (6.655 U/mg). Hence ammonium nitrate was optimize as nitrogen source for lipase production and were used throughout the study.

Table 4.5 Effect nitrogen sources on extracellular lipase activity

Nitrogen source	Incubation time (h)	Enzyme activity (U/ml/min)	Protein assay (mg/ml)	Specific activity (U/mg)
Ammonium Sulphate	4	0	0.722	0
	8	0	0.813	0
	12	2	0.997	2.006
	24	4	1.172	3.412
	36	2	0.873	2.290
Ammonium Nitrate	4	0	0.76	0
	8	2	0.822	2.433
	12	6	0.915	6.557
	24	**8**	**1.202**	**6.655**
	36	6	1.101	5.449
Urea	4	0	0.732	0
	8	2	0.877	2.280
	12	2	0.912	2.192
	24	4	1.133	3.53
	36	2	0.915	2.185
DAP	4	0	0.655	0
	8	2	0.731	2.735
	12	2	0.911	2.195
	24	4	1.210	3.305
	36	2	0.861	2.322

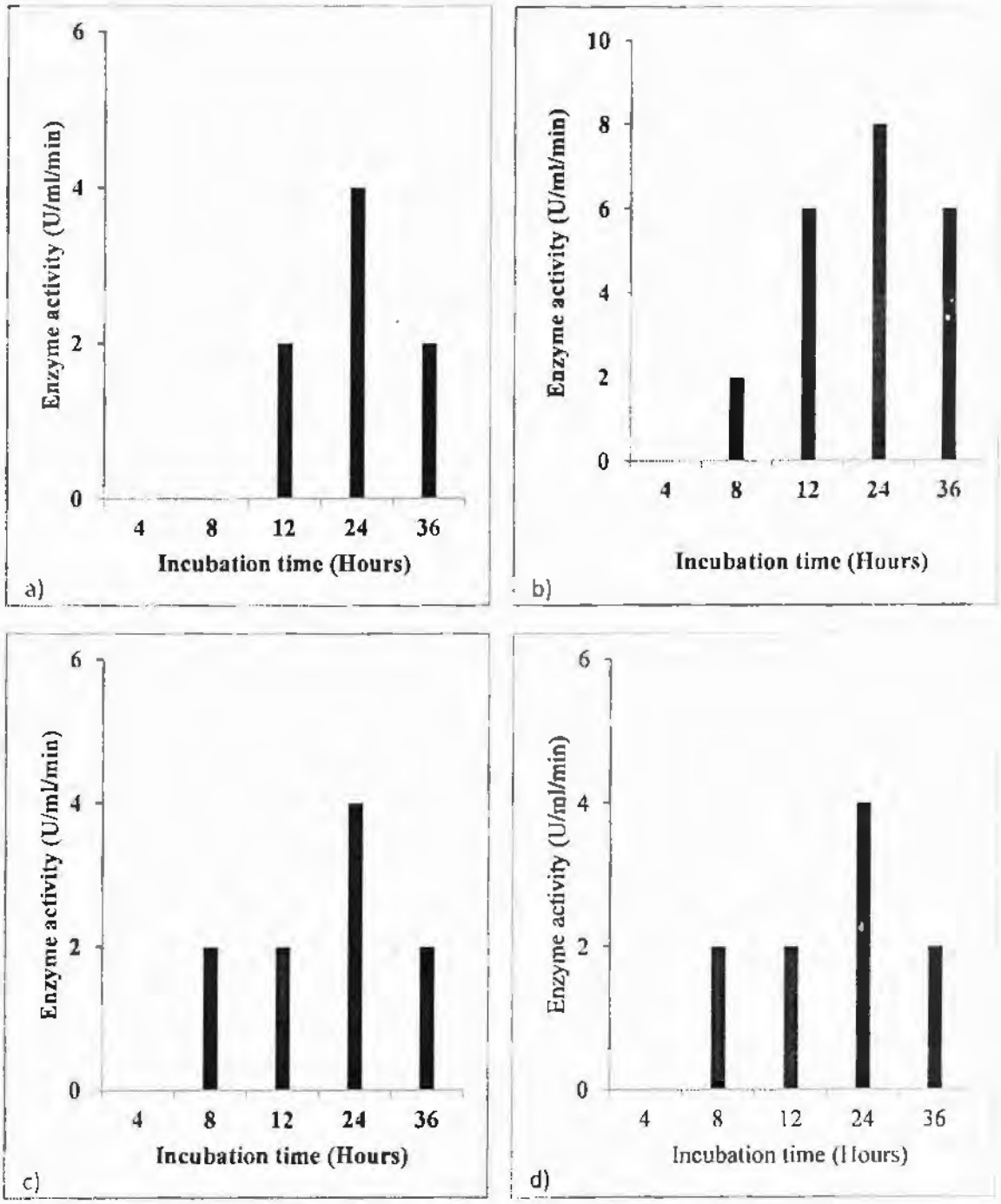


Figure 4.18 Effect of nitrogen source on extracellular lipase activity a) Ammonium sulphate b) Ammonium nitrate c) Urea d) DAP

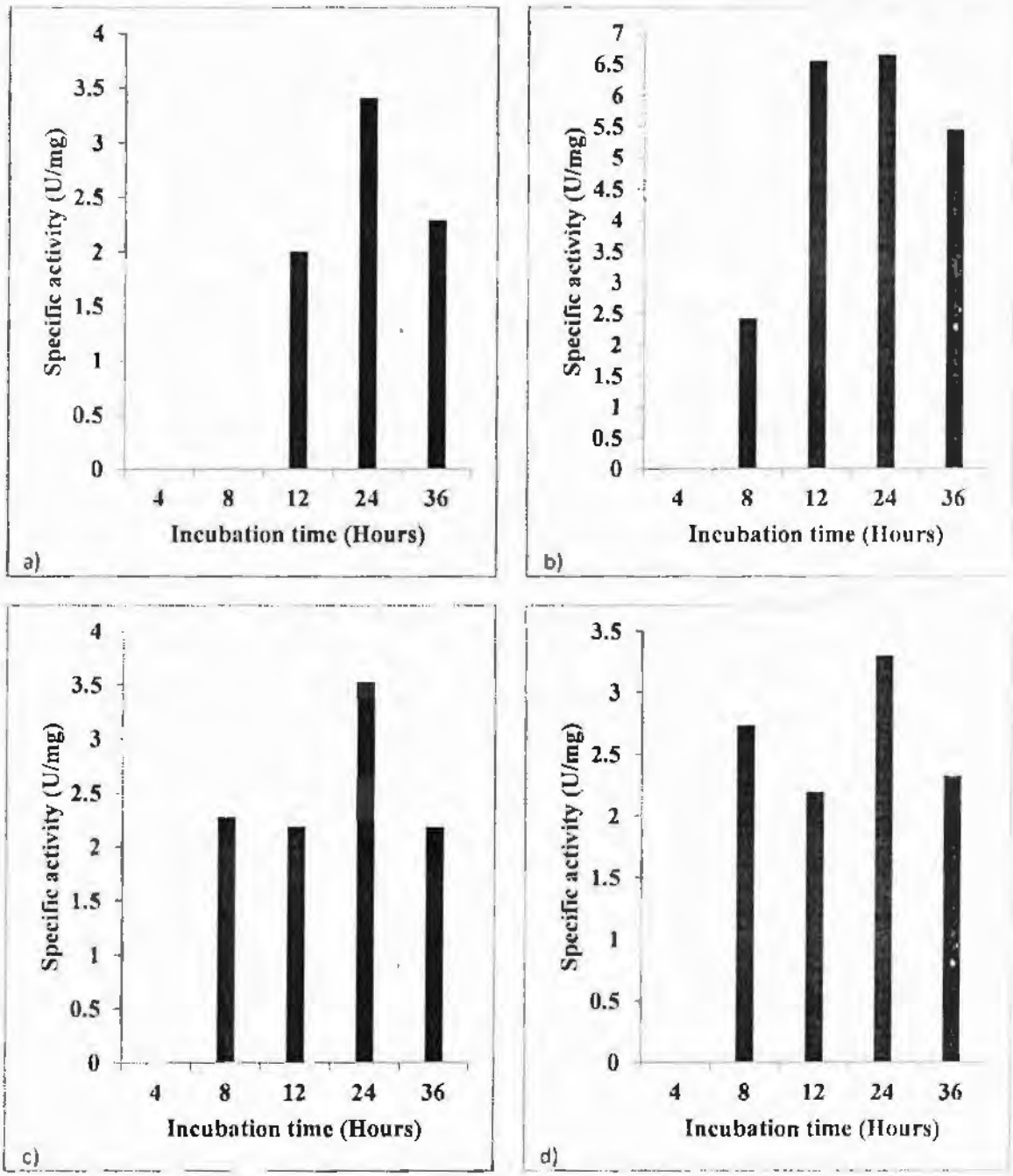


Figure 4.19 Effect of nitrogen source on specific activity a) Ammonium sulphate b) Ammonium nitrate c) Urea d) DAP

4.2.6 Effect of the pH of the medium on lipase production

Effect of pH of the fermentation medium on lipase production by *Pseudomonas aeruginosa* strain was investigated in shake flask fermentation. The initial pH of the fermentation medium was varied from pH (4.0 to 7.0). The result for the effect of pH on extracellular lipase enzyme activity are shown in (figure 4.20 and table 4.6), while their effect on specific activity are shown in (figure 4.21). The activity of extracellular lipase enzyme was found maximum (10 U/ml/min) when the pH of the medium was maintain at pH (7.0). Hence the pH 7.0 was optimized for maximum extracellular lipase activity and used throughout the study. The selected *Pseudomonas aeruginosa* strain is reported for maximum protein production (1.339 mg/ml) at pH (7.0) after 24 h of incubation period with maximum specific activity (7.468 U/mg).

Table 4.6 Effect of pH on extracellular lipase production

pH	Incubation time (h)	Enzyme activity U/ml/min	Protein assay (mg/ml)	Specific activity U/mg
4.0	4	0	0.646	0
	8	0	0.778	0
	12	2	0.822	2.433
	24	2	1.108	1.805
	36	0	0.778	0
5.0	4	0	0.512	0
	8	0	0.619	0
	12	2	0.69	2.898
	24	2	0.753	2.656
	36	0	0.511	0
6.0	4	0	0.651	0
	8	0	0.759	0
	12	2	0.819	2.442
	24	4	0.998	4.008
	36	2	0.837	2.389
7.0	4	2	0.711	2.812
	8	4	0.89	4.494
	12	8	1.102	7.259
	24	**10**	**1.339**	**7.468**
	36	8	1.132	7.067

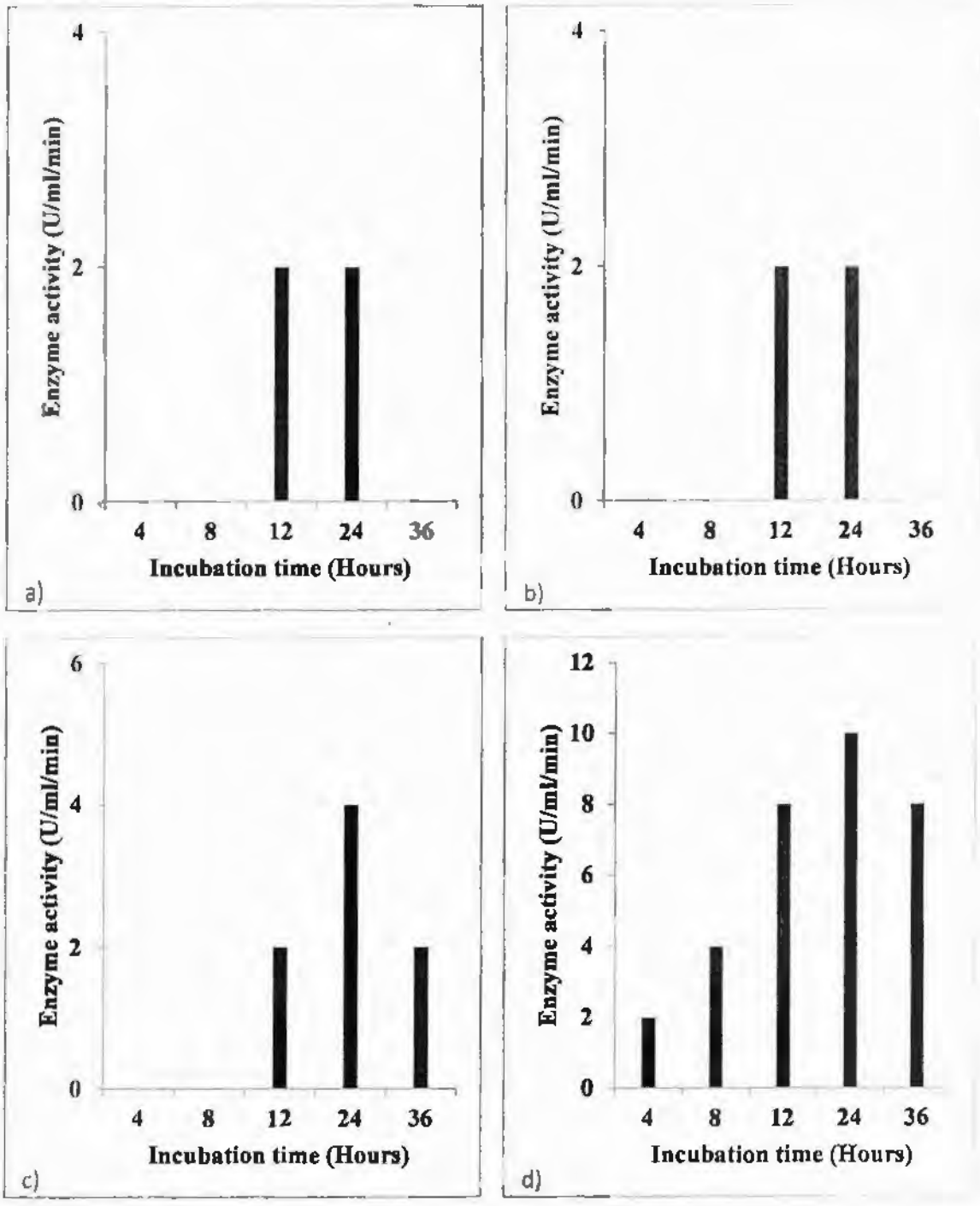


Figure 4.20 Effect of pH on extracellular lipase activity a) pH 4.0 b) pH 5.0 c) pH 6.0 d) pH 7.0

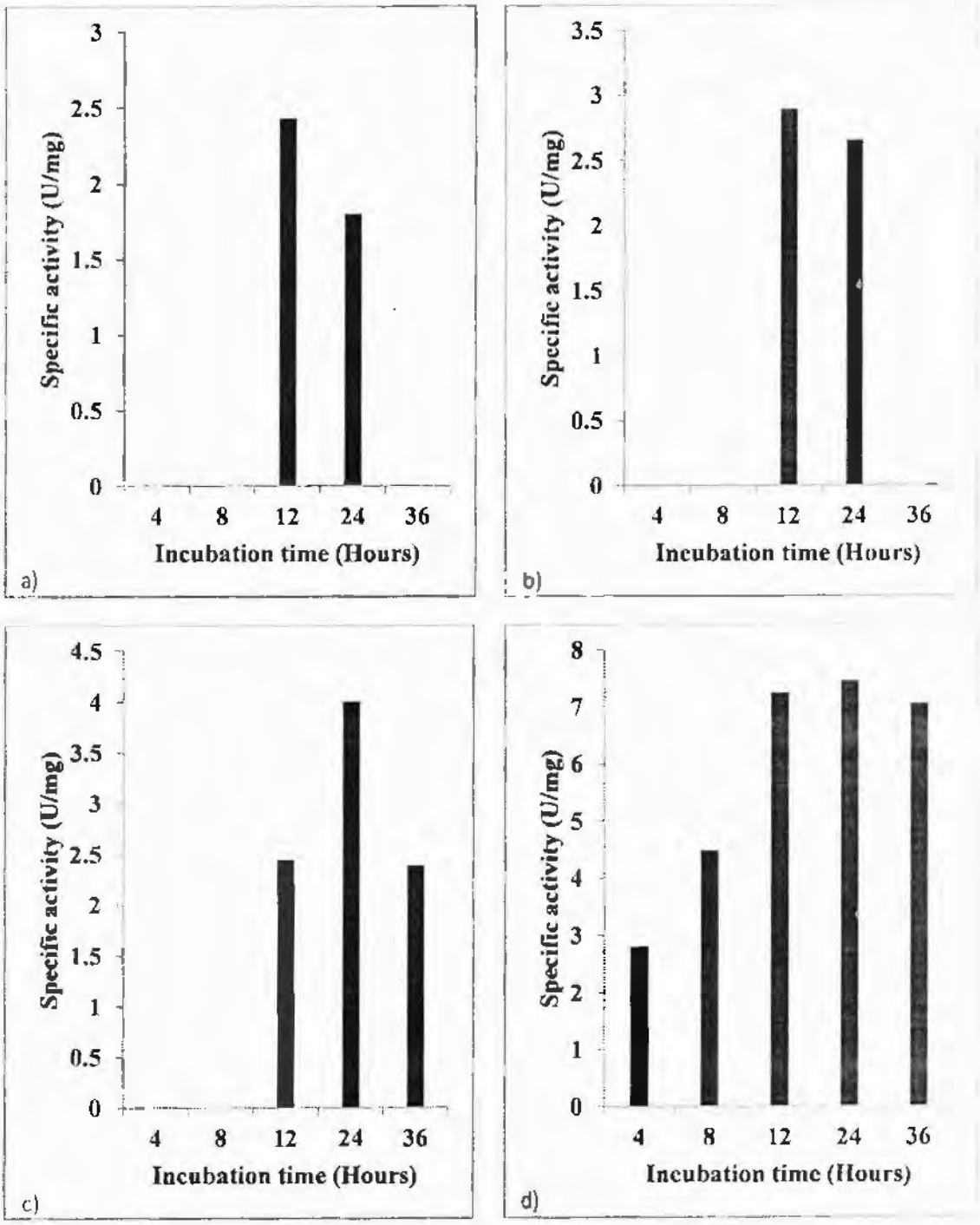


Figure 4.21 Effect of pH on specific activity a) Ph 4.0 b) pH 5.0 c) pH 6.0 d) pH 7.0

4.2.7 Effect of the incubation temperature on lipase production

Effect of incubation temperature on extracellular lipase production by *Pseudomonas aeruginosa* strain was investigated and lipase activity at different temperature i.e. 30, 35, 40, and 45 °C was evaluated. Results for the effect of incubation temperature are shown in (figure 4.22 and table 4.7), while their effect on specific activity are shown in (figure 4.23). Maximum lipase activity (12 U/ml/min) was reported by *Pseudomonas aeruginosa* strain at 35 °C. The selected *Pseudomonas aeruginosa* strain is reported for maximum protein production (1.411 mg/ml) at 45 °C after 24 h of incubation period with maximum specific activity (8.504 U/mg) at 35 °C after 24 h of incubation period. Hence 35 °C was optimize for maximum lipase enzyme activity and were used throughout the study.

Table 4.7 Effect of temperature on extracellular lipase production

Temperature (°C)	Incubation time (h)	Enzyme activity (U/ml/min)	Protein assay (mg/ml)	Specific activity U/mg
30	4	0	0.867	0
	8	2	0.89	2.247
	12	4	1.102	3.629
	24	6	1.339	4.48
	36	4	1.120	3.571
35	4	2	0.721	2.773
	8	6	1.133	5.295
	12	8	1.271	6.294
	24	**12**	**1.411**	**8.504**
	36	8	1.251	6.394
40	4	0	0.581	0
	8	2	0.699	2.861
	12	4	0.831	4.813
	24	6	0.921	6.514
	36	4	0.831	4.813
45	4	0	0.799	0
	8	0	0.933	0
	12	2	1.311	1.796
	24	4	1.42	2.816
	36	2	0.913	2.190

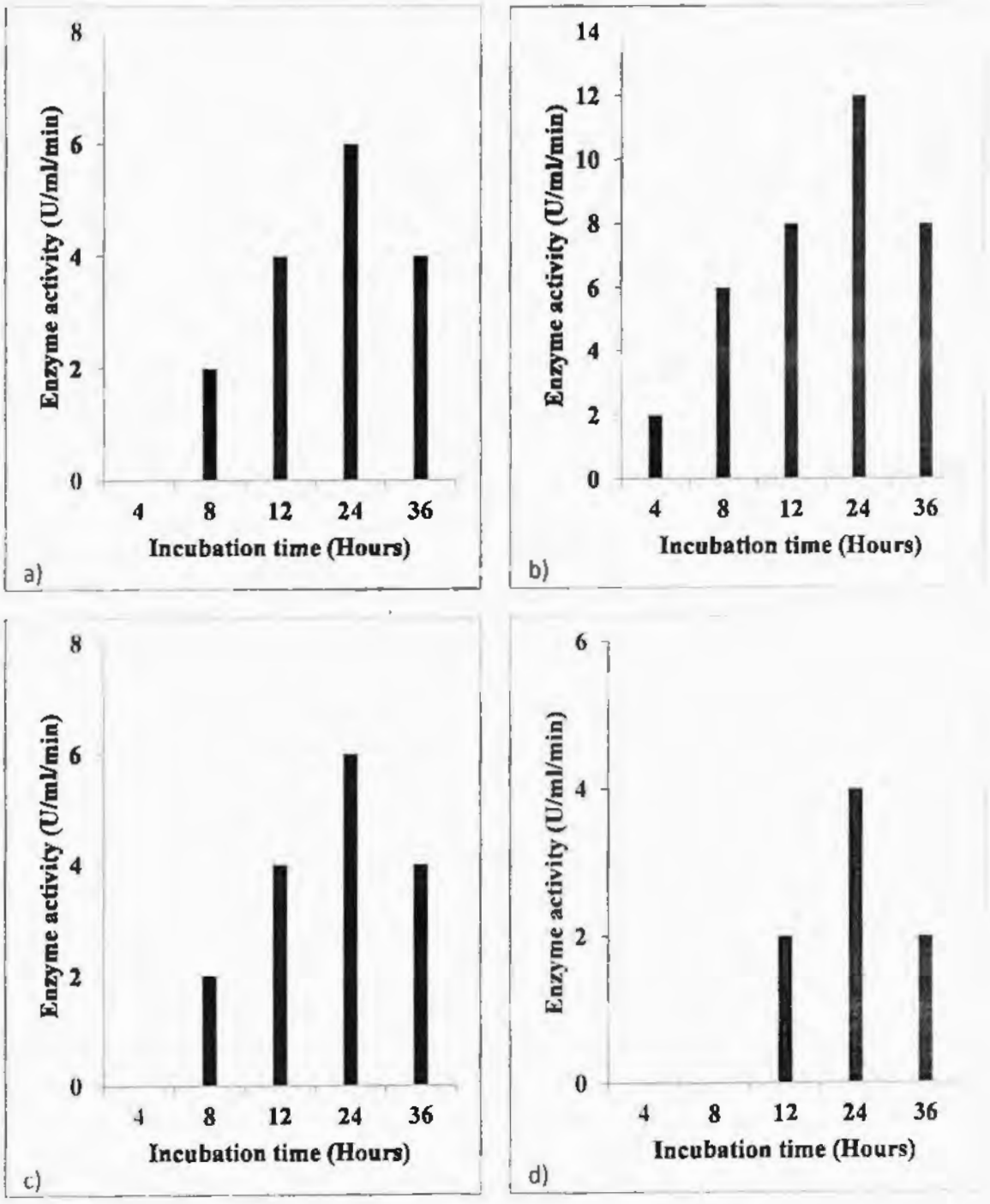


Figure 4.22 Effect of incubation temperature on extracellular lipase activity a) 30 °C b) 35 °C c) 40 °C d) 45 °C

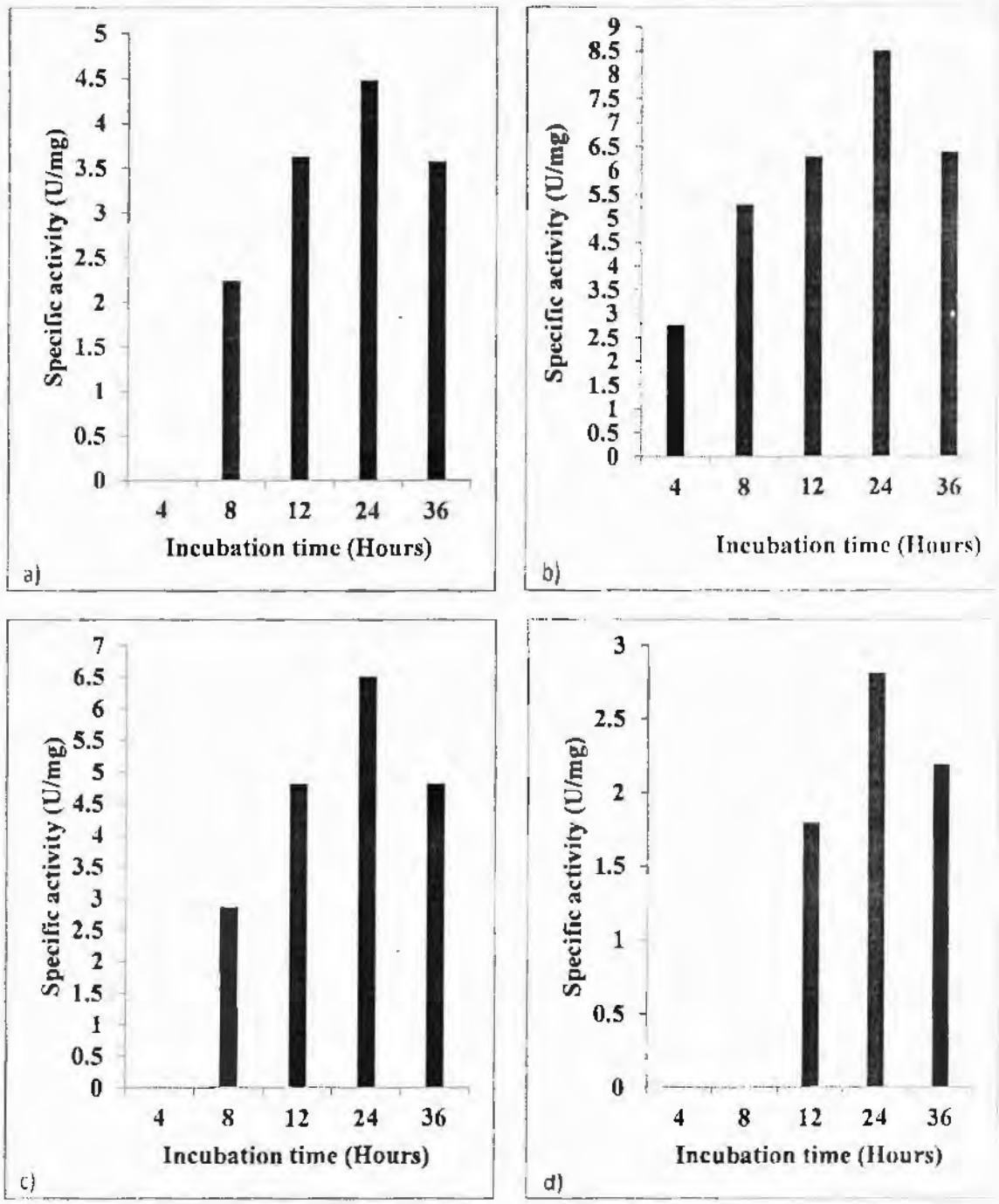


Figure 4.23 Effect of incubation temperature on specific activity a) 30 °C b) 35 °C c) 40 °C d) 45 °C

4.3 Partial purification of extracellular lipase

Ammonium sulphate precipitation was performed to purify extracellular lipase enzyme.

4.3.1 Ammonium sulphate precipitation

Solid ammonium sulphate of different concentration were separately added to the 50 ml of crude enzyme extract in numbered Erlenmeyer flasks to get 20 %, 30 % and 40 % saturation. Each saturation was kept overnight at 4 °C. The supernatant and pellet were collected by centrifuge the each saturated crude enzyme extract at 4000 rpm for 5 minutes. Both the pellet and supernatant were assayed for extracellular lipase activity. The result of 20 %, 30 % and 40 % saturation are shown in (figure 4.24 and table 4.8). The lipase activity of (66 U/ml/min) with maximum specific activity of (34.21 U/mg) was found at 20 % saturation supernatant which specifies partial purification of extracellular lipase enzyme and was used for characterization.

Table 4.8 Partial purification of extracellular lipase

Salt conc. (%)	Enzyme activity (U/ml/min)	Protein assay (mg/ml)	Specific activity (U/mg)
20	**66**	**1.929**	**34.21**
30	50	1.489	33.57
40	45	1.439	31.27

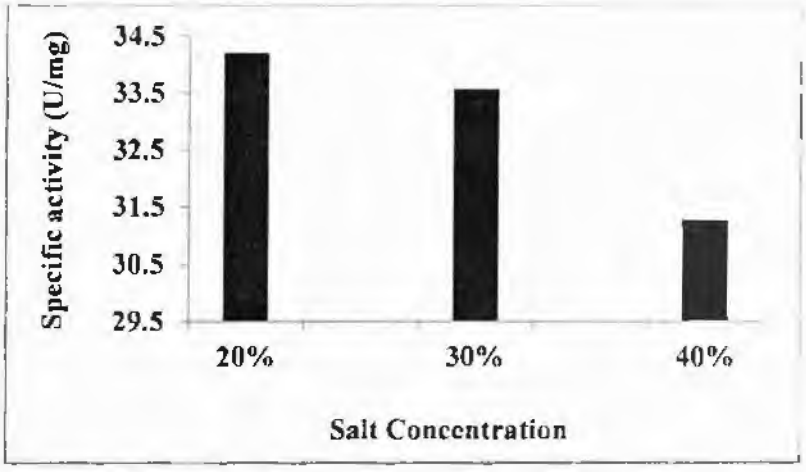


Figure 4.24 Ammonium sulphate precipitation

4.4 Characterization of extracellular lipase

The extracellular lipase enzyme was purified from culture supernatant through ammonium sulphate precipitation and was then characterized.

4.4.1 Effect of pH on lipase activity

The effect of pH on purified extracellular lipase enzyme activity was studied by incubating the lipase enzyme in citrate buffer with pH rang of (4.0 to 7.6). Results for the effect of pH on extracellular lipase activity are shown in (figure 4.25 and table 4.9). The present enzyme was most active in the pH rang of (5.6 to 7.2) with maximum activity (68 U/ml/min) at pH (7.0). The extracellular lipase activity was decrease below the pH (5.6) and above the pH (7.0).

Table 4.9 Effect of pH on lipase activity

pH	Enzyme activity (U/ml/min)
4	46
4.4	50
4.8	54
5	54
5.2	56
5.4	60
5.6	62
6	64
7	**68**
7.2	66
7.4	62
7.6	58

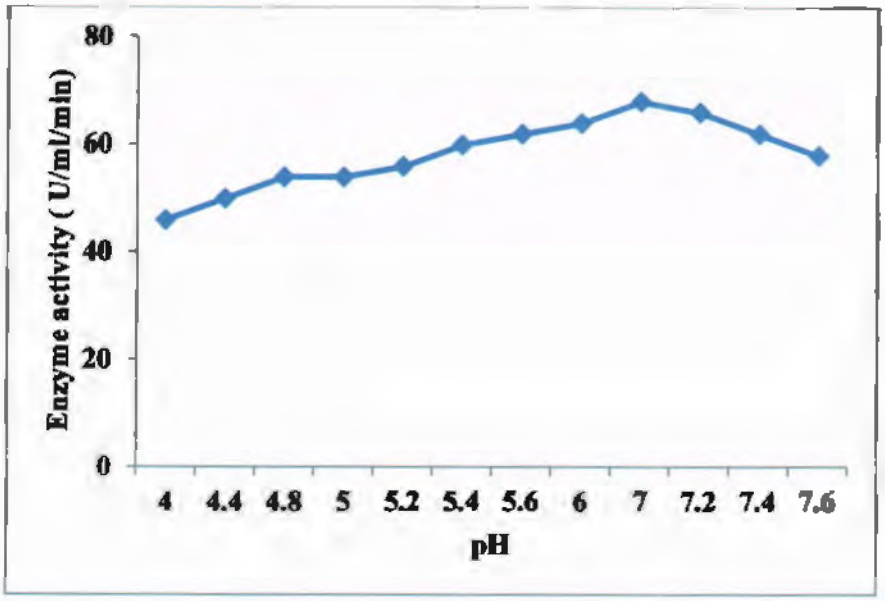


Figure 4.25 Effect of pH on lipase activity

4.4.2 Effect of temperature on lipase activity

The reaction mixture was incubated with different temperature range from 25 to 80 °C. The results for their effect are shown in (figure 4.26 and table 4.10). Highest activity (72 U/ml/min) was obtained at 55 °C. The activity of the present enzyme was dropped above 60 °C.

Table 4.10 Effect of temperature on lipase activity

Temperature (°C)	Enzyme activity (U/ml/min)
25	50
30	52
35	54
40	58
45	62
50	68
55	**72**
60	64
65	60
70	54
75	46
80	42

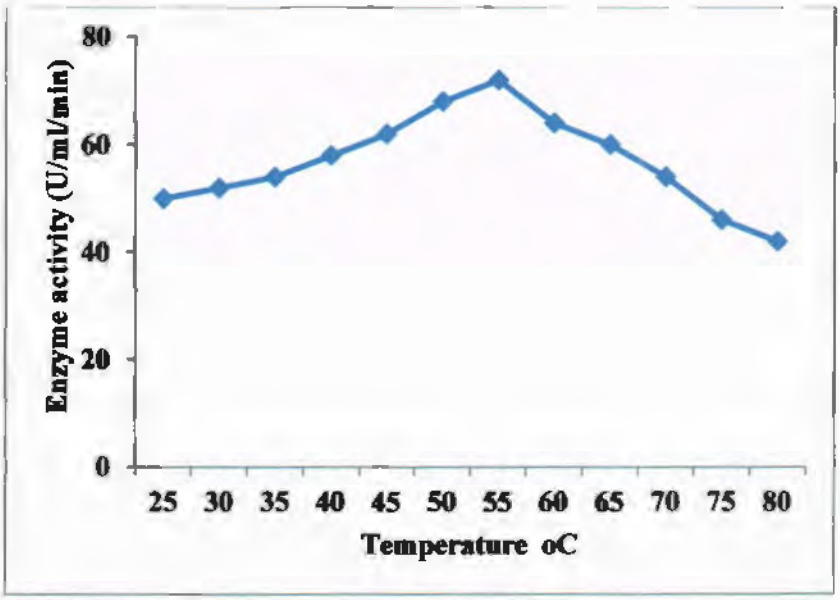


Figure 4.26 Effect of temperature on lipase activity

4.4.3 Effect of different concentration of olive oil on lipase Activity

The effect of different concentration of olive oil on extracellular lipase enzyme activity was studied by incubating the lipase enzyme with different olive oil concentration ranges from (0.1 to 0.9 ml) for 10 min. Result for the effect of different concentration of olive oil on extracellular lipase enzyme activity are shown in (table 4.11). The enzyme activity was gradually increased from (0.1 to 0.9 ml) concentration of olive oil and show maximum enzyme activity (68 U/ml/min) at 0.9 ml concentration of olive oil. Michaelis-Menten kinetics was characterized by two parameters, K_m and V_{max}. The K_m and V_{max} of lipase obtained by Lineweaver Burk plot shown in (figure 4.27) while using olive oil as substrate. The K_m and V_{max} of lipase measured by Line weaver Burk plot was K_m of 0.28 ml. and V_{max} of 87.71 U/ml/min.

Table 4.11 Effect of olive oil concentration on lipase activity

Olive oil concentration (ml)	Enzyme activity U/ml/min
0.1	42
0.2	46
0.3	50
0.4	52
0.5	56
0.6	58
0.7	61
0.8	66
0.9	68

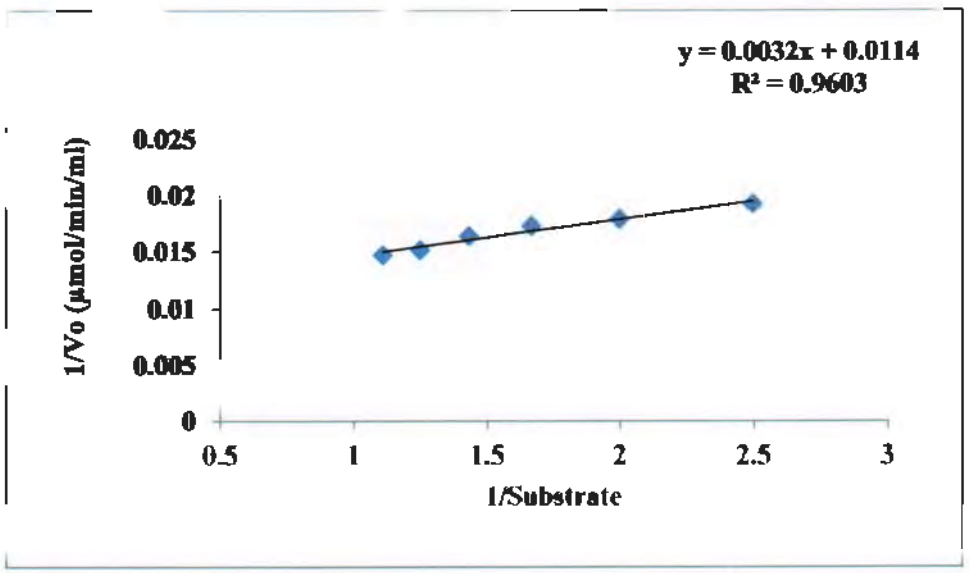


Figure 4.27 Lineweaver Burk plot of *Km* and *Vmax*

4.4.4 Effect of various organic solvent on lipase activity

Lipase enzyme was incubated with Ethanol, n-hexane, acetone and methanol at concentration of 30 % (v/v) for 10 minutes. Results for control and the effect of ethanol, methanol, acetone and n-hexane are shown in (figure 4.28 and table 4.12). Maximum activity of extracellular lipase (40 U/ml/min) was obtained when the enzyme was

incubated with methanol, while the minimum activity of extracellular lipase (20 U/ml/min) was obtained by incubating the enzyme with n-hexane.

Table 4.12 Effect of organic solvent on lipase activity

Organic solvents	Enzyme activity (U/ml/min)	(%) Residual activity
Control	52	100
Ethanol	34	65.38
Methanol	**40**	**76.92**
Acetone	36	69.23
n-hexane	20	38.46

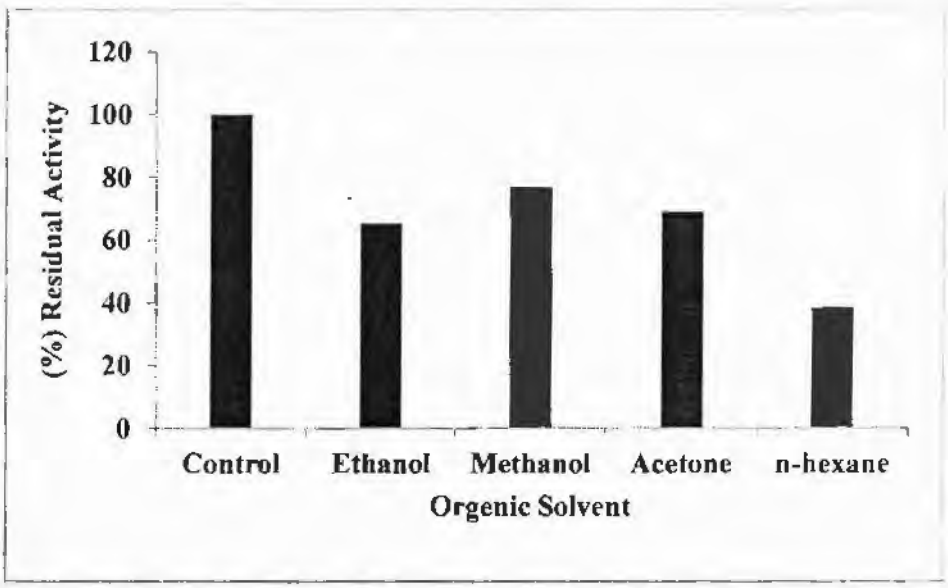


Figure 4.28 Effect of organic solvent on lipase activity

4.4.5 Effect of different substrate on lipase activity

Olive oil substrate in a reaction mixture was replaced by other substrate like mustard oil, Apricot oil and tween 80 to study their effect on lipase activity. The result for the selected above substrate on lipase activity are shown in (table 4.13, figure 4.29). The enzyme show maximum activity (68 U/ml/min) when incubated with tween 80 for 10 min. The enzyme show minimum activity (38 U/ml/min) when incubated with Apricot oil for 10 min.

Table 4.13 Effect of substrates on lipase activity

Substrate	Enzyme activity (U/ml/min)
Olive oil	58
Tween 80	**68**
Mustard oil	46
Apricot oil	38

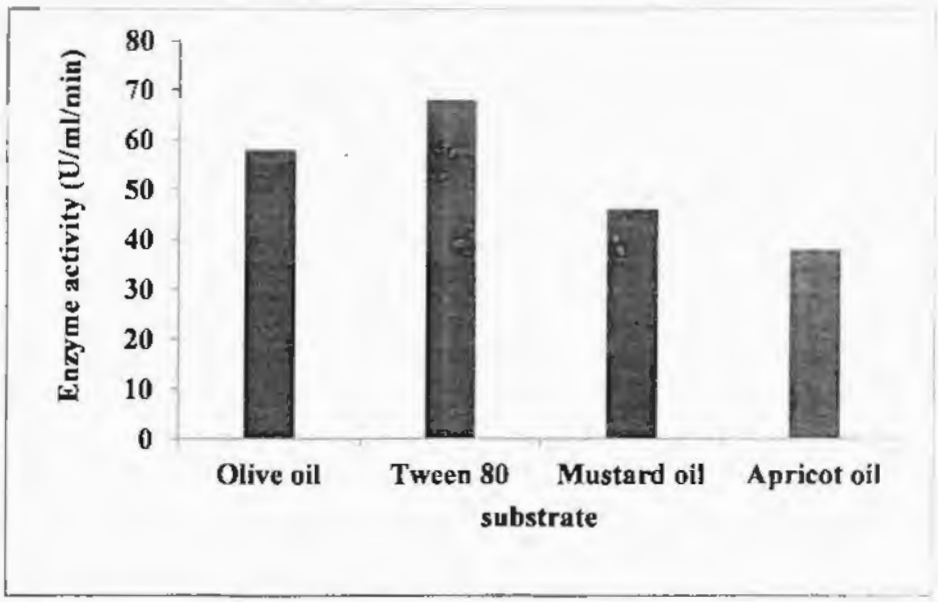


Figure 4.29 Effect of different substrates on lipase activity

4.4.6 Thermal denaturation of lipase enzyme

Enzyme thermo stability was observed in temperatures ranging from (65 to 90 °C) for 30 min. The result for thermal denaturation are shown in (table 4.14), ln (% Residual Activity) of the enzyme are shown in (figure 4.30). The k_d value and his graph for the thermal denaturation of extracellular lipase are respectively shown in (table 4.15) and (figure 4.31). The enzyme activity was stable at 65 °C temperature after 30 min incubation period showing highest enzyme activity (58 U/ml/min).The enzyme activity was gradually decrease above 65 °C. The enzyme was unstable and denature at 90 °C after 30 min incubation showing the minimum lipase enzyme activity (6 U/ml/min). The activation energy (E_a) of 65.6 kJ/mol for lipase enzyme was calculated.

Table 4.14 Thermal denaturation of extracellular lipase enzyme

Temperature °C	5	10	15	20	25	30
65	70	66	64	62	60	58
70	68	64	62	60	56	54
75	54	50	48	44	42	40
80	48	44	42	40	38	36
85	38	34	30	28	26	22
90	20	16	14	12	10	6

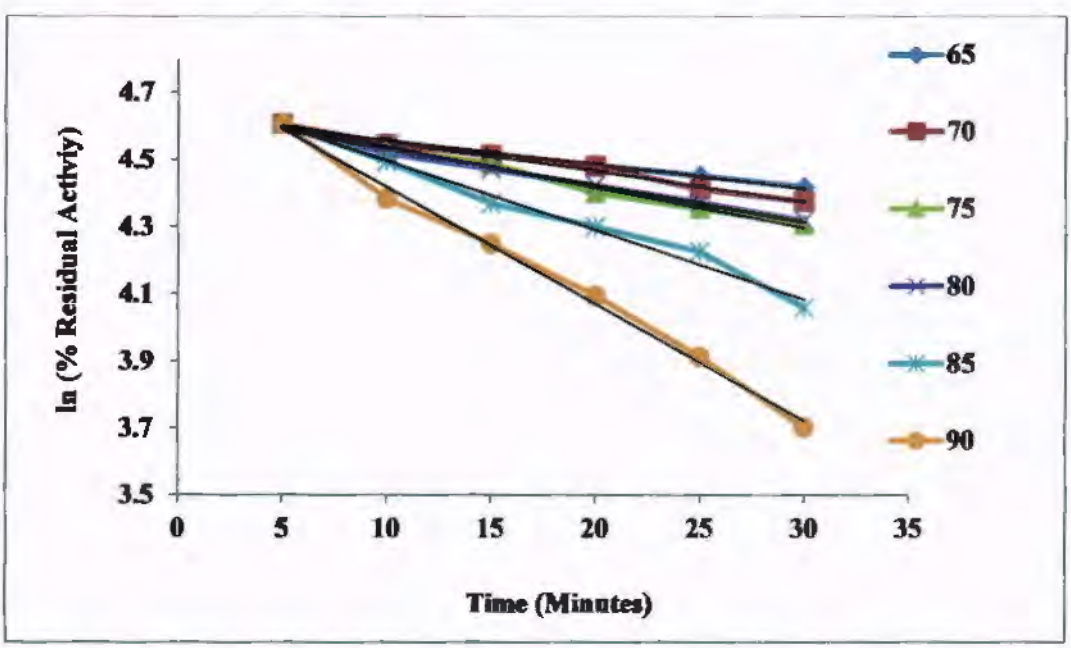


Figure 4.30 ln (% Residual Activity) of extracellular lipase enzyme

Table 4.15 k_d value of extracellular lipase enzyme

Temperature °C	K	k_d
65	338	0.0072
70	343	0.0091
75	348	0.0121
80	353	0.011
85	358	0.0206
90	363	0.0433

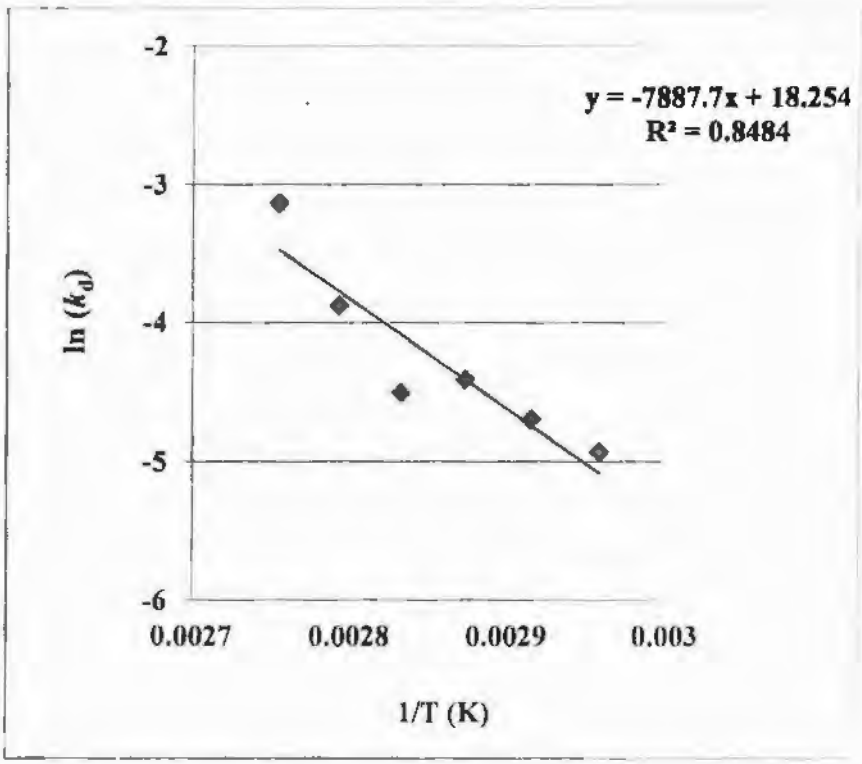


Figure 4.31 k_d value graph for extracellular lipase enzyme

DISCUSSION

5. DISCUSSION

Microbes are very important and diverse group of organisms that ranges in size from unicellular to multicellular organisms. All these microbes have an important role in many natural processes like medical, pharmaceutical and industrial processes like fermentations. Some of the microorganism like bacteria, algae and fungi are having many biological application most importantly *Pseudomonas* is the group which have a wide range of industrial applications. It belongs to a complex group called *Pseudomonadaceae* which contain number of heterogeneous organism and it is approximately containing 211 sp. in which only 56 are classified to separate genus (Pandey *et al.*, 1999).

The biological features of *Pseudomonas* sp. they survive in the oxygen presence means oxygen have vital role in the life cycle of such microorganisms. some other features includes they are non-spore forming, Gram negative having an extra peptidoglycan layer and structurally they are rods in shape which may varies in structure straight or slightly curved and are 0.5 – 1.0 μm by 1.5 – 5.0 μm. They have the ability of movement and have proper locomotory organ having one or pair of flagella which are polar means they are carrying charges over it. The respiratory process in such organism is oxygen dependent and they also need oxygen for their metabolic processes while in other cases nitrate is also used by these organisms as an alternative source of energy to allow anaerobic growth. Mostly species are catalase positive (Funke and Bernard., 2011).

Narasimha *et al.*, 2011 reported that lipase can also be produced by *Pseudomonas* sp. and such bacterial strains were isolated from the area having oil constituents that are from groundnuts and when they were biologically studied it were characterized as *Pseudomonas* sp. based on its morphological, physiological and biochemical characteristics. To produce lipases from such organisms they were provided with best condition which were suitable for their growth and life cycle and such condition were optimized by keeping different carbon and nitrogen sources in the medium maintained at pH-7 through submerged fermentation. Numbers of studies were carried out like studying their chemical and physiologically features. The conditions includes Olive oil, Glucose and Ammonium sulphate were served as best carbon and nitrogen sources for lipase

production by *Pseudomonas* sp. grown at 30 °C for approximately 24 h incubation period was given.

The current study was conducted for the isolation, production and characterization of lipase enzyme from *Pseudomonas* sp. Various growth parameters and fermentation conditions like time course study, effect of inoculum size, effect of carbon source and its different concentration, nitrogen source, effect of various pH (citrate buffer of 4-7) and temperature were optimized for most favorable lipase production through submerged fermentation. The enzyme was subjected to 20 to 40 % of salt ammonium sulphate precipitation concentration. The purified lipase enzyme characterization was done for various kinetic parameters. The thermostability and effect of pH, temperature, organic solvent and different concentration of substrate on purified extracellular lipase enzyme activity was investigated. The aim of the proposed research was to optimize conditions for maximum lipase production on less cost that may be available for industrial uses.

Zouaoui and Bouziane, (2012) isolated *Pseudomonas* sp. from an urban waste water at SidiBel abbes, Algeria. He reported the morphological and biochemical characteristics of the isolated strain. Saeed *et al.*, (2005) isolated *pseudomonas* sp. and he characterized it as *pseudomonas aeruginosa* on the basis of physiological structure, biochemical analysis and the genetic analysis that includes the study of 16S rRNA sequencing. Pitt and Simpson, (2006) evaluated that blue-green pigment is indicative of *Pseudomonas aeruginosa* and they are Gram negative rod shaped and rarely associated with human infection and they are catalase positive *Pseudomonas* sp. Soesanto *et al.*, (2011) also investigated that *Pseudomonas florescence* produce greenish yellow soluble pigment (Pyoverdin) and morphology of *Pseudomonas fluorescens* P60 was Gram negative rod shape biochemically the isolated *Pseudomonas fluorescens* P60 was catalase positive.

In current study the two *Pseudomonas* sp. were isolated and grown on nutrient agar medium. These two species were identified as *Pseudomonas aeruginosa* and *Pseudomonas florescene* on the basis of their cultural characteristic. *Pseudomonas aeruginosa* produce a greenish blue soluble pigment (Pyocyanin). When observed observed under fluorescent light, fluorescent color greenish blue was appeared around the dense culture of *Pseudomonas aeruginosa*. Similarly *Pseudomonas florescene* produce a

greenish yellow soluble pigment (Pyoverdin) was observed under fluorescent light. fluorescent color a greenish yellow was appeared around the dense culture of *Pseudomonas florescene*. Both the species were reported as Gram negative rod shaped, catalase test positive and coagulase test negative.

In present study *Pseudomonas aeruginosa* strain was used which was obtained from Dr. Bashir Ahmad. Lipase enzyme was isolated from this *Pseudomonas aeruginosa* strain. Different parameters for lipase production, purification and characterization were evaluated. Result showed that maximum lipase enzyme activity (4 U/ml) was obtained at 24 h incubation and by increasing the incubation period from 24 h lipase enzyme activity decreased. Tembhurkar *et al.*, (2012) used *Pseudomonas* strain which gave lipase activity using olive oil as a substrate after 72 h. More incubation effect negatively lipase production. Similarly Saeed et al., (2005) reported that *Pseudomonas aeruginosa* ps-x produce maximum extracellular lipase (74.66 U/ml) after 48 h incubation period by using tween 80 as inducer for lipase enzyme production. The proposed study for optimization of incubation period shows no resemblance to the reported study.

Effects of different inoculum size for extracellular lipase activity by *Pseudomonas aeruginosa* strain were reported in proposed study. It was investigated that the inoculum size of 10 % yielded maximum extracellular lipase activity (6 U/ml) with maximum specific activity (4.316 U/mg) with 24 h incubation time. In contrast (Pagu *et al.*, 2013) reported that *Pseudomonas* sp. yielded maximum lipase activity (108 U/ml) using 6 % inoculum at 30 ^{o}C for 24 h. As a part of the study to optimize best carbon source and nitrogen source for lipase enzyme production by *Pseudomonas aeruginosa* were also evaluated. In present study 3 % olive oil cakes was reported as best carbon source and yielded maximum lipase enzyme activity (6 U/ml) after 24 h incubation period. Similarly best nitrogen source ammonium nitrate yielded maximum lipase activity (6 U/ml) after 24 h incubation. Faisal *et al.*, (2015) also worked on the same species but instead of mustered oil cakes he used gingelly oil cakes GOC and he illustrated that the specie give the highest lipase production (107.44 U/gds) and the specie used was *Pseudomonas* sp. Narasimha *et al.*, (2011) reported that ammonium sulphate was the best nitrogen source yielded maximum lipase (8 U/ml) from *Pseudomonas* sp.

In present study it was reported that *Pseudomonas aeruginosa* at pH 7 give optimum lipase activity of (10 U/ml). This results are in accordance with Kojima and Shimizu., (2003). They obtained optimum lipase activity of *Pseudomonas fluorescens* HU 380 at pH 7. In present study Maximum lipase activity (12 U/ml/min) was reported by *Pseudomonas aeruginosa* strain at 35 °C. In contrast Kathiravan *et al.*, (2012) reported that *Pseudomonas aeruginosa* yielded maximum lipase activity (6 U/ml) with optimum temperature 40 °C.

In current study crude lipase enzyme extract were subjected to ammonium sulphate precipitation. It was investigated that lipase activity of (66 U/ml/min) with maximum specific activity of (34.21 U/mg) was found at 20 % saturation supernatant. In contrast Zouaoui and Bouziane, (2012) reported *Pseudomonas aeruginosa* lipase was subjected to purification and maximum lipase activity (21.5 U/ml) with maximum specific activity (25.65 U/mg) were detected at 40 % ammonium sulphate. In present study the pH and temperature effect on lipase activity was measured on at various pH values (4 to 7.6) and temperature (25 to 80 °C). It was reported that the present enzyme was most active in the pH rang of (5.6 to 7.2) with maximum activity (68 U/ml/min) at pH (7.0) and this property can be made the lipase applicable at neutral pH conditions. In contrast (Mobarak-Qamsari *et al*, 2011) reported that *Pseudomonas aeruginosa* KM110 lipase was most active at pH (6.0) with maximum activity (0.6 U/ml). Similarly Gupta *et al.*, (2004) reported lipase optimum activity at pH 7.

In current study it was reported that lipase enzyme maximum activity (72 U/ml/min) was obtained at 55 °C. In contrast Mobarak-Qamsari *et al.*, (2011) reported that the temperature preference of *Pseudomonas aeruginosa* KM110 lipase reveals highest activity values (0.5 U/ml) at temperature 35 °C. Similarly Gilbert *et al.*, (1991) reported that *Pseudomonas aeruginosa* EF2 lipase having optimum temperature 50 °C. In present study thermal stability of purified lipase was investigated at various temperatures ranging from (65 to 90 °C). It was reported that the enzyme was found to be completely stable at 65°C after 30 min and maintain residual activity (4.45 %) after 30 min. The stability of the enzyme decreased sharply after 30 mnt of incubation at 70 °C temperature. This study indicates that *Psudomonas aeruginosa* lipase is a thermophilic enzyme.

Borkar *et al.*, (2009) reported that the *Pseudomonas aeruginosa* SRT 9 lipase was found to be thermostable which can withstand the temperature upto 55 °C after 2 h. The enzyme (78 %) of the initial activity maintained at 65 °C after 1 h incubation and (46 %) activity after 2 h incubation at 70 °C. Similarly Mobarak-Qamsari, 2011 reported that *Pseudomonas aeruginosa* KM110 thermostability was performed by determining the residual activity after incubation at different temperatures on various times. The enzyme retain (80 %) residual activity after 3 h of storage at 45 °C and retained lowered residual activity (40 %) after 3 h at 65 °C temperature. In present study Michaelis–Menten kinetics were characterized by two parameters K_m and V_{max}. The K_m and V_{max} of lipase measured by Line weaver Burk plot when olive oil was used as substrate and revealed K_m of 0.28 ml and V_{max} of 87.71 U/ml/min. In contrast to the proposed study Dey *et al.*, (2014) reported the Lineweaver-Burk plots for the determination of K_m and V_{max} value of purified extracellular lipase from *Pseudomonas* ADT3. The reported data for K_m and V_{max} were (0.260 mM) and (144.93 U/mg/min) respectively.

In present study lipase enzyme substrate specificity were evaluated by subjecting the enzyme to different substrate. It was investigated that the enzyme showed highest activity toward tween 80 (68 U/ml) and olive oil (58 U/ml). In contrast Borkar *et al.*, (2009) investigated that *Pseudomonas aeruginosa* SRT 9 lipase enzyme also showed good activity towards trilaurin and tripalmitin with relative activities of 60 % and 76 % respectively. In proposed study the *Pseudomonas aeruginosa* lipase enzyme were evaluated for stability in organic solvents. The results revealed that lipase enzyme was highly stable in methanol for 10 min at 30 °C and reported maximum enzyme activity (40 U/ml). Bharti and Sharma, (2015) incubated *Pseudomonas aeruginosa* lipase with organic solvents and reported that the enzyme does not loss its activity and retained 90 % of its activity after 12 h incubated with organic solvent like methanol, ethanol, hexane and xylene. In proposed study the result for the effect of organic solvent on lipase is similar to the reported study.

Conclusion and future prospects

The study results obtained showed that inoculum size of 10 %, carbon source olive oil cake at 3 % concentration, ammonium nitrate as nitrogen source, initial medium of pH 7.0 and fermentation temperature of 35 °C for 24 h gave maximum lipase enzyme production. The lipase enzyme can be produced on industrial large scale from *Pseudomonas* species using olive oil cake, found in abundance in Pakistan which will be economically feasible.

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5. REFERENCES

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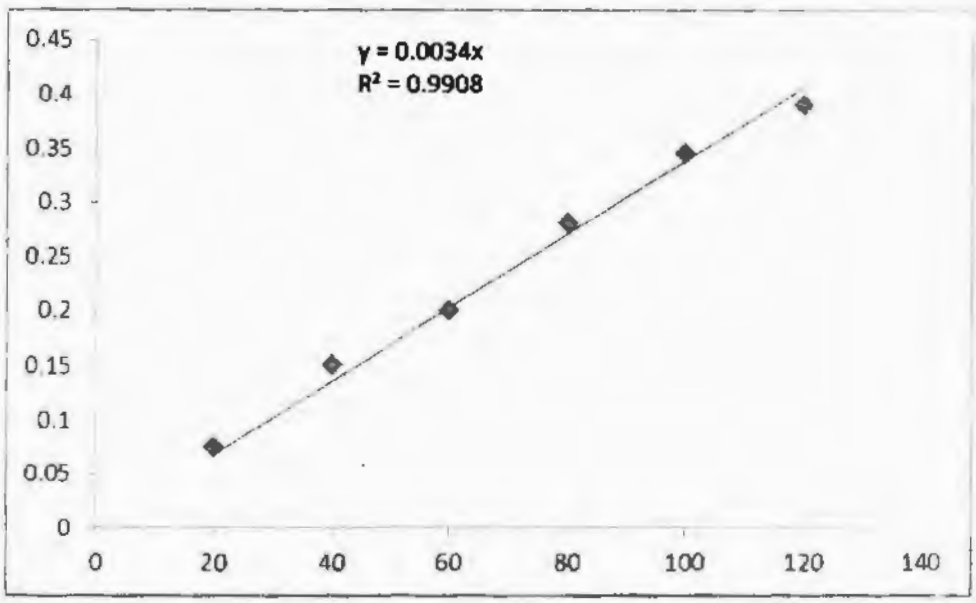
APPENDICES

APPENDIX I

BSA Standard Curve for Protein Estimation



Abs. (595 nm)

Concentration of BSA (µg/ml)

APPENDIX II

CONCENTRATION OF AMMONIUM SULPHATE

Initial concentration of ammonium sulphate (percentage saturation at 0 °C)	Final concentration of ammonium sulphate, % saturation at 0°C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulphate (grams) to be added to 1 liter of solution																
0	107	136	166	197	229	262	295	331	366	404	442	483	523	567	611	659	707
5	80	109	139	168	200	232	266	300	336	373	411	450	491	533	578	624	671
10	54	82	111	141	171	203	236	270	305	342	379	418	458	500	545	589	636
15	26	55	83	113	143	174	207	240	275	310	348	386	426	466	510	555	600
20	0	27	56	84	115	145	177	210	244	280	316	354	392	433	476	519	565
25		0	27	57	85	117	148	182	214	248	284	321	360	401	442	485	529
30			0	28	57	87	119	150	184	217	253	289	328	367	408	451	495
35				0	28	58	88	120	153	187	221	258	295	334	374	416	459
40					0	29	59	90	122	155	190	225	262	300	340	381	424
45						0	29	60	91	125	158	193	229	267	306	347	388
50							0	30	61	93	127	161	197	233	272	312	353
55								0	30	62	94	129	163	200	238	277	317
60									0	31	63	96	131	166	204	242	283
65										0	31	64	98	134	170	208	247
70											0	32	66	100	136	173	212
75												0	32	67	102	139	176
80													0	33	68	104	141
85														0	34	69	106
90															0	34	71
95																0	35
100																	0

Source: Protein purification techniques (A practical approach) by Simon Roe, Second edition (2001), Oxford University Press. Page no: 137.