Production and Characterization of Lipase from *Humicola* species.

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

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Dated: ____________

FINAL APPROVAL

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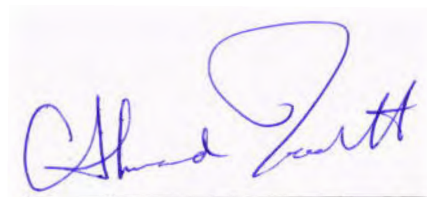

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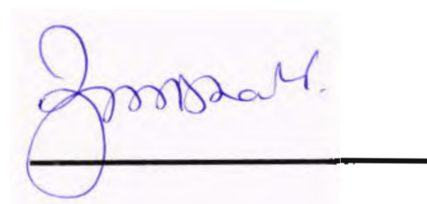

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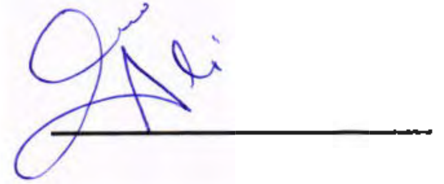

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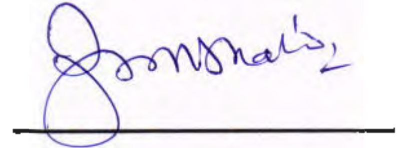

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A thesis submitted to Department of Bioinformatics and Biotechnology,
International Islamic University, Islamabad as a partial Fulfillment of requirement of the award of the
Master in Sciences of Biotechnology
(MSBT)

This humble effort is

Dedicated

To my beloved

Parents, Brothers, Sisters and especially late brother Aftab Sarwar

Without their unrelenting supervision
I could not have completed this task

DECLARATION

I hereby solemnly declare that the work **"Production and Characterization of Lipase from *Humicola* species"**. 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: 10-10-16

Fiaz Ahmed

165-FBAS/MSBT/F-14

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ABBREVIATIONS

AOC	Apricot oil cake
ASD	Aillary seborrheic dermatitis
AV	Acne vulgaris
CCRD	Central composite rotatable design
CLA	Conjugated linoleic acid
DAP	Diammonium phosphate
DEAE	Diethylaminoethanol
FFAs	Free fatty acids
kDa	Kilodaltons
K_m	Michaelis-Menten constant
MOC	Mustered oil cake
MWF	Metal working fluids
NaTDC	Sodium taurodeoxycholate
OOC	Olive oil cake
PPEs	Personal protective equipments
PDA	potato dextrose agar
PAGE	Polyacrylamide gel electrophoresis
PUFAs	Polyunsaturated fatty acids
PCA	Polycarboxylates
pNP	Para-nitro phenyl palmitate
RSM	Response surface methodology
SDS	Sodium dodecyl sulphate
SSF	Solid-state fermentation
SmF	Submerged state fermentation
TAG	Triacylglycerol
TOC	Taramera oil cake
TLL	*Thermomyces lanuginosus*
V_{max}	Maximum velocity

ABSTRACT

The current study was conducted for the isolation, production and characterization of lipase enzyme from *Humicola* species. Six different fungal species were isolated from the different soil sample (Vermi-zote, Elephant dung , plant composed material, white mushroom, oily soil) collected from the vicinity of Islamabad. The isolated fungi consist of *Humicola grisea, Humicola fuscoatra, Rhizomucor, Aspergillus, Penicillium purpurogenum. Humicola grisea* was selected for lipase production. For the optimum lipase production various growth parameters and fermentation conditions were optimized including time course study up to 120 h, effect of carbon sources (Apricot oil cake, Olive oil cake, Taramira oil cake, Mustard oil cake and its different concentration, nitrogen source (Urea, DAP, Ammonium nitrate and Ammonium sulphate), effect of various pH (citrate buffer of 4 to 7) and temperature range from 40 °C , 45 °C, 50 °C and 55 °C for optimum lipase production. The study results showed that at 72 h using 1 % of inoculum, 2 % of olive oil cake, 2 % ammonium nitrate, and pH 6 was found best for the maximum lipase production. The crude enzyme was purified by ammonium sulphate precipitation method using different salt concentration. It was found 40 % of ammonium sulphate concentration was found best for lipase enzyme precipitation. The characterization of purified lipase enzyme was done for the determination of kinetic parameters. 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 to 7. The present enzyme was most active in the range of pH 6 with maximum activity 390 U/ml/min. Effect of temperature on purified extracellular lipase enzyme activity was studied by incubating the enzyme at different temperature ranges from 30 °C to 80 °C. Maximum extracellular lipase enzyme activity was 250 U/ml/min) was obtained at 45 °C. The activity of the present enzyme was dropped above 55 °C. Thermo stability of the enzyme was optimized by incubating the partially purified extra cellular lipase with different temperatures range from 50 °C to 95 °C) for 30 min. The values of K_m and V_{max}, as calculated from the Line weaver Burk plot, were 0.4 g/ml and 285.7 U/ml/min, respectively.The activation energy Ea calculated for lipase enzyme denaturation calculated was Ea 49.7 kJ/mol.

INTRODUCTION

1. INTRODUCTION

The word enzymes is derived from the Greek word 'en' meaning 'in' and 'zyme' meaning 'yeast' or 'leaven'. Enzymes have been important research venture for researcher in the field of biochemistry and biotechnology which has boosted knowledge of enzymes for industries. Today these enzymes are used in almost every step of life such as in research products like in medicine, food, research and diverse applications in industries. Currently approximately 4000 enzymes are known and out of these only 200 enzymes are used commercially. Majority of the industrial enzymes are microbial origin because of improved production and their applications as biocatalysts in biotechnological processes (Sharma *et al.*, 2001).

Lipase enzyme has the ability to breakdown long chain of carban atom that is triacylglycerides into mono and diacylglyceride and free fatty acid (Fig 1.1) (Verger, 1997). Lipases also act on esters as a result it slow down the process of production from the environment having low concentrations of water (Sharma *et al.*, 2002).

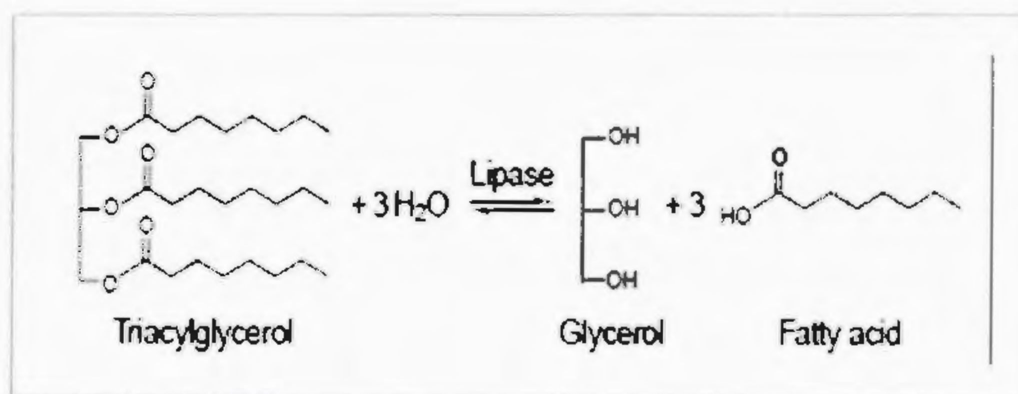

Fig 1.1: Hydrolytic and syn thetic action of lipases (Jaeger and Reetz, 1998)

Lipase has more potential having extensive range of pH, positional specificity and thermostablity (Saxena *et al.*, 1999). Lipase are widely used in the industrial field due to having ability of non specific reaction at all sites, it can react at any position of the substrate as a result we able to get different varieties from the lipase like synthesis of cocoa butter, taste enhancer the resolution of chiral drugs and biofules (Saxena *et al.*, 1999).

1.1 Structure and mechanism of lipases

To be aware of the Working mechanism of lipase on molecular level in the different organisms is very important. Sarda and Desnuelle, (1958) described the lipase activity toward insoluble substrate in term of kinetic term on the basis of interfacial activation. There are three active site of amino acid where one after another forms catalytic activity namely, aspartic glutamic acid and histidine. Substrate hydrolysis starts with a nucleophilic attack by the catalytic-site- Ser oxygen on the carbonyl carbon atom of the ester bond, leading to the formation of a tetrahedral intermediate stabilized by hydrogen bonding to nitrogen atoms of main chain residues that belong to the so called 'oxyanion hole'. An alcohol is liberated, leaving behind an acyl-lipase complex, which is finally hydrolysed with liberation of the fatty acid and regeneration of the enzyme (Fig 1.2)

Lipases are serine hydrolases and contain the consensus sequence G–X1–S–X2–G as the catalytic moiety, where G = glycine, S = serine, X1 = histidine and X2 = glutamic or aspartic acid (Svendsen., 1994). Knowledge of the three dimensional structures of lipases plays an important role in designing and engineering lipases for specific purposes.

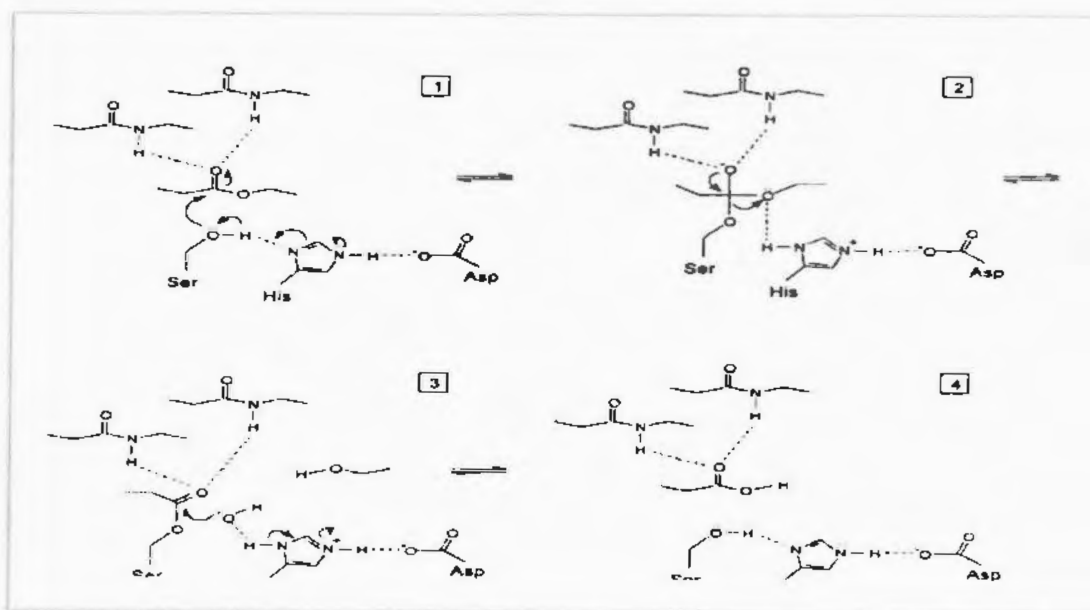

Fig 1.2: Reaction mechanism of lipases (Jaeger *et al.*, 1999)

1.2 Substrate specificity of lipase

Specificity of substrate can be studied in to three parts i.e. positional specificity, fatty acid specificity and partial glycerides specificity (Rahman *et al.*, 2006). Specificity of lipases depends upon three things that is composition of substrate, molecular possessions of enzyme and feature disturbing to the substrate respectively (Saxena *et al.*, 1999).

1.2.1 Positional specificity

In the positional specificity lipase brake down large component into small component like triglycerides in to diaglycerides, monoglycerides, glycerol and free fatty acid .Fatty acid can be released from the all three position of triglycerides and secondly it can be released from 1-3 position of glycerides (Illanes, 2008). There are some microbial species that shows 1-3-(regio)-specificity and partial stereo specificity in *H. lanuginosa* studied 3-(regio)-specificity (Saxena *et al.*, 1999) and Candida cylindracea species on the brake down of triacylglycerols. Alcohols and ester can separated optically in the presences of lipase (Illanes, 2008).

1.2.2 Fatty acid specificity

Lipolytic activity of microorganisms to breakdown fatty ester depending upon the substrate availability such as rates of treptomyces rimosus lipase show maximum hydrolysis in the substrates p-Nitro phenyl-caprylate (C8), p-NP- caprate (C10) and p-NP-laurate (12) representing the enzyme inclination for average acyl chain lengths. p-NP-palmitate and p-NP-myristate were also excellent sources but p-NP-stearate ate breakdown at significantly slower pace. Lipase gives its maximum activity on specific chain substrate that is above than 70 % and less than 30 % (Rahman *et al.*, 2006) the lipases from various *Rhizopus* species were reported to show maximum hydrolytic activity with the medium chain fatty acids (Saxena *et al.*, 1999).

1.2.3 Partial glycerides specificity

In addition to triglycerides, lipases can hydrolyze mono and triglycerides into fatty acids and glycerol. Lipase that hydrolyses monoglycerides is called monoglyceride lipases (E.C 3.1.1.23) (Shimada *et al.*, 1994).

1.3 Distribution and production of fungal lipases enzyme

Lipase enzyme is uni versally distributed in almost all organisms which comprise bacteria, archea and eukaryotic with plant animal and fungi. Microorganism with approaching to produce lipase found in the different habitat like industrial waste, soil contaminated, vegetable

oil, dairy product seed and rubbish food. Due to too much importance of enzyme in our daily life researcher are studying enzyme on its different aspects and their publication helpful in the industries (Thakur, 2012). In 1856 Clade Bernard exposed lipase enzyme from the animal pancreatic juice which has converted insoluble oil into soluble product. In the past lipase enzyme was usually obtained from the animals to assist as a digestive for individual. It was not easy to get enzyme in a broad range from the animals so microbial enzyme was introduced having a lot of potential. Microbes are known as a best source for enzyme production because these can easily be grown in the laboratory in a short time. Microbes can be easily manipulate genetically when ever required (Hasan *et al.*, 2006). Enzyme obtained from microorganisms has massive biotechnological prospective having extensive specificity of substrate, stability in the organic solvent. Lipases do not require cofactor for reaction. (Jaeger and Reetz, 1998). Filamentous fungi are fascinating source of lipases as they produce extracellular enzymes (Gutarra *et al.,* 2009). Lipases can be produced by two technique Submerged Fermentation (SmF) and Solid-State Fermentation (SSF), fungi production yields maximum at SSF on the other hand yeast and bacteria gives maximum production on SSF (Gutarra *et al.,* 2009). As comparing to SmF the SSF taken as first priority for the production of enzyme due to low cost of fermentation media, easy to handling, simpler equipments and control systems, lower energy utilization (Hosseinpour *et al.,* 2012).

1.4 *Humicola* sp. lipase

Although both Thermophlic , Mesophilic and psycrophiles species. can produce lipase but protein obtained from microorganisms which can tolerate in high temperature as a result thermo stable enzyme obtained which is the demand of industries. (Imamura and Kitaura, 2000). Although lipase obtained from mesophylic and psychrophlic species also used in the different field of industry but the Thermo-stable enzyme have lot of advantages like low risk contamination during isolation to purification, more yield, and lower stickiness. With the help of thermo stable enzyme we can make biopolymers and biodiesel it also helpful in the field of pharmaceuticals, makeup, and flavors. According to (Razak *et al.*, 1997) a very small number of fungal lipases can show their reaction at temperatures above 40 °C. There is some species like *Humicola lanuginosa* were reported as 45 °C to 60 °C, (Maheshwari *et al.*, 2000), *Rhizopus* strain active at 50 °C, *Geotrichum* 60 °C (Ginalska *et al.*, 2004) From the *Humicola lanuginosa* Lipase was purified by the adding the salt acetone precipitation. The method applied was the

steps of chromatographic filtrate its molecular weight was calculated as 39 kDa on Sephadex G-100, indicating that enzyme is a monomer with a pH of 6.6. Lipase range 30-60 kDa was observed in the majority lipases from *Penicillum* sp. (Ferrer *et al.*, 2000) that *Humicola grisea* lipases almost denature at the 95 °C after 30 Minutes. Bacteria which are identified as a best lipase producer includes *Serratia marcescens, Pseudomonas aeruginosa* and *Pseudomonas fluorescens, Bacillus prodigiosus, B. pyocyaneus* and *B. fluorescens among the moulds Penicillum* genus have been renowned as most excellent lipase sources (Christakopoulos *et al.*, 1992). *Humicola* sp. lipase articulated in *A.oryzae* (Lypolase TM) is examples of lipases which validate a high degree of specificity for the external site of triglycerides. Such lipases used in washing detergents and for specific adjustment of triglycerides (Damaso *et al.*, 2008).

1.5 Industrial application of lipase

With the rapid development of biotechnological techniques now it's easy for researcher to work on the enzyme. Due to the fast development of this technology enzyme almost used in the every step of human being. Like in industries different enzyme used for the production of biodiesel to synthesis of fine chemicals (Fernandez, 2010). Having the potential of esterification, and transesterification reaction in non eqous environment lipase enzyme used in the various application in the food, leather industry, washing powder (detergent), textile industry, makeup, paper and pharmaceutical industries (Houde *et al.*, 2004). Some important application which used in our daily life is mentioned one by one.

1.5.1 Oil / fat processing industry

Regiospecificity is playing its vital role in the field of lipase skill for the alteration of oils and fats to make high-value additional products. By changing its length of fatty acid we can convert less valuable product in to valuable product for example through reaction low cost oil that is palm mid fraction convert in comparable of cocoa butter, human milk fat substitutes, biodiesel obtained from vegetable oil (Jaeger and Reetz, 1998). A chain length of fatty acid i.e. gl ycerol backbone can be influenced by physical factors and substrate (Sharma *et al.*, 2002). in the processing of oleochemical during hydrolysis, glycolysis and alcoholysis lipases, conditional upon the substrates, can catalyze acidolysis (where an acyl moiety is uprooted between an acyl glycerol and a carboxylic corrosive), alcoholysis (where an acyl moiety is dislodged between an acyl glycerol and a liquor) and transesterification (where acyl moieties are traded between two

acylglycerols) (Sharma *et al.*, 2002). Lipases, which are commercially prepared from fungal source, were used in the synthesis of long chain fatty acids. These structured glycerides were synthesized by lipase catalyzed, interestrification between tricaprylin and peanut oil (Soumanov *et al.*, 1997). Due to the high cost of enzyme applications of lipases for normal oils and fats are still limited. Therefore, positional specificity of lipases has the priority and will be the target property to be exploited for commercial and industrial progress, because no chemical method has such specificity to shows such a potential.

1.5.2 Food industry

Likewise, protease, amylase, cellulose and xylanase the lipases enzyme are used in the food industry for the biocatalysis of stereoselective transformation. Lipase from *Rhizomucor miehei* is utilized to deliver a cocoa margarine alternate from less expensive palatable oil, the oleate solely involves the sn-2 position however palmitate instead of stearate prevails at the sn-1 and sn- 3 positions. Saxena *et al.*, (1999) and Pastou *et al.*, (2000) investigated that Endeavors in protein designing the lipases enzymes are used for acquire enhanced on the lipid surface as a negative charges, to release the top and enact the catalyst, and to expand dependability to elevated pH and from polar surfactants. Lipases and α-amylases alongside two miscellaneous eminence flavors and three microbial starters were employing to plan twenty four breads. So the blend of catalysts and starters brought about utilized unaccompanied (Pastou *et al.*, 2000). Lipases and α-amylases along with two different quality flavors and three microbial starters were used to formulate 24 breads. (Martinez *et al.*, 1999). *Humicola lanuginosa* lipase (Lipolase; Novo Industry) is being used in detergent formulations in conjunction with other microbial enzymes e.g. protease and amylase. Lipases are used to accelerate flavor production in blue cheese made with pasteurized milk. These lipases are contaminated with proteases. Selected animal and microbial lipases were added directly to the cheese curds along with *Penicillium rogueforti* spores and salts (Undurraga *et al.*, 2001). Sample of Baranje semi hard cheese was manufactured on industrial scale with different amount of lipolytic enzyme (Hiol *et al.*, 2000).

1.5.3 Organic compound industry

The specificity of lipases makes it conceivable to acquire mixes, which are hard to get ready by routine concoction techniques (Rezanka, 1991; Bell *et al.*, 1981). For the purpose of utilizations of saccharides, peptides or the arrangement of optically dynamic mixes Organic

compound dissolve through transsesterification and interesterficaion . Lipases speedup the water break down and merge of esters shaped from glycerol and elongated chain unsaturated fats. It has also been found that the different lipases behave differently in different organic solvents with different level of resistance in different reaction systems (Zhang *et al.*, 2011) Lipases differ in their sensitivity towards different organic solvents and are generally more unstable in polar water miscible-solvents than in water-immiscible solvents (Xu *et al.*, 2010). Therefore, efforts are being made to screen the enzymes which can work efficiently with high activity in different types of toxic organic solvents. Some bacterial strains are capable to survive in the presence of short chain alcohols like butanol and also in other toxic solvents like benzene and toluene. Two organic solvent tolerant bacteria *Enterococcus faecalis* and *Clostridium sporogenes* were screened in a medium supplemented with acetone or butanol (5 %; v/v) and *S. haemolyticus* was screened in a medium overlaid with 15 % benzene and 85 % cyclohexane. *S. haemolyticus*was able to grow in the presence of cyclohexane, benzene and toluene Nielsen et al., (2008). The numerous utilizations of lipases incorporate strength natural unions, hydrolysis of fats and oils, alteration of fats, flavor improvement in nourishment handling, determination of racemic blends, and substance examinations.

1.5.4 Chemical industry

Lipases are flexible biocatalyst that accomplishes a capacity of bio-conversion reactions, such as lysis of water, alcohol acid and amine along with interesterification. These lipase qualities resultes a vital part in the assembling of excellent chemicals in synthetic industry (Pandey *et al.*, 1999). Development of lipase based technologies for the synthesis of novel compounds is rapidly expanding the uses of these enzymes as well. Liese *et al.*, (2006) and Ortega *et al.*, (2004) reported interesterification (acidolysis) of fully hydrogenated soybean oil with conjugated linoleic acid (CLA) in a batch reactor at 75 °C. Lipases from *Candida antarctica*, *Rhizomucor miehei*, *Pseudomonas* sp. and *Thermomyces lanuginosa* were used at 5 % (wt/wt) of the total substrate load. The lipase from *Rhizomucor miehei* produced the fastest reaction rates, and the greatest extent of incorporation of (CLA) residues in acylglycerols was achieved in 12 h. Lipases from *C.antarctica* and *T. lanuginosa* produced slower initial rates, and maximum extents of incorporation of CLA residues were achieved in 24 h. The lipase from *Pseudomonas* sp. produced the slowest initial rate. The corresponding maximum extent of incorporation was reached in 48 h. Differential scanning calorime try analysis of the

triacylglycerol (TAG) fractions produced by *C. antarctica*, *R. miehei*, and *T. lanuginosa* lipases after purification by solid phase extraction showed little variation in melting point (60.4 °C, 62.8 °C, and 60.1 °C, respectively). By contrast, the corresponding TAG fraction produced by the *Pseudomonas* sp. lipase melted at 48.4 °C. The positional distribution of the TAGs produced by the lipase from *Pseudomonas* sp. differed appreciably from those produced by the other enzymes. (Macedo *et al.*, 2004) reported that microbial lipases (E.C. 3.1.1.3) from *Geotrichum* sp. and *Rhizopus* sp. were tested as catalysts in the esterification of isoamyl alcohol and butyric acid in a solvent-free system. Response surface methodology (RSM) based on a five level, two variable central composite rotatable design (CCRD) was used to optimize this process with respect to important reaction variables-substrate molar ratio (RM) temperature and lipase concentration (L). The variables RM and L were the most significant in the process. An increase in alcohol concentration from 1:2 to 4:1 caused a decrease in yield to 18.45 % after 48 h of reaction time and a variation of L from 1 to10 % caused an increase in yield to 32.2 %. The optimum conditions found to achieve maximum ester yield (75 %) are: RM=1.5:1; L=5.5 % (w/w), 40 °C after 48 h of reaction time. (Karadzic *et al.*, 2006) studied the stability of extracellular lipases and its activity in organic solvents and suggested that it is highly suitable as biotechnological tool in water restricted medium with a variety of applications including organosynthtic reactions and the control and prevention of metal working fluids (MWF) purification on metal industry.

1.5.5 Pharmaceutical industry

Lipases enzyme is the manufacture of chiral building for pharmaceuticals, agrochemicals and pesticides. Making of single enantiomers of medication intermediates has turned out to be progressively imperative in medicinal companies (Patel, 2002). Lipase from *Candida antarctica* (Novozyme (R) 435) has been used for the kinetic resolution of racemic flurbiprofen by the method of enantioselective esterification with alcohols (Zhang *et al.*, 2005). Baclofen, chemically (*RS*)-beta-(aminomethyl)-4-chlorobenzenepropanoic acid, which is used as muscle relaxant and in the pain therapy, produces two isomers and lipase from *C. cylindracea* has been used in the resolution of racemic mixture (Muralidhar *et al.*, 2001). chiral molecules include: (1) the synthesis of (3R-cis)-3-(acetyl o xy)-4-phenyl- 2-azetidinone-2 for the semi-synthesis of Paclitaxel (taxol) 5, an anticancer compound; (2) synthesis of chiral (exo)-7-oxabicyclo heptane-2,3-dimethanol monoacetate ester 9 for the chemoenzymic preparation of a thermboxane A2

antagonist; (3) the enzymic synthesis of S-3-benylthio-2- methylpropanoic acid, a key chiral intermediate for the synthesis of ant hypersensitive drugs captopril 10 or zofenopril 13.

1.5.6 Detergents industry

Biotechnology based cleaning agents such as enzyme based agents, are widely used in industries. It was observed that more or less than 1000 tons of lipases are sold every year (Cardenas *et al.*, 2001). Scientist trying to get more stable lipase which are tollrate to extreme temperature and pH conditions from microbial lipase. The enzyme based detergents have better cleaning properties as compared to synthetic detergents. They are active at low washing temperatures and environment friendly also (Kumar *et al.*, 1998). The enzyme containing detergents also improve the fabric quality and keeping color bright. The domestic dishwashing detergents contain different chemicals like chelating agents, surfactant, polycarboxylates (PCA), phosphonates, active chlorine compounds etc. These substances have high environmental risks, particularly via sewage biosolids, poor biodegradability, increased organic load to sewage works, Thermostable enzymes are usually stable in solvents and detergents; therefore, they also have considerable potential for many biotechnological and industrial applications (Haki and Rakshit, 2003) Lipase in detergent demand increasing in commercially because of (a) low substrate specificity to be able to hydrolyze (fats) (b) resistance toward harsh washing condition (30-60 °C, pH 10-12) and (c) resistance to damaging surfactants and enzymes. Hydrolysis fat, which is used in detergents both house hold and industrial laundry (Ito *et al.*, 1998). It makes environment friendly by removing. The enzyme hydrolyzes the original fats (triglycerides) into more soluble monoglycerides, triglycerides, free fatty acids and glycerol (Sharma *et al.*, 2002). *Humicola* sp. stains was commercialized by Novo Nordisk company developed (1988) for textile industry, unfortunately it can't full fill its demand due to less production However, by introducing gene from *Humicola* sp in to *Aspergillus oryzae*, helpful toward commercialization showing improved washing performances and energy savings (http://www.efbweb.org/topics/genetic/menu.htm).Lipases which are active at alkaline media are preferred for laundry detergents.

1.5.7 Diagnostic tool

In the medical sector, lipases are important drug targets or marker enzymes. Their presence and high levels can indicate certain infection or disease and thus lipases can be used as diagnostic tools. In blood serum, the levels of lipases can be used as diagnostic tool in the

detection of acute pancreatitis and pancreatic injury. Despite the most accurate laboratory indicators for pancreatitis, serum amylase and lipase levels are still used in the diagnosis of this disease (Lott and Lu, 1991). In *Vibrio cholerae* infections, kanagawa haemolysin, slime, lipase, and colonial opacity have also been considered as virulence markers. Lipases derived from some pathogenic bacteria such as *Propionibacterium acnes, Corynebacterium acnes* and *Staphylococcus aureus* have als o been found to affect skin in acne patients (Hasan *et al.*, 2006).

1.5.8 Waste treatment

In aerobic waste processes, lipases are utilized to remove layers of fats continuously from the surfaces of aerated tanks to allow oxygen transport (to maintain living conditions for biomass). For this purpose, *Candida rugosa* lipase can be used. Clearing and prevention of fat blockage and effective breakdown of solids in waste systems are important in many industrial operations such as degradation of organic debris - for this purpose, commercial mixture of lipase, cellulase, amylase, inorganic nutrients is utilized, and cleaning of holding tanks, saptic tanks, grease traps. In food processing industry, leather industry and in the poultry waste processing, effective treatment is also necessary. In lipid-rich wastewater treatment, *P. aeruginosa* LP602 cells and lipase together were shown to be usable. Some environmental problems such as breakdown of fats in anaerobic digesters can be solved by bacterial lipases. During anaerobic digestion process, macromolecules are broken down into simpler compounds in the presence of extracellular enzymes. The increased sulphide concentration generated during the sulphate reduction process stimulates the enzymes (proteases, lipases and glycosidase) leading to enhanced solubilization of primary sewage sludge (Hasan *et al.*, 2006).

1.5.9 Pulp and paper industry

Lipases play important role in paper industry. Lipase remove pitch from the pulp and help to making paper. Lipase of *Candida rugosa* was used by Nippon Paper industries (Japan) which hydrolysis up to 90 % of w ood triglycerides (Sharma *et al.*, 2002).

1.5.10 Biodiesel synthesis.

Lipase has an important application in the field of bioenergy especially in the production of biodiesel demand increasing in the world wide due to lack of limited fossil fuel and environmental pressure. Biodiesel gained its importance due to environmentally friendly with emission of low carbon dioxide and greenhouse gases (Colla *et al.*, 2010; Jegannathan *et al.*, 2008). Currently there are three main biodiesel feed stock in the form of plant oil included

soybean oil (Yuan *et al.*, 2008) Jatropha oil (Pudi *et al.*, 2007) palm oil (Halim *et al.*, 2009) cotton seed oil, sunflower oil (Dizge *et al.*, 2008) animal fat like tallow, lard, grease (Ngo *et al.*, 2008) and waste cooking oil and industrial waste oil (Dizge *et al.*, 2008). Biodiesel production in different countries depends on their stock at national conditions (Zhang, 2008). Biodiesel can be produced from the different feed stocks by the process of transesterification by using catalyst either chemical or enzymatic. Chemical transesterification using alkaline catalyst give high yield of biodiesel in a short reaction time however it consume high energy and glycerol recovery is difficult while enzymatic catalyst like lipase are effective in the process of transesterfication in both aqous and non aqous system on the other hand product cost of lipase is greater than alkaline (Pizarro and Park, 2003; Fuduka *et al.*, 2001). Immobilized lipase different techniques like adsorption, encapsulation, entrapment, covalent bonding and cross linking played an important role in the production of biodiesel production. These method help to improve lipase stability and allow for repeated utilization. (Cao, 2005; Salis *et al.*, 2008) *Thermomyces lanuginosa* and *C. antarctica* were used as biocatalysts in preparation of simple alkyl ester derivatives of restaurant grease (Hsu *et al.*, 2002).

1.6. Aims and objective

The present study is aimed to achieve the following objectives:

- Isolation and identification of *Humicola* sp.from soil samples collected from Islamabad.
- Optimization of carbon and nitrogen sources for lipase production.
- Optimization of growth temperature and media pH for lipase production.
- Partial purification of lipase from *Humicola* sp.
- Calculation of various kinetic parameters of partially purified lipase.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Lima *et al.*, (2003) isolated *Penicillium aurantiogriseum* fungal sp. from soybean oil. It was cultured on submerged medium with 1 % olive oil and 0.5 % yeast extract which gave 0.4 U/mL lipase activities through volumetric assay. The end product of various nitrogen sources (yeast extract, peptone, meat peptone, casein, ammonium sulfate in the culture medium including potassium nitrate and other important mineral salts) was observed for the maximum lipase activity. After 72 h with the ammonium sulfate the lipase production of calculated 13 U/ml was obtained. The lipase activity was increased up to 18 U/ml with the increase in ammonium sulfate concentration. Various carbon sources were used like olive, corn, soy and sunflower oils. The maximum lipase activity was obtained at 1 % of olive oil giving 3625 U/ml after 48 h. The enzyme activity was obtained on different temperature ranging from 26 to 32 °C, the maximum lipase activity was optimized at 29 °C. Under optimized conditions, the dry bran gives 40 U/g of lipase activity.

Cihangir and Sarikaya, (2004) isolated *Aspergillus* sp. fungi from the soil and effect of different growth parameters was optimized for lipase production. Under optimized carbon, nitrogen sources, temperature range, pH and incubation period, *Aspergillus* sp. shows maximum lipase production on olive oil as carbon sources and peptone as nitrogen sources at of pH 5.5 and 30 °C.

Fickers *et al.*, (2004) performed experiment on lipase activity from fungal sp. *Yarrowia lipolytica* and its mutant sp. The Tryptone N1 as nitrogen sources and oleic acid as carban sources was selected for the production of extracellular lipase. When tryptone N1 concentration increased in a culture media its enzymatic activity was also increased. It was observed that in the presence of oleic acid its enzymatic activity decreased before it is released in the media. In their growth phase lipase is bounded to cell due to which the decrease occurs in it.

Lin and Ko, (2005) investigated that a thermostable lipase from *Antrodia cinnamomea* had a novel alkaline-resistant. Two media was used for the synthesis of enzyme on submerged culture that is (modified ME and modified YM). With the addition of glucose lipase production increased when it have 8 % of the custom-made ME and YM media. The manufacture of lipase was improved extraordinarily from zero to 3.35 U/ml and from 0.59 to

6.17 U/mL, correspondingly. However the action of lipase decreases after certain concentration limit. From both modified media YM medium was better than modified ME medium for enzyme synthesis. For the crude lipase maximum pH 11 and temperature 60 °C. Lipolytic activities of enzyme remain same at the pH 7 and 12 having temperature 80 °C.

Gopinath *et al.*, (2005) conducted research on 34 wild fungal sp. to investigate extracellular enzyme of different fungus from the surrounding environment. The substrate used for it was edible oil mill wastes. The techniques apply for it was serial dilution technique for isolation and rapid screening for the production of gelatinolytic activity extra cellular enzymes like lipase, amylase, protease and cellulose. Along with all species of *Aspergillus versicolor* showed amlyatic activity and *Penicillium citrinum* displayed the amylolytic activity. Cellulolytic activity expressed in the *Absidia corymbifera.*15 Fungal species *Ab. corymbifera, As. fumigatus, As. japonicus, As. nidulans, As. terreus, Cun. Verticillata, Cur. Pallescens, F.oxysporum, Geotrichum candidum, M.racemosus, P.Citrinum, P.Frequentans, Rhizopus stolonifer* and *Trichoderma viride* revealed extreme lipase activity.

Colen *et al.*, (2006) isolated 59 fungal sp from Brazilian savanna soil in plate containing agar medium having olive oil emulsion and bile salts. Out of 59 species, Twenty-one strains reported as a lipase producer strain, eleven strains fungal sp. gives good result on solid-state fermentation and submerged liquid state fermentation (shaken cultures). *Colletotrichum gloesporioides* selected as the most productive strain, which gives 27,700 U/l of lipase under optimum conditions.

Karadzic *et al.*, (2006) studied extracellular lipase of extremophile. Its optimized pH and temperature was 11 and 70 °C. SDS-PAGE was used to calculate 26 the molecular mass of purified lipase was to be 54 kDa. Enzyme activity was effected by Zn^{+2}, Hg^{+2} and Cu^{+2} and to some and Mg^{+2}.

Savitha *et al.*, (2007) studied different fungal genera to check lipase activity by applying the screening method Rhodamine B and the substrate used as sunflower oil as carbon sources. *Mucor* sp. was found to inducible, alkalophilic and thermo stable display on full fluorescence zone at 350 nm. Out of 32 fungal species, only three species belonging to *Aspergillus* sp. and one *Mucor* sp. shows ability for lipase production.

Aloulou *et al.*, (2007) studied the different effect of detergents and pH on the two fungal lipase (*Yarrowia lipolytica*) (YLLIP2) and *Thermomyces lanuginosus*) (TLL). There finding helpful for lipase application enzyme especially in the replacement therapy of patients with the lack of pancreatic enzyme because of high detergent concentrations and highly pH values variable. They encounter in the GI tract. The strategies applied for it trioctanoin emulsions as well as monomolecular films banquet at the air water boundary, TLL is more sensitive as compared to YLLIP2 however it can protected in the presence of monomers and minimizing temperature. At pH 7.0 interfacial binding and activities on substrate trioctanoin of YLLIP2 and TLL were inhibited by sodium taurodeoxycholate (NaTDC). *Yarrowia lipolytica*) sp. in the substrate trioctanoin in addition to NaTDC remains active at pH 6.0 but not *Thermomyces lanuginosea* (TLL). *Yarrowia lipolytica* (YLLIP2) need strong interfacial binding while TLL can be detected in water phase. YLLIP2 become strong when at oil water bounding at low pH with detergents.

Kempka *et al.*, (2008) investigated that lipase obtained from the agroindustrial waste is cheap sources for the commercialization. It was also observed that different inductor shows positive result on the production of lipase on the other hand the temperature and initial moisture may affect its production. The moisture of substrate 55 % and temperature 27.5 °C were determined and optimized in the response surface methodology.

Thirunavukarasu *et al.*, (2008) experimented the efficacy of *Cryptococcus* sp. (CS2) lipase for the exclusion of triglyceride from fabrics with soil and olive oil consecutively to use the enzyme as a preservative in detergents. In this process single wash cycle was used as a The (CS2) yeast produce tool of response surface methodology (RSM). Novel lipase cutiase-like enzyme which was attuned with various cationic, ionic and non-ionic surfactants as well as oxidizing agents and commercial detergents. Five levels of four factorial central composite rotatable design (CCRD) was used to calculate the interactive effects of lipase concentration, buffer pH, detergent concentration and washing temperature on the optimum conditions were 0.5 % of detergent, lipase 1000 U, pH of 8.0, and The conditions advanced after RSM were 0.5 % cleanser, 1000 U of lipase, cushion removal of triglyceride soil from cotton fabric. After RSM the pH of 8.0 and washing temperature of 37 °C.The study results appeared under particular ideal conditions utilized had huge effect on oil expulsion from cotton fabric5in the nearness of lipase when contrasted with the treatment

results with cleanser wash alone. The result was 19.6 and 43.1 % higher at 20 and 37 °C separately.

Romdhane *et al.*, (2010) confined a contagious sp. *Talaromyces thermophilus* and watched that its lipase action is like *Thermomyces lanuginosea.* A 39 kDa monomeric lipase (TTL) was refined with the assistance of ammonium sulfate precipitation, gel filtration and anion trade chromatography respectively. At the pH 9.5 and temperature 50 °C the lipolytic action was calculated against tributyrin and olive oil emulsion as substrate, about (7300 ± 122) and (9868 ± 139) U/mg. TTL appears on trioctano in maximum particular exercises were observed to be (24,110 ± 390 U/mg).TTL was observed to be to some degree steady and dynamic on long chain triglycerides at pH 9.5 Interestingly, TTL was observed to be impervious to interfacial denaturation since it didn't require any cleanser to demonstrate its most extreme movement on immaculate triglycerides. These outcomes are especially noteworthy for lipase applications, specifically when variable applications, specifically when variable temperatures and high pH qualities can be experienced and it's bent to show abnormal states movement within the sight of different surfactants and similarity with some business wash specialists and blanch operators.

Cavalcanti *et al.*, (2011) performed an experiment to produce biodiesel by the method of hydroesterfication, *Thermomyces lanuginosa* (TL100L) lipase was used to produce free fatty acid by the process of esterification without alcohol in the presences of catalyst niobic acid or without catalyst. Experiment shows that in the presence of niobic acid only after one hour yield 92 % free fatty acid obtained while in the absence of niobic acid quantity of yield was 89 % after 48 h. The most excellent result was obtained for the enzyme catalyzed hydrolysis under the reaction conditions of 50 % (v/v) soybean oil and 2.3 % (v/v) lipase (25 U/mL of reaction medium) in distilled water at 60 °C. 89 % alteration rate to FFAs was obtained after almost 48 h of reaction. In esterification reaction, the best result was obtained at FFA/methanol molar ratio of 1:3 niobic acid catalyst at a concentration of 20 % (w/w FFA) at and 200 °C which yielded 92 % alteration of FFAs to soy methyl esters after 1 h of reaction, Simple reaction medium was used to study both hydrolysis and the esterification with high substrate concentrations.

Kovalenko *et al.*, (2013) conducted experiment to study lipase biocatalyst activity from *Thermomyces lanuginosus* strain through immobilization SiO_2 xerogel composed of

carbon nanotubes having multi walled bulb like structure with different diameter. A lipase property like structure and dispersity of biocatalysts of nanocarbon of the strain on the basis of *E.coli* BL (DE3) was studied in cell suspension and in immobilized state. Recombinant intracellular lipase activity of dry cell having on high thermostability on tributyrine hydrolysis gives 50 U/mg average values. Upon heating in olive oil at 100 °C, the inactivation constant and the half-life inactivation time comprised 6×10^{-3} per min and 2 h, respectively exceeding by one order the thermostability of lipase in a buffer solution. Biocatalysts that contained aggregated thick carbon nanotubes with a diameter of 20-22 nm had the maximum initial activity 250 U/g.

Esteves *et al.,* (2014) studied the extracellular lipase production from phytopathogenic Botryosphaeriaceae on different temperature. Hydrolytic and oxidative activities were studied 70 % strain produce at the same time cellulases, laccases, xylanases, pectinases, pectin lyases, amylases, lipases, and proteases. Several enzymes of Mesophilic fungi were noted as thermostable. *Botryosphaeriales* lipase which is significantly more active than cellulases from *Neofusicoccum mediterraneum* CAA 001 and from *Dothiorella prunicola* CBS 124723, lipases from *Diplodia pinea* (CAA 015 and CBS 109726), and proteases from *Melanops tulasnei* CBS 116806 were more active at 70 °C. Due to significant thermal activity these fungi may be used for industrial application like food and the health industries (proteases), the pulp-and-paper and biofuel industries (cellulases), or even in the detergent industry (lipases, proteases, amylases, and cellulases).

Fabiszewska *et al.*, (2014) evaluated the production of lipase from *Yarrowia lipolytica* KKP 379 fungi. Extracellular lipase activity increased in the late exponential phase due to the lower accumulation of lipase molecules in cell walls. The extracellular and cell bound lipase production was observed on different pattern ,which was found in highest in the early exponential phase and extracellular lipase activity increased due to the lower accumulation of lipase molecules in cell walls in the late exponential phase. Olive oil as a carbon sources was best for *Y. lipolytica* KKP 379 for the production of lipase while by the addition of glucose, dodecane and olive oil had effect on positive effect on biomass yield. Lipase production was also possible from microbiological utilization of Dodecane and/or glycerol with the addition of some activators such as olive oil in the culture medium.

Yaswanth *et al.*, (2015) investigated that species which has ability to produce lipase helpful as a remediation from oil contaminated soil. A bacterial species *Stenotrophomonas maltophilia* which belong to family of Xanthomonadaceae was selected on the basis of highest of lipase production. *Stenotrophomonas maltophilia* was identified on the basis of 16S rRNA and biochemically. For the enzyme production carbon and nitrogen sources (sesame oil and tryptone) was optimized. On optimized condition (175µmoles/ml) enzyme calculated from the species. He observed that Na^{+} ions promote enzyme production on the other hand Hg^{2+} ions repressed the lipolytic activity. Best enzyme produced having parameter on 80% ammonium sulfate, pH 6 and at 50°C.its molecular weight was calculated as 45 kDa based on SDS-PAGE. He reported the value of 0.727 K_m and V_{max} of 156.6µmoles/ml. Arrhenius plot study for energy of activation showed as 11.07 $Kjmol^{-1}$ K^{-1} for this lipase.

MATERIALS AND METHODS

3. Material and Method

3.1. Chemicals

The chemical used in this research were of analytical grade.

3.2. Collection of carbon substrate

The raw materials as carbon sources like carbon apricot oil cake was obtained from Gilgit Baltistan Hunza oil industry (Pvt) Ltd, Mustered oil cake and Taramera oil cake were collected from Adnan oil manufacturer at Rawalpindi (Raja Bazar), Olive oil cake was obtained from PMAS-University of Arid Agriculture Rawalpindi.

3.3. Collection of samples

The soil samples of vermi-zote, Elephant dung, plant composite material, white mushroom, as well as industrial waste were collected in bottles using pre-sterilized spatula from different places of Islamabad and Rawalpindi, Pakistan. Soil samples collected from different areas, Islamabad are shown in the table 3.1.

Table 3.1: Soil samples collected from different areas, Islamabad

Sample name	Area
Vermi-zote	National Agricultural Research Centre
Elephant dung	Zoo (Islamabad)
Plant composite material	National Agricultural Research Centre
White mushroom	National Agricultural Research Centre
Oily soil	Oil industries (Pvt) Ltd at Rawalpindi (Raja Bazar)

3.4. Isolation of *Humicola* sp.

3.4.1. Serial dilution

In serial dilution, 20 ml tubes were used and labeled as 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. Each tube was filled with 9 ml distilled water. On the other hand 1 gram of soil sample was added in the first tube (10^0) containing 9 ml of water. The tube was shaked well and 1 ml sample from the first tubes was transferred into next tubes up to 10^{-6} one by one with the help of micropipette.

3.5. PDA media composition

The potato dextrose agar (PDA) media used for production of *Humicola grisea*. The recipe of PDA is shown in table 3.2.

Table 3.2: PDA Media used for production of *Humicola grisea*

Chemical	Amount
Distill water	100 ml
Potato infusion	100 ml
Agar technical	1.5 g
Glucose	1.5 g

3.6. Streaking method

Personal protective equipments (PPEs) were regularly used in laboratory while conducting the experiments. Laminar hood was clean and sterilized by smearing 70 % ethanol before conducting the experiments. Other instruments like petri plates, spreader, pipettes, media and spatula were sterilized in autoclave for 15 minutes at 121 °C under pressure of 15 Pascal (pa). The antibiotics ampicolax 250 mg was added in the media before pouring it in petri plate, which resists the imminent bacterial contamination. Minimum 20 ml M media poured and waited for 30-40 min until its solidification. All petri plates were labeled 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 100 µl each diluted soil sample was dropped with the help of micropipettes in each plate and gently spreaded by means of spreader. The culture plates were incubated in different temperature range of 40 °C, 45 °C and 50 °C and were individually observed after every 24 hour.

3.7. Identification of fungal species

These isolated fungi were purified by sub culturing on fresh plates with help of sterilized wire loop. Isolated fungi were examined under microscope. One drop of distill water and fungal spore having mycelia was picked by sterilized wire loop and placed on slide than covered by slip. The Fungi were identified by their specific characteristics of spores and mycelium by comparing it with the literature. During research work from isolation to optimization it was observed that *Humicola* sp. growth processes is slow as compared to other fungal sp. like *Aspergillus* sp., *Rhizomucor* and *Pencillium*.

3.8. Slant formation and streaking

For the purpose of inoculums production from targeted *Humicola grisea* and to curtail contamination, first of all slants were prepared in 20 ml tubes by adding sterilized potato dextrose agar (PDA) media. Slants were assured in special slant tray until it becomes fixed. After that a full wire loop of fungal spore streaked in the slant in zigzag formation. The streaked slants were incubated at 45 °C. After five days a complete fungal growth was obtained on the slant. These slants were shifted in t o refrigerator having temperature 4 °C as a result its additional growth cease.

3.9. Spore suspension

Spore suspension was prepared by the adding of 0.9 % saline water in 4-7 days old slant followed by gentle scratching of slant surface with sterilized wire loop. 1 ml of spore suspension was used for inoculums production on different carbon and nitrogen sources.

3.10. Enzyme assay for the production of lipase enzyme

Enzyme activity was determined by using Titrim atric Assay as described by Fatima *et al.*, (2015).

3.11. Enzyme assay reagents

Following reagents were used for the assay. 0.1 M Phosphate buffer, Enzyme solution, Tween 80, Olive oil, Alcohol, Acetone, Phenolphthalein indictor and 0.1 N NaOH .

3.11.1 Procedures

Enzyme assay was performed by taking 0.5 ml of 0.1 M Phosphate buffer, 0.5 ml enzyme solution, 01 drop of Tween 80 and 01 ml olive oil in 100 ml flask. This mixture was put in the incubator at 45 °C for 10 minutes at 150 rpm. After 10 mints immediately 01 ml of acetone and alcohol 1:1 was added in the mixture to stop further reaction. 4-5 drop of phenolphthalein indictor was also added in the solution than it was titrated with 0.1 N NaOH. The concentration of NaOH used was carefully noted during titration and enzyme unit was calculated by fallowing formula.

$$\text{Enzyme unit (U/ml/min} = \frac{\Delta V \times N \times 1000 \times DF}{V\ \text{sample} \times T\text{min}}$$

Where,

ΔV is Volume of NaOH used

N is Normality of NaOH

DF is Dilution factor used for extraction buffer

V is Enzyme extract

Tmin is the time of incubation

3.12. Different parameter used for the production of lipase enzyme

For the production of lipase enzyme from *Humicola grisea* fallowing factor were studied in the current research

3.12.1. Selection of carbon sources

For the optimization of carbon sources four substrates were selected i.e. Apricot oil cakes, Olive oil cake, Taramera oil cake and Mustered oil cake. Before conducting experiment formostly, these substrates were grinded with the help of mortar and pestle to transform in to powder form. 01 g of substrate containing 5 ml Vogel media was autoclaved. The recipe for Vogel media is given in table 3.3. 01 ml inoculum of *Humicola grisea* from spore suspension was introduced in 150 ml flask. The flasks were labeled than shifted in to incubator. Each substrate enzyme activity was checked after 24, 48, 72, 96 and 120 hour. From all of the mentioned carbon sources, olive oil cake was selected as a best carbon source for the production of lipase enzyme because of its highest enzyme activity.

3.12.2. Optimization of carbon source concentration

For the optimization of carbon sources concentration optimization, 1g, 2g and 4g of substrate containing 5 ml Vogel and Johnson Agar was autoclaved. The recipe for Vogel media is given in table 3.3. 01 ml inoculum of *Humicola grisea* from spore suspension was introduced in 150 ml flask. The flasks were labeled than shifted in to incubator. Each substrate enzyme activity was checked after 24, 48, 72, 96 and 120 hour. From all of the mentioned carbon sources cocentrations, best carbon source concentration was selected for the production of lipase enzyme because of its highest enzyme activity.

3.12.3. Selection of nitrogen source

Four nitrogen sources were selected to tested enzyme activity for the optimization i.e. Ammonium sulphate, Ammonium nitrate, Urea and DAP. Nitrogen a source were added by the fallowing ration 0.17 % ammonium sulfate, 0.43 % ammonium nitrate, 0.32 % Urea and 0.7 % DAP in Vogel media (Roe, 2001). The recipe of Vogel media is given in table 3.3. All the

substrate autoclaved in 250 ml flask than and 01 ml spore suspension introduced. In every 24 hour enzyme assay was performed to check enzyme activity. From the entire substrate highest enzyme activity was noted in urea

Table.3.3: Vogel's media used for the production of lipase enzyme on different carbon and nitrogen sources for *Humicola grisea* (Vogel, 1956)

Chemical	Amount
1 %	Glucose
0.5 %	Tri-sodium citrate
0.5 %	KH_2PO_4
0.4 %	$(NH_4)_2 SO_4$
0.2 %	NH_4NO_3
0.1 %	yeast extract
0.02 %	$MgSO_47H_2O$

3.12.4. Optimization of temperature

Humicola grisea was cultured in different range of temperature 40 °C, 45 ° C, 50 °C, and 55 °C against substrate respectively. The growth appears at the 45 °C after 48 hour. After 48 hour its color turns from white to black gradually. Above and low from this rang of temperature its growth rate effect. Ultimately high enzyme production was observed in this rang of temperature

3.12.5. Optimization of pH

Citrate buffer of pH 4.0 to pH 6.0 was used on the selected carbon and nitrogen optimized substrate. Enzyme unit were calculated against each pH after 24, 48, 72, 96 and 120 h. The tritematric method applied for enzyme assay was similar as discussed as above 3.7. Highest enzyme unit was obtained at citrate buffer of pH 6.0.

3.13. Protein assay for substrate

Protein assay was performed to check concentration of protein in the substrate by Bradford method using spectrophotometer (Kruger, 1994). Absorbance of the crude extract substrate noted at 595 nm having, 0.1 % sample extract having 1 % Bradford reagent. Test

sample was taken into small glass tubes and incubated for 10 mints at 45 °C. The recipe of Bradford reagent is shown in the table 3.4. Bradford reagent was stirred overnight in a dark bottle and stored at 4 °C.

Table.3.4: The recipe of Bradford reagent to check concentration of protein in the substrate (Bradford, 1976)

Reagent	Quantity
Distilled water	1000 ml
Coomassie blue g -250	100 mg
Phosphoric acid 85 %	100 ml
Ethanol 95 %	50 ml

3.14. Partial purification of lipase

Crude extract was taken by adding 50 ml citrate buffer having pH 6.0 with the help of cloth and filter paper. After filtration properly a specific concentration of Ammonium sulfate added in the solution for overnight. Initially 20 % ammonium sulphate was dissolved in the enzyme solution. After overnight 20 % ammonium sulphate containing solution was carried to centrifugation for 5 minutes at 4000 rpm. After centrifugation enzyme assay and protein assay was performed of both pellet and supernatants. Supernatants were shifted in to dialysis bag for 24 hour. After dialyzing again enzyme assay and protein assay was performed. By fallowing above procedure ammonium sulphate of 30 % and 40 % was added. After calculating units we have selected 40 % which shows a significant result. Ammonium sulphate concentration table shown in the appendix II (Bokhari *et al*., 2009).

3.15. Characterization of Lipase

To study *Humicola grisea* lipase potential on different parameter was checked by comparing against experimental and controlled test each i.e. thermal denaturation of lipase, effect of temperature on enzyme assay, effect of on different olive oil concentration and effect of pH on lipase. For optimization of different parameter from crude extract enzyme unit calculated in the presence of undiluted enzyme while characterization of lipase for different parameter purified enzyme was diluted in to 5x, 10 x, 15 x and 20 x. 10x was selected as best dilution

range for characterization.

3.15.1. Effect of temperature on enzyme activity

Effect of temperature on *Humicola grisea* lipase enzyme was executed in shaking incubator at 150 rpm for 10 minutes. Lipase assay was carried on different range of temperature at constant time which includes 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C and 60 °C. Every 10 minutes through titrematric assay enzymatic activity was calculated. Maximum Enzyme unit (U/ ml/min) was examined on the 45 °C.

3.15.2. Effect of pH on lipase

On optimized temperature (45 °C) having 10 minutes incubation period in shaking incubator at 150 rpm an experiment was conducted in the presences of different buffer as pH 4.0, pH 4.4, pH 4.8, pH 5.0, pH 5.2, pH 5.4, pH 5.6, pH 6.0 and pH 7.0. Maximum lipase activity observed at pH 6.0.

3.15.3. Effect of lipase on different olive oil concentration

Olive oil substrate was used during enzyme assay as lipase inducer. Effect of oil concentration on enzymatic activity was performed on optimized temperature that is 45 °C. Olive oil concentration checked from 0.1 µl to 1.0 µl, (0.2 µl, 0.3 µl, 0.4 µl, 0.5 µl, 0.6 µl, 0.7 µl, 0.8 µl, 0.9 µl). Maximum Enzyme unit (U/ml/min) was recorded at 1.0 µl.

3.15.4. Thermal denaturation of *Humicola grisea* lipase

Lipase Thermal denaturation was conducted in incubator having a small sterilized tube in which 0.1 µl samples was taken on specified temperature i.e. 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, 85 °C, 90 °C and 95 °C for different time period 5, 10, 15, 20, 25 and 30 Minutes respectively. Temperature was monitored by the help of thermometer and time was noted by the help of stopwatch. Every 5 minutes the sample immediately shifted into the 100 ml flask contains enzyme assay mixture. Enzyme unit (U/ml/min) calculated against titration.

RESULTS

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4. RESULTS

4.1. Isolation and screening of thermophilic fungal sp.

Soil samples were collected from the vicinity of Islamabad and Rawalpindi, Pakistan. Six different fungal species were isolated from soil samples mentioned in the Table (4.1) by serial dilution method in the infectious disease lab of International Islamic University Islamabad. The fungal colonies appearing on the potato dextrose agar (PDA) media plates were picked and shifted to the slants of potato dextrose agar (Figure 4.1 and Figure 4.2).

Fig 4.1: Different soil sample dilution

Fig 4.2: *Humicola grisea* growth on PDA slant

Table 4.1: Soil samples collected from different areas and isolated fungal species

Sample name	Area	Isolated species
Vermi-zote	National Agricultural Research Centre	*Humicola grisea* *Humicola fuscoatra*
Elephant dung	Zoo (Islamabad)	*Rhizomucor* sp.
Plant composed material	National Agricultural Research Centre	*Aspergillus* sp.
White mushroom (*Agaricus bisporus*)	National Agricultural Research Centre	*Penicillium purpurogenum*
Oily soil	Oil industries (Pvt) Ltd at Rawalpindi (Raja Bazar)	*Aspergillus* sp.

4.2. Identification of different fungal species

Different fungal sp. was identified by the help of external morphology and microscopic image having specific characteristics of spores and mycelium by comparing it with the literature (Watanabe, 2002) among different isolated species, *Humicola* sp. growth period vary. *Humicola grisea* growth cycle complete in approximately 7 days by following two consecutive steps. After 48 h of growth, the culture plate looks white in color figure (4.3) with the passage of time spore formations start and color changes in to black figure (4.4). Microscopic images of different fungi (Figure 4.5, 4.6, 4.7, 4.9) appeared on slide having their specific characteristics of spores and mycelium.

Fig 4.3: *Humicola grisea* after 48 hours on PDA media

Fig 4.4: *Humicola grisea* after 7 days on PDA media

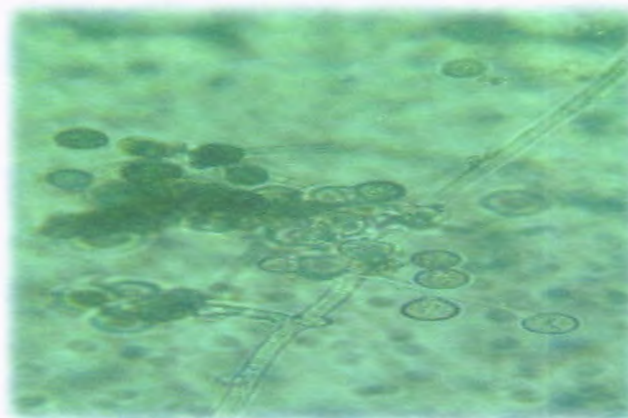

Fig 4.5: Microscopic view of *Humicola grisea*

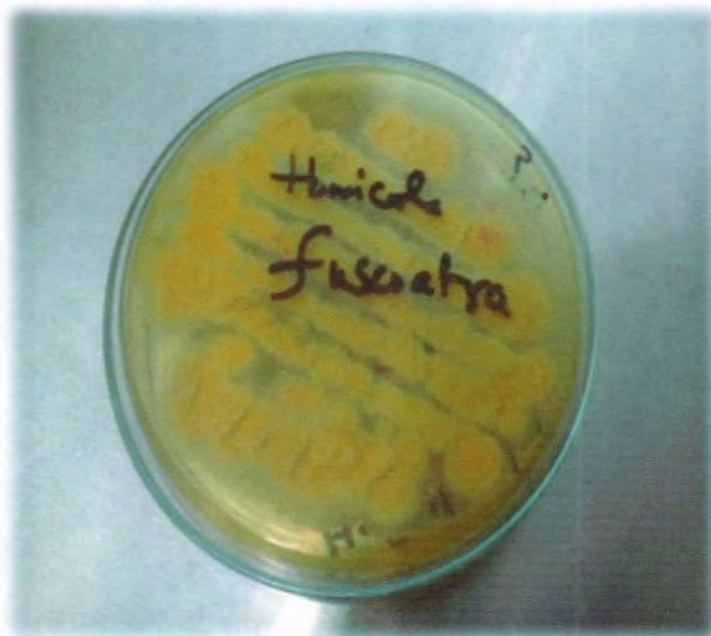

Fig 4.6: *Humicola fuscotra* on PDA media

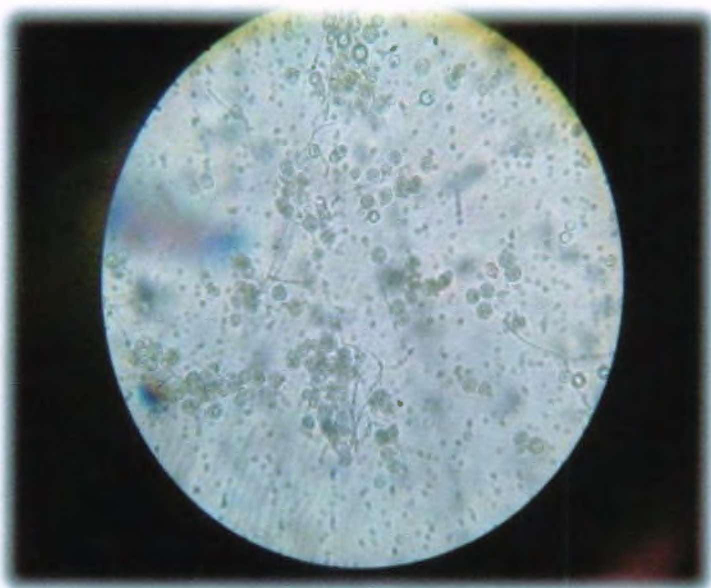

Fig 4.7: Microscopic view of *Humicola fuscotra*

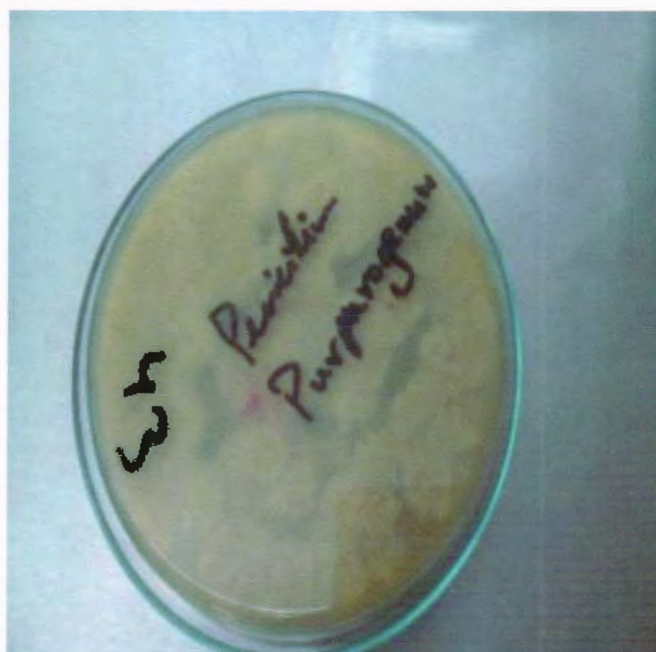

Fig 4.8: *Penicillium purpurogenum* on PDA media

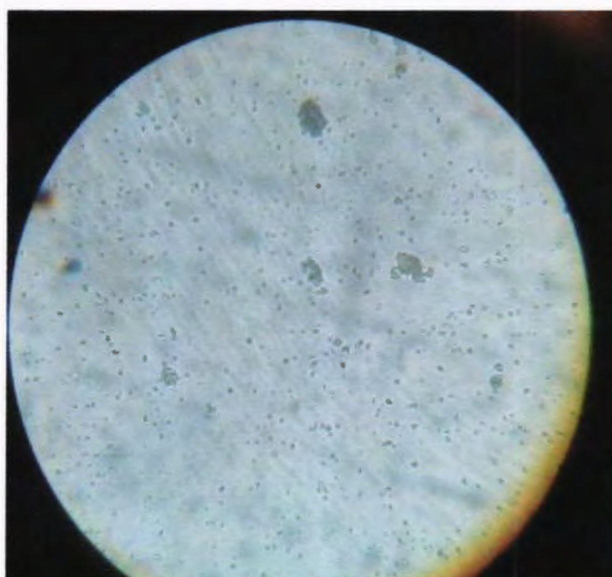

Fig 4.9: Microscopic view of *Penicillium purpurogenum*

4.3. Optimization of different parameter for lipase production

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

4.3.1. Selection of carbon sources used for lipase enzyme production

Humicola grisea was grown on different carbon sources like Apricot oil cakes, Olive oil cake, Taramera oil cake and Mustered oil cake for 5 days at 45 °C. After every 24 h the enzyme assay and protein assay was done for each substrate to investigate lipase and protein activity on different time interval i.e. 24 h, 48 h, 72 h, 96 h and 120 h respectively. Lipase and protein production results are shown in the table 4.2. From the chosen carbon substrate lipase unit was calculated and selected highest activity taking average of all units after 120 h. Table 4.2 showed results for lipase and protein production at various carbon substrates. Fig 4.10 indicates that highest enzyme unit was produced on olive oil cake at 72 h was 8 U/ml/min. While Fig 4.11 showed the highest protein unit that is 0.507 mg/ml was produced on olive oil cake at 72 h.

Table 4.2: Effect of different carbon sources source on lipase production from *Humicola grisea*

Carbon sources	Time (h)	Enzyme Activity (U/ml/min)	Protein (mg/ml)	Specific activity (U/mg)
Apricot oil cake (AOC)	24	0	0.052	0
	48	2	0.181	11.04
	72	4	0.312	12.82
	96	2	0.189	10.58
	120	2	0.177	11.29
Mustered oil cake (MOC)	24	2	0.176	11.63
	48	6	0.439	13.66
	72	8	0.509	15.71
	96	4	0.314	12.69
	120	2	0.186	10.75
Taramera oil cake (TOC)	24	0	0.027	0
	48	2	0.197	10.15
	72	8	0.548	14.59
	96	4	0.351	11.39
	120	2	0.184	10.86
Olive oil cake (OOC)	24	2	0.191	10.47
	48	6	0.427	14.05
	72	**8**	**0.507**	**15.77**
	96	6	0.429	13.98
	120	4	0.325	12.31

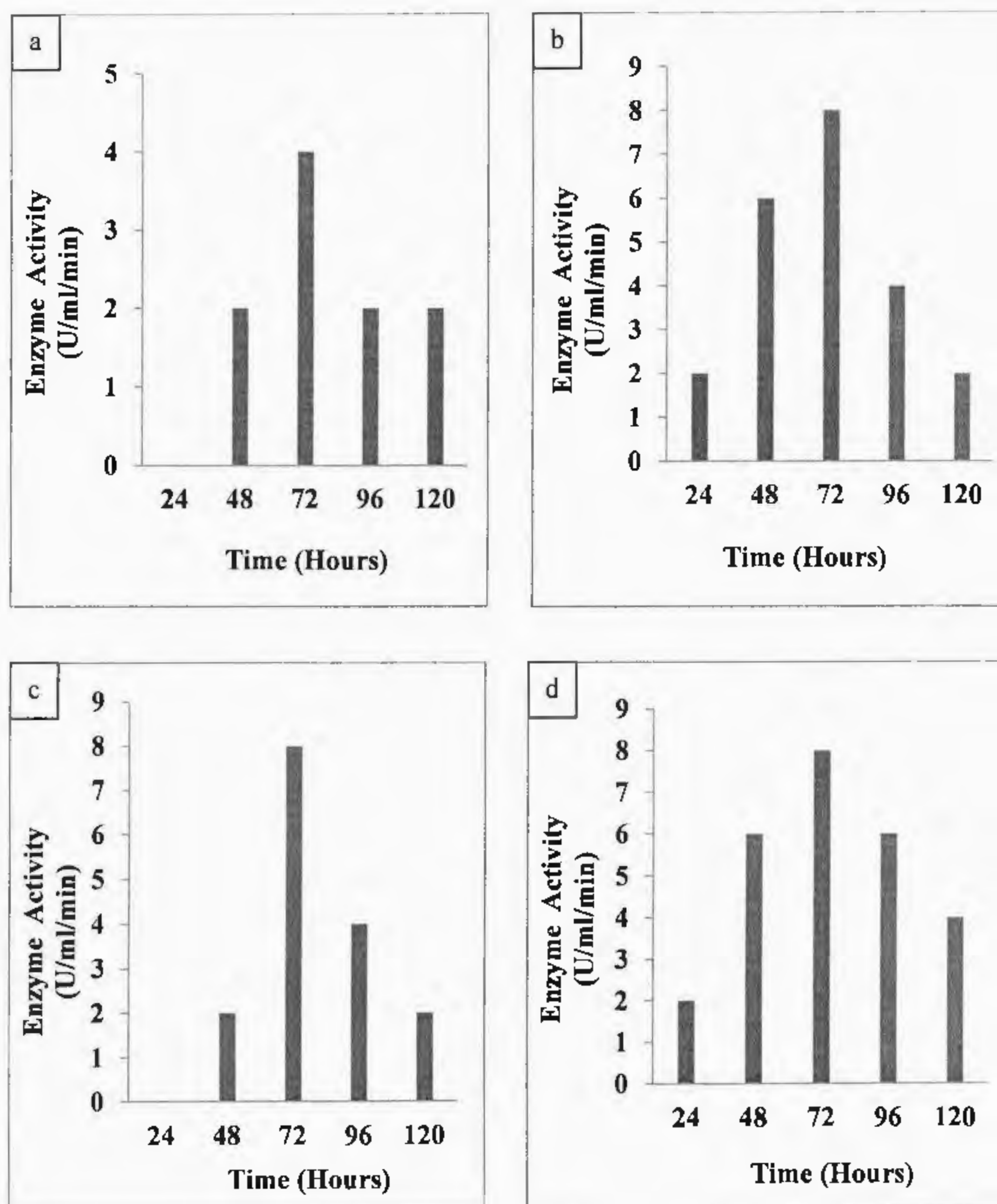

Fig 4.10: Lipase enzyme activity from *Humicola grisea* by (a) Apricot oil cake (b) Mustered oil cake (c) Taramera oil cake (d) Olive oil cake

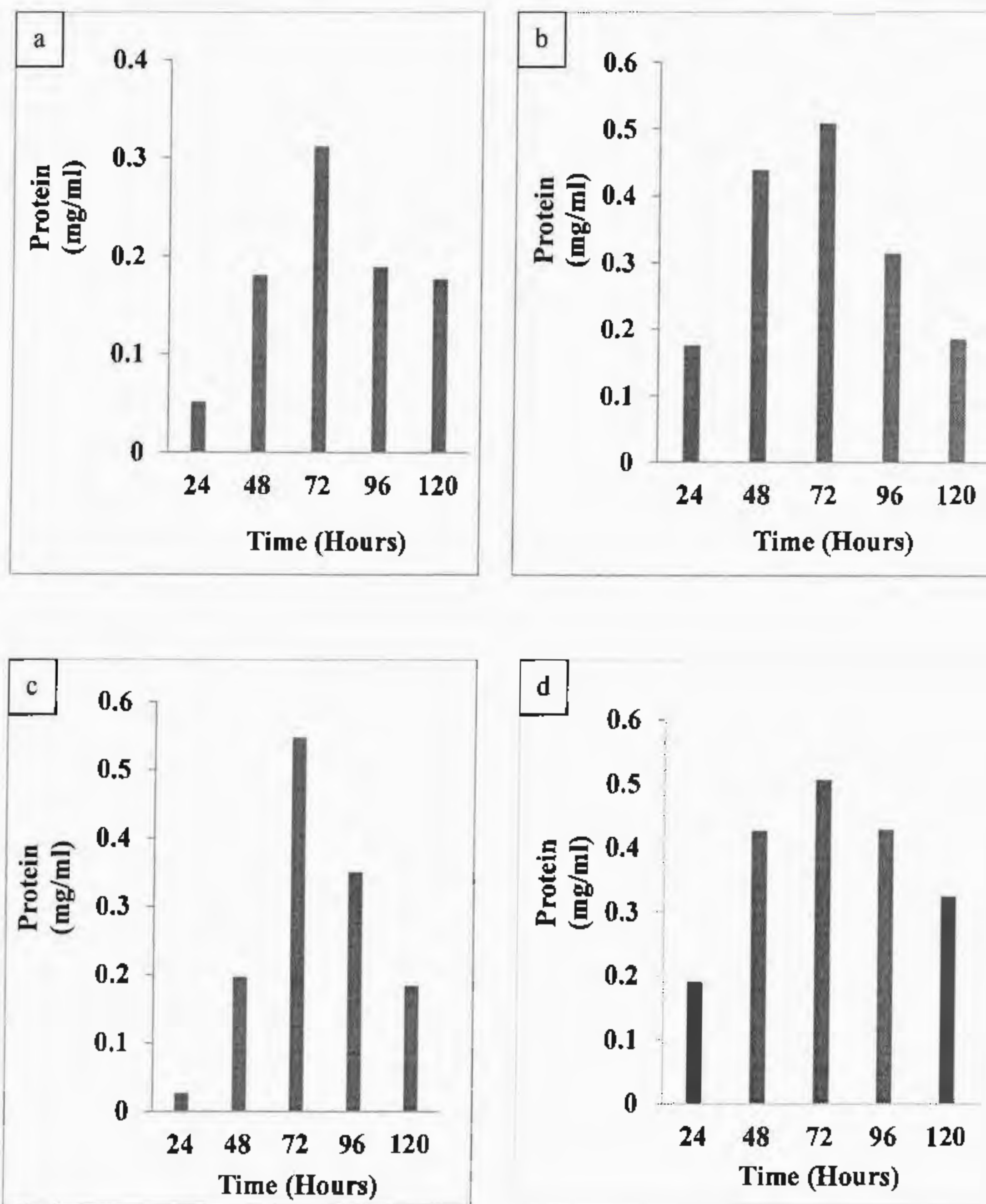

Fig 4.11: Protein activity from *Humicola grisea* by (a) Apricot oil cake (b) Mustered oil cake (c) Taramera oil cake (d) Olive oil cake

4.3.2. Optimization of carbon source concentration used for lipase enzyme production

Humicola grisea was grown on different carbon sources concentration of olive oil cake for 5 days at 45 °C. After every 24 h the enzyme assay and protein assay was done for each concentration to investigate lipase and protein activity on different time interval i.e. 24 h, 48 h, 72 h, 96 h and 120 h respectively. Table 4.3 showed results for lipase and protein production at various olive oil cake concentration. Fig 4.12 indicates that highest enzyme unit that is 8 U/ml/min was produced on 2 % olive oil cake at 72 h while Figure 4.13 showed that highest protein unit 0.545 mg/ml was produced on 2 % olive oil cake at 72 h.

Table 4.3: Effect of different Olive oil cake concentration on lipase production from *Humicola grisea*

Olive oil cake (OOC) Concentration	Time (h)	Enzyme activity (U/ml/min)	Protein (mg/ml)	Specific activity (U/mg)
1 %	24	2	0.197	10.15
	48	4	0.334	11.97
	72	6	0.412	14.56
	96	4	0.327	12.23
	120	2	0.184	10.86
2 %	24	2	0.195	10.25
	48	6	0.494	12.14
	72	**8**	**0.545**	**14.67**
	96	4	0.305	13.11
	120	2	0.192	10.41
4 %	24	2	0.187	10.69
	48	4	0.314	12.73
	72	6	0.413	14.52
	96	4	0.343	11.66
	120	2	0.194	10.62

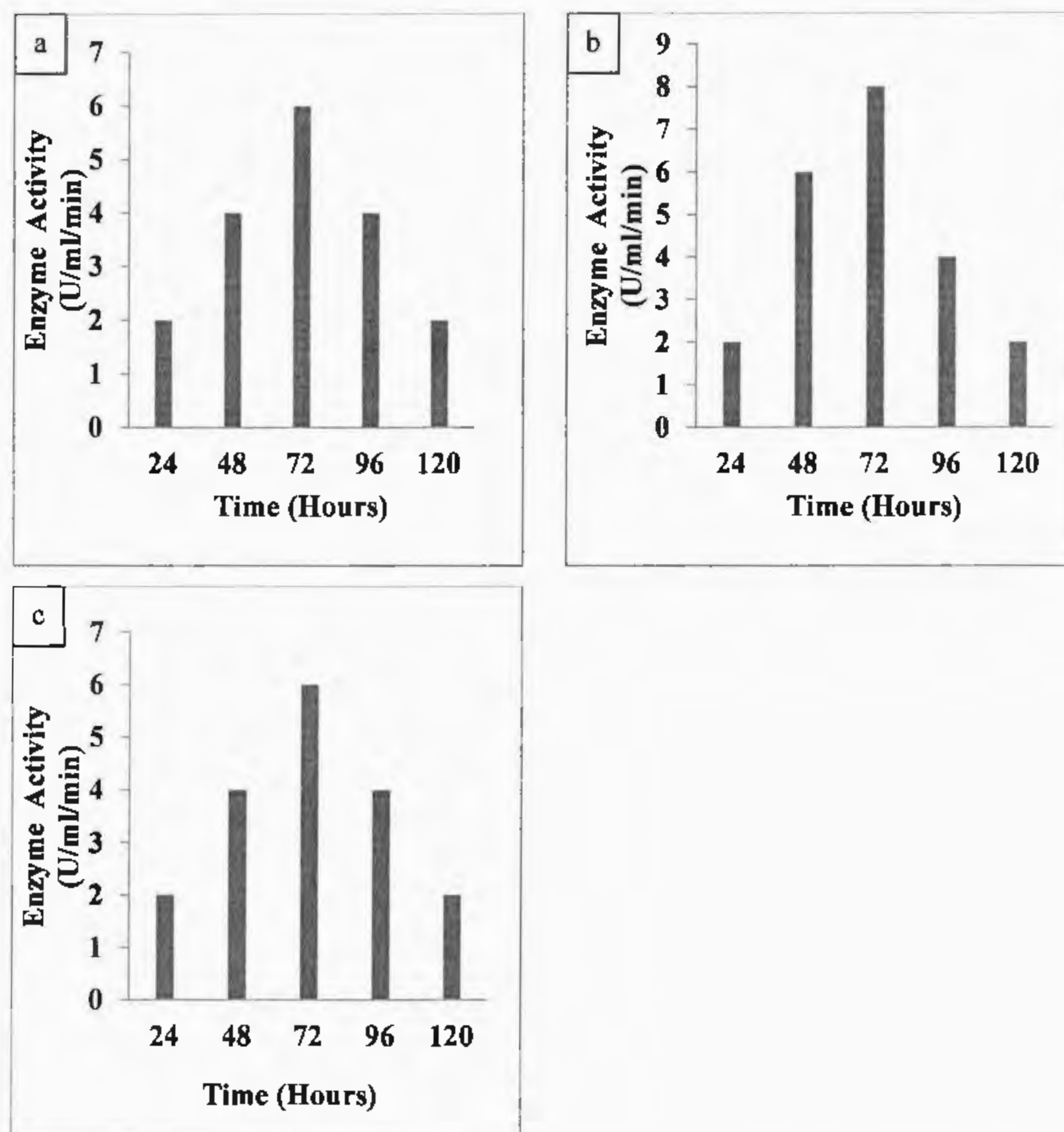

Fig 4.12: Lipase enzyme activity from *Humicola grisea* by (a) 1 % Olive oil cake (b) 2 % Olive oil cake (c) 4 % Olive oil cake

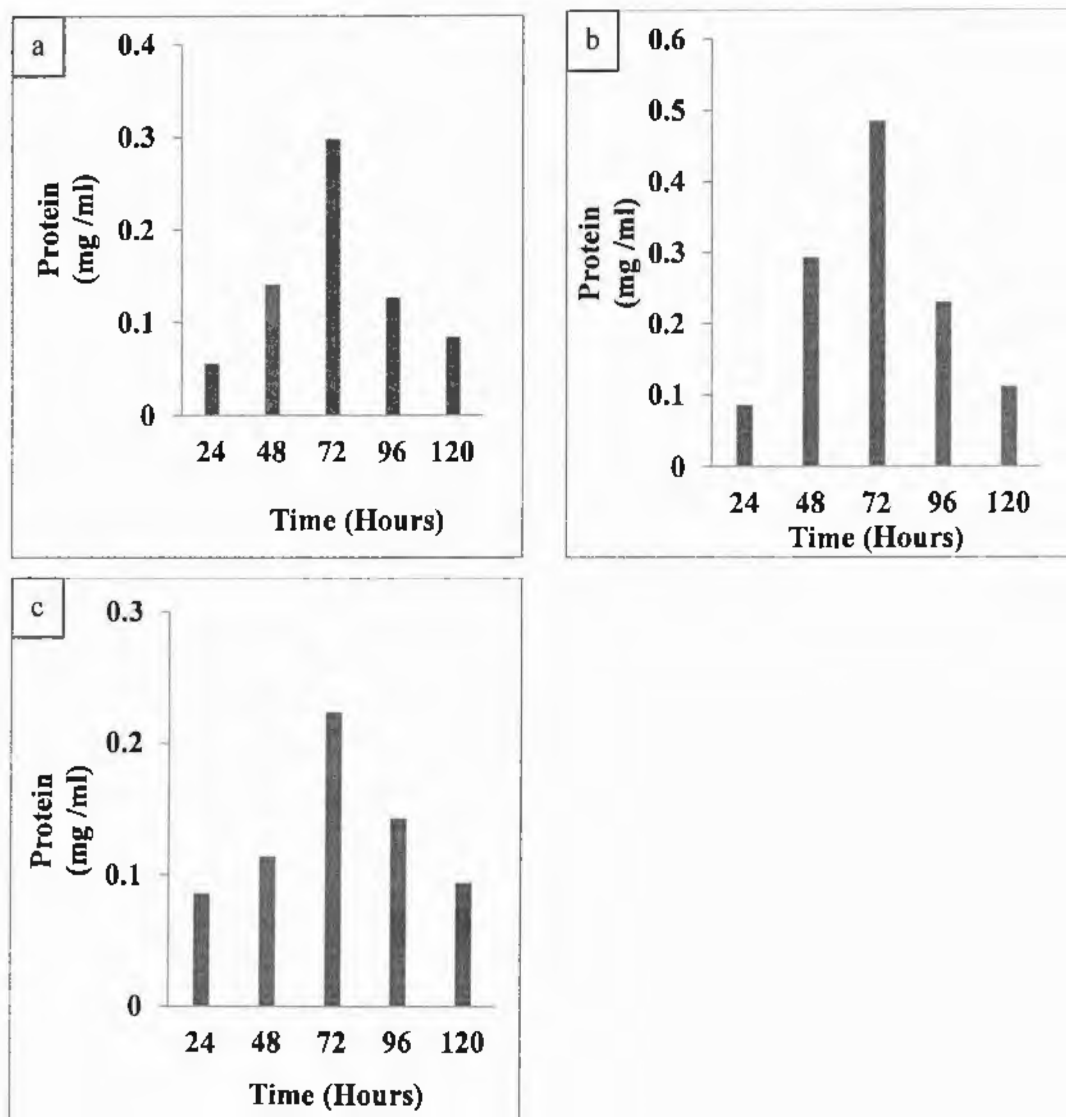

Fig 4.13: Protein activity from *Humicola grisea* by (a) 1 % Olive oil cake (b) 2 % Olive oil cake (c) 4 % Olive oil cake

4.3.3. Selection of nitrogen sources for lipase enzyme production

Humicola grisea was grown on different nitrogen sources 2 % such as (Urea, DAP, Ammonium sulphate, Ammonium nitrate) in the presence of the selected carbon substrate (olive oil cake) for 5 days. After every 24 h each nitrogen source was checked against lipase and protein production. Table 4.4 showed results for lipase and protein production at various nitrogen sources. Fig 4.14 indicates that highest enzyme unit 8 U/ml/min was produced on urea at 72 h while Figure 4.15 showed that highest protein unit 0.511 mg/ml was produced on urea at 72 h.

Table. 4.4: Effect of different nitrogen sources for lipase production from *Humicola grisea*

Nitrogen sources	Time (H)	Enzyme Activity (U/ml/min)	Protein (mg/ml)	Specific activity (U/mg)
Urea	24	4	0.376	10.63
	48	6	0.421	14.25
	72	**8**	**0.511**	**15.65**
	96	4	0.354	11.29
	120	2	0.185	10.81
DAP	24	2	0.179	11.71
	48	2	0.197	10.15
	72	6	0.449	13.36
	96	4	0.312	12.82
	120	2	0.197	10.15
$(NH_4)_2SO_4$	24	0	0.083	0
	48	2	0.187	10.69
	72	4	0.332	12.04
	96	2	0.195	10.25
	120	0	0.076	0
NH_4NO_3	24	0	0.037	0
	48	2	0.192	10.41
	72	4	0.322	12.42
	96	2	0.187	10.69
	120	0	0.092	0

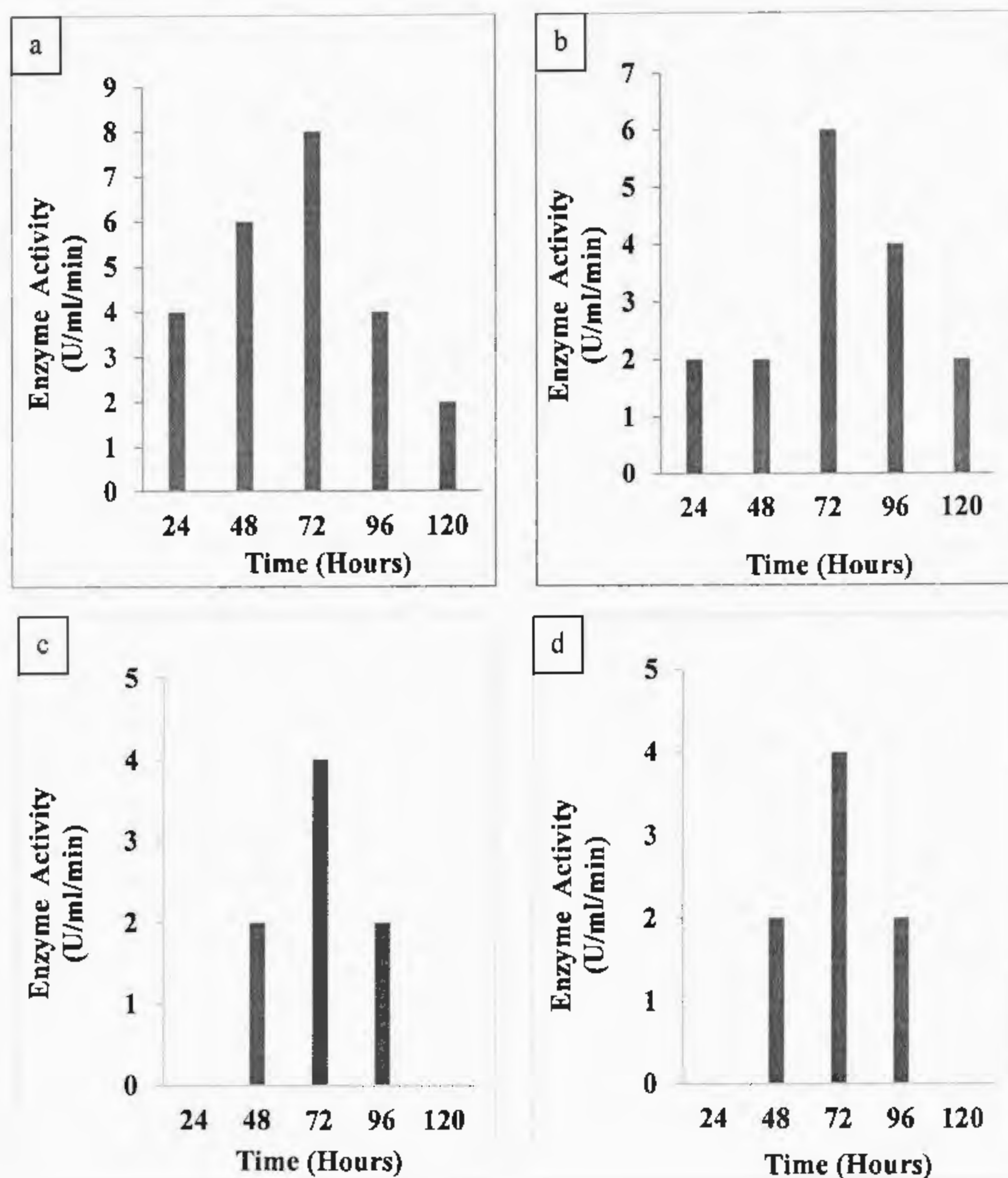

Fig 4.14: Lipase enzyme activity from *Humicola grisea* by (a) Urea (b) DAP (c) $(NH4)_2 SO_4$ (d) $NH_4 NO_3$

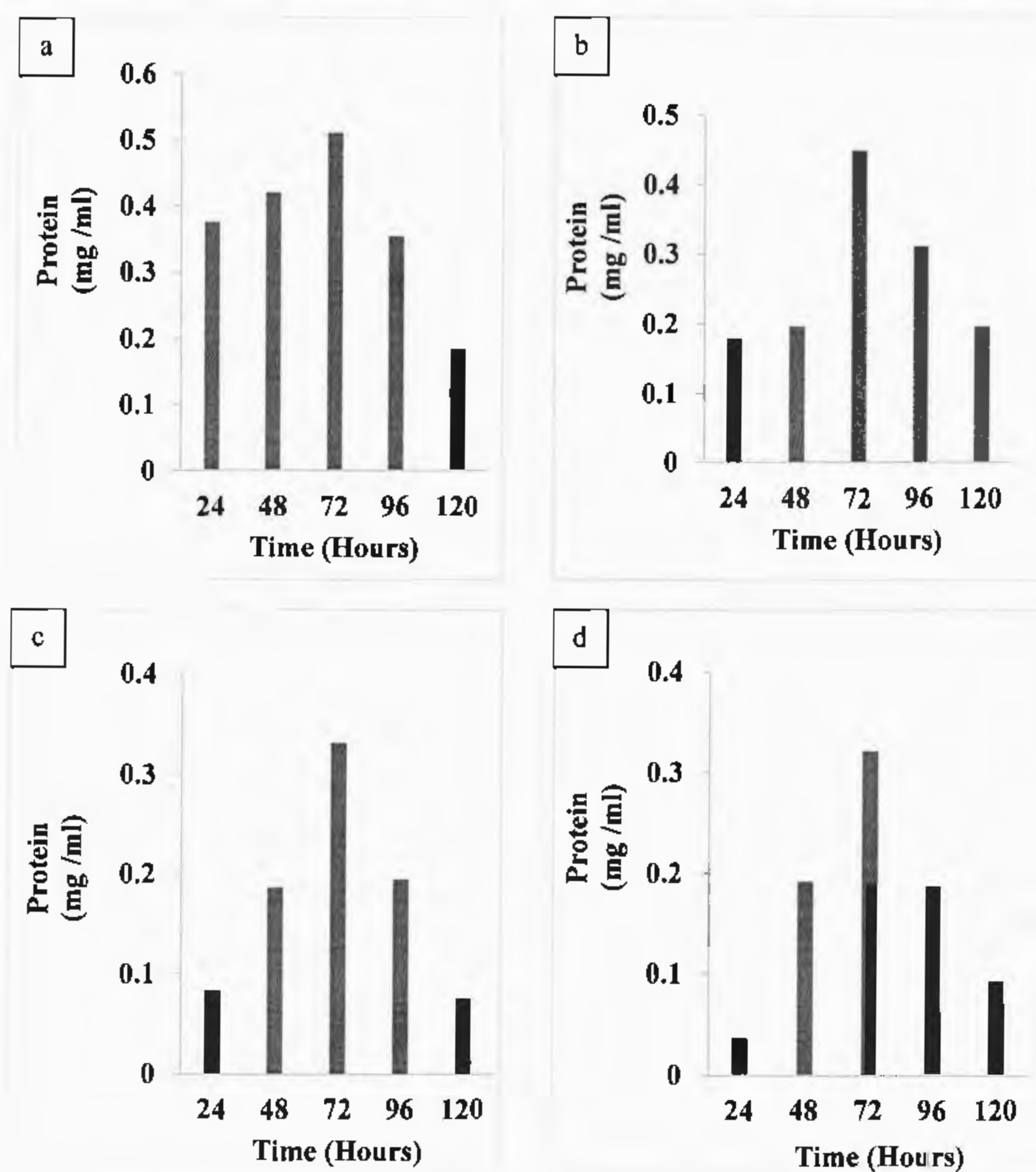

Fig 4.15: Protein activity from *Humicola grisea* by (a) Urea (b) DAP (c) $(NH_4)_2SO_4$ (d) NH_4NO_3

4.3.4 Optimization of pH for lipase enzyme production

Effect of different pH range (from pH 4 to pH 7) was investigated for lipolytic activity against with optimized carbon and nitrogen source. Table 4.5 showed results for lipase and protein production at various pH citrate buffers. Fig 4.16 indicates that highest enzyme unit 12 U/ml/min was produced on optimized carbon and nitrogen sources at 72 h while figure 4.17 showed that highest protein unit 0.732 mg/ml was produced on urea at 72 h.

Table 4.5: Effect of different range of pH on enzyme activity

pH range	Time (h)	Enzyme Activity (U/ml/min)	Protein (mg/ml)	Specific activity (U/mg)
pH 4	24	2	0.189	10.58
	48	4	0.362	11.04
	72	6	0.415	14.45
	96	4	0.357	11.2
	120	4	0.344	11.62
pH 5	24	2	0.188	10.63
	48	6	0.443	13.54
	72	10	0.628	15.93
	96	6	0.427	14.05
	120	4	0.351	11.39
pH 6	24	2	0.189	10.58
	48	8	0.546	14.65
	72	**12**	**0.732**	**16.39**
	96	6	0.433	13.85
	120	4	0.329	12.15
pH 7	24	2	0.185	10.81
	48	4	0.356	11.23
	72	6	0.414	14.49
	96	4	0.359	11.14
	120	2	0.192	10.41

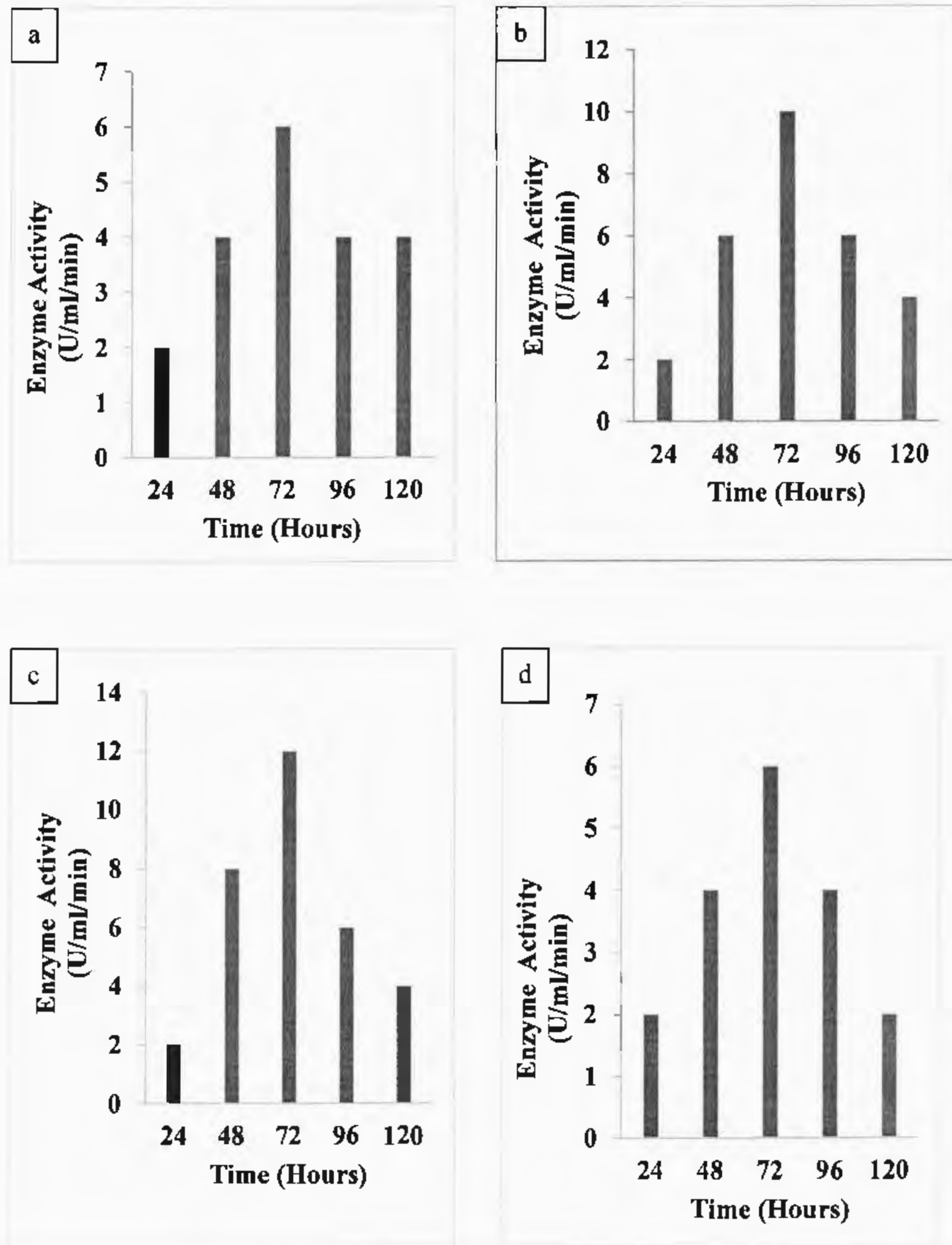

Fig 4.16: Lipase enzyme activity from *Humicola grisea* at (a) pH 4 (b) pH 5 (c) pH 6 (d) pH 7

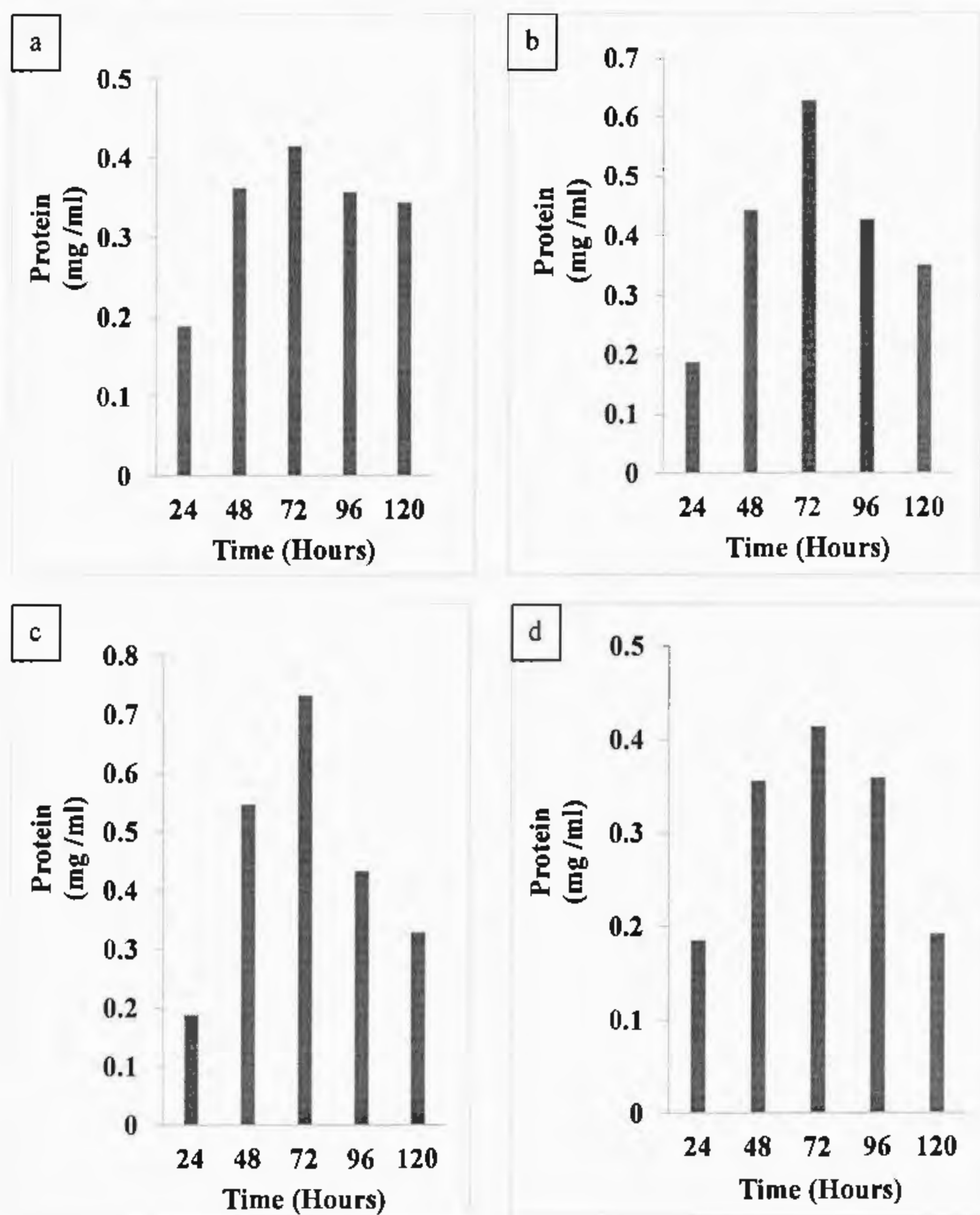

Fig 4.17: Protein activity from *Humicola grisea* at (a) pH 4 (b) pH 5 (c) pH 6 (d) pH 7

4.3.5 Optimization of temperature for lipase enzyme production

Effect of temperature on *Humicola grisea* was grown on different temperature from 40 °C, 45 °C, 50 °C, 55 °C for maximum lipase production. The experiment was carried out for 120 h and production of enzyme was observed after every 24 h. Table 4.6 showed results for lipase and protein production at various temperatures. Fig 4.18 indicates that highest enzyme unit 8 U/ml/min was produced on 45 °C at 72 h while Figure 4.19 showed that highest protein unit 0.521 mg/ml was produced on 45 °C at 72 h.

Table 4.6: Effect of different rang of temperature on enzyme activity from *Humicola grisea*

Temperature	Time (h)	Enzyme activity (U/ml/min)	Protein (mg/ml)	Specific activity (U/mg)
40 °C	24	0	0.046	0
	48	2	0.187	10.69
	72	4	0.322	12.42
	96	2	0.178	11.23
	120	0	0.034	0
45 °C	24	2	0.178	11.23
	48	6	0.426	14.08
	72	**8**	**0.521**	**15.35**
	96	4	0.333	12.01
	120	2	0.199	10.05
50 °C	24	2	0.182	10.98
	48	4	0.341	11.73
	72	8	0.537	14.89
	96	4	0.326	12.26
	120	2	0.191	10.47
55 °C	24	0	0.091	0
	48	2	0.177	11.29
	72	6	0.449	13.36
	96	2	0.175	11.42
	120	0	0.123	0

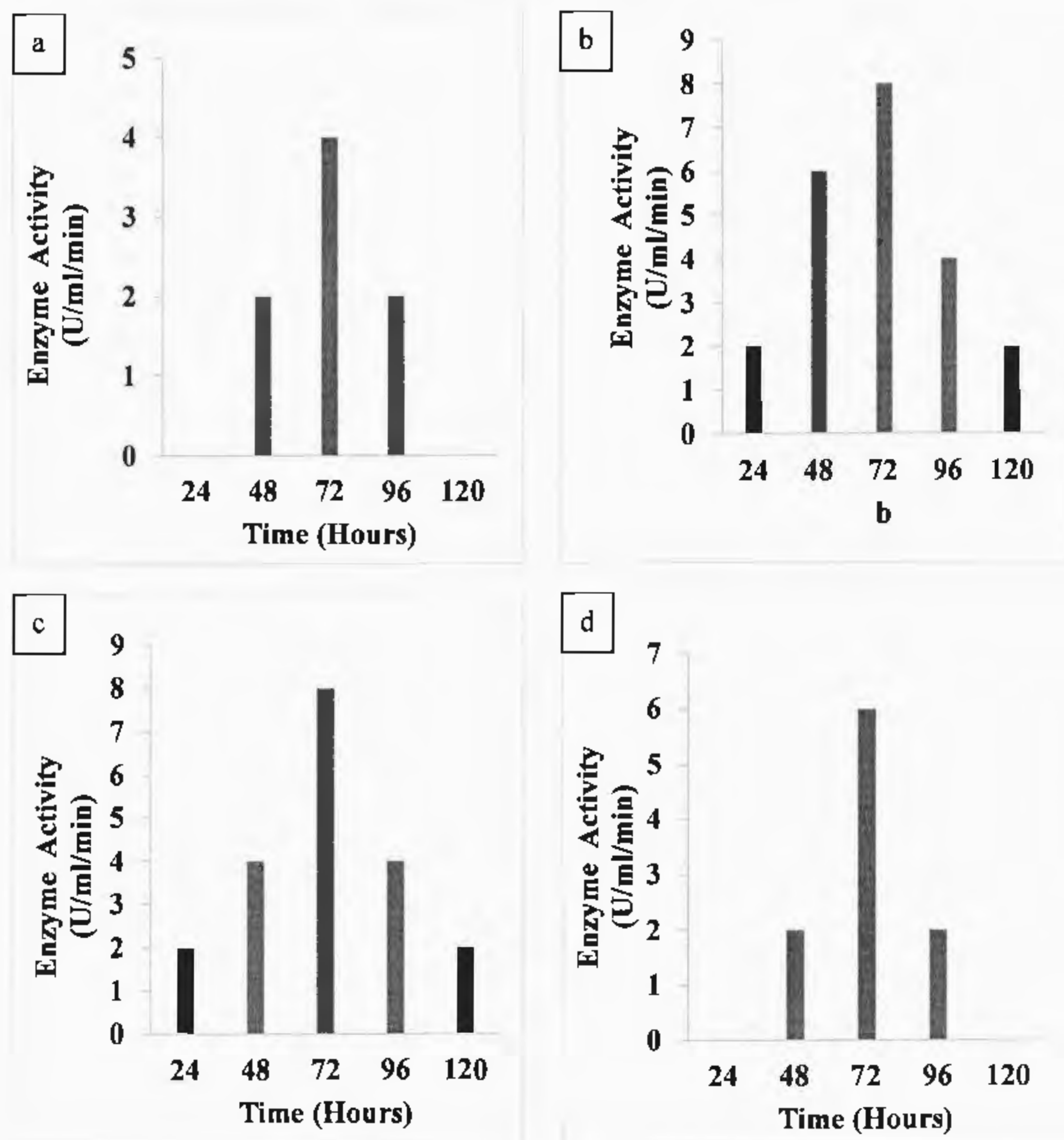

Fig 4.18: Lipase enzyme activity from *Humicola grisea* on different temperature (a) 40 °C (b) 45 °C (c) 50 °C (d) 55 °C

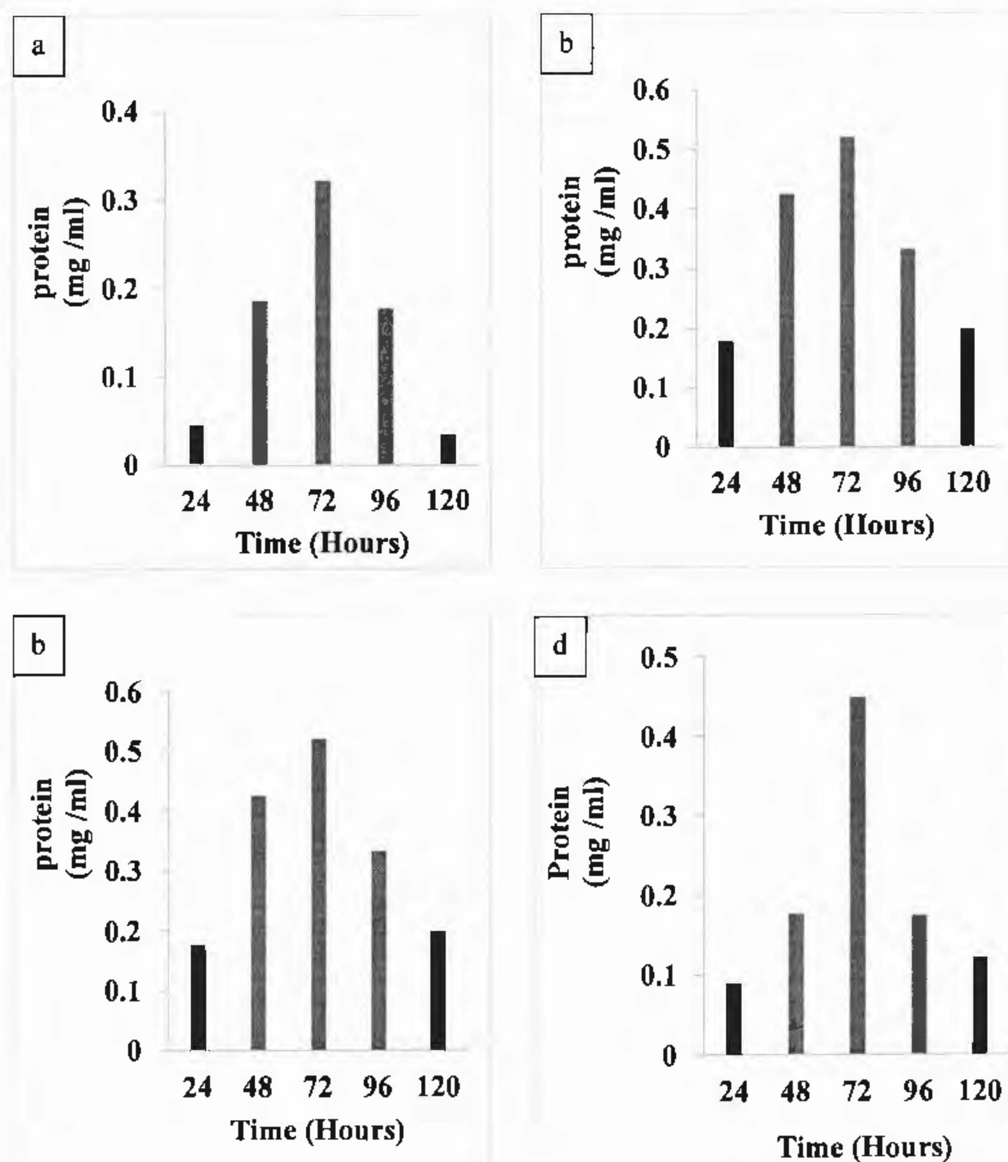

Fig 4.19: Protein activity from *Humicola grisea* on different temperature (a) 40 °C (b) 45 °C (c) 50 °C (d) 55 °C

4.4 Partial purification of lipase enzyme

Purification of lipase enzyme was done by ammonium sulphate precipitation after acquired optimized condition for *Humicola grisea* (Zia, 2006). Ammonium sulphate different concentration (shown in the appendix III) i.e. 20 % 30 % and 40 % was added in the crude extract Saxena *et al.*, (2003) and left overnight. Enzyme and protein assay was performed and units calculated for crude extract before and after by adding different concentration of ammonium sulphate after overnight. Enzyme activity of crude extract calculated after 72 h was 6 (U/ml/min) and protein 0.413 (mg/ml). Similarly enzyme unit calculated against 20 % 30 % and 40 % was shown in the table 4.7. After 3 days enzyme assay of optimized 40 % was again performed and calculated 86 (U/ml/min) and protein 2.184 mg/ml was noted. Dialysis of salted enzyme was done by the help of dialyzing bag. After dialysis enzyme assay was performed for 20 % 30 % and 40 % and the pellets. Result showed in the table 4.8 indicates that there was no lipolytic activity in 20 % and 40 %.

Table 4.7 Enzyme precipitation from *Humicola grisea* on different concentration of ammonium sulphate

Ammonium sulphate Concentration %	Enzyme Activity (U/ml/min)	Protein (mg/ml)	Specific activity
20 %	14	0.871	**16.07**
30 %	24	1.547	**15.51**
40 %	26	1.615	**16.09**

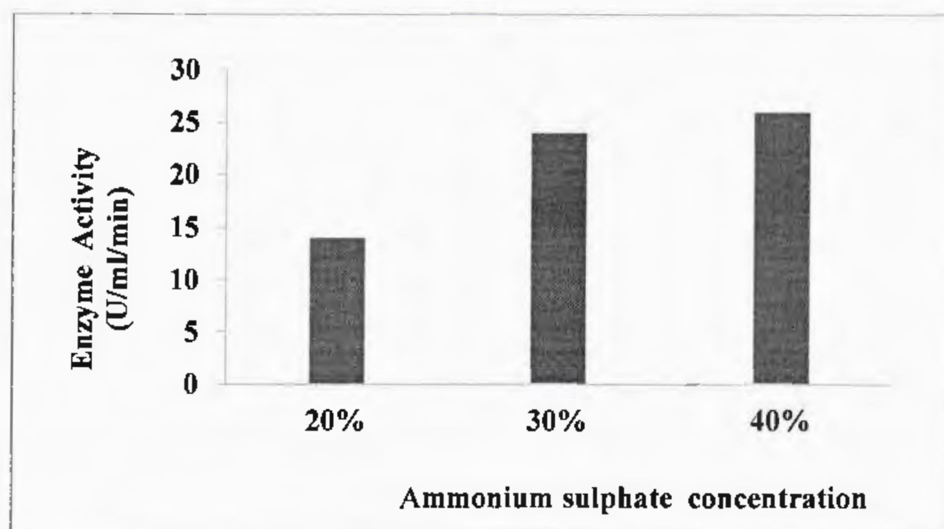

Fig 4.20: Enzyme precipitation after adding different ammonium sulphate Concentration

Table 4.8 Enzyme precipitation of 20 - 40 % pellets after dialysis

Concentration %	Enzyme Activity (U/ml/min)	Protein (mg/ml)	Specific activity
20 %	0	0.017	0
30 %	2	0.194	10.309
40 %	0	0.025	0

4.5. Dilution result of purified enzyme

After purification enzyme from *Humicola grisea* lipase lipolytic activity was found in highly concentrated form. Concentrated enzyme was diluted from 5 X to 20 X. It was observed that 10 X dilution show favorable activity. 10 X dilution contain 0.1 ml purified enzyme sample and 0.9 ml citrate buffer. The result was shown in the table 4.9.

Table 4.9 Different dilutions results of purified enzyme

Enzyme concentration	Enzyme Activity (U/ml/min)
5 X	550
10 X	250
15 X	190
20 X	180

4.6. Characterization of purified lipase of *Humicola grisea*

The dialysed enzyme was used to find out various kinetic parameters as follows.

4.6.1. Effect of different olive oil concentration

The effect of oil concentration on enzymatic activity of *Humicola grisea* was observed on optimized temperature 45 °C. Olive oil concentration tested from 0.1 µl to 1.0 µl shown in table 4.10. Results showes that lipase enzyme activity unit increase with the increase of oil concentration. Maximum enzyme unit was calculated at 0.9 µl i.e. 240 (U/ml/min). Table 4.15 data was plotted in the form of Line weaver-Burk plot figure 4.25. The K_m and V_{max} values for lipase were calculated as 0.4gm/ml and 285.7 U/ml/min.

Table 4.10 Effect of different olive oil concentration

Olive Oil concentration (gm/ml)	Enzyme unit (U/ml/min)
0.1	60
0.2	80
0.3	120
0.4	140
0.5	150
0.6	170
0.7	190
0.8	210
0.9	240

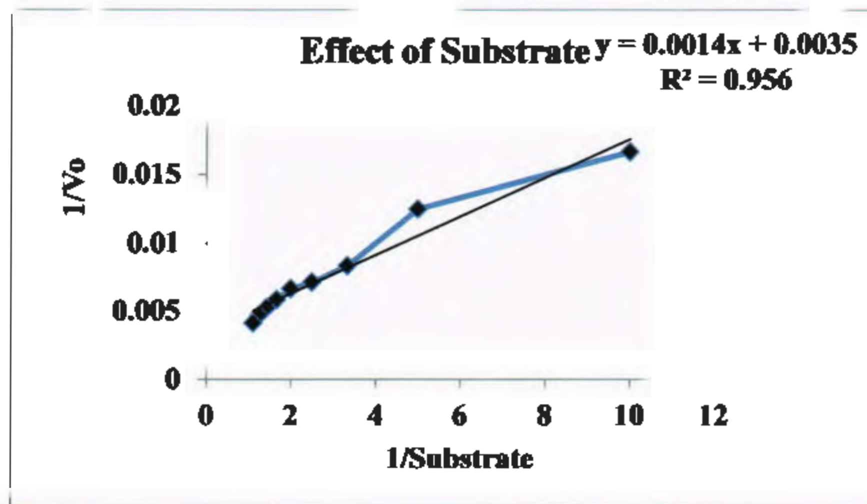

Fig 4.21: Lineweaver-Burk plot for enzyme activity on various olive oil substrate concentrations

4.6.2 Effect of pH on enzyme assay

The effect of pH on lipase enzyme from *Humicola grisea* activity was observed on various pH buffers from pH 4 to pH 7. The effect of pH was shown on the table 4.11. Figure 4.24 showed that maximum lipase activity was measured in the pH 6 after 10 minutes of incubation period.

Table 4.11 Effect of pH on enzyme activity

pH	Enzyme (U/ml/min)
4.0	120
4.4	170
4.8	150
5.0	150
5.2	240
5.4	270
5.6	290
6.0	390
7.0	210

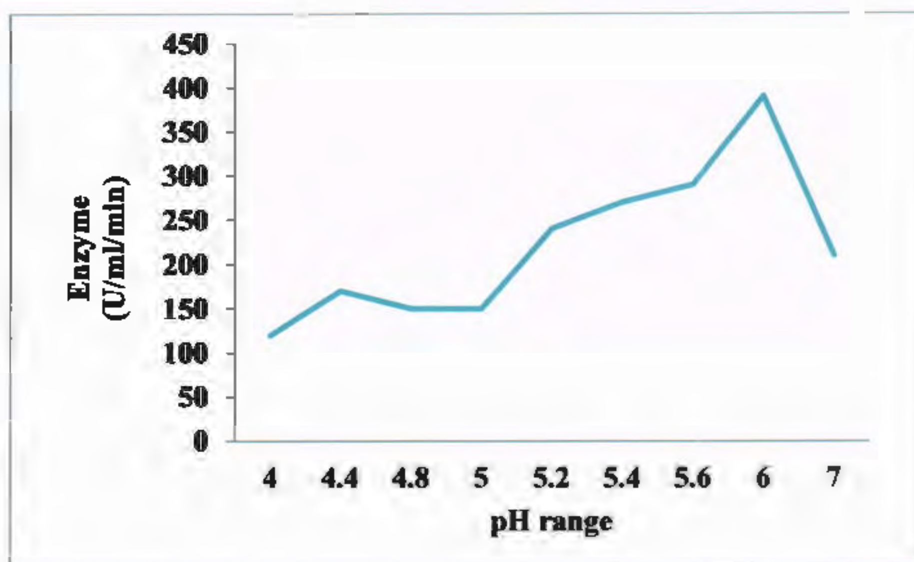

Fig.4.22: Effect of pH on enzyme activity

4.6.3 Effect of temperature on enzyme assay activity

The effect of temperature on Lipolytic assay activity of *Humicola grisea* at different temperature was observed i.e. from 30 °C to 80 °C. The time interval for each temperature for assay was 10 mints. Result showed in the table 4.12 and fig 4.23, that highest activity of lipase was observed 45 °C.

Table 4.12: Effect of temperature on enzyme assay

Temperature °C	Enzyme unit (U/ml/min)
30 °C	80
35 °C	100
40 °C	130
45 °C	250
50 °C	220
55 °C	210
60 °C	200
65 °C	120
70 °C	100
75 °C	100
80 °C	90

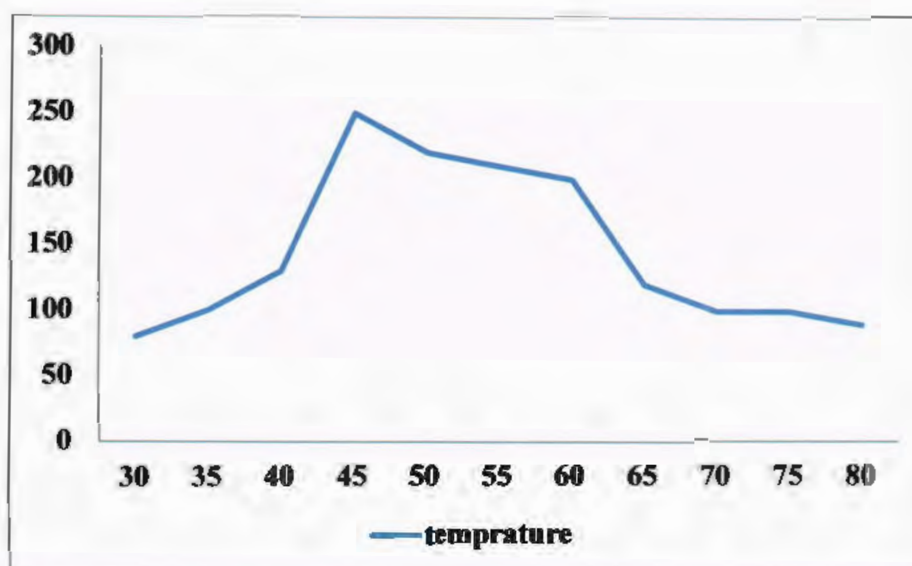

Fig.4.23: Effect of temperature on enzyme assay activity

4.6.4 Thermal denaturation of lipase from *Humicola grisea*

The thermal denaturation of lipase from *Humicola grisea* was observed on particular temperature ranging from 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, 85 °C, 90 °C and 95 °C for different incubation time period 5, 10, 15, 20, 25 and 30 minutes respectively. Result of lipase denaturation was shown in the table 4.13. Figure 4.24 shows that *Humicola grisea* lipases almost denature at the 95 °C after 30 Minutes. Table 4.13 data were fitted to the 1st order plot and examined. The k_d values for lipase at various temperatures was calculated as shown in table 4.14. The thermal irreversible denaturation value for lipase was E_a 49.7 kJ/mol which was calculated using Arrhenius plot (Figure 4.25)

Table 4.13 Irreversible thermal denaturation of lipase enzyme

Temperature °C	5 Mins Enzyme activity (U/ml/min)	10 Mins Enzyme activity (U/ml/min)	15 Mins Enzyme activity (U/ml/min)	20 Mins Enzyme activity (U/ml/min)	25 Mins Enzyme activity (U/ml/min)	30 Mins Enzyme activity (U/ml/min)
50 °C	560	540	523.33	510	485	466.66
55 °C	540	520	483.33	475	450	433.33
60 °C	525	490	470	445	422	410
65 °C	510	480	455	430	405	390
70 °C	460	430	410	385	370	345
75 °C	380	350	325	310	284	266
80 °C	360	330	286.66	260	234	203.33
85 °C	320	260	220	200	175	140
90 °C	290	225	190	150	115	80
95 °C	230	150	90	60	45	30

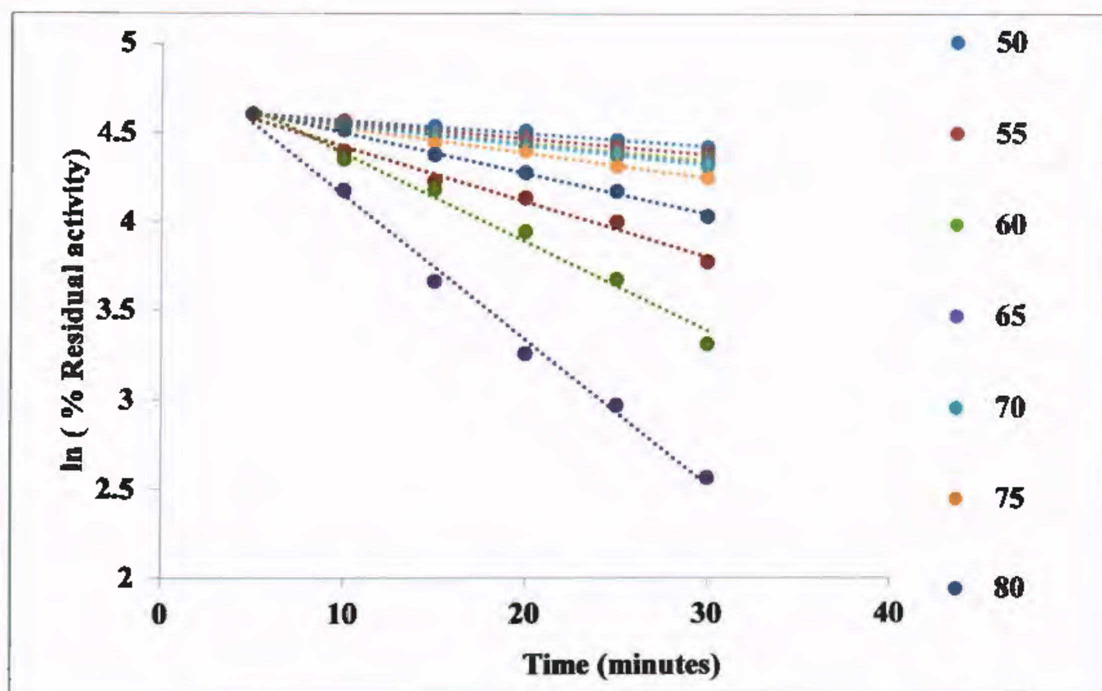

Fig 4.24: Irreversible thermal denaturation of lipase enzyme

Table 4.14 k_d value of lipase at various temperatures

Temp °C	K	1/T	k_d
50 °C	323	0.00309597	0.0072
55 °C	328	0.00304878	0.0089
60 °C	333	0.00300300	0.0100
65 °C	338	0.00295857	0.0109
70 °C	343	0.00291545	0.0112
75 °C	348	0.00287356	0.0141
80 °C	353	0.00283286	0.0228
85 °C	358	0.00279329	0.0310
90 °C	363	0.00275482	0.0497
95 °C	368	0.00271739	0.08125

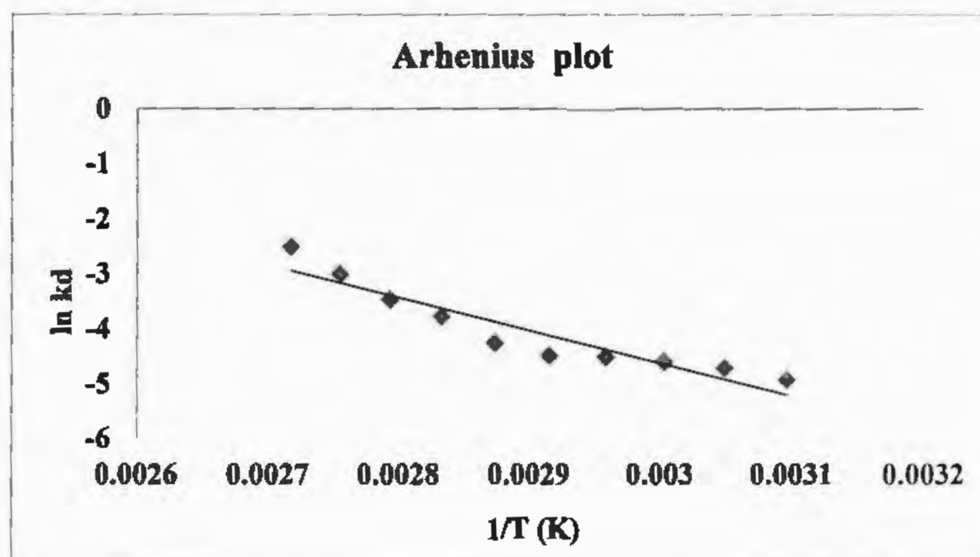

Fig 4.25: Arrhenius plot for calculation of (E_a) for lipase enzyme

DISCUSSION

5. DISCUSSION

Humicola is a genus of hyphomycetes related to the family Chaetomiaceae, which consist of fungi frequently isolated from soil and plant waste (Seifert *et al*., 2011). The genus *Humicola* has more than 20 species, among the twenty species *H. fuscoatra* and *Humicola grisea* isolated in the environment. They are famous having the composition of the strongly cellulolytic fungi. Although *Humicola grisea* known as non pathogenic but in rare cases it was observed disease in a human case of peritonitis, as well as a case of *Humicola*-associated hypersensitivity pneumonitis (Kita *et al*., 2003). In both cases, *H. fuscoatra* was identified morphologically as the causative organism but not confirmed molecularly. However it was successfully treated with antifungal therapy.

Humicola sp. were isolated by different researchers from different sources using the techniques applied by Cooney and Emerson (1964). Alexopolus and Maltas (1979) isolated *Humicola grisea* from cow dung, collected from different areas of Guntur and East Godavari. The collected sample was incubated for 24 h at 45-55 °C. Tabata *et al*., 1993) isolated *Humicola grisea* a soil sample collected at Hachinohe, Aomori, Japan. The media used for fungal growth was potato - glucose agar, corn meal agar, malt extract agar and yeast extract soluble starch (YpSs) agar. *Humicola grisea* was grown after 14 days at 25°C. Nazir *et al*., 2007) isolated this fungus from compost material plant.

Burns *et al*., 2015 isolated *Humicola* sp. during examination of biopsy samples of patience which was grown at 40 °C. *Humicola grisea* was isolated from the soil sample of vermi-zote collected from NARC Islamabad. In current research the fungus was grown for 7 days at temperature 45 °C in infectious disease lab, international Islamic university Islamabad. Morphologically *Humicola grisea* growth appears in the Petri dish plate rapidly to form white to gray colonies. *Humicola* sp. composed of two types of spore i.e. large, dark, more or less globose blastoconidia produce singly on vegetative hyphae (Damaso *et al*., 2008).

In the current study lipase enzyme was successfully produced from the *Humicola grisea* in the solid state fermentation (SSF) using olive oil cake as carbon sources and taking urea as a nitrogen sources with Optimized temperature 45 °C and pH 6. Our foremost objective was to produce lipase enzyme from the industrial waste at reason able cost for industrial use. As microbes are very sensitive therefore

environmental condition was monitored to achieve targeted result from the species. Fermentation media play important role for the production of enzyme as well as for the growth of fungi. In the fermentation media carbon sources nitrogen source, temperature and pH affect the fungal growth.

In the current study, four carbon sources that is Olive oil cake, Mustered oil cake, Taramera oil cake, Apricot oil cake, the Olive oil cake was used. The results showed that olive oil cake gave best lipolytic activity of 86 (U/ml/min) and protein 2.184 mg/ml from *Humicola grisea*. Likewise olive oil was optimized by different scientist for different fungal sp. like Lima *et al.*, (2003) observed lipolytic activity of 12.5 U/mL on carbon sources (olive oil) from *P. aurantiogriseum* after 72 h of fermentation. Iftikhar *et al.*, (2011) also investigated the effect of different additional oils such as olive oil, sunflower oil, soybean oil, coconut oil, almond oil and mustard oil on the production of lipase by *Penicillium chrysogenum* having 1% of different oils such as olive oil, coconut oil, almond oil, mustard oil, soybean oil and sunflower oil were added to the fermentation medium as an additional source. The maximum extracellular lipase activity (64.77 and protein activity was 0.155 U/mL) was obtained when olive oil was added to the fermentation medium while soybean oil and sunflower oil showed an inhibitory effect.

To increase lipase production by the fungus and considering that high nitrogen concentrations are typically used for the production of fungal lipases. Similarly in the current study four nitrogen sources named urea, ammonium sulphate, ammonium nitrate and DAP were used to optimize lipolytic activity. Thus it was found that urea is the best nitrogen sources. The results are in accordance with Ramini *et al.*, (2010) who found that urea showed higher lipolytic activity. (Imandi *et al.*, 2010) performed an experiment to check the effect of nitrogen sources for lipolytic activity on ammonium sulfate, urea, peptone and yeast extract. Among all the nitrogen sources urea yield maximum lipase activity. Urea was best lipolytic source in the six different yeast culture (*Yarrowia Lipolytica*).

The most essential medium pH which effect fungal growth and enzyme production level. In the current study various pH conditions were used to optimize lipolytic activity Highest lipolytic activity was obtained from *Humicola grisea* at pH 6 in after 120 h. A significant activity was also observed in the pH 4 and pH 5. Arima *et al.*,

(1972) investigated various pH and found pH 8.0 optimum for lipase production from *Humicola lanuginosa*. Ibrahim *et al*., (1987) conduct experiment on lipase production from *Humicola lanuginosa*. The pH of 7.0 was found optimum for enzyme activity.

In the current study various temperatures were used to optimize lipolytic activity. It was observed that at 45 °C *Humicola grisea* produce maximum lipase production after 72 h and afterwards enzyme production level decreases. It might be due to effect of high temperature which acts as inhibitory for the production enzyme as well as for the growth of microorganisms (Lima *et al*., 2003). Lin and Ko, (2005) investigated on thermostable lipase from *Antrodia cinnamomea* found temperature of 60 °C optimum for lipase production. Karadzic *et al*., (2006) studied extracellular lipase of extremophile. Its optimized temperature was found 70 °C.

Aloulou *et al*., (2007) studied the effect of different detergents and pH on the fungal lipase (*Yarrowia lipolytica*) (YLLIP2) and *Thermomyces lanuginosus*) (TLL). He observed that with the addition of sodium taurodeoxycholate (NaTDC at pH 7.0 activities on substrate inhibited in both species while at pH 6.0 *Yarrowia lipolytica*) sp. remains active but not *Thermomyces lanuginosus* (TLL).

Humicola grisea catalytic activities differ with subject to carbon. It was observed that if favorable carbon source provide to *Humicola grisea* it shows highest activity within 72 h, after that it activity start to decrease. In the current study it was also observed that optamized carban, nitrogen (C/N) sources combination not explain a significant lipolytic result however the parameter of pH greatly affects in the lipolytic activity. Optimization of pH is very important as compared to other parameter like carbon source, nitrogen source, temperature and pH.

In present study thermal stability of an enzyme was investigated and it was reported the enzyme was completely stable 50 °C after 30 minutes and maintain residual activity (4.5 %) and sharply the enzyme stability decrease after 30 minutes at 55 °C. The activation energy Ea calculated for lipase enzyme denaturation calculated was Ea 49.7 kJ/mol. Sharma *et al*., (2002) studied that extracellular lipase of a new thermophilic *Bacillus* sp. RSJ-1 and they have calculated *Km* 2.2 mg/ml *V*max 1429 U/ml with the help of Lineweaver–Burk plot, similarly Yaswanth *et al*., (2015) investigated a bacterial species *Stenotrophomonas maltophilia* and reported the value of 0.727 *Km* and *Vmax* of

156.6 µmoles/ml. in the current study. The K_m and V_{max} values from *Humicola grisea* lipase was calculated with the help of Lineweaver-Burk plot as 0.4gm/ml and 285.7 U/ml/min respectively.

Conclusion and future prospects

The study results obtained showed that carbon source olive oil cake at 2 % concentration, urea concentration as nitrogen source, initial medium pH of 6.0 and fermentation temperature of 45 °C for 72 h gave lipase production of 12 Enzyme (U/ml/min). The lipase enzyme can be produced on industrial large scale from *Humicola* species using oil cakes, found in abundance in Pakistan which will be economically feasible.

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APPENDICES

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.

APPENDIX I

BSA Standard Curve for Protein Estimation