Production, Purification and Characterization of Psychrotolerant Alkaline Lipase and Protease and their Application in Detergent Formulation

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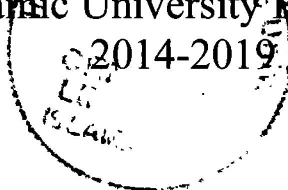

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Microbial proteases

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بسم الله الرحمن الرحيم

A thesis submitted to the Department of Biological Sciences, International Islamic University, Islamabad in the partial fulfillment of the requirements of the degree of Doctor of Philosophy in Biotechnology

DEDICATION

This doctoral dissertation is dedicated to my beloved parents whose constant prayers, support and guidance enabled me to achieve this milestone

DECLARATION

It is hereby declared that the work present in this doctoral dissertation is my own effort except where otherwise acknowledged and that the dissertation is my own composition. No part of the dissertation has been previously presented for any other degree.

Date 27/06/2018

Yasir Ali

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LIST OF ABBREVIATIONS

Abbreviation	Definition
His6	Polyhistidine
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
(NH4)2SO4	Ammonium sulfate
$HgCl_2$	Mercury (II) chloride
$CuSO_4$	Copper (II) sulfate
$ZnSO_4$	Zinc sulfate
$FeCl_3$	Iron (III) chloride
EDTA	Ethylenediaminetetraacetic acid
PMSF	Phenyl methane sulfonylfluoride
DMSO	Dimethyl sulfoxide
p-CMB	4-Chloromercuribenzoic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid)
DIFP	Diisopropyl fluorophosphate
MFP	ABC-type protease exporter membrane fusion protein
OMP	Outer membrane protein biogenesis
CFB	Complement factor B
RFLPs	Restriction Fragment Length Polymorphism
HGC	High-G+C
CFB	HGC/Cytophaga-Flavobacterium-Bacteroides
LGC	Low-G+C
DIFP	Diisopropyl fluorophosphate
SDS	Sodium dodecyl sulfate
PMF	Proton-motive force
β-ME	2-Mercaptoethanol
DEAE	Diethylaminoethyl cellulose
CM	Carboxymethyl cellulose
DO	Dissolved oxygen
ORF	Open reading frame
NH4NO3	Ammonium nitrate
NH4Cl2	Ammonium chloride
Na2CO3	Sodium carbonate
BSA	Bovine serum albumin
NaK tartarate	Potassium sodium tartarate
TCA	Trichloroacetic acid
DTT	Dithiothreitol
Km	Michaelis–Menten kinetics
Vmax	Maximum velocity

Kcat	Turnover rate of an enzyme-substrate complex to product and enzyme
E(t)	Enzyme quantity in moles
Kcat/Km	Catalytic efficiency
pNPP	4-Nitrophenyl palmitate
p-NP	4-Nitrophenol
NaBO3	Sodium perborate
NaClO2	Sodium chlorite
E_a(P)	Activation energy
$NiCl_2$	Nickel (II) chloride
kDa	Kilo Dalton
rRNA	Ribosomal Ribose nucleic acid
H2O2	Hydrogen per oxide
SDS-PAGE	Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis
C_2H_4INO	Indoacetamide
ATPase	Adenyl pyrophosphatase
ASP	Aspartate
mM	Milli moles per litre
Rpm	Revolution per minute
His	Histidine
KCl	Potassium Chloride
Mn	Manganese
Ser	Serine
Tween 20/80	Polyoxyethylenesorbitane monooleate

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Yasir Ali

ABSTRACT

Proteases are important enzymes for various applications of industrial importance. Microbial proteases show an imperative task in different biotechnological processes. The purpose of the present study was to screen, isolate and production optimization of cold-active alkaline protease producing psychrotrophic bacteria from soil and water samples collected from Juglot, Jutial and Rakaposhi glacier Gilgit, Northern Areas of Pakistan. Soil samples were serially diluted and 0.1ml of sample was spread on skim milk agar plates, at 15 to 25°C for 48 hrs. Total four of the bacterial colonies from glacier soil and water showed clear zone around the colony indicating protease activity. Among these, *Stenotrophomonas sp.* strain PAK-01 produced highest protease activity (5559 unit/ml) and the isolated thermolabile alkaline protease producing bacterium was identified as *Stenotrophomonas* sp. (NCBI GenBank Accession no. MG662181) on the basis of 16S ribosomal RNA. Factors influencing the maximum hydrolytic catalysis of extracellular thermolabile alkaline protease by *Stenotrophomonas* sp. PAK-01 were optimized through the procedure of the one-factor at a time manner. Highest catalytic activity was observed toward temperature was 25°C (4469 unit/ml) at pH 9 (4516 unit/ml) in fermentation production medium with a 24-hour old inoculum (5765 U/ml) alongside 5 % size of the inoculum (5754 U/ml) and constant agitation at 150 rpm for 96 hours incubation (6886 unit/ml). *Stenotrophomonas* sp. PAK-01 exploited multiple sources of carbon for its highest proteolysis and glucose (5833 unit/ml) was observed the best inducer for the highest catalytic activity followed by sucrose and galactose. Amongst the numerous organic and inorganic nitrogen nutrients experienced highest proteolytic activity value (6965 unit/ml) was examined by the supplementation of yeast extract in the basal fermentation production medium. Overall activity of thermolabile protease secretion from *Stenotrophomonas* sp. strain PAK01 in the basal production medium observed was induced in the existence of Ca^{2+} (5231 unit/ml) and Mg^{2+}(3984 unit/ml). These metal ions enhanced the enzymatic activity and had stimulatory effect on enzyme production. Amongst the ions examined, $HgCl_2$, $CuSO_4$ and $ZnSO_4$ constrained the enzymatic activity, while $FeCl_3$ had no observable effect on enzymatic activity. The above results indicate that this bacterial isolate can be use as biotechnological tool for industrial purpose.

Pry gene extracted from *Stenotrophomonas sp.* strains PAK-01 translating alkaline protease was amplified from genomic DNA correspondingly, cloned in pET28a plasmid and afterward nucleotide genomic DNA was sequenced. Sequence analysis of the pET28a /protease gene revealed an open reading frame of 1740 base pair and exhibited a 96 % resemblance to the Pr2 nucleotide sequence encoding trypsin of *S. maltophilia* strain and accessible in GenBank database (JF317278). Interpreting the analyzed 1740 bp nucleotide sequence of the serine protease gene was translated into amino acid sequence and was observed a polypeptide chain of 578 monomers of amino acid with molecular weight of 58 KDa. Protein comprise numerous domains discovered in serine peptidase belonging to family of peptidase protein S8 domain in the position of 172 to 448 and proprotein-processing peptidases C-terminal domain to combined basic monomers of amino acids and was examined between the protein sequence at position of 501 and 567 aa. PrY gene cloned in pET28a plasmid multiple cloning region of T7 promoter with N-terminal His_6 residues and subsequently expressed in *Escherichia coli* BL21 (DE3) in the existence of 1 mM IPTG. Recombinant PrY gene protease protein was expressed as a soluble fraction in *E. coli* BL21 (DE3) and purified by nickel chelate chromatography followed another technique of Sepharose column chromatography. Purified monomer proteins were observed from the protein analysis. Specific activity of 245 U/mg with yield recovery of 48% was observed from the overall purification procedure.

Our study was designing to isolate a novel psychrotrophic bacteria that has the ability to produce alkaline stable protease from temperate region of Gilgit-Baltistan. Universal primers of 16s RNA was used for the identification of the selected strain PAK-03. Enzyme purification was obtained using distinctive techniques of purification such as $(NH_4)_2SO_4$ precipitation, dialysis, ion exchange and gel permeation chromatography and then characterized by assessing its stability and activity on the basis of different parameters and its formulation in washing detergent was performed by washing analysis using proteinaceous pigments (Blood and Yolk). Strain PAK01 was identified as *Stenotrophomonas* sp. with a gene bank accession no MG662181. SDS PAGE analysis of PAK-01 supernatant showed that the purified thermolabile protease has a molecular size of about 58 kDa. The highest catalytic efficiency of enzyme was achieved at cold environment in comparison with other commercial proteases and does not exposed the characteristics of thermostability. To check the catalytic activity of enzyme, the maximum hydrolysis for protease enzyme was

observed at 9 pH and showed resistance against wide- ranging pH distending from 6 to 10. Enhanced catalytic activity was experimented when bivalent cations such as magnesium chloride $MgCl_2$ and calcium chloride $CaCl_2$ are supplemented in the reaction mixture and hydrolytic activity was inhibited by the supplementation of iron sulfate and copper sulfate (Fe^{2+} and Cu^{2+}). The thermolabile alkaline protease was delicate to denaturing agents such as EDTA and PMSF and was resilient regarding to H_2O_2, SDS, DTT and mercaptoethanol.

Thermolabile molecules enzyme from *Stenotrophomonas* sp. PAK-01 with optimum hydrolysis at 25 to 30°C and buffer of Tris-HCl was discovered active in detergents at concentration of 5 % of 10 mg/ml (purex and xtra) and also functional in 500 mg/l of sodium hypochlorite at low environment such as 25°C. It preserved 51 % and 41 % hydrolysis even subsequently after an incubation with (5 % of 15 mg/ml) (purex and xtra) at 24 hours at 25°C. Blood plasma pigments from test fabric were removed within 15 min in 100 U/ml molecule at Tris-HCl buffer with 1 % of detergent (purex) and it acquired 100 U/ml molecules of enzymes to eradicate egg yolk from test fabric (cotton) within 15 min only. It is concluded that protease isolated from this psychrotrophic bacterium can be a better option in detergent industry. An extracellular cold active lipase producing psychrotrophic *pseudomonas peli* (PAK-03) isolated from the soil samples collected from Rakaposhi glacier, in northmost territory of Gilgit Baltistan, Pakistan and was identification as *pseudomonas peli* by 16S rRNA nucleotide sequencing. Psychrotrophic *pseudomonas peli* produced maximum thermolabile alkaline lipase at 25°C after 96 h in alkaline conditions by using yeast extract (3923 unit/ml), tributyrin (3557 unit/ml) as a substrate. $CaCl_2$ stimulated activity by 3766 U respectively. whereas $ZnSO_4$ and $FeCl_3$ strongly inhibited the activity.

A novel organic solvent stable lipase gene yLip (GenBank ID MH338242), was amplified from *Pseudomonas peli* (MH338242). Cloned yLip was expressed in BL21 (λDE3) gave protein of molecular mass 32-kDa soluble protein after induction with 1 mM IPTG and was purified by standard procedure. It maintained thermal stability from 4 to 50°C and half of the catalytic activity was inhibited subsequently 60 min of incubation at 60°C. The activity in organic solvents was increased in the existence of DMSO and ethanol. Present results suggest YLip as thermolabile lipase with comparatively maximum thermal stability and remarkable resistance toward organic solvents. Purified thermolabile lipase enzyme from *Pseudomonas peli* PAK-03 was

obtained by distinctive techniques of purification such as $(NH_4)_2SO_4$ precipitation, dialysis, ion exchange and gel permeation chromatography.

Alkaline lipase secreted by *Pseudomonas peli* PAK-03 was purified 68.42-fold with an overall yield of 20.52 %, and a specific activity of 1381 U/mg beside molecular mass of purified protein was estimated to be approximately 32 kDa by SDS-PAGE analysis. While the lipase was active at a temperature range of 15–40°C, it exhibited maximum catalytic activity at 30°C, at pH 9. Thermostability analysis of the enzyme was experimented at different temperature from 4 to 75°C. Cold adapted enzyme demonstrated stability at low temperature 4°C and after the incubation of reaction mixture at 240 minutes observed retained 38 % of the residual activity. Half of its activity was inhibited at 55°C and preserved 50 % of its residual preliminary activity by incubation of the reaction mixture at 90 min and the thermolabile enzymes was supposed to have stability toward high temperature. Highest residual activity was observed at pH 9 and by increasing the alkaline condition of the reaction mixture the enzyme stability observed was progressively deteriorated. $CaCl_2$ was found to have stimulated effect on catalysis of enzyme. Whereas the enzyme preserved its catalysis levels toward the existence of a diversity of organic solvents, DMSO boosted this. Extraordinary stability against diverse ions, solvents, high alkalinity and activity at low temperatures make the alkaline thermolabile lipase of *Pseudomonas peli* PAK-03 a contender for industrial applications. Lipases as an additive to the washing detergent formulation are the enzymes of best choice for laundry cleaner industries due to outstanding to their triacylglyceride eradicating potential from dirtied cloth which ultimately decreases the practice of peroxide-based bleaches cleansers in the detergent formulation. In this research, *pseudomonas peli* PAK-03 secreted lipase was purified and evaluated for its triacylglyceride eradicating potential by formulating a presoak mixture so as to usage of lipase as an improved in washing detergent formulations. Alkaline lipase stability toward the effect of different marketable detergents, oxidizing compounds and surfactants were investigated in a preliminary assessment to aimed at its further potential in the industrial environment. It was observed that Purified lipase exhibited decent stability in the existence of varied chemical compounds. Laundry competence was observed to be boosted while exploiting alkaline lipase with 0.6 % nonionic detergent as compared towards anionic detergent. Washing performance exploiting against 0.6 % wheel alongside 60 U lipase at 25°C in 40 min consequences in maximum removal of oil was 71 % from dirtied cloth. Therefore, present-day

analysis begins the innovative objective in bio detergent sector for making of chemical-free detergent formulation by means of alkaline bacterial lipase.

CHAPTER.1

1. INTRODUCTION

Cold-active protease enzymes from psychrophilic bacteria are the significant group of industrial enzymes. Their occurrence in the ice field (Margesin *et al.*, 1991), polar region (Vazquez *et al.*, 1995) and the marine animal intestine (Hoshino *et al.*, 1997) is documented extensively. Not only due to their biotechnological efficacy for the innovative applications, including additives in detergents, food processing or pharmacy, nonetheless also because these cold- active enzyme characterizes the lesser natural limit of the stability of protein and is one of the crucial tools for studies concerning the protein folding (Feller *et al.*, 1996). Microorganism living in cold ecosystem and mesophile that can endure cold atmosphere, are known to produce thermolabile molecules at low temperatures of extraordinary activity; these are called thermolabile enzymes. Psychrotolerants from the earlier descriptions indicate of getting more microorganisms that can tolerate cold habitations like Antarctica as compared to obligate psychrophile (Vaz *et al.*, 2011, Antony *et al.*, 2016 and Hatha *et al.*, 2013). At glacial atmospheres probabilities of collecting psychrotolerants and psychrophiles are evident and found in alpine soils, high mountains and glaciers (Maharana and Singh, 2018). Thermolabile molecules acquire three broad-spectrum physiognomies: maximum reaction activity, infrequently elevated k_{cat}/K_m and K_m comparative to their mesophilic organisms endure temperature less than 40°C, and relatively have the potential to tolerate thermostability (Russell, 2000).

Saline tolerant proteases are of numerous concerns of their hydrolytic reaction and constancy in basic environment (Saeki *et al.*, 2007 and Maurer, 2004). Resistant of these molecules to alkaline environment have functions in feather practices, food treating, detergents, silk cementing, drugs, biosynthesis and biotransformation and environmental biodegradation (Sareen and Mishra, 2008, Jellouli *et al.*, 2009, Kumar and Bhalla, 2004 and Bhaskar *et al.*, 2007). In microorganisms, the assembly of extracellular protease depends on many factors like the presence of glucose and Carbon/Nitrogen ratio (Gupta *et al.*, 2002) and the occurrence of metal ions (Varela *et al.*, 1996). Other than this, many other aspects, including the inoculum density, aeration, incubation time, pH and temperature also have influence on the production of protease (Gupta *et al.*, 2002). Addition of alkaline protease to detergents may

greatly enhances (35–40%) the effect of cleaning and may also upsurges the surface-active substances intake, which ultimately mends the ecological situation (Grebeshova *et al.*, 1999). The stability of enzyme also depends on the presence of surfactants, oxidizing agents, bleaches and many more additives that might have been found during formulation (Greene *et al.*, 1996).

The cold- adaptive *Stenotrophomonas sp.* isolated from northern area of Pakistan have the potential to produce thermolabile hydrolytic molecules in the fermentation medium and was acknowledged as *Stenotrophomonas* sp. on 16s rRNA sequence basis (accession number MG662181submitted in genebank). The Cold-adapted proteases are hereby utilized to optimize some industrial processes and it can be also used for the development of new technologies owing to minimum energy cost and inputs by the removal of heat inactivation step cost (Cavicchioli *et al.*, 2002 and Margesin *et al.*, 2003). To check the detailed diversity of microbes from the temperate regions in Gilgit-Baltistan, Pakistan, it is well known that there is a great possibility for the utilization of alkaline protease giving bacterium which can be used for its efficient applications in cold washing. Hence, the present investigation was carried out in order to quarantine the microbes which produces the cold active alkaline protease enzyme. This work will unveil the catalytic possessions of protease enzyme from *Stenotrophomonas* sp. Pak1 (the optimum standards of temperature, pH activity/stability and thermostability, and compatibility with components of detergents), with a view to its use in various branches of industry. Thermolabile alkaline protease from *Stenotrophomonas* sp. isolated from soil and water samples of Juglot (N-35°41'062' E-74°37'261"), Jutial (N-35°54'276" E-74°19'841) Rakaposhi glacier (N-36°14'368 E-74°26'576"and altitude of 2,688m situated in the Gilgit and Hunza-Nagar region of the largest valley type glacier in Gilgit-Baltistan, Pakistan (Nafees *et al.*, 2014).

1.1. Microbes Producing Cold-Active Proteases

Microorganisms producing thermolabile proteases from freezing habitations such as, alpine territories, polar areas, deep ocean, glacier loams and ice, arctic and sub-Antarctic deposits, low temperature desert mud territories on earth. Enzymes produced from thermolabile microorganisms and their potentials was reassessed from different periods (Georlette *et al.*, 2004, Cavicchioli *et al.*, 2002 and Gerday *et al.*, 2000). Morita (Morita, 1975) defined psychrophiles. Psychrophiles have the ability to

survive at 15 °C with a best temperature of cell biomass at around 20 °C and have the potential to tolerate 0 °C. Psychrotolerant microbes in contrast, best conditions for growth at 20 °C or exceeding (Feller, 2003). (Feller, 2003) psychrophile microbe was quarantined from oceanic to diverse categories of microbes as gram-negative have a diverse range including *Pseudoalteromonas, pseudomonas, Moraxella, planococcus Psychrobacter, Exiguobacterium Polaromonas, Xanthomonas Psychroflexus, etc.*)

(Oh *et al.*, 1999) isolated *Azospirillum* sp. from different geographical regions from mountain soil producing cold active hydrolytic protease. (Baghel *et al.*, 2005) who studied *Bacillus licheniformis* from polar glacier loam, *Clostridium* from polar Antarctic territory (Alam *et al.*, 2005), *Colwellia* from ocean frost and sub-antarctic sediments (Wang *et al.*, 2008), (Kuddus and Ramteke, 2008) who observed *Curtobacterium* from polar glacier mud, *Exiguobacterium* from freezing wasteland loam (Kasana and Yadav, 2007), *Pedobacter cryoconitis* from glacier ice (Margesin *et al.*, 2005), *Penicillium* from oceanic ecosystem (Zhu *et al.*, 2009), *Pseudomonas* sp. from deap sea (Zeng *et al.*, 2003), gamma-proteobacteria from Antarctic (Denner *et al.*, 2001), (Larsen *et al.*, 2006) studied *Serratia* sp. from shoreline, (Margesin and Schinner, 1991) observed *Xanthomonas maltophilia* from high-altitude and (Kristjansson *et al.*, 1999) who investigated *Vibrio* sp. from oceanic.

For the cold-active hydrolytic enzymes (Yu *et al.,* 2011) investigated microorganisms from Antarctica. *Sulfitobacter* sp, *Photobacterium*, *Pseudomonas*, *Shewanella*, *Bizionia*, *Flavobacterim*, *Salinibacterium* were found to secrete thermolabile proteolytic enzymes. Loam from polar glacier of Gangotri (Kuddus *et al.*,2011) observed thermolabile protease secreting *Stenotrophomonas* sp. Microorganisms excreted thermolabile proteases are enumerated in Table 1. (Buia 1997) observed a variety of Preliminary data obtained from extremophiles secreted biocatalyst from Antarctic conditions. Quantitative measurements designated the existence of protease, amylase, phosphatase, and β- galactosidase demonstrating durable freezing conditions acclimatization in numerous strains (Table 1.1). Since this investigation is obvious that polar bacteria, particularly isolated from freeze, are worthy sources of thermolabile enzymes (Buia, 1997).

Table 1.1: Microbes secreting thermolabile alkaline protease

S. No.	Organisms	Molecular weight (kDa)	Optimum Temp. (°C)	Optimum pH	Reference
		Properties of the protease			
1	*Alcaligenes faecalis*	-	30	8.8	Thangam and Rajkumar *et al.*, 2000
2	*Alkaliphilus transvaalensis*	30	40	12.6	Kobayashi *et al.*, 2007
3	*Alteromonas haloplanktis*	76	20	9	Suzuki and Odagami, 1997
4	*Aspergillus ustus*	45	32	9	Damare *et al.*, 2006
5	*Azospirillum sp.*	48.6	40	8.5	Oh *et al.*, 1999
6	*Bacillus* sp.	-	30	9.6	Kaur *et al.*, 2001
7	*Bacillus* spp.	-	40	10.5-11	Okuda *et al.*, 2008
8	*Bacillus amyloliquefaciens*	45	-	10	Son and Kim, 2003
9	*Bacillus cereus*	-	20	9	Joshi *et al.*, 2007
10	*Bacillus* licheniformis	31	50	10	Toyokawa *et al.*, 2010
11	*Bacillus pumilus*	-	30	11.5	Kumar, 2002
12	*Beauveria bassiana*	-	37	10	Rao *et al.*, 2006
13	*Candida humicola*	-	37	10	Ray *et al.*, 1992
14	*Clostridium* sp.	46	37	7	Alam *et al.*, 2005
15	*Colwellia* sp.	60	35	8-9	Wang, *et al.*, 2005
16	*Curtobacterium luteum*	115	20	7	Huston *et al.*, 2004
17	*Colwellia psychrerythraea*strain	71	19	6-8.5	Kuddus and Ramteke, 2008
18	*Engyodontium album*	-	25	11	Chellappan *et al.*, 2006
19	*Escherichia freundii*	55	25	10	Nakajima *et al.*, 1974
20	*Exiguobacterium* sp	36	40	8	Kasana and Yadav, 2007
21	*Flavobacterium*	49	30	8-11	Zhang *et al.*, 2011
22	*Flavobacterium balustinum*	70	40	7-9	Morita *et al.*, 1998

23	*Leucosporidium antarcticum*	34.4	30	8	Turkiewicz *et al.*, 2003
24	*Pedobacter cryoconitis,*	27	40	8	Margesin *et al.*, 2005
25	*Penicillium chrysogenum*	41	35	9	Zhu *et al.*, 2009
26	*Planomicrobium* sp.	-	35	9	Sheng *et al.*, 2011
27	*Pseudoalteromonas* sp.	34	35	8	Xiong *et al.*, 2007
28	*Pseudoalteromonas* sp.	28	30	8	Wang *et al.*, 2008
29	*Pseudoalteromonas* sp.	-	20	8	Vazquez *et al.*, 2008
30	*Pseudoalteromonas* sp.	65	25	9	Chen *et al.*, 2007
31	*Pseudomonas* sp	45	40	-	Vazquez *et al.*, 2004
32	*Pseudomonas* sp.	-	20	-	Chessa *et al.*, 2000
33	*Pseudomonas strain* DY-A	-	40	10	Zeng *et al.*, 2003
34	*Pseudomonas aeruginosa*	-	40	9	Patil, and Chaudhari, 2011
35	*Pseudomonas lundensis*	48	30	10	Yang *et al.*, 2010
36	*Pseudomonas fluorescens*	-	35	5	Koka, and Weimer, 2000
37	*Pseudomonas fluorescens.*	47	40	8	Hamamato *et al.*, 1994
38	*Pycnoporus cinnabarinus*	-	30	4	Meza *et al.*, 2007
39	*Roseobacter* sp.	-	40	9	Shanmughapriya *et al.*, 2008
40	*Serratia marcescens*	58	40	8	Tariq *et al.*, 2011
41	*Serratia marcescens*	56	40	8	Morita *et al.*, 1997
42	*Serratia proteamaculans*	50	30	8	Mozhina *et al.*, 2008
43	*Shewanella* strain	44	15	9	Kulakova *et al.*, 1999
44	*Stenotrophomonas*	55	15	10	Saba, *et al.*, 2012
45	*Stenotrophomonas maltophilia*	75	20	10	Kuddus and Ramteke, 2011

46	*Streptomyces* sp.	-	30	10	Tokiwa *et al.*, 1999
47	*Streptomyces alboniger*	-	37	9-11	Lopes *et al.*, 1999
48	*Teredinobacter turnirae*	-	25	7	Elibol and Moreira, 2005
49	*Trichoderma atroviride*	24	25	6	Kredics *et al.*, 2008
50	*Vibrio* sp.	47	25	8.6	Kristjansson *et al.*, 1999

1.2. Catalytic mechanism

Principal performer in the hydrolytic machinery is the catalytic triad in the catalytic position of the serine proteases wherever hydrolysis follows and is conserved in all the serine peptidase family. Triad is a synchronized arrangement comprising of Aspartic acid 102, Histidine 57 and Serine 195. These 3 strategic amino acids monomers separately performance an indispensable part in the splitting potential of the proteases. Although the amino acid members of the catalytic position are positioned faraway from one another on the structure arrangement of the protein, because of the folding, they are appropriate adjacent to each other in the enzyme heart. Specific geometry of the catalytic residues is greatly distinctive to their precise task and was exhibited that the location of 4 points of the catalytic residues differentiating purpose of the comprising enzyme (Ivan *et al.*, 2009). Mechanism of serine peptidase is given in (Figure 1.1).

1.3. Classification of Proteases

Enzyme Commission categorized, proteases classification in group such as 3 (hydrolytic enzyme biocatalyst), and 4 hydrolytic biocatalyst sub-group. Enzyme was apportioned with 2 comprehensive factions upon their potential to catalyze exopeptidases external peptide bonds or (endopeptidases). However, in industrial purposes exopeptidases biocatalyst are exploited, endopeptidases biocatalysts are considered as scientifically imperative. Exopeptidases biocatalysts segregated as aminopeptidases that naturally hydrolyzed amino acids chains at the N-terminal and the polypeptides chain at the C-terminal was observed to be catalyzed by carboxypeptidases. Other qualities used in categorizing proteases into dissimilar clusters comprising as incidence of incriminated moieties at positions comparative to susceptible bond (Ward, 1985), and their optimum pH, substrate explicitness containing as collagenase and homologized to their beforehand categorized biocatalysts contained, biocatalysts pepsin and trypsin enzymes. Morihara (1974) catalogued trypsin-like proteinases as serine proteases, Myxobacteria extracted proteinases, alkaline proteinases and proteinases biocatalysts extracted from staphylococcal. Hartley *et al.* (1960) categorized endopeptidases into 4 distinctive factions because of their functional position and stability against multifarious inhibitors. Characteristics of the biocatalysts were reviewed in (Table 1.2).

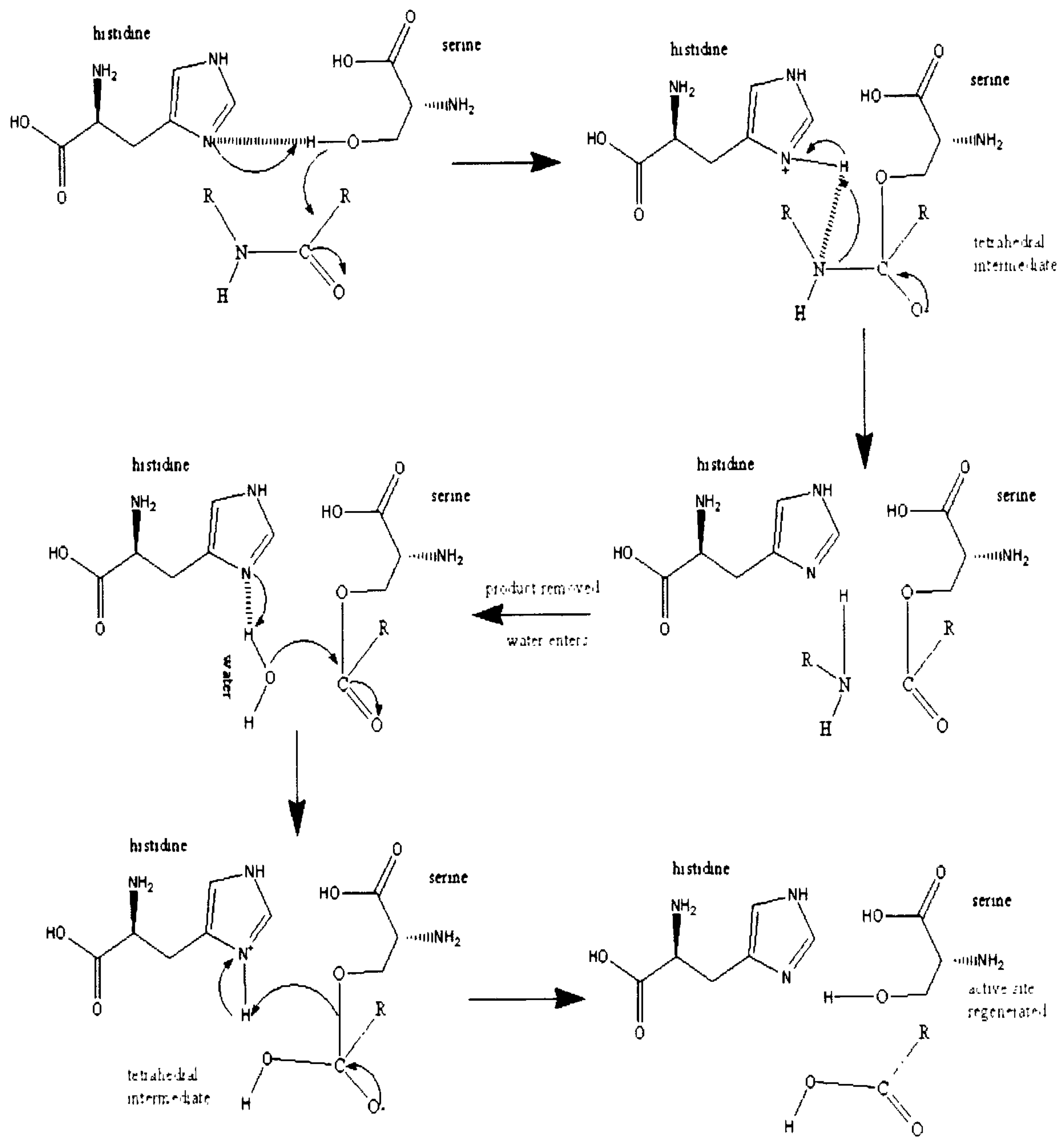

Figure 1.1: Serine protease mechanism (Ivan *et al.*, 2009)

Table 1.2: Classification and biochemical characteristics of endoproteases.

Endoprotease	EC No.	Mol. Mass Range (kDa)	Opt. pH	Opt. Tem. (°C)	Metal ion	Active Site Residues	Major Inhibitors
Carboxyl proteases	3.4.23	30-45	3-5	40-55	calcium	Aspartate	Pepstatin
Cysteine protease	3.4.22	34-35	2-3	40-55	-	Aspartate	C2H4INO, *p*-CMB
Metallo-proteases	3.4.24	19-37	5-7	65-85	Zinc, Calcium	Phenylalanine	Chelating agents such as EDTA, EGTA
Serine proteases	3.4.21	18-35	6-11	50-70	Calcium	Serine, histidine and aspartate	DIFP, EDTA, PMSF, soybean trypsin inhibitor, indole, phenol, triamino acetic acid and phosphate buffers

Microorganisms existing in extreme cold temperature are considered as psychrophiles having potential to propagate at temperature below 20°C however additional group is existing as psychrotrophic microorganisms having highest growth at temperature of 37°C in the mesophilic range and the potential to cultivate also at close to freezing temperature (Moyer and Morita, 2007). Earlier research indicates of receiving more psychrotrophic from temperate habitats similar to Antarctica than optimum temperature at 10 °C for growth (Antony *et al.*, 2016; Hatha *et al.*, 2013). The probabilities of attainment psychrophiles and psychrotrophic microorganisms are more observable at polar circumstances and can also be discovered in elevated mountains, ocean, alpine soils and glaciers (Maharana and Singh, 2018).

Lipases are triacylglycerol hydrolases performing different chemical reactions such as transesterification, esterification, chemical synthesis underneath water-restricted conditions and stereospecific chemical breakdown of optically active esters (Berglund and Hutt 2000; Soni and Madamwar 2000; Ferrer *et al.*, 2005). Lipases functioning at low temperature covering a wide spectrum of purposes of biotechnology such as detergents formulation, food production additives, biotransformation, pollutants eradicate from soiled regions, functions in molecular biology, and expression of heterologous gene in psychrophilic microorganisms to preclude appearance of inclusion elementary bodies (Joseph *et al.*, 2008). Thermolabile lipases has numerous capabilities in positions of lower activation energy overheads and microbial impurity in the different industrialized procedures (Marshall, 1997; Alquati *et al.*, 2002). During the preceding three decades the enzymes have the potential of being functional in the existence of organic solvents has acknowledged much consideration. Lipases from microorganisms' have biocatalyst function as in solvents having non-aqueous properties proposal novel capacities such as thermodynamic stabilities changing in favor of synthesis, substrate specificity monitoring by solvent engineering, facilitating the utilization of substrates with hydrophobic properties and improving resistance toward high temperature of the hydrolytic enzymes (Koops *et al.*, 1999).

In the reactions of chemical breakdown and synthesis, lipases validate region and stereo-selectivity, constructing them as a suitable applicant for the construction of optically functional composites (Snellman *et al.*, 2002). Pseudomonas is observed as an extraordinary producer for thermolabile lipase (Maharana and Ray, 2013, 2014a,

2015b; Zeng *et al.*, 2004). Furthermore, other bacterial species for enhanced hydrolytic lipase production are belonged to the genera of Microbacterium, Acinetobacter, *Bacillus, Aeromonas Staphylococcus, Psychrobacter* and *Moraxella* (Joseph *et al.*, 2008). Growth of the microorganisms depends on numerous chemical and physical aspects similar to temperature range, pH such as salinity, substrates specificity, different sources of carbon and nitrogen, amino acids and minerals salts. Production of enzyme is directly proportionate to the microorganism's growth but in most situations, enzyme production is maximum in the fermentation medium by the microbes are an inactive form, consequentially in inferior hydrolytic activity (Maharana and Ray, 2015a). Such circumstance accomplished because of the deviations in nutrient accessibility, temperature and pH. Addition to these observations, there are several additional molecules that display feedback inhibition of production of hydrolytic enzyme. Moreover, diverse microbes exhibited wide-ranging affinity against substrates catalyzed their specific mechanism for enzymatic hydrolytic reactions and production. Consequently, production optimization of different parameters for enzymes is fundamental for innumerable microbes and must be associated for the potent.

1.4. Mechanism and Structure of lipase

Lipases are stated fundamentally as triglycerides ester bonds- cleaving enzymes which hydrolyze the catalysis of linear long chain of triacylglycerols to product in glycerin simple compound and fatty acid with in the existence of surplus H_2O (Figure 1.2). Correspondingly, esterases hydrolyze the reverse catalysis and resulted in the production of triacylglycerols because of the non-aqueous circumstances (Jaeger *et al.*, 1999, Gupta *et al.*, 2004, Pascale *et al.*, 2008). Though a various assortment of genetically distinctive fat hydrolyzing enzymes are discovered in nature environment and characterize numerous categories of protein 3 dimensional structure and lipolysis, most of the lipase enzyme are assembled on the basis of an alpha/beta hydrolase folding (Winkler *et al.*, 1990, Schrag and Cygler, 1997, Egmond and Bemmel, 1997 and Withers-Martinez *et al.*, 1996) (Figure 1.3) that functioning as chymotrypsin-like catalysis machinery exploiting a catalytic triad in the active site of the lipase comprising of a serine residue that function like nucleophile alongside a histidine base, and an aspartic acid residue domain (Brady *et al.*, 1990 and Lowe, 1992). Functioning site comprising serine nucleophilic

residue of lipase enzyme is positioned in a greatly conserved motif of Gly-X-Ser-X-Ser in manifold tandem replicas in molecules (Pascale *et al.*, 2008; Joseph *et al.*, 2008)

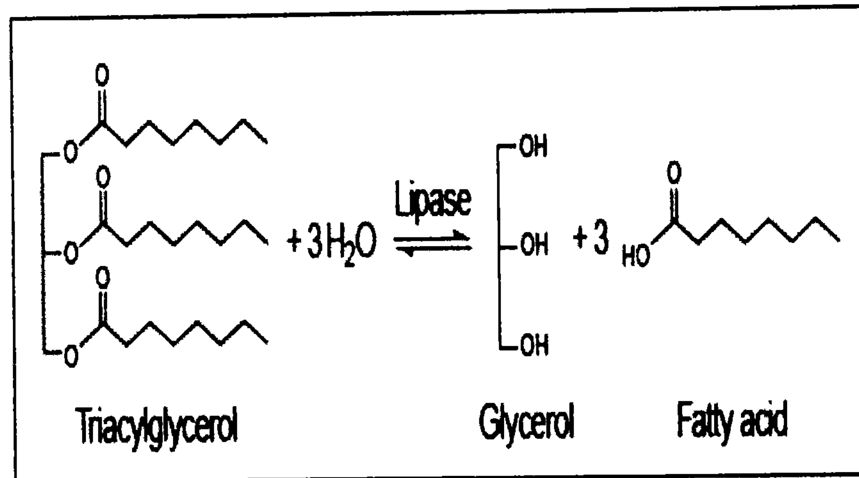

Figure 1.2: Hydrolytic and Synthetic Actions of Lipase (Source: Jaeger and Reetz 1998)

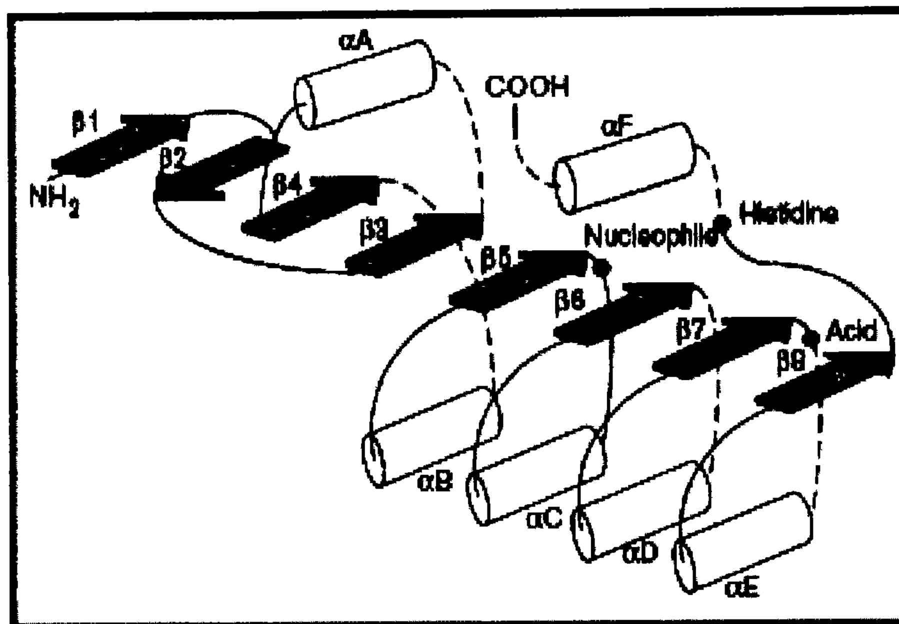

Figure 1.3: The α/β hydrolase fold (Source: Jaeger, *et al.* 1999)

Conclusively, roughly some of the *Pseudomonas* lipases showed calcium binding motif in the protein domain (Figure 1.4). Neighboring to the catalytic triad active site calcium binding site is positioned, nonetheless not associated to hydrolytic endeavor and supposed to perform function in equilibrium to the usual 3-D structure of the enzyme molecule.

1.5. Bacterial Lipases

A diversity of microorganisms from both Gram-positive and Gram-negative secreted lipase. Gram-negative bacteria was observed to be the highest production of lipase enzyme and the largest group of genus *Pseudomonas* belong to Gram-negative family which comprises at least 7 lipase secreting species, that includes *P. putida, P.fragi, P. alcaligenes, P. glumae, P. cepacia, P. aeruginosa* and *P. fluorescens* (Jaeger *et al.*, 1994, Kojima *et al.*, 2003). On the basis of protein homologies *Pseudomonas* secreted lipases are classified into 3 main groups and about some biological characteristics (Zhang *et al.*, 2008). Group I comprises enzyme from, *P.alcaligenes, P.aeruginosa* and *P.fragi.* Group 1 proteins are comprised of roughly 285 amino acid residues alongside a molecular mass of 30 Kdalton. Similarly, comprise two residues of cysteine which create S-S bond and signal sequence at N-terminal site of the protein and require molecular chaperones help the amino acid non-covalent folding under ATP-relying mechanism and secretion.

Lipase protein in Group II comprise 320 amino acids sequence with mass of 33 kda, N-terminal protein signal sequence beside one disulfide bridge. *P.cepacia and P.glumae* secreting lipase protein are paradigms from group II. Group III comprising nearby 475 amino acids protein sequence with mass of 50 kda. Lipases secreting from *P.fluorescens* are paradigms of this group (Arpigny and Jaeger, 1999). Type II secretion pathway follow by lipases enzyme belong in Group I and II although lipases proteins fitting to this group III are produced through type I secretion method (correspondingly designated ABC transporters (Rosenau and Jaeger, 2000). Though group III transporters mechanism of lipases protein have not N-terminal protein structure, thus comprise a C-terminal directing signal system accountable for the production of protein through ABC transporters (Figure 1.5) (Amada *et al.*, 2000; Duong *et al.*, 1994).

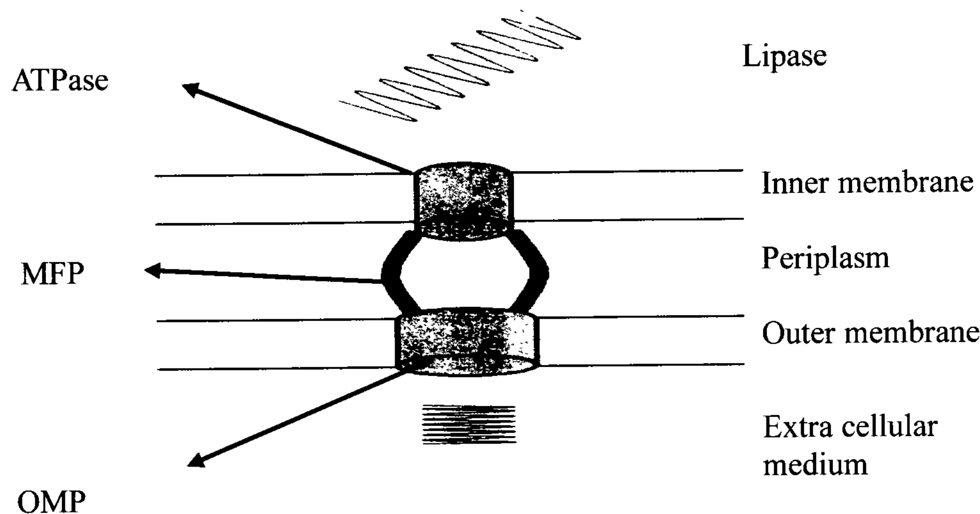

Figure 1.5: Type I secretion pathway (Source: Rosenau and Jaeger, 2000)

Lipases secreted by Group I and II mechanism have the signal peptides at the N-terminal that is used for effective transfer of proteins across the membrane of the cytoplasm to the periplasm through Sec- dependent machinery. This procedure comprehends Sec translocase is a complex of proteins subunits which distinguishes the N-terminal site of protein 3-D structures. However, across the mitochondrial membrane protein residues is secreted and the signal peptides on N-terminus is detached and peptide residues interrelate alongside its particular foldase chaperones (Rosenau *et al.*, 2004). Structural configuration of Lipases transformed into vigorous isomerism in between inner and outer cytoplasm membrane. Furthermore, this procedure supported by the disulfide bridge peptides that hydrolyzes establishment of S-S bridge with functional group. Otherwise, unusual tertiary arrangement might be destroyed by the restriction enzymes presents in the periplasmic membrane. Conclusively, lipases proteins conveyed across the exterior membrane through assistance of a complicated machinery termed secretion that comprises diverse peptides establishing type II secretion intervened- secretion (Figure 1.6). Likewise, *Pseudomonas* species other usual lipase secreting gram-negative species are *Alcaligenes*, *Chromobacterium*, *Burkholderia* and *Achromobacter* (Gupta and Rathi, 2004). Lipase secreting Gram-positive bacterial strains includes *Staphylococcus, Streptomyces* and *Bacillus* species. Maximum beneficial lipase secreting genus

exploited in industry is *Bacillus* amongst other gram-positive strains of bacteria (Jaeger *et al.*, 1994).

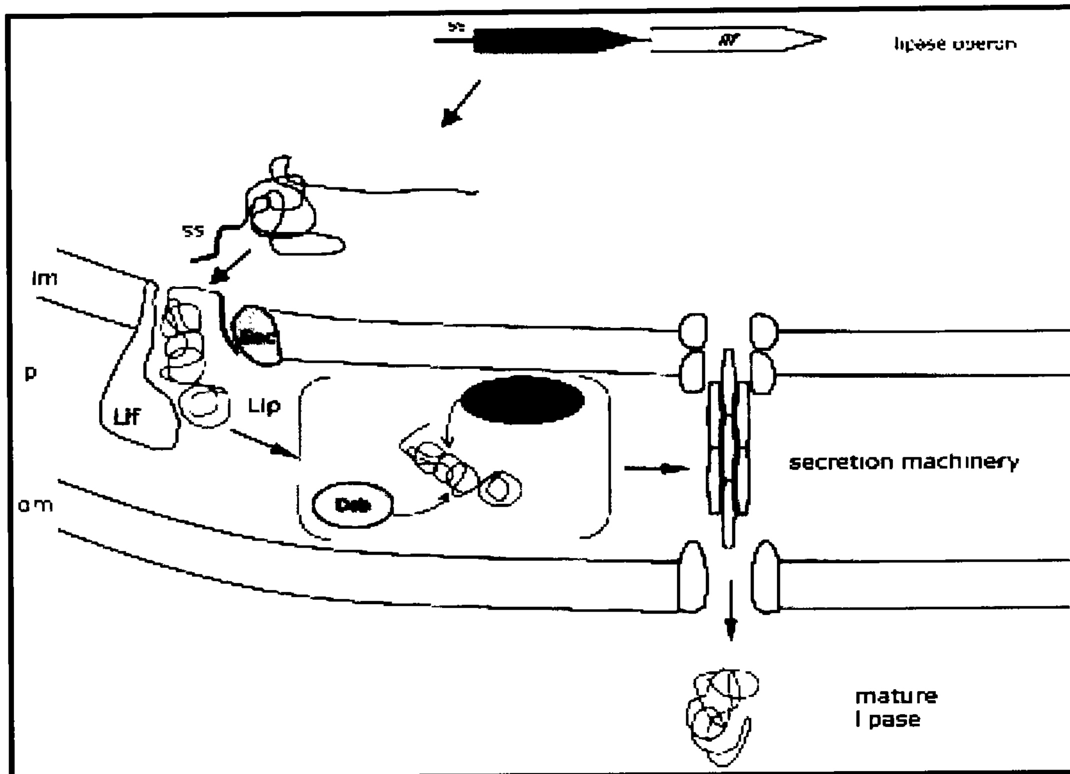

Figure 1.6: Model for *Pseudomonas aeruginosa* lipase secretion pathway (Source: Rosenau and Jaeger 2000)

1.6. Goals and objectives

The primary goal of the present research was to explore the Karakorum glaciers of Pakistan for isolation of psychrotrophic microorganisms in assessment of their proficiency for biotechnological applications. Following were the objectives of present-day research.

i. To isolate protease and lipase producing psychrotrophic bacterial strains from soil and water samples from glaciers of Pakistan.
ii. To identify the selected thermolabile protease and lipase producer bacterial strains, 16S rRNA nucleotide sequence analysis was applied.
iii. Cloning, expression and characterization of thermolabile alkaline protease and lipase encoding gene from *Stenotrophomonas sp.* and *pseudomonas peli* strain.
iv. Screening of the selected isolates for their ability to produce protease and lipase enzymes.
v. Production of thermolabile alkaline protease and lipase in shake flask by submerged fermentation on diverse media
vi. The other purpose of this research was to optimize different parameters for the maximum production of cold active alkaline protease and lipase enzyme
vii. Alkaline protease and lipase purification were conducted through various methods such as ammonium sulfate precipitation, dialysis, ion exchange chromatography and gel permeation chromatography
viii. Determination of molecular mass through SDS-PAGE analysis.
ix. Purified protease and lipase enzyme were characterized for stability analysis and investigating various other parameters such as effect of pH, temperature, metal ions, surfactants, chelators and inhibitors and organic solvents on the catalytic activity of enzymes.
x. Kinetic studies were evaluated of purified enzyme.
xi. Evaluation of cold active alkaline lipase and protease for use in detergent formulation

1.7. Problem Statement, Research Gap, and Research Hypothesis

i. Existing detergents in the market consist ingredients including dyes, salts and soda which damage the shine and quality of fiber and hence fabric as a whole.

ii. They also cause a significant addition to the sanitary pollution. Further, they cause the causticity and hence allergies. Besides these issues, the existing detergents are expensive.

iii. To the best of our knowledge, there is no formulation of biodetergents in the market so far.

iv. To tackle the issues, cost effective and environment friendly biodetergents should be fabricated and launched in the market.

v. The sustainable cost and quality of the fabric can be maintained by using the detergents designed through bio-based material of low economical value.

vi. The biodetergents consisting hydrolase enzymes particularly the lipase and protease can help to sort the issue.

vii. Washing at low temperature in home settings and higher pH can be sorted by extracting the lipase and protease from low temperature active bacteria and proliferating at higher pH.

viii. Hence a formulation of a biodetergent added with low temperature and alkaline pH active lipase and protease is proposed during this study.

CHAPTER. 2

2. REVIEW OF LITERATURE

Neutrophile or mesophiles is an organism that live at neutral pH or moderate temperature for existence in contrast to extremophiles that thrives in extreme condition for life, respectively. Extreme environment provides extraordinarily basic, acidic, dry, hot, cold and under immense pressure niche for life. Predominantly, extremophiles are microbes present in distinct but numerous genetic lineages of archaea and bacteria. Extremophiles receive attributes based upon their habitats like, salt (halophiles), extremely dry conditions (xerophiles), pH (neutrophiles, acidophiles, alkaliphiles), temperature (psychrophiles, thermophiles), rocks (endoliths), high sugar contents (osmophiles) and higher atmospheric pressure (piezophiles). Other than their applications as biompass, prototypical organisms to explore novel genes and biochemical pathways, due to their unique characteristics attained during adaptation to harsh conditions of environment, extremophiles are potent sources of metabolites as demonstrated by Burg (2003). Morita, (2001) classified bacteria based upon lower, optimum and upper limit of temperature for growth and also to the habitats they thrive in. Psychrotrophs, might have the potential to survive at temperature near to freezing temperature, and the temperature where they have the highest catalytic activity was observed equal to 30°C; henceforth they might believe as being cold-tolerant mesophilic microorganisms (Russell, 2006). In contrast psychrophiles usually nurture toward at or underneath 0°C and have the best cell mass temperature equal or lower than 15°C and higher frontier of equal or lower than 20°C. Moyer and Morita (2007) defined psychrotrophic microorganisms are cold-stable that have the best and maximal cell biomass temperatures exceeding toward 15 and 20°C whereas, psychrophiles as "cold loving extremophilic bacteria or archaea having an optimal temperature against 15°C and encouraging temperature toward 20°C and temperature at 0°C or lesser the cell biomass observed was not so encouraging for growth, respectively. Higher specific activity and thermolability at low cost of energy are important adaptations of psychrophiles.

The biomolecules isolated from these bacteria are very important, biotechnologically. The millions of years old glaciers have entombed ancient simple life that may be representative of primitive life on earth, transferred across the other

psychrophiles. (Hamilton and Lenton, 1998) reported that distilled droplets up in air contain bacteria as typhoons, winds, birds and aircrafts can contaminate the upper atmosphere up to acceptable limit for microbial dispersion. Sattler *et al.*, (2001) isolated bacteria actively cultivating and reproducing at temperatures at or below 0°C, from supercooled cloud droplets collected from high altitudes. Phytoplankton and microorganisms isolated from watery sites through procedures of bubble-burst particularly in 'white-caps', these frequently themselves instigated by airstreams convective. *Pseudomonas syringae*, discharge heat dynamism of phase transformation and facilitating to indigenous air drives that might use for lobbing and for lateral scattering of their producers. Hamilton and Lenton, (1998) suggested the 'phase change catalysis' as means of dispersal for nuclei amongst their marine, continental or epiphytic growing locates.

2.5. Water and snow

Darwish and Bloomfield, (1995) described the methodology for sampling from temperate and cold waters. For vegetative cells and for spore's cells water samples are purified throughout membrane screens of 0.5 m and 0.2 m. Freshly fallen snow can be collected without extraneous contamination in sterile bags and melted slowly at lower than 5°C prior to filtration as for water samples. From deposits of the Oceanic of Japan and coastline ocean frost Romanenko, (2004) isolated psychrotrophic Gram-negative aerobic strains, *Psychrobacter arenosus* bacteria and *Psychrobacter maritimus* sp. and. Li *et al.*, (2009) analyzed 463 clones and identified 13 diverse core ancestries of microorganisms such as Bacteroidetes, *Stenotrophomonas,* Proteobacteria Chloroflexi, *Exiguobacterium.* Actinobacteria Firmicutes, Verrucomicrobia, Spirochetes and Planctomycetes,) by using 16S rRNA sequencing technique. The community composition was different among sampling sites, which potentially was related to geochemical differences.

YongQin *et al.*, (2007) found that as compared to Antarctic bacteria were abundant in snow and augmented with elevation had no association to biochemical aspects. *Bacteroides* (CFB) family entirely governed in polar meltwater, whereas CFB, β-*Proteobacteria* group governed in serac frost, and *Actinobacteria and* β-*Proteobacteria* subject in frost surface. Microorganisms in the part of frozen water on Everest intently interrelated to microbes from land, marine environments and eukaryotes and other freezing exospheres.

2.6. Permafrost soils and ice

For collecting and handling permafrost soils and ice cores, and for their decontamination number of protocols was developed by (Rogers *et al.*, 2004; Christner *et al.*, 2007). (Zheng *et al.*, 2006) isolated psychrotrophic, halophilic *Psychrobacter* sp. from prehistoric Siberian deposits. Christner *et al.*, (2006) examined the possible sampling measures and limnological conditions from subpolar River of Antarctica and psychrophilic microorganisms was isolated from East Antarctic Ice Layer. Sinclair and Stokes, (1964) Isolated obligately anaerobic low temperature active bacteria from soil, mud, and sewage. The organisms grew well at zero degree centigrade in liquid and on solid media and grew only in the absence of oxygen. All of the organisms were classified as Clostridia on the basis of shape, sporulation, flagellation, and strictly anaerobic growth. Dominant *Arthrobacter* genus of bacterial family culturable inhabitants in alpine sediments. The pyschrophillic culturable microorganisms from high-altitude sediments in Mountains located in northwestern China was reported by Bai *et al.*, (2006).

2.7. Extraterrestrial sources

(Chyba, 2000) examined that the filterate of meltwater from Europa's ice, microbial life could exist up to detectable limit Samples obtained from other cold planets, belongings of astronauts and space shuttles in contact with extraneous environment, and meteorites may be explored for isolation of psychrophiles.

2.8. Glaciers

(Miteva, 2008) reported that the bacteria in dormant and vegetative forms are present under ice of glacier and adapt the ecosystem with any one or combination of different mechanisms of adaptation. A glacier is a large mass of moving ice that flows slowly over the land. Glaciers are usually permanently enduring body of ice, comprising fundamentally of re-crystallized snowflake, exhibits sign of down gradient slope or external drive because of the influence of gravity. During the process of formation of glaciers, small droplets nuclei, airborne, dust, mud and snow; all contribute to add the spores, microbes or their vegetative configurations which are entombed for years. Some of the compromised candidates adapt this ecosystem to survive at such stress of the environment. (McNamara and Kane, 2009) observed portion of earth terrain remains beneath frozen water part known as cryosphere which is majorly formed by glaciers like Arctic, Antarctic and mountain glaciers.

2.9. Polar glaciers

The psychrotrophic bacteria from Lapland northernmost territory comprising forestry topsoil, glacial alpine loam, watercourse, river and swamp deposits, lichens with snowflake algae was characterized by (Mannisto and Haggblom, 2006). Most of the *Proteobacteria, Actinobacteria, Stenotrophomonas* and other *Bacteroides* group observed because of the biochemical characterization, microscopy and 16S ribosomal RNA analysis of nucleotide sequence. The commonly isolated Gram-negative bacteria include *Pseudomonas* sp., *Burkholderia* sp., *Pedobacter sp., Stenotrophomonas sp, Collimonas sp., Janthinobacter sp., Exiguobacterium sp., Sphingomonas sp and Duganella sp.* From Antarctic sea ice (Bowman, 1998) isolated a psychrotrophic, halotolerant bacterium, *Pseudoalteromonas prydzensis* sp. On the basis of 16s ribosomal RNA-genome sequences dependent mono and paraphyletic analysis to signify a discrete genospecies clade of clusters at the frontier of the *Pseudoalteromonas* species non-pigmented mono and paraphyletic clade.

Sogin *et al*., (2006) observed that the microbial diversity of North Atlantic and diffuse flow hydrothermal vents, a very ancient ''rare biosphere'' that may represent a source of genomic innovation. Shivaji, (2005) illustrated the *aquaticus and vallis* species of psychrobacter isolated from different sites of Antarctica. Zhang *et al*., (2008) collected ice samples from Skavrvsnes, Antarctic Lakes, isolated bacteria at 4°C and 20°C and characterized them by the analysis of universal primers of 16S rRNA nucleotide sequence. Phylogenetically, the isolates were members of *Flavobacteria*, *Bacillus*, *Actinobacteria*, and *proteobacteria*. lakes under Antarctic sheets may contain some of the most unusual, extreme microbial ecosystems was reported by (Christner and Priscu, 2008). Tindall (2004) studied the diversity in the ice and water samples of Antarctica, using cultivation and molecular techniques. Junge *et al*., (2004) experimented that the bacterial abundance, activity and diversity of *Cytophaga-Flavobacteria-Bacteroides* and *Archaea,* at 0 to -20C in winter ice samples of Arctic sea.

From a pond in Antarctica thermolabile *rubra* and *aurea* species of genus *Leifsonia* were isolated by (Reddy 2002). using 16S rRNA analysis (Bowman, 1997) studied the phylogenetic diversity and ancestor relationship of pscychrophilic from polar Ice. Overall, four phylogenetic groups, two subdivisions of *Proteobacteria* and the (CFBs) were found. These psychrophilic strains on the 16S rDNA genome

sequence evaluation belonged to the diverse groups of *Shewanella*, *Exiguobacterium*, *Marinobacter* and *Planococcus*. Psychrotrophic bacterial species were observed of the diverse genera of *stenotrophomonas*, *Halomonas*, *Pseudomonas*, *Hyphomonas*, *Arthrobacter*, *Exiguobacterium Psychrobacter Planococcus*, and *Halobacillus*.

(Bozal, 2003) characterized two novel gamma-proteobacteria isolated from Polar glaciers of Antarctic environments. Major microbial communities from Antarctic sea ice were collected, isolated and studied by (Garrison *et al.*, 1986) however the mechanisms for cold adaptations were explored by (Nichols *et al.*, 2004) who attributed the cold adaptation of unsaturation of its lipid contents in membrane. For cold-activity of enzymes the structural adaptation is important (Sheridan *et al.*, 2000). Bacteria were retrieved from Guliya ice and Lake Vostok ice. 16S rRNA genome sequences technique used and the isolates were observed as species of the *Stenotrophomonas sp.,* proteobacteria, *Planococcus bacterial sp.,* actinobacteria and *Brachybacteria*, *Sphingomonas*, *Methylobacterium* and *Paenibacillus* respectively. (Christner *et al.*, 2002, 2003 and 2008) experimented that the direct elaboration of 16S rRNA from Guliya and Lake Vostoc ice melt water evidenced the presence of *Pseudomonas, Acinetobacter,* proteobacterial and a member of the family of (CFB), respectively. Borriss *et al.*, (2003) isolated and characterized oceanic psychrophilic from polar Arctic ocean ice. 16S rDNA genome sequences revealed that the three phage bacterial hosts presented maximum resemblance to diverse species of *frigidimarina*, *hibernum* and *psychrerythraea* correspondingly. Sheridan *et al.*, (2000) they determined low temperature activity by comparing the enzymes having different optima, purified from phylogenetically related organisms. Xiang *et al.*, 2005 isolated psychrophilic different bacterial species from diverse territories of ice core glacier on the Pamirs Plateau, China. Small subunit 16S ribosomal RNA nucleotide sequences suggested that 11% were psychrophilic bacteria though 82% were psychrotrophic bacteria. (Gupta and Prakash, 2014) observed that the cold active enzymes from psychrophiles are particularly important due to flexible structure and tremendous potential for use in low temperature processes. The obtained sequence obtained nucleotide sequence compared with the NCBI databases through BLAST which showed 100% similarity with *Pseudomonas vancouverensis* as because of the 16s rDNA genome sequencing technique.

(Park *et al.*, 2014) collected samples from Arctic Chukchi Ocean excursion coast of the Korean RV Araon icebreaker, diverse species of bacteria were isolated. Only 16% exhibited protease hydrolytic activity identified as *Pseudoalteromonas. Alteromonas* and *Staphylococcus*. From the Phylogenetic analysis observed that these microbial strains were associated with the *Pseudoalteromonas*. (Moschonas *et al.*, 2011) experimented PCR and 16S rRNA sequencing for the identification of psychrophilic or psychrotrophic microorganisms and assessed genetic characteristics using (RFLP) analysis to manipulates dissimilarities in DNA homologous sequences and PCR (SR-PCR). 25 bacterial species were recognized with *gasigenes* and *estertheticum* species of genus *Clostridium*. Irina *et al.,* (2007) studied the contamination free sampling method and examined bacterial diversity of a deep ice bore hole at east of Antarctica above Vostok. *Sphingomonas* genus was observed using 16s rRNA analysis, Chattopadhyay and Jagannadham, (2001) isolated psychrotrophic bacterium from soil samples of polar Antarctic region.

2.10. Non-Polar Glaciers

During study of glacial history, Meiners, (2005), found that the great Karakorum main ridge retreated about 8 kilometers at Baltar and Kukuar glaciers (Pakistan) since 1915 till to date. As hypothesized by Kuhle, (2005) the geomorphological and glacial perspective of the Karakorum morain history, confirm connection to Hunza and Bar glacier.

Kim *et al.*, (2008) isolated ice-active substance producing, psychrophilic bacterium *Moritella dasanensis* sp. nov., from a polar glacier in Kongsfjorden and identified using 16s ribosomal RNA nucleotide sequence analytical technique. Using ARDRA and genome Sequence technique 16S rRNA Segawa (2005) studied the bacteriological diversity from snow of Tateyama Mountains. The samples of snow contained psychrotrophic and psychrophilic microbes such as *Cryobacterium psychrophilum* and *Variovorax paradoxus, Janthinobacterium lividum*.

Genomic diversity in polar Glaciers was investigated by (Liu *et al.*, 2009) through exploration of molecular procedure of 16S rRNA and cytometry approaches. Bacterial diversity found, included 15 usual genera of bacterial scattered broadly in polar glaciers. Within South American mountain glacier (Campen *et al.*, 2003) studied the microbial consortia metabolizing. Thermolabile bacteria from John Evans polar Glacier was studied by Foght *et al.*, (2004). The 16S rRNA gene RFLPs of 341

clones were consisted of proteobacteria, Bacteroidetes and Actinobacteria. A second water sample had proteobacteria, Bacteroidetes and a deposit of sample was governed by β-proteobacteria and phylum of Bacteroidetes. Zhu *et al.*, (2003) isolated psychrophilic species *xinjiangense* and *omnivorum* of genra *Flavobacterium* from polar glacier of China and recognized by using universal primers of 16s ribosomal RNA.

The four facultatively psychrophilic were isolated by Yumoto *et al.*, (2001) from Japan. The isolates were catalase, oxidase, nitrate, gelatinase positive but amylase negative, have the potential to survive at 4°C and was observed that bacteria exhibited no characteristics of thermostability. The 16S ribosomal RNA sequencing designated that the bacteria belonging to categories of *Pseudomonas*. Baghela, (2005) studied the cold active excreting thermolabile protease microorganisms from polar Glacier India. The genetic variability and widespreading psychrotrophic microorganism were examined by (Zhang *et al.*, 2007) in the frozen sediments of Himalayan Plateau. Liu *et al.*, (2001) studied diversity of culturable bacteria from East Rongbuk Glacier melt water and found that the major micro biota included members of Proteobacteria, Actinobacteria, and Firmicutes. Based upon Biology bioassay and growth test patterns, psychrophilic bacteria could be divided into three categories: temperature dependent but flexible in consumption of diverse carbon substrates secondly survive to diverse kind of temperature and weak biomass.

(Yao *et al.*, 2006) Isolates from Malan Ice Cap indicated that micro flora consisted of Proteobacteria, different family domains such as HGC, CFB and LGC classified by means of the 16S 30S small subunit ribosomal RNA sequence analysis. Microbial population varied along the depth of glacier core, morphologically, that was correlated to environmental changes directly. The 16S rDNA analyses of the bacteria isolated from refrigerated beef suggested *Pseudomonas* spp. as the wide spreading group nearly in all conditions. *Panacisl* and *brennerii* species of *Pseudomona* was the wide spreading in taxonomic group, but sequences with highest similarity 16S 30S small subunit ribosomal RNA sequence to *lundensis*, *beteli* and *koreensis* species of *Pseudomonas* were also uncovered (Olofsson, 2007).

2.11. Metabolism and adaptations for cold environment

Cold active bacterial strains owe the ability to cope with the challenges like, reduced enzyme activity, transformed carrying of nutrients, diminished transcription rate, protein synthesizing and cell cycle, decreased membrane fluidity, thermolabile protein denaturation; incorrect protein folding, and ice formation within cell, survive in such conditions. (D'Amico *et al.*, 2006) Due to the extreme conditions of the different environment these extremophiles successfully evolved their genetic makeup to survive and reproduce against the harsh conditions. Psychrotrophic microorganisms productively evolved structures to overcome against low conditions. psychrophiles and psychrotrophs bacterial organisms due to the molecular and biochemical properties have the potential to survive tremendously freezing circumstances are categorized (Jermy, 2009).

Konings, (2002) studied that the role of the cell membrane in survival of bacteria in extreme environments were examined and concluded that with temperature and other factors permeabilities to charged ions of all organic membrane growth intensification. Psychrotrophic and mesophilic microorganisms have the potential regulating the permeabilities lipid conformation in such a way that permeability to charged ions such as proton at the particular growing conditions retain low and persistent. Transportation of biochemicals through the cell membrane is generally regulated by prime ATP motivated transport mechanism.

Bakermans, (2003) studied the isolation and metabolism of spore forming, cold active bacteria from Siberian freezing sediments. The cells capable of evolving at -10°C revealed physicochemical variations at the basic-structural level. Chintalapati, (2004) studied the cold adaptation of psychrophiles in the fluidity of the membrane by special fatty acids. Additional strategies include, the protein strength of the cell membrane, category of carotenoids translated by cell, by altering lipid head group, polypeptide amino acid length and a two-component signal transduction pathway. Sabri *et al.*, (2007) isolated *Rhodotorula aurantiaca* and studied the consequence of extremophiles conditions on cell biomass of psychrophilic and psychrotrophic cell members of microorganisms. Cell biomass kinematic factors was investigated for psychrophilic and psychrotrophic strain. Awano, (2007) studied the mutant, cold sensitive *E. coli*, and by using transforming of conditions from 37°C to 15°C, observed its correlation to induction of cold shock proteins. Two other low

temperature stimulating proteins, specifically an RNA *protein* regulates folding or unfolding macromolecular structures, exonuclease can also accompaniment the freezing inducible purpose of *csdA*.

2.12. Microbes Producing Cold-Active Proteases

Microorganisms producing thermolabile proteases from freezing habitations such as, alpine territories, polar areas, deep ocean, glacier loams and ice, arctic and sub-Antarctic deposits, low temperature desert mud territories on earth. Enzymes produced from thermolabile microorganisms and their potentials was reassessed from different periods (Georlette *et al.*, 2004, Cavicchioli *et al.*, 2002 and Gerday *et al.*, 2000). Morita (Morita, 1975) defined psychrophiles. Psychrophiles have the ability to survive at 15 °C with a best temperature of cell biomass at around 20 °C and have the potential to tolerate 0 °C. Psychrotolerant microbes in contrast, best conditions for growth at 20 °C or exceeding (Feller, 2003). (Feller, 2003) psychrophile microbe was quarantined from oceanic to diverse categories of microbes as gram-negative have a diverse range including *Pseudoalteromonas, pseudomonas, Moraxella, planococcus Psychrobacter, Exiguobacterium Polaromonas, Xanthomonas Psychroflexus, etc.*)

(Oh *et al.*, 1999) isolated *Azospirillum* sp. from different geographical regions from mountain soil producing cold active hydrolytic protease. (Baghel *et al.*, 2005) who studied *Bacillus licheniformis* from polar glacier loam, *Clostridium* from polar Antarctic territory (Alam *et al.*, 2005), *Colwellia* from ocean frost and sub-antarctic sediments (Wang *et al.*, 2008), (Kuddus and Ramteke, 2008) who observed *Curtobacterium* from polar glacier mud, *Exiguobacterium* from freezing wasteland loam (Kasana and Yadav, 2007), *Pedobacter cryoconitis* from glacier ice (Margesin *et al.*, 2005), *Penicillium* from oceanic ecosystem (Zhu *et al.*, 2009), *Pseudomonas* sp. from deap sea (Zeng *et al.*, 2003), gamma-proteobacteria from Antarctic (Denner *et al.*, 2001), (Larsen *et al.*, 2006) studied *Serratia* sp. from shoreline, (Margesin and Schinner, 1991) observed *Xanthomonas maltophilia* from high-altitude and (Kristjansson *et al.*, 1999) who investigated *Vibrio* sp. from oceanic.

For the cold-active hydrolytic enzymes (Yu *et al.*, 2011) investigated microorganisms from Antarctica. *Sulfitobacter* sp, *Photobacterium*, *Pseudomonas*, *Shewanella*, *Bizionia*, *Flavobacterim*, *Salinibacterium* were found to secrete thermolabile proteolytic enzymes. Loam from polar glacier of Gangotri (Kuddus *et*

al., 2011) observed thermolabile protease secreting *Stenotrophomonas* sp. Microorganisms excreted thermolabile proteases are enumerated in Table 2.1.

(Buia, 1997) observed a variety of Preliminary data obtained from extremophiles secreted biocatalyst from Antarctic conditions. Quantitative measurements designated the existence of protease, amylase, phosphatase, and β-galactosidase demonstrating durable freezing conditions acclimatization in numerous strains (Table 2.2). Since this investigation is obvious that polar bacteria, particularly isolated from freeze, are worthy sources of thermolabile enzymes (Buia, 1997).

2.13. Classification of Proteases

Enzyme Commission categorized, proteases classification in group such as 3 (hydrolytic enzyme biocatalyst), and 4 hydrolytic biocatalyst sub-group. Enzyme was apportioned with 2 comprehensive factions upon their potential to catalyze exopeptidases external peptide bonds or (endopeptidases). However, in industrial purposes exopeptidases biocatalyst are exploited, endopeptidases biocatalysts are considered as scientifically imperative. Exopeptidases biocatalysts segregated as aminopeptidases that naturally hydrolyzed amino acids chains at the N-terminal and the polypeptides chain at the C-terminal was observed to be catalyzed by carboxypeptidases. Other qualities used in categorizing proteases into dissimilar clusters comprising as incidence of incriminated moieties at positions comparative to susceptible bond (Ward, 1985), and their optimum pH, substrate explicitness containing as collagenase and homologized to their beforehand categorized biocatalysts contained, biocatalysts pepsin and trypsin enzymes. Morihara, (1974) catalogued trypsin-like proteinases as serine proteases, Myxobacteria extracted proteinases, alkaline proteinases and proteinases biocatalysts extracted from staphylococcal. Hartley *et al.*, (1960) categorized endopeptidases into 4 distinctive factions because of their functional position and stability against multifarious inhibitors. Characteristics of the biocatalysts were reviewed in (Table 2.3).

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Table 2.1: Microbes secreting thermolabile alkaline protease

S. No.	Organisms	Molecular weight (kDa)	Optimum Temp. (°C)	Optimum pH	Reference
1	Alcaligenes faecalis	-	30	8.8	Thangam and Rajkumar *et al.*, 2000
2	Alkaliphilus transvaalensis	30	40	12.6	Kobayashi *et al.*, 2007
3	Alteromonas haloplanktis	76	20	9	Suzuki and Odagami, 1997
4	Aspergillus ustus	45	32	9	Damare *et al.*, 2006
5	Azospirillum sp.	48.6	40	8.5	Oh *et al.*, 1999
6	*Bacillus* sp.	-	30	9.6	Kaur *et al.*, 2001
7	*Bacillus* spp.	-	40	10.5-11	Okuda *et al.*, 2008
8	*Bacillus amyloliquefaciens*	45	-	10	Son and Kim, 2003
9	*Bacillus cereus*	-	20	9	Joshi *et al.*, 2007
10	Bacillus *licheniformis*	31	50	10	Toyokawa *et al.*, 2010
11	*Bacillus pumilus*	-	30	11.5	Kumar, 2002
12	*Beauveria bassiana*	-	37	10	Rao *et al.*, 2006
13	*Candida humicola*	-	37	10	Ray *et al.*, 1992
14	*Clostridium* sp.	46	37	7	Alam *et al.*, 2005
15	*Colwellia* sp.	60	35	8-9	Wang *et al.*, 2005
16	*Curtobacterium luteum*	115	20	7	Huston *et al.*, 2004
17	*Colwellia psychrerythraea*strain	71	19	6-8.5	Kuddus and Ramteke, 2008
18	*Engyodontium album*	-	25	11	Chellappan *et al.*, 2006
19	*Escherichia freundii*	55	25	10	Nakajima *et al.*, 1974
20	*Exiguobacterium* sp	36	40	8	Kasana and Yadav, 2007
21	*Flavobacterium*	49	30	8-11	Zhang *et al.*, 2011

22	*Flavobacterium balustinum*	70	40	7-9	Morita *et al.*, 1998
23	*Leucosporidium antarcticum*	34.4	30	8	Turkiewicz *et al.*, 2003
24	*Pedobacter cryoconitis,*	27	40	8	Margesin *et al.*, 2005
25	*Penicillium chrysogenum*	41	35	9	Zhu *et al.*, 2009
26	*Planomicrobium* sp.	-	35	9	Sheng *et al.*, 2011
27	*Pseudoalteromonas* sp.	34	35	8	Xiong *et al.*, 2007
28	*Pseudoalteromonas* sp.	28	30	8	Wang *et al.*, 2008
29	*Pseudoalteromonas* sp.	-	20	8	Vazquez *et al.*, 2008
30	*Pseudoalteromonas* sp.	65	25	9	Chen *et al.*, 2007
31	*Pseudomonas* sp	45	40	-	Vazquez *et al.*, 2004
32	*Pseudomonas* sp.	-	20	-	Chessa *et al.*, 2000
33	*Pseudomonas strain* DY-A	-	40	10	Zeng *et al.*, 2003
34	*Pseudomonas aeruginosa*	-	40	9	Patil, and Chaudhari, 2011
35	*Pseudomonas lundensis*	48	30	10	Yang *et al.*, 2010
36	*Pseudomonas fluorescens*	-	35	5	Koka, and Weimer, 2000
37	*Pseudomonas fluorescens.*	47	40	8	Hamamato *et al.*, 1994
38	*Pycnoporus cinnabarinus*	-	30	4	Meza *et al.*, 2007
39	*Roseobacter* sp.	-	40	9	Shanmughapriya *et al.*, 2008
40	*Serratia marcescens*	58	40	8	Tariq *et al.*, 2011
41	*Serratia marcescens*	56	40	8	Morita *et al.*, 1997
42	*Serratia proteamaculans*	50	30	8	Mozhina *et al.*, 2008
43	*Shewanella* strain	44	15	9	Kulakova *et al.*, 1999
44	*Stenotrophomonas*	55	15	10	Saba, *et al.*, 2012

45	*Stenotrophomonas maltophilia*	75	20	10	Kuddus and Ramteke, 2011
46	*Streptomyces* sp.	-	30	10	Tokiwa *et al.*, 1999
47	*Streptomyces alboniger*	-	37	9-11	Lopes *et al.*, 1999
48	*Teredinobacter turnirae*	-	25	7	Elibol and Moreira, 2005
49	*Trichoderma atroviride*	24	25	6	Kredics *et al.*, 2008
50	*Vibrio* sp.	35	40	8.5-9	Hamamato *et al.*, 1995
51	*Vibrio* sp.	47	25	8.6	Kristjansson *et al.*, 1999

Table 2.2: Optimal temperature and relative activity of cold-active enzymes from a variety of Antarctic sea ice bacteria. Adapted from Buia, 1997; and Nichols *et al.*, 1999.

Bacterial strains	Enzymes	Optimal Temp. (°C)	Residual activity (%)	
			10°C	40°C
Colwellia demingae	Protease (azocasein)	28	75	25
	Protease (azoalbumin)	30	39	30
	Trypsin	14	90	29
	Phosphatase	23	90	85
Pseudomonas strain	Protease	20	10	40
Penicillium chrysogenum	protease	35	15	50
Cytophaga-like strain	Protease (azocasein)	20	68	65
	Protease (azoalbumin)	27	70	55
	Trypsin	30	72	60
	β-galactosidase	15	100	46
	amylase	25	65	60
	Phosphatase	19	85	85
Colwellia-like strain	Trypsin	12	100	53
	Phosphatase	17	85	85
	β-galactosidase	26	75	70
Pseudoalteromonas sp.	Protease	29	55	37
	Trypsin	22	90	23

Cytophaga-like strain	Phosphatase	19	85	85
Shewanella gelidimarina	β-galactosidase	24	65	<20
Stenotrophomonas maltophilia	Protease	20	4	40

Table 2.3: Classification and biochemical characteristics of endoproteases

Endoprotease	EC No.	Mol. Mas Range (kDa)	Opt. pH	Opt. Tem. (°C)	Metal ion	Active Site Residues	Major Inhibitors
Carboxyl proteases	3.4.23	30-45	3-5	40-55	calcium	Aspartate	Pepstatin
Cysteine protease	3.4.22	34-35	2-3	40-55	-	Aspartate	C2H4INO, *p*-CMB
Metallo-proteases	3.4.24	19-37	5-7	65-85	Zinc, Calcium	Phenylalanine	Chelating agents such as EDTA, EGTA
Serine proteases	3.4.21	18-35	6-11	50-70	Calcium	Serine, histidine and aspartate	DIFP, EDTA, PMSF, soybean trypsin inhibitor, indole, phenol, triamino acetic acid and phosphate buffers

2.14. Isolation of Alkaline protease producing microorganism

Hydrolytic proteases are biocatalyst abundant in nature environment hydrolyze bio chemical reactions catalyzing polypeptides chains into diverse amino acids (Sharma *et al.*, 2010). At basic pH stable proteases are essential and abundant in type. By streaking on nutrient agar Petri dishes in hifg pH conditions and assessment for the preferred features from various sources. Hydrolytic protease biocatalysts excreting bacteria extracted from diverse foundations includes egyptian soda lake (Ibrahim *et al.*, 2007), hot springs, Jordan (Akel *et al.*, 2009) for quantitative screening of protease secreting microbes was explored, Alkaline lonar lake Maharastra, (Mothe, V.R. Sultanpuram, 2016). Soil samples collected from different tannery (Rao and Narasu, 2007, Chellapandi, 2010), and industrialized surplus such as leather surplus (Ellouz *et al.*, 2003, Almas *et al.*, 2009, Srinivasan *et al.*, 2009), wood factory (Shafee *et al.*, 2005), milk dispensation plant (Chu *et al.*, 2007) industry of detergents (Naidu *et al.*, 2005), food treating industrialized effluent (Sangeetha *et al.*, 2010).

Protease producers was collected from the samples of soil in Kurukshetra (Kumar *et al.*, 2014) Thai fish sauce (Yossan, *et al.*, 2006), and sugarcane molasses (Younis *et al.*, 2009), vegetable surplus (Jaswal *et al.*, 2008), tarnished and fresh meat (Gupta *et al.*, 2008) animal slurry ((Gupta *et al.*, 2008; Alves *et al.*, 2005; Sharmin and Rahman, 2007), and from the buffalo leather (Shankar *et al.*,2011). Additional foundations expended for alkaline biocatalysts manufacturers comprise mangrove taster residue (Zambare *et al.*, 2007), manure slush sample (Venugopal and Saramma, 2007), compost from the dead animal's vestiges (Chen *et al.*, 2004) meat remaining polluted soil (Nadeem *et al.*, 2007) and soil from rooster discarded position (2010).

2.15. Properties of alkaline proteases

Numerous bacteria generating alkaline proteases was considered comprehensively so grounded because their characteristics so that they can be used for specific purposes. Hydrolytic Proteases need to acquire hydrolysis and sensitivity against comparatively antagonistic circumstances for industrial applications. Research endeavor has aims to searched out an enzyme is vigorous in activity and multipurpose in functions. Essential assets are summarized in (Table 2.4).

Table 2.4: Alkaline protease producing microorganisms and their characteristics

Microorganism	Opt. pH	Opt. Temp. (°C)	Molecular Mass (kDa)	Other properties	References
Bacillus pseudofirmus	11	60	24	Stable in oxidants and surfactants	Gessesse *et al.*, (2003)
Nesterenkonia halobia	10	70	23	Calcium for activity and stability not required	Gessesse *et al.*, (2003)
Vibrio metschnikovii	12	60	29	Stable in oxidants and surfactants	Mei and Jiang, (2005)
Stenotrophomonas maltophilia	9	20	75	Stable in detergents	Kuddus and Ramteke, (2011)
Penicillium chrysogenum	9	50	41	Enhanced by divalent Mg and Ca	Zhu *et al.*, 2009
Pseudomonas strain	10	40	25	Stable in reducing agents dithiotreitol.	Zheng *et al.*, 2003
Xenorhabdus nematophila	8.5	30	39	Solvent tolerant	Mohammad, (2007)
Pseudomonas aeruginosa	8	60	38	SDS and tween 80 tolerant	Najafi *et al.*, (2005)
Pseudomonas thermaerum	8	60	43	Solvent stable	Gaur *et al.*, (2010)
Serratia marcescens subsp	10	50	58	Stimulatory effect by Tween 40	Liang *et al.*, (2010)
Pseudomonas aeruginosa	8	35	56	strong dehairing activities	Zambare *et al.*, (2011)

Bacillus halodurans	**11**	**70**	**29**	Stable up to 70 °C	Shrinivas and Naik, (2011)

2.16. Production and optimization of psychrotrophic alkaline protease

Shi *et al.*, (2005) isolated pyschrotrophic bacterial strain from diverse samples gathered from the polar region of China that are capable of producing cold-adapted protease. 30S small ribosomal subunit 16SrRNA genome sequence procedure for thermolabile enzyme excreted was acknowledged as *Bacillus cereus*. Best temperature observed was 15°C for thermolabile secretion and survive amongst 0° C and 38°C whereas favorable conditions for biomass was 25°C. thermolabile enzyme was metallo-protease and molecular mass observed was 34 kD. For protease production different fermentation conditions were also investigated. For optimum protease production casein observed optimum source of nitrogen whereas glucose and starch existed appropriate carbon.

A bacterial isolate with significant proteolytic activity was isolated by (Shine *et al.*, 2016) from desert soil of Riyadh, Saudi Arabia. Isolate was identified as *Bacillus cereus*. Factors influencing the use of the one aspect at a period technique, maximum secretion of extracellular alkaline protease by *Bacillus cereus* were optimized. when the optimum incubation temperature was 45̊ C in growth medium with a pH of 9 and 4% inoculum, and continuous agitation at 170 rpm for 24 hours resulting in a maximum alkaline protease enzyme yield was achieved. Best sources of carbon for its alkaline proteases production was fructose whereas sucrose and galactose followed. Among the various organic and inorganic examined sources, maximum secretion of enzyme was observed by yeast extract supplemented in the fermentation production medium.

Salwan *et al.*, (2010) investigated genotypic diversity of hydrolytic enzyme secreting microorganisms related with polar glacier. From the pure culture one hundred nine strains have the capability of protease production. Catalytic enzyme excretion observed was highest at low temperature and high pH. 30S small subunit 16S ribosomal RNA phylogenetic analysis exposed selected bacteria was related to three classes i.e. *Gammaproteobacteria* and *Actinobacteria* and *Alphaproteobacteria*, and existed as associated with the genera *Stenotrophomonas, Pseudoxanthomonas, Pseudomonas* and *Serratia*. For most of isolates in plate assay, highest enzyme secretion happened of biomass growth condition amongst 25 degrees C. Calcium, dithiothreitol and beta-mercaptoethanol showed stimulatory effect on protease

hydrolysis, However Manganese, Hg, PMF, sodium, Zn and EDTA showed no stimulatory effect on protease activity

Margesin *et al*., (2005) isolated polar glacier bacteria *Pedobacter sp.,* and have the potential to secret an extracellular protease which was purified and characterized. Best temperature observed for enzyme secretion was 15°C. highest enzyme excretion observed with wheat dredge, citrate and soybean meal as protein supplements. Enzyme was perceived as metalloprotease. Thermolabile enzyme observed active at 20-30 degrees C and was deactivated at 50 degrees C nonetheless experimented tolerant to frequent freezing and defrosting. Against thermal denaturation, metal ions such as calcium observed was no protecting consequence. In pH range of 7 to 10 20% of the activity was inhibited. (Kuddus and Ramteke, 2008) extracted an extracellular protease from the novel psychro-tolerant bacterium from polar glacier. whilst fermentation production comprehending skim milk at 15°C throughout 120 hours consequential highest secretion of thermolabile enzyme.

Pratibha *et al*., (2016) isolated Psychrotrophic bacterium producing cold active protease from polar glacier acknowledged as *Bacillus sp* using technique 16s rRNA. Bacillus sp. one of the best psychrotrophic strain out of the ten selected protease producing isolates was further characterized. The bacterial strain exhibited highest catalysis at high basic conditions 10 pH, 20°C. thermolabile protease at lower temperature and lower thermal stability by high-catalytic efficiencies was characterized. Hydrolytic enzyme significance its comprehensive variety functions in cleanser industries, foodstuff industries, and tannery, bioremediation, meat tenderizers, protein hydrolyzates, bio-film degradation, pharmaceuticals industry. The results indicate that cold active alkaline protease have enormous implications in detergent industry.

At low temperatures psychrotrophic bacteria can produce enzymes. Thermolabile enzyme excretion by psychrotrophic *Rheinheimera sp.* quarantined by (Tehran *et al*., 2016) was optimized by the surface methodology. Cold active enzyme has widespread biotechnological prospective as competed the properties of mesophilic microorganisms. Primary, the consequences of diverse environmental conditions on the biomass and enzyme excretions one-parameter at interval technique. Highest secretion of enzyme was observed with the optimized environmental physicochemical conditions such as cultivation period 120 h, widespread temperature range 15 to 37°C,

wide-ranging pH from 6 to 11, skim milk application 0-2%, and spore size 0.5-3%. Using response surface methodology through 27 experiments the overall parameters consequences of the 4 general alterable parameters occurred to assessed inside 96 hours. Astronomical biomass concentration was distinguished at 72h of fermentation period, 20°C, 7 pH citrate buffer, skim milk 2%, and 3% spore size and maximum enzyme production (533.74 unit per ml) was achieved and inoculum size of 3 percent at a method of one factor at a time.

Wang *et al.*, (2016) isolated and purified to acquire highest catalytic activity enzyme excreting bacteria. 16S rDNA method was used for the identification of *Stenotrophomonas maltophilia*, evacuated thermolabile proteases. Develop the implementation rate of catalytic enzyme, submerged fermentation conditions for excreting proteases was optimized and best enzyme-secreting circumstances such as pH of 7 citrate buffer, culture condition at 35 °C, growth time of 36 hours, outfit liquefied aggregate of 60 ml /100 ml.

2.17. Purification and characterization of thermolabile alkaline protease

Xiong *et al.*, (2007) purified thermolabile protease biocatalyst to homogeneousness and its molecular weight was 34000 Da assessed by SDS-PAGE technique. KDa by mass spectrometric analysis protein E1 was observed 32.411 kDa. From the purified enzyme molecules characterization, it was observed that Phenylmethyl sulfonylfluoride (PMSF) exhibited no inhibition influence on the catalytic activity of thermolabile enzyme, however was observed moderately constrained by EDTA-Na. In one study, Zeng *et al.*, (2003) purified the extracellular protease from a psychrophilic bacteriological strain. They acknowledged this species as *Pseudomonas*. Two temperatures optimized viz; protease-producing temperature and the optimal growth temperatures for the studied strain were 10°C. they also found that the enzyme protease was only produced at 20°C temperatures. Moreover, the protease enzyme was highly active at 40°C with the pH 10.0. In their study, they found that the enzyme has serine residue in its catalytic triad so because this catalytic enzyme was constrained by diisopropyl fluorophosphate and phenylmethyl sulfonylfluoride. Catalytic enzyme was subtle to urea, guanidine HCl and SDS. The enzyme was also resistant to dithiotreitol and active to N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide and N-succinyl-Ala-Ala-Pro-Leu- p-nitroanilide. Molecular mass noticed was 25000 Da.

Anbu *et al.*, (2013) experimented partial purification of novel protease from *Exiguobacterium profundum* by $(NH_4)_2SO_4$ and additional purified by diverse column chromatography approaches. Purification observed 10.23-fold with a percent yield of fourteen percent and mass of protein observed was anticipated 52000 Da. Catalytic activity observed amongst different pH buffer from 7 to10 and was stable at condition of 50°C. Catalytic activity observed was enhanced by calcium chloride but was inhibited by magnesium sulfate, and silver nitrate and cobalt chloride. Serine residue was present at the catalytic triad of protease which was constrained by PMSF. Preincubation of enzyme with diverse surfactants such as Triton X-100, trailed by Tween 80 and SDS it was observed that thermolabile biocatalyst steady in both of the ionic and nonionic surfactant. After preincubation with 1% oxidizing agent the thermolabile enzyme retained its catalytic activity and was stable. Activity of the thermolabile molecules of enzyme in the incidence of oxidizing elements, surfactants, and organic solvents exhibited hypothetical function in commercial washing industry.

Waghmare *et al.*, (2015) experimented the purification of active enzyme extracted from *S. maltophilia* was completed by using $(NH_4)_2SO_4$ trailed by chromatography procedure. Catalytic activity of enzyme showed activity at 40°C and in basic pH condition 9. After preincubation with diverse metal salts it was observed that Calcium, Magnesium and iron absolutely subjugated the hydrolysis of substrate. Hydrolytic activity in the presence of solvents like ethanol, methanol, isopropanol at 25% (v/v) concentration in reaction mixture it was observed that the enzyme catalyzed the substrate actively but when the concentration at 37.5% activity observed was slightly suppressed. enzyme with these characteristics be appropriate for commercial surfactant and therapeutic industries.

Kuddus *et al.*, (2008) purified enzyme with specific activity was observed 8,090 units/mg subsequently after 34.1- purification fold. 115000 Da enzyme biomass experimented was a metalloprotease and catalytic hydrolysis of substrate in the presence of EDTA and EGTA observed was constrained and highest hydrolytic activity at 20 degrees C and at citrate buffer of pH 7 and stable in different buffer pH range of 6 to 8. Enzyme catalysis of substrate observed was active afterward 3 hours at 4 to 20°C. Nevertheless, the enzyme disclosed no thermostability properties, by manganese ions activity was enthused and was inactivated toward copper ions.

Kuddus and Ramteke, (2008) observed 8090 u/mg and 34-fold of purification and catalytic activity was stimulated in the presence of Zn and Cr in the reaction mixture. Protein size was 115 kD enzyme was a metalloprotease and highest residual activity was observed at 20°C and pH 7. After enzyme was exposed to 3 hours at 4°C to 20°C No hammering of catalysis was observed in enzyme hydrolysis and no thermostability exhibited by enzyme. Attachment of manganese ions to the catalytic site stimulated the activity; nonetheless, deactivated by copper.

Kasana and Yadav, (2007) observed 7.1fold of purification mass was observed to be 36000 Da. Highest catalytic hydrolysis was exhibited against 50°C and 8 tris-hcl buffers. Attachment of these metal to the active site of the enzyme Mg, Ca, Zn, and Mn showed stimulated the activity, in the presence of EDTA and PMSF the molecules of enzyme was stable, although a 20% induce the activity when reducing agents was mixed in the reaction mixture such as dithiothreitol and beta-mercaptoethanol.

Saba *et al.*, (2012) observed 55000 Da and the catalytic enzyme observed was purified to 18.45 folds of purification. Highest activity was estimated when enzyme was incubated against the casein. Enzyme disclosed stability at wide-ranging buffer of pH from citric acid 6 to tris-hcl 12. Enzyme determined no thermostability characteristics. Hydrolytic activity of enzyme against protein was estimated at temperature range from 10 to 60°C. Greatest laundry competence results exhibited at cold temperatures designate enzyme could be utilized for cold laundry determinations of sensitive materials.

Datta *et al.*, (2017) observed that hydrolytic activity of enzyme afterward 48 hours incubation in citrate buffer of 7 to tris-hcl of 12 exhibited activity against substrate and was also showed stability in the existence of 0-5% NaCl in the reaction mixture. In the reaction mixture Cu, Mn, Co, and Ca when bind to the active site of the enzyme showed no inhibitory effect. Firmness at intense basic conditions of pH, temperatures, and exhibited the hydrolysis of substrate against surfactants and marketable detergents recommends its achievable function in washing detergents.

2.18. Cloning and Expression of Cold-Active Proteases

Biotechnology functions making enzyme production process very economical has revolutionized the enzyme industry. Insufficient challenges were made in cloning of thermolabile protein and gene expression in hosts. (Joo *et al.*, 2007) amplified a chromosomal nucleotide sequence from complementary DNA archive of the *Periserrula leucophryna* and translating for a cysteine protease and the amplified positive nucleotide genome expressed in BL21 DE3 exploiting the T7 promoter procedure by transcribing the cloned gene inserted into plasmid that have phage T7 open reading frame promotor where the RNA polymerase bind and started to expressed the gene of concern, and the protein was purified and characterized toward diverse physicochemical conditions. 498 bp gene fragment was cloned constructing cDNA exploiting disintegrated primers resulting from nucleotide sequence of cysteine protease. From the consequence, nucleotide sequence was observed comprise 2591 base pair which translated 283 polypeptide chain. 31800 Da mass of protein and the matured protein was estimated 25000 Da. Gene encrypting protein cloned from the parent to target plasmid comprised T7-7 promotor after PCR procedure exploiting the specific primers, incorporating instigation and stop codons. Recombinants protein observed expressed 13% required proteins in the BL21 for 8 recombinants. cysteine protease observed around 25000 Da. Highest hydrolysis observed towards 9 pH and 35°C representing alkaline group of proteases.

Jankiewicz *et al.*, (2016) observed 1740 bp of nucleotide sequence precursor form of the enzyme was cloned. amino acid sequence was deduced which comprises the conserved section of the protein domain motif of the peptidase families S8 with S8A subfamilies of subtilisin, Kexin with S8B and sedolisin with S53 domains by mass spectrophotometric analysis and the serine residue catalytic triad at the active site of the enzyme. (Hajji *et al.*, 2010) studied that the genes such as cDNA alpES1 and alpES1 translate protein amplified from chromosomal genome and cDNA, incorporated in plasmid and then observed for genome sequencing. Mass spectrophotometric analysis observed that gene of interest alpES1 of 1212 bp encrypting polypeptide chain of 403 comprising 21-aa monomers, 100-amino acid monomers and a 282- amino acid monomers and molecular mass observed with 28500 Da. Desired gene cloned in destination plasmid of pET30 in BL21. Expressed purified protease observed 32000 Da.

Bacillus cereus protease gene extracted by (Esakkiraj *et al.*, 2016) and inserted in DH5a. 1700 bp gene size observed on gel doc. and exploiting BL21(DE3) for more expression. 0.1 mM strength of IPTG observed for highest activity regarding 3 h at 37 C. Affinity chromatography trailed by SDS technique caused in two bands. 1st one was a predecessor protein with 70000 Da and the next was a mature protein with 28000 Da. (Iqbal *et al.*, 2015) studied Geobacillus stearothermophilus chromosomal gene responsible for translating serine protease protein was extracted and expressed in BL21 (DE3) exploiting pET-22 plasmid for encoding our desired protein as an expression vector.

Sareen *et al.*, (2005) studied Bacillus licheniformis chromosomal gene of 1725 bp responsible for translating protease protein of 63200 Da was extracted and expressed in BL21 (DE3) exploiting pET-22 plasmid for encoding our desired protein as an expression vector. Apr 46 comprises of 500 polypeptide chain of amino acid 55000 Da. Expressed protein sequence observed was 50% resemblance to acknowledged serine proteases with conserved motifs.

Strain producing extracellular proteases was isolated by Li *et al.*, (2013) and screened on casein plate and was identified by biochemical and morphological tests and by 16S rDNA sequence analysis. ORF of the protease was acquire by degenerate primers designing and genome walking method were used. Protease having precursor and mature peptide were recombinant expressed in BL21 (DE3). After active protease purification, the characteristics and the catalytic ability were detected using synthetic peptide as the substrates such as succinyl-Ala-Ala-Pro-Phe-p-nitroanilide. Isolated strain was named as *Bacillus sp.* after identification. Protease was 1149-base pair extended and encoded a protein of 382 polypeptide monomers comprised with a 30-residual signal peptide, a 275-residual mature protein, and a 77-residual propeptide, and the encoded protein was one of the members of subtilisin of serine proteases. Serine proteases precursor auto processed mediated by the propeptide when pro-serine proteases was recombinant expressed in BL21 (DE3). High catalytic efficiency of the enzyme exhibited towards synthesis substrates with highest activity at 70 degrees C and pH 9 - 10. Subtilisin of serine proteases was active throughout a range of pH 7.0 to 10.0 and was thermal stable at 25°C – 60°C.

From Bacillus licheniformis 2709 Alkaline protease gene was cloned by (Tang *et al.*, 2004) cloned in expression vector. Expression was contracted in Bacillus

subtilis Recombinant, observed an extraordinary expression strain. recombinant protease observed have 65% increased catalytic activity as compared to the original strain. 30500 Da protein size was estimated.

2.19. Application of cold active alkaline protease

(Kumar and Bhalla, 2004) studied hydrolytic purified protease extracted from *Bacillus* sp. with highest catalytic activity observed was at *p*H 9 tris-hcl buffer and condition of 50°C was observed active in 5% strength of commercial surfactant. Stability was estimated in strength of 500 mg/l of sodium hypochlorite active compounds. 22 % of the catalytic activity was inhibited after 1 day of incubation with commercial surfactant 30°C. Yolk straining was detached after 10 min in concentration of enzyme with 100 Unit/ml with 1% commercial surfactant and 30 min was estimated to eradicate plasma blemishes with 100 U/ml strength of enzyme. (Kanmani *et al.*, 2011 studied application analysis was conveyed out with the purified molecules of enzyme extracted from *Bacillus cereus* and because of the potential of purified enzyme incorporated in to the pigment elimination practice. (Manni *et al.*, 2010) observed that the hydrolytic protease showed activity concerning anionic and non-ionic surfactants and catalytic activity was stimulated by oxidizing compounds. Purified enzyme incubation with several marketable detergents exhibited exceptional compatibility. So, because of the exceptional characteristics become an ultimate choice for purpose in detergent formulations constructions.

Ali *et al.*, (2011) extracted and characterized against numerous surfactants and compatibility toward particular marketable detergents and peroxide based bleach purified proteases molecules exhibited catalytic activity. Residual activity was observed with 50% inhibited as compared to control hydrolytic activity subsequently 30 days of incubation at condition of 30 °C in the existence of strength of 25% dimethylformamide, diethyl ether, dimethyl sulfoxide, and hexane, the extracellular hydrolytic protease enzyme was stable. (Rajkumar *et al.*, 2011) isolated Bacillus megaterium producing an alkaline serine protease from the red alga and manipulated as an ingredient in the commercial surfactant moderately purified protease was observed to be an appropriate for laundry garments remarkably with plasma blemished.

Aishwary *et al.*, (2013) studied extraction, purification and function of protease secreted from *Alcaligenes sp.* For their wide range of industrial applications

proteases are enzymes well known such as food, detergent, pharmaceutical etc. High basic pH stable purified protease is recognized to be a functioning constituent of the marketable surfactants. Compatibility with other surfactants the purified enzyme observed was utilized its capability to eradicate the proteinaceous blemish. (Niyonzima and More, 2014) observed that 50% of the catalytic activity was inhibited because of the 1 day of incubation at 28, 60, and 90°C in the manifestation of marketable enzymatic and nonenzymatic surfactants. Super Wheel-enzyme mixture reaction have the potential to entirely eradicate plasma streak as compared to detergent alone, Outstanding chemistry with detergents distinctly recommended that enzyme in detergent and the catalytic activity was also observed against the extreme physicochemical conditions such high pH and temperatures, comprehensive substrate specificity, activity against bleaching compounds, detergents and oxidizing elements.

2.20. Cold active lipases

Cold adapted hydrolytic extracellular lipase secreted by psychrotrophic microorganisms which function effectively with extraordinary frequencies of hydrolysis at cold temperatures in dissimilarity from mesophilic or thermophilic microbes' demonstrations with low or no catalytic activity at low truncated temperature. Structural features observed evolved gradually so that they consult an extraordinary attitude of flexibility, exceptionally observed that around catalytic triad are decoded into low-substrate correspondence, low activation energy, and extraordinary specific activity at low truncated temperature. Lipases with a maximum catalytic activity is transferred against the freezing temperatures with a simultaneous diminution in thermostability. Characteristics biotechnologically and industrially important enzyme was gathered and assembled from knowledge accessible in the literature existing descriptions on thermolabile lipases from the restricted resources were concentrated on extraction, purification and purified molecules characterization trailed by amplification of desired gene, creating recombinants and desired protein expression and sequencing analysis for conformational analysis. Thermolabile lipases encrypting chromosomal genes were extracted and inserted into E. coli bacterium and exploited for expression of proteins. Gerday *et al.*, (1997) experimented that an enormously unpredictable circumstance for the encoding of thermolabile lipases inside their host bacterial organism (Feller *et al.*, 1990, 1991a).

2.21. Sources of cold active lipases

Fundamentally disseminated microorganisms stable at extreme freezing temperatures nearby 5 °C or low are secreted thermolabile lipase for existing. Thermolabile lipases extracted from diverse microorganisms having high catalytic hydrolysis toward substrate at freezing temperatures and endeavors was completed to observed time to time these diverse extremophilic microorganism secreting thermolabile enzymes. Thermolabile lipase secreting psychrotrophic and psychrophillic bacteria is portrayed in Table 2.5.

(Sullivan and Palmisano, 1984; Delille, 1993) studied that in the sea, ice deposits comprise diverse bacterial reckoning that was documented as extraordinary as 10^6 /ml. Extremophiles from diverse polar Antarctic regions were isolated and characterizes a perpetually freezing and persistent low temperature habitation. oceanic bacteria were observed have the conceivable source of thermolabile lipases. *Bacterium Aeromonas* hydrophila growing at cold and moderate temperature excreted thermolabile lipase enzyme (Pemberton *et al.*, 1997).

Isolated bacteriological groups were confirmed from profound- oceanic residues wherever observed extreme low temperature. (Lee *et al.*, 2003) isolated *Aeromonas sp.*, (Zeng *et al.*, 2004) isolated *Psychrobacter sp.* and *Pseudoalteromonas sp.*, (Alquati *et al.*, 2002) experimented bacterial genera including *P. fragi,* (Dieckelmann *et al.*, 1998) studied *Pseudomonas fluorescens and* and *S. marcescens* by (Abdou, 2003) isolated from food samples and refrigerated milk have potential to produce cold active lipase. Cold regions such as glaciers are additional haunt for thermolabile lipase secreting microorganisms. Psychrophillic microorganisms were found in soil and ice residues in Alpine polar region, which produces lipases. The widespread use of refrigeration postulates a numerous assortment diversity of nutrient lush environment for some of the distinguished psychrotrophic microorganisms causing food decomposition.

Table 2.5: Bacteria producing cold active lipases

Microorganism	Source	References
Acinetobacter sp.	Siberian soil	Suzuki *et al.*, (2001)
Acinetobacter sp.	Ns	Breuil and Kushner, (1975)
Achromobacter lipolyticum	Ns	Khan *et al.*, (1967)
Aeromonas sp.	Sea sediments	Lee *et al.*, (2003)
Aeromonas hydrophila	Marine Habitat	Pemberton *et al.*, (1997)
Bacillus sphaericus	Gangothri Glacier	Joseph, (2006)
Corynebacterium paurametabolum	Naukuchiatal lake	Joshi *et al.*, (2006)
Moraxella	Antarctic Habitat	Feller *et al.*, (1990)
Moraxella	Antarctic Habitat	Feller *et al.*, (1991a)
Photobacterium lipolyticum	Marine Habitat	Ryu *et al.*, (2006)
Pseudoalteromonas sp.	Deep sea sediments	Zeng *et al.*, (2004)
Pseudoalteromonas sp.	Antarctic marine	Giudice *et al.*, (2006)
Pseudomonas sp.	Subterranean environment	Rashid *et al.*, (2001)
Pseudomonas sp.	Alaskan soil	Choo *et al.*, (1998)
Pseudomonas sp.	Ns	Tan *et al.*, (1996)
Pseudomonas fluorsescens	Refrigerated milk samples	Dieckelmann *et al.*, (1998)
Pseudomonas fluorsescens	Refrigerated food	Andersson, (1980)
Pseudomonas fluorsescens	Refrigerated human placental extracts	Preuss *et al.*, (2001)

Pseudomonas fragistrain	BCCM™/LMG	Alquati *et al.*, (2002)
Pseudomonas fragistrain	Ns	Aoyama *et al.*, 1988
Psychrobacter okhotskensis	Sea coast	Yumoto *et al.*, (2003)
Psychrobacter sp.	Deep sea sediments	Zeng *et al.*, (2004)
Psychrobacter sp.	Antarctic Habitat	Kulakovaa *et al.*, (2004)
Psychrobacter immobilisstrain	Antarctic Habitat	Arpigny *et al.*, (1997)
Psychrobacter sp.	Antarctic Habitat	Zhang *et al.*, (2007)
Psychrobacter sp.	Antarctic Habitat	Parra *et al.*, (2007)
Serratia marcescens	Raw milk	Abdou, 2003
Staphylococcus epidermidis	Frozen fish samples	Joseph *et al.*, 2006
Bacillus sphaericus	Gangotri glacier	Joseph *et al.*, 2013
Pseudomonas Sp. Adt3	Soil sample of Svalbard, Arctic region	Dey *et al.*, 2014
Pseudomonas fluorescens	Soil samples, Mount Erciyes, Turkey	Gokbulut and Arslanoglu, 2013
Halomonas sp.	Antarctic sea	Jadhav *et al.*, 2013
Cryptococcus sp.	East Antarctica	Maharana and Sing, 2018

2.22. Fermentation conditions for cold active lipase production

Extracellular thermolabile lipase enzyme observed was extremely persuaded by dietary and physicochemical aspects such as agitation speed, diverse buffer concentration, nitrogen stock, temperature, dissolved oxygen, carbon source and stimulators. (Dieckelmann *et al.*, 1998; Lee *et al.*, 2003) studied cold active lipase production by Submerged fermentation procedure. Table 2.6 presented catalogue of miscellaneous enzyme secretion considerations for diverse thermolabile lipase excreting bacteria.

(Zeng *et al.*, 2004) observed thermolabile lipase excreting bacteria Pseudoalteromonas sp. Secretion of thermolabile lipase in fermentation medium observed was best with different physicochemical parameters conditions at 25 °C for 14 days of incubation with supplements of yeast extract and olive oil and Tween 80 as inducers. (Jadhav *et al.*, 2013) studied the best physicochemical parameters conditions for fermentation excretion of thermolabile lipase from *Halomonas* sp. Maximum excretion of thermolabile lipase was observed in the fermentation production after incubation at condition of 10 °C and olive oil, peptone as carbon and nitrogen inducers in the production medium.

(Joseph *et al.*, 2011) studied various optimizations of parameters for cold active enzyme production. Maximum thermolabile lipase secretion was observed with Groundnut oil cake substrate preferred and against 15 ± 1°C after incubation of 2 days. Ammonium nitrate and glucose supplementation as surplus nitrogen and carbon source stimulated excretion of thermolabile lipase in production medium. Highest activity was observed by the addition of mustard oil cake along with Groundnut oil cake in the fermentation process.

Table 2.6: production parameters for cold active lipase

Microorganism	pH	Temp. (°C)	Incubation period	C source	N source	References
Aeromonas sp.	8	10	8 days	Tributyrin	Tryptone, yeast extract	Lee *at al.*, 2003
Acinetobacter sp.	8	4	7 days	Soyabean oil	Tryptone, yeast extract	Suzuki *et al.*, 2001
Bacillus sphaericus	8	15	48 h	Tributyrin	Peptone	Joseph, 2006
Corynebacterium paurometabolum	8.5	25	48 h	Soyabean oil	$NaNO_3$, KNO_3	Joshi *et al.*, 2006
Microbacterium phyllosphaerae	8	15	36 h	Tributyrin	Peptone	Joseph, 2006
Moraxella	8	25	48 h	Tributyrin	Peptone	Feller *et al.*, 1990
Pseudoalteromonas sp.	8	25	14 days	Olive oil	yeast extract	Zeng *et al.*, 2004
Pseudomonas sp.	7.2	-5	3 days	Tween 80	Tryptone, yeast extract	Rashid *et al.*, 2001
Pseudomonas sp	7.6	4	4 days	Tributyrin	Tryptone, yeast extract	Choo *et al.*, 1998
Psychrobacter sp.	8	25	14 days	Tween 80	yeast extract	Zeng *et al.*, 2004
Serratia Marcescens	7	6	3 days	Tributyrin	Skim milk	Abdou, 2003
Halomonas sp	7	10	48 hrs	Olive oil	peptone	Jadhav *et al.*, 2013
Micrococcus roseus	8	10-15	48 hrs	Glucose	Ammonium Nitrate	Joseph *et al.*, 2011
Pseudomonas sp	8.5	22	-	Olive oil	-	Dey *et al.*, 2014
Bacillus sphaericus	8	15	48 hrs	Lactose	-	Joseph and Ramteke, 2013

Bacillus sphaericus	8.5	15	-	Fructose	Peptone	Maharana and Sing, 2018

2.23. Purification and characterization of cold active lipases

Purification patterns for diverse thermolabile lipases are founded on several phase with different approaches. Though, innovative practices were established because of the extraordinary yield purification retrieval. Depends on the biochemical properties of diverse lipase excreted by the extremophiles one has to strategy the procedure for different purification procedures (Saxena *et al.*, 2003). Thermolabile lipases importance is comprehensively acknowledged in a large quantity of purposes (Houde *et al.*, 2004). Thermolabile Purified enzyme is necessary for the biocatalytic reactions of cosmetics, reasonable chemicals, bio detergents and in pharmacological productions. A catalogue of thermolabile purified lipases and consequences achieved during different phases of purification conclusions are assumed in (Table 2.7).

Thermolabile lipase characterization and enzyme catalytic effectiveness analysis considered in relationships with physicochemical parameters conditions of pH and stability conditions where maximum catalytic activity was analyzed, best temperature, thermostability properties of thermolabile lipase and effect of diverse chelating compounds, wide-ranging inhibitors, metal ions and solvents. Thermolabile lipases secreted from extremophilic microbes have observed highest catalytic activity at 20 °C and showed stability at a wide-ranging temperature. Nevertheless, these enzymes don't have thermostability properties (Table 2.8).

Table 2.7: Thermolabile lipase purification procedures

Organisms	Purification techniques	Fold increase or yield purification or U/mg	References
Aeromonas sp	QAE Sephadex column	54/8	Lee *et al.*, 2003
Bacillus sphaericus	DEAE cellulose	18/5	Joseph, 2006
Microbacterium phyllosphaerae	DEAE cellulose	22/8	Joseph, 2006
Moraxelle sp	AcA 34 column, Ultrafiltration	Ns	Feller, 2006
Pseudomonas sp (Gene amplified and inserted into BL21 and purified)	DEAE cellulofine	38/17	Choo *et al.*, 1988
Psychrobacter sp	DEAE Sepharose CL-48 and Sephadex	Ns	Zhang *et al.*, 2007
Serratia marcescens	CM Cellulose, DEAE cellulose Sephadex G-150	20/45	Abdou, 2003
Aspergillus nidulans	Phenyl-sepharose chromatography	Ns	Mayordomo *et al.*, 2000
pseudomonas fluorescens	Sephadex G-100 size-exclusion chromatography, and ultrafiltration	41/55	Gokbulut and Arslanoglu, 2013
Bacillus sphaericus	DEAE cellulose column chromatography	17.74	Joseph *et al.*, 2013
Pseudomonas sp	Ammonium sulfate and dialysis	527.8 U/mg	Dey *et al.*, 2014
Stenotrophomonas maltophilia	DEAE cellulose column chromatography	60.5-fold	Li *et al.*, 2013
Yarrowia lipolytica	DEAE cellulose column chromatography	20-fold	Yadav *et al.*, 2011

Yersinia enterocolitica	DEAE cellulose column chromatography	26-fold	Ji *et al.*, 2015
Pichia lynferdii,	DEAE anion-exchange chromatography column	7.4 folds /12.56%	Bae *et al.*, 2014
S. marcescens	Q-Sepharose and SP-Sepharose chromatography	2.50-fold	Abdou, 2003

Table 2.8: Characterization of thermolabile lipase

Organisms	Optimum Temp.(°C)/pH	Stability Temp./pH	MW (Kda)	Comments	References
Acinetobacter sp	20/Ns	Ns/ Ns	Ns	Broad specificity against the acyl group of ethyl esters	Suzuki *et al.*, (2001)
Aeromonas sp	35/Ns	50/ Ns	50	Average chain acyl group p-nitrophenyl esters to be decent substrate; Amplified activity with detergents	Lee *et al.*, (2003)
Bacillus sphaericus	15/8	30/8	40	Active in presence of organic solvents and stable with detergents	Joseph, (2006)
Microbacterium phyllosphaerae	20/8	35/8	42	Existence of organic solvents catalytic activity compatible with detergents	Joseph, (2006)
Pseudo-alteromonas	20-30/7-8	Ns/ Ns	85	Enzymes were 60% stable at 4 °C	Zeng *et al.*, (2004)
Pseudomonas sp	35/8	Ns/ Ns	50	Maximum activity with p-nitrophenyl caprate	Rashid *et al.*, (2001)
Pseudomonas sp (recombinant)	45/8	5-35/6-9	33	Strongly constrained by Zn^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}	Choo *et al.*, (1998)

Psychrobacter sp.	20-30/7-8	Ns/ Ns	85	Enzymes were 60% stable at 4 °C	Zeng *et al.*, (2004)
Psychrobacter sp (recombinant)	30/9	Ns/7-10	Ns	Ca2+ and Mg2+ stimulated activity	Zhang *et al.*, (2007)
Serratia marcescens	37/8	65/6.6	52	Detected 90% activity at 5 °C	Abdou, (2003)
Aspergillus nidulans	40/6.5	Low thermal stability/ Ns	29	Resemblance against esters of short- and middle-chain fatty acids	Mayordomo *et al.*, (2000)
Pseudomonas fluorescens	45/8	15-65	43	wide-ranging substrate specificity proceeding on p-nitrophenyl esters with C8-C18 acyl groups and Ca and Ni stimulated catalysis	Gokbulut and Arslanoglu, 2013
Bacillus sphaericus	15 °C/8	20–30/6-9	Ns	Stability in presence of acetone and DMSO	Joseph *et al.*, 2013
Pichia lynferdii,	15/8	5-50/6-8.5	80	Highest activity was highly dependent on pH	Bae *et al.*, 2014
Yersinia enterocolitica	37/9	0-60/6-9	34.3	strongly inhibited by Zn, Cu, SDS, EDTA, and PMSF	Ji *et al.*, 2015

2.24. Biotechnological approaches in cold active lipase

In the field of enzymology an emerging area of research is to search for diverse and unique thermolabile enzyme excreted from extremophilic bacteria through recombinant amplified DNA fragment inserted into plasmid, engineering in protein manufacturing, evolution directing in protein domain and the metagenomic methodology. At the end of lag phase lipase biotechnology has just reached the beginning of the exponential phase. Restructuring lipase gene in term of qualitative improvements and protein could accomplished by utilizing already recognized DNA manipulation technology and engineering in polypeptide domains. Development of Strain throughout diverse levels of mutations in nucleotide arraignments and schematizing different concentrations of nutrient in reaction medium for the highest secretions of thermolabile lipases for the measureable augmentation requirements. Thermolabile lipase genes were extracted, and the interconnected conclusions was conveyed out (Table 2.9).

Table 2.9: Gene isolation and characterization

Microorganisms	Studies conducted	References
Moraxella sp	Sequence homology of Human HSL gene with Antarctic bacterium	Langin *et al.*, 1993
Pseudomonas fluorescens	Isolation of lipase-encoding gene lip A	Dieckelmann *et al.*, 1998
Cloning, expression and sequencing		
Pseudomonas fragi	Molecular cloning and nucleotide sequencing of the lipase gene	Kugimiya *et al.*, 1986
Pseudomonas fragi	Cloning, sequencing and expression of the lipase gene	Aoyama *et al.*, 1988
Pseudomonas sp.	Gene cloning and sequencing	Choo *et al.*, 1998
Moraxella sp	Sequencing of lipase gene	Feller *et al.*, 1991b
Moraxella sp	Cloning and expression in E. coli of three lipase-encoding genes	Feller *et al.*, 1991a
Psychrobacter immobilis	Cloning, sequencing and structural features of lipase gene	Arpigny *et al.*, 1993
Psedomonas fluorescens	Cloning and sequencing of DNA encoding Phospholipase C	Preuss *et al.*, 2001
Psedomonas sp.	Gene cloning	Rashid *et al.*, 2001
Pseudomonas fragi	Heterologous expression, and molecular modeling	Alquati *et al.*, 2002
Psychrobacter sp.	Gene cloning, expression and characterization	Kulakovaa *et al.*, 2004
Pseudomonas fragi	Molecular properties, mutagenesis and overexpression of cold active lipase	Lafranconi *et al.*, 2005
Photobacterium lipolyticum	Isolation of a new cold-adapted lipase and gene cloning	Ryu *et al.*, 2006
C. antartica	Functional expression of lipase B in *Eschericha coli*	Blank *et al.*, 2006
Psychrobacter sp.	Cloning, expression, and characterization of a cold-adapted lipase gene	Zhang *et al.*, 2007

Moritella sp.	Cloning and expression of lipP, a Gene encoding a cold-adapted lipase	Yang *et al.,* 2008
Pseudomonas mandelii	Cloning, expression and characterization of lipS Gene	Kim *et al.*, 2013
Stenotrophomonas maltophilia	Cloning and characterization of LipSM54 Gene	Li *et al.*, 2016
Bacillus pumilus	Two BPL1 and BPL2 recombinant lipase genes strains were expressed in *Bacillus subtilis*	Litantra *et al.*, 2013
Bacillus subtilis	expressed the lipase gene in BL21(DE3) using pET-28 plasmid	Asoodeh *et al.*, 2014
Yarrowia lipolytica	lipase genes expressed in BL21 and assessed for hydrolyzing toward p-nitrophenyl-palmitate.	Zhao *et al.,* 2011
Cohnella sp	gene from the thermophilic bacterium was expressed in BL21	Golaki *et al.*, 2015
Yersinia enterocolitica	novel thermolabile lipase gene expressed in BL21	Ji *et al.*, 2015

2.25. Industrial applications of cold active lipases

Thermolabile lipases that showed higher catalytic activity at extreme conditions such as cold temperature, inhibited activity at high temperature and uncommon specificities offers chances for biotechnological operation. Certainly, thermolabile enzyme, alongside with microorganisms that are used to host certain nucleotide sequences covering a wide-ranging continuum of biotechnological functions.

Comprise as detergents formulation of different peroxide-based bleaches ingredients for cold laundry, fermentation procedures as a preservative in food productions, cheese making, bakery, meat pounding, used of organisms that secreted biocatalyst to eradicate ecological ravages, modification of substances in body and molecular ecology functions, Incorporation of the foreign gene in the heterologous different host (Feller *et al.*, 1996). Hypothetical functions of cold-active lipases are existing in (Table 2.10).

Table 2.10: Applications of thermolabile lipases

Field of application	purpose	References
Pharmaceutical functions	Esterification of docosahexaenoic acid to docosahexaenoate	Shimada *et al.,* (2001)
	Manufacture into citronellol and lauric acid from citronellol laurate	Ganapati and Piyush, (2005)
Manufacturing of chemical	Manufacturing of Ester, modification and production of peracids	Zhang *et al.,* (2003)
	Manufacturing of butyl lactate by transesterification	Pirozzi and Greco, (2004)
	Manufacturing of amides	Slotema *et al.,* (2003)
Food production	Reacting of protein monomers in reaction mixture and crystallizing in fish, enhancement in food coarseness, essence modification	Cavicchioli and Siddiqui, (2004)
Environmental functions	Production of biodiesel through biocatalysts	Chang *et al.,* (2004)
	Deprivation of lipid ravages	Ramteke *et al.,* (2005)
	Bioremediation and degradations of pollutants	Gerday *et al.* (2000), Suzuki *et al.,* (2001)
Domestic application	Thermolabile proteins as Detergents constituent's formulation and cold-water laundry application	Gerday *et al.* 2000; Joseph 2006 and Wang *et al.*, 2012
	Thermostable lipase excreted from Serratia marcescens and its function detergent formulation	Garcia *et al.*, 2018
	Lipase extracted from *Staphylococcus arlettae* was evaluated for its triacylglyceride eradicating potential to use as an improver in washing detergent expressions	Chauhan *et al.*, 2013
	Commercial detergent compatibility	Lailaja and Chandrasekaran, 2013
	washing test analysis revealed that biosurfactant and lipase observed being resilient amputation of oil.	Zarinviarsagh *et al.*, 2017

CHAPTER. 3

3. MATERIAL AND METHODS

Section # 1

3.1. Isolation and identification of extracellular cold active protease from psychrotrophic *Stenotrophomonas sp.* PAK01

3.1.1. Samples collections

Samples of water and soil were collected from three diverse localities of polar glacier: Juglot (latitude 35°41'06", longitude 74°37'26"), Jutial (latitude 35°54'276", longitude 74°19'841") and Rakaposhi (latitude 36°14'368", longitude 74°26'576") Gilgit Pakistan, Northern Areas of Pakistan (Figure 3.1) (Figure 3.2a and 3.2b). Microbiological prospects like sterility of instruments, personnels and handling of samples were performed according to standard microbiological techniques. A total of 20 samples were collected from 10 different sites. Samples of water and soil were collected carefully with intense care and transferred to respective portable ice boxes. The materials included a manual drill, sampler, sterile gloves and sample bags, pH strips, thermometer, GPS, Ice cabins, ethanol, methylated spirit, spray bottles, tissues and cotton, organized Petri plates with nutrient agar medium.

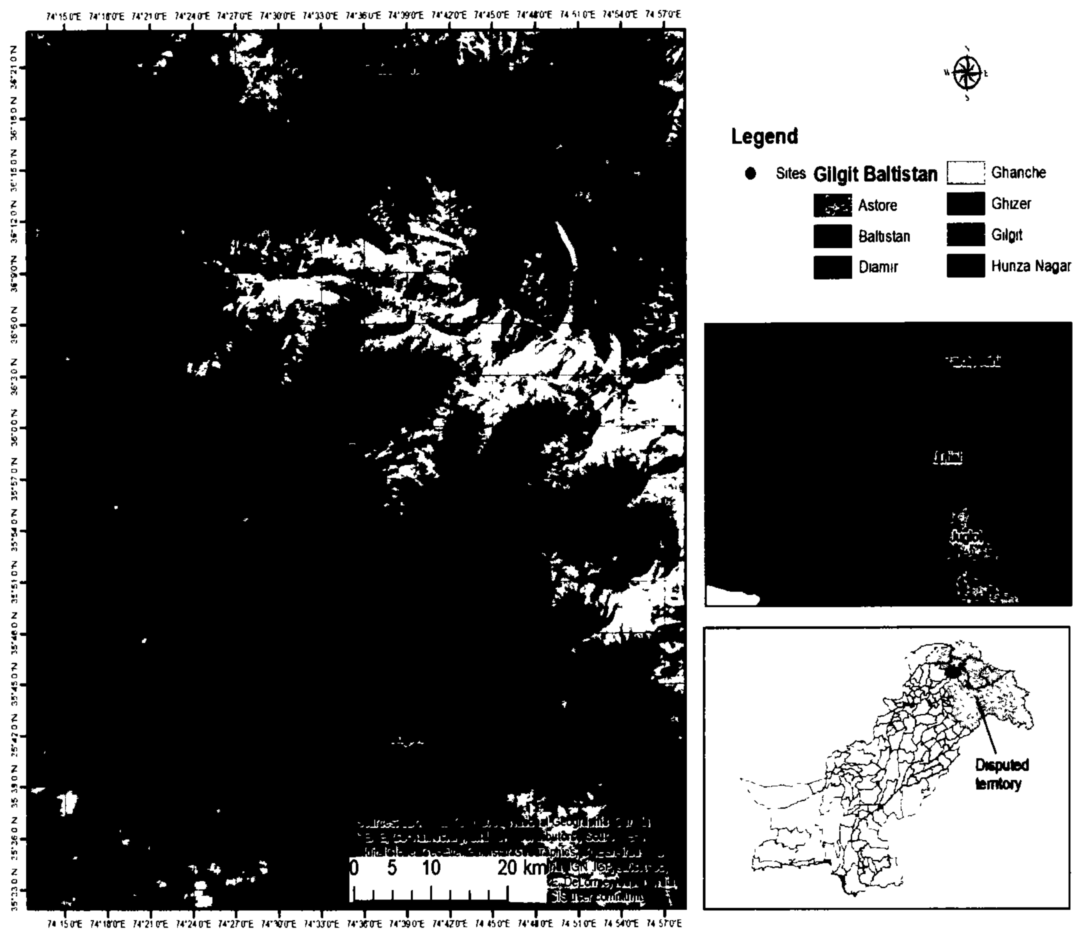
Geographic coordinates, height and atmospheric pressure were recorded using a GPS. Dissolved oxygen (DO) was measured by using Portable Dissolved Oxygen Meter. The pH was recorded using pH indicator strips. The ice was cut into pieces and collected in sterile sample bags. The water samples were obtained in sterile bottles by opening their lids inside the water. The soil samples were collected in sterile bags. All samples were conveyed to the laboratory in intact physical conditions. The soil and water samples were preserved at 4°C while ice was preserved at -20°C.

3.1.2. Sampling, Isolation and screening of thermolabile protease producing bacteria

From the samples of water and soil the isolation of diverse categories of psychotropic bacteriological stains were collected. Total of 40 different samples were collected from 15 different spots (Table 3.1). In order to isolate psychrotolerant bacteria, soil and water samples were collected from vicinity of Rakaposhi glacier, northern Baltistan, Pakistan during 2017. *Stenotrophomonas sp.* PAK01 was isolated

in laboratory of Applied microbiology and biotechnology, International Islamic University Islamabad from the soil samples gathered from Gilgit Baltistan Pakistan. Samples of soil from distant sites was diluted serially and Streak on the exterior of nutrient agar with reaction mixture (peptone 0.5%, NaCl 0.5%, beef extract at concentration of 0.3%, agar 1% and Tris-HCl buffer of 9 pH) for screening of protease producing bacteria 20 °C for 24 hr. Out of twenty strains, five bacterial stains were observed to have the potential to grow at low temperature and alkaline pH. Quantitative screening of the selected five bacterial strains was experimented in submerged fermentation for 48 hrs at 20 °C in alkaline conditions at pH 9. After incubation bacterial strain that have the potential to catalyze maximum substrate of Azocasein was selected and stored at -20 °C for further analysis. Further processing was done in the chemical engineering department of Taxas A&M university, **USA.**

The microorganisms isolated in this study were *Stenotrophomonas sp.* (PAK01), *Exiguobacterium* sp. (PAK02), *Planococcus* sp. (PAK10), *Pseudomonas peli* (PAK03) and *Pseudomonas aeruginosa* (PAKP1) in Applied Microbiology and Biotechnology Laboratory (AMBL), Department of Bioinformatics and Biotechnology, International Islamic University Islambad, Pakistan.



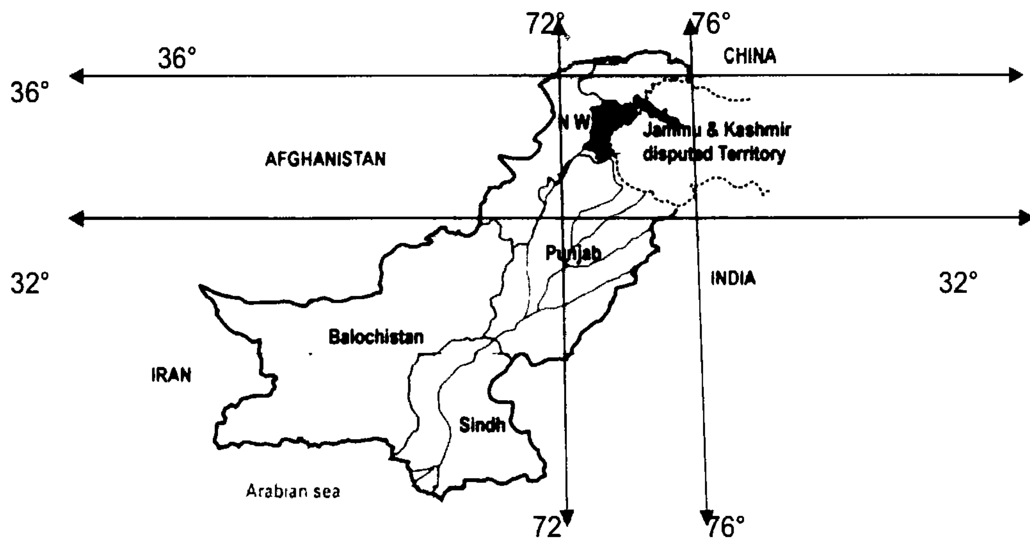


Figure 3.1. Geographical Location of the sampling site: (Indus Basin Report, 2005).

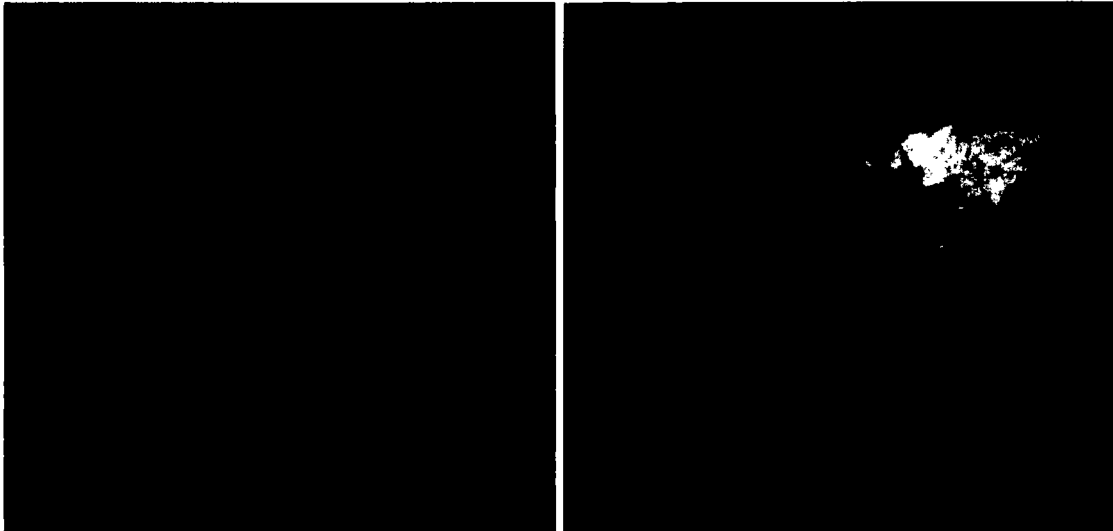


Figure 3.2a: Rakaposhi Glacier **Figure 3.2b:** Juglot Glacier

Figure 3.2a and 3.2b: Site of sampling at Rakaposhi and Juglot glaciers. Adapted from record of glacial lakes, glaciers and documentation of potential glacial lake outburst floods, In: Indus Basin-Pakistan (2005).

Table 3.1: Isolation of psychrophilic bacteria from various samples collected from glaciers

Source		Sample Codes	Isolates
Glacial samples and isolates	**Rakaposhi Glacier: Ice**	1.7	PAK01
	Juglot Glacier: water	2.3	PAK02
	Jutial Glacier: soil	3.6	PAK10
	Rakaposhi Glacier: soil	4.5	PAKP1

3.1.3. Genomic bacterial DNA Extraction

Isolates were identified on the basis 16S rRNA sequencing. Isolation of genomic Bacteria DNA was done by Thermoscientific GeneJET Genomic DNA Purification Equipment and saved at –80 °C for additional molecular exploration.

3.1.4. Amplification and sequencing of 16S ribosomal RNA gene

Genomic DNA of selected bacteria was extracted from pure fresh culture of 24 hours and nearly the complete nucleotide stretches of PCR amplification of 16S rRNA of complete fragment of 1500bps was experimented. Forward and reverse primers used for amplification were 5′-AGAGTTTGATCATGGCTCAGA- 3′ and 5′-GTTACCTTGTTACGACTT-3′- equivalent to 8 to 28 and 1493 to 1510 and are segments of 16S ribosomal RNA genome of *E. coli* and so are valuable for amplification of 16S ribosomal RNA genomic DNA from diverse classes of bacteria.

50 µl of reaction mixture for each PCR comprised: purified genomic DNA of 20 ng, Taq DNA polymerase for elongation at a concentration of 2.5 Units, 10 X concentrated 5 µl volume of Taq polymerase buffer (100 mM and 500 mM of Tris-HCl and KCl at pH of 8), forward primer and reverse primer of 27F to 1492R each universal primer of 10 pmoles concentration and dNTP of 200µM and magnesium chloride at quantity of 2 mM was utilized. PCR reaction comprises initial step of denaturation for 3 minutes at 95 °C, trailed by final denaturation at 94 °C for 25 runs for 60 seconds, annealing temperature at 55 °C of amplification of primers for 60 seconds and last amplification step of elongation/extension at 72 °C for 60 seconds. Final elongation/extension for 5 minutes at 72°C was experimented. Sample of 5 µl of the positive amplified product of gene was then examined by electrophoresis technique comprised of 1 percent agarose gel with DNA visualize dye ethidium bromide and then envisioned underneath Gel doc or UV transilluminator. The PCR product was than purified for further analysis using the DNA Clean & Concentrator™-5 and stored in -20°C.

3.1.5. Sequencing and analysis of Phylogeny

16S rRNA amplified nucleotide of particular hydrolytic protease yielding psychrotrophic bacteria was sequenced. To acquire the 1500 bps complete nucleotide sequence of the cleansed amplicon of 16S ribosomal RNA was then referred to the Laboratory of MCLAB California, USA. The attained genomic mapping was examined using NCBI software (BLAST). Sequences were matched and aligned with

other 16S ribosomal RNA sequences added in the GenBank database of NCBI (Altschul, *et al.*, 1997) and for the documentation of bacteria software of Ribosomal Database Project II was analyzed. CLUSTAL W was utilized for the BLAST sequences aligned (Thompson, *et al.*, 1997). Alignment of the BLAST nucleotide sequences was then physically checked and modified. Pairwise evolutionary gaps for the relationship between microorganisms were processed exploiting the Cantor and Jukes calculation applied in the MEGA7 database and a tree of phylogenetic was created by the technique of joining neighbor software accessible online (Tamura, *et al.,* 2007). PAK-01 *Stenotrophomonas sp.,* nucleotide sequences were used for making phylogenetic pyramid. A complete of 100 bootstrapped replications of different values were tested to conclude a measure for every node on the consensus tree diagram. 16S rRNA nucleotide sequences were submitted to NCBI Gene bank using Submission Portal, online software by NCBI and accession numbers were assigned to the submitted partial sequences.

3.1.6. Sequence submission to Genbank and Accession Numbers

After interpretation, respective sequences were submitted to NCBI Genbank using Submission Portal, online software by NCBI and accession numbers were assigned to the submitted partial sequences.

3.1.7. Molecular Cloning and Expression of alkaline thermolabile protease gene in BL21 cells

3.1.7.1. Microorganisms

BL21 (λDE3) was used as an expression strain. BL21 wild cells were cultured in aerobic respiration condition in Luria Bertani broth with reaction mixture composed of peptone 5 g/500ml, yeast extract 0.25g/500ml and NaCl 0.25g/500ml) at 37 °C. Suitable extent of antibiotic(s) (50 µg/ml kanamycin) was required for differentiating between other non-recombinant BL21 wild cells. Genomic DNA was isolated from *Stenotrophomonas sp.* PAK01 cells using a Quick-DNA Fungal/Bacterial Miniprep (Zymo Research) molecular Equipment. Mix & Go E. coli Transformation Kit & Buffer Set (Zymo Research) was demonstrated for gene cloning, and the vector pET28a was used for gene expression. Zyppy™ Plasmid Miniprep kit (Zymo Research) was used for plasmid preparation.

3.1.7.2. Cloning of the thermolabile alkaline protease gene

Nucleotide genomic DNA was extracted from cells of *Stenotrophomonas* PAK01 MG662181 strain utilizing a Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). PCR specific primers were designed utilizing the nucleotide sequence of pr2 gene, which interpreting the serine protease of S8 domain family of peptidase with serine as a catalytic residue in the active site from *S. maltophilia* strain PS5 and available in GeneBank, Pr2 gene (accession number JF317278)). The PrY genomic DNA was amplified from wild cells of *Stenotrophomonas* PAK01 by thermal cycle of polymerase chain reaction (PCR). The genomic DNA of interest was amplified utilizing the Forward primers FPPG: 5′-GCGCATCCATATGTTTGTTTAACTTTAAGAAGGAGA**ATGTCCCAGGTAACGCAACCG** -3′ (Nde1 position highlighted and the N- terminal fragment of LipS gene in bold-face style) and primers used for reverse amplification was RPPG: 5′-GCGCATCAAGCTT**TCAGTACTGGGCGTTGAGGGTCAC** -3′ (with *Hind III* locate highlighted and the section of C-terminal of PrY gene in bold-face style) were designed for the amplification of the entire open reading frame (ORF) ylip gene. Digested PCR fragment of PrY gene was cleaned from other impurities using kit of DNA Clean and Concentrator ™ (Zymo Research) and inserted between the Nde1and *Hind III* sites of the pET28 DNA vector separated from *E. coli* using Zyppy™ Plasmid Miniprep kit (Zymo Research) creating recombinant plasmid pET28-PrY

using for expression. The resulting recombinant pET28 bacterial plasmid was transformed into chemically competent recombinant cells of *E. coli* BL21 (DE3) by utilizing kit of (Zymo Research) Mix & Go Transformation & Buffer Set. Amino acid sequence linker and His_6 came from pET28 vector positioned on N terminus of PrY. Recombinant cells were confirmed by nucleotide sequencing.

3.1.7.3. Sequence analysis of nucleotide

Search for homology nucleotide sequences was completed by BLAST software (http: //www.ncbi.nlm.nih.gov/BLAST/). Alignments of Numerous sequences of nucleotides were by online software of ClustalW programe (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3.1.7.4. Enzyme and protein assays

The enzyme hydrolysis was measured with casein following method explained with some amendments of (Chen *et al.*, 2002). Purified enzyme molecules of 1 ml were mixed with reaction mixture (1 ml casein substrate at concentration of 2 % in 50 mM Tris–HCl buffer) and kept for 10 min at 30°C. 2 ml 0.4 M trichloroacetic acid was used to precipitate the undigested proteins in the reaction mixture. Centrifugation for 5 min at 10,000g was conducted to remove the undigested protein that was not catalyzed by thermolabile protease molecules and clear supernatant of 1 ml was deactivated with sodium hydroxide 1 ml of 1N and alkaline reagents of 5 ml incubated with 1 ml of Folin phenol reagent solution at 30 °C for 15 min and absorbance was measured at 660 nm.

3.1.7.5. Expression and purification Analysis of Pry gene

Gene information for the expression of functional product of protease Pry gene in *E. coli BL21* (λDE3), a positive transformant holding pET28a -PrY protease gene was selected from a particular colony and cultured in the nutrient broth medium for 12 hours at 37 °C in LB medium supplemented with 50 µg/ml kanamycin. For additional research, inoculation with cloned cells was conducted into 250 ml fresh nutrient broth/kanamycin mixture and cultured aerobically at 37 °C when the cell biomass reached to an optical density of A_{600} with 0.6–0.8 and subsequently after 0.6 OD at 600 nm was gotten Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was subsequently used for the induction of protein expression with concentration of 1 mM and cells were grown for further 16 hours.

After incubation, the cells were harvested from induced cells with the assistance of centrifugation at 10000 × *g* for 15 min at 4 °C and was suspended in 50 mM Tris-HCl buffer with pH 8 followed by sonication at 4°C. Supernatant was collected from the cell debris subsequently after centrifugation at 15000 x g for 20 minutes and the compound concentration of imidazole was corrected to 5mM for purification of crude enzyme through nickel-chelated affinity chromatography procedure. 1-mL HisTrap column was used to purified Pry. Pry enzyme bound to column was eluted operating a step-gradient (binding buffer contains 100–500mM imidazole). Dialysis buffer was utilized to remove Imidazole with (pH 10, 25 mM KCl, 6% glycerol). AKTA Explorer system utilized the Anion-exchange chromatography purification procedure organized with a 1 mL HiTrap Q- Sepharose FF column fixedness with dialysis buffer and KCl with linear gradient was used equal to 25 to 1,000 mM concentration. Protease activity exhibiting fractions were gathered. Purification stages at 4 °C were performed. The purified protease enzymes were kept at -80 °C for further experiment in chemical engineering department of Taxas A&M university, USA.

3.1.8. Production optimization of extracellular thermolabile alkaline protease from *Stenotrophomonas sp.*

3.1.8.1. Fermentation of Extracellular crude protease enzyme

The production of extracellular crude enzyme was done in two steps.

3.1.8.2. Inoculum preparation

The inoculum was prepared on two different media having following compositions.

Medium # 1: (PG Nyc 1 broth)

Ingredients	g/ml
NaCl	0.5
Yeast Extract	0.5
Casein	0.5
Peptone	0.3
Gelatin	0.3
D/ water	100mL

Medium # 2: (Ohta *et al.*, 1995)

Ingredients	g/ml
Yeast Extract	0.5
Peptone	0.1
Casein	0.1
Gelatin	0.1
KH_2PO_4	0.1
D/ water	100ml

In two 250 ml Erlenmeyer flasks, 100 ml of nutrient broth media dispensed, and the pH was adjusted by using 0.1 N NaOH and 0.1N HCl. The media were then autoclaving at 121°C for 20 min at 15 Ibs pressure. After autoclaving, 2 to 3 loopful of the isolated *Stenothrophomonas sp.* bacterial strain was inoculated aseptically in both media containing flasks. The flasks were then kept at shaking incubator at 20°C at 150 rpm for 24hrs.

3.1.8.3. Fermentation for Crude Enzyme Production

Batch cultures for enzyme production were done in shake flasks. Various parameters were optimized for thermolabile protease production in shake flasks and various production media having different compositions were used to check the maximum production of enzyme activity. the medium with maximum production of enzyme was selected for further optimization studies in shake flasks.

3.1.8.4. Selection of Media for Protease Production

Medium #1: (Kim *et al.*, 2002)

Ingredients	g/ml
Soluble strarch	1
Tryptone	0.5
Yeast Excract	0.5
MnCl2	0.05
KH2PO4	0.1
CaCl2	0.05
D/water	100

Medium #2: (Do Nascimento and Martins, 2004)

Ingredients	g/L
MgSO4	0.5
K2HPO4	0.2
KCL	0.3
NH4NO3	10
Peptone	1.0
Trisodium Citrate	10

Medium # 3: (Kumar and Parrack, 2003)

Ingredients	g/L
$NH4Cl2$	0.5
NaCl	0.5
$K2HPO4$	0.3
$MgCl2$	0.1
Yeast Extract	0.1
$KH2PO4$	0.4

Medium # 4: (Rahman *et al.*, 2003)

Ingredients	g/l
$CaCl2$	0.5
$K2HPO4$	0.2
$MgSO4$	0.5
KCl	0.2
NaCl	0.1

Medium # 5: (Thangam and Rajkumar *et al.*, 2003)

Ingredients	g/l
Soya meal	5
Peptone	5
K2HPO4	0.2
MgSO4	0.5
NaCl	0.5
CaCl2	0.5

Medium # 6: (Johnvesly and Naiket, 2003)

Ingredients	g/l
Citric Acid	10
NaNO3	10
K2HPO4	5
MgSO4	0.3
NaCl	5
CaCl2	0.2
Na2CO3	10

Medium # 7: (Kuddus and Ramteke, 2011)

Ingredients	g/l
Glucose	10
peptone	5
Yeast extract	5
KH2PO4	1
MgSO4	0.2

Medium # 8: (Saba *et al.*, 2012)

Ingredients	g/l
soymeal	15
Crude protein	45
Crude fate	6
carbohydrate	19
NaCl	8
Sodium glutamate	1
Yeast extract	5
KH2PO4	1.5
MgSO4	0.3

3.1.8.5. Quantitative estimation of protease

200 ml of production media was poured in separate 500 ml Erlenmeyer flask and the uncontaminated fermentation broth pH of was regulated by addition of decontaminated Na2CO3 or Tris-HCl solution at pH 9. Submerged fermentation production media was kept in refrigerated incubator shaker for approximately 2 days at 20°C after inoculation with 1%- and 24-hours old spore suspension. Hydrolytic activity of thermolabile protease enzyme in unit per ml was estimated after centrifugation at 10,000 x g for 10 minutes. Catalytic activity was researched against Azocasein as substrate specificity by the customized scheme of (Carrie Cupp- Enyard 2008). Lowry *et al.*, (1951) method was evaluated for assessment of Protein concentration in incubated fermentation production medium.

3.1.8.6. Production of thermolabile protease enzyme

Production factors for the highest hydrolytic activity of thermolabile protease enzyme were targeted to analyze the significance of a specific parameter at a time and as standardized conditions demonstrating it later before optimizing the subsequent parameter. Incubation period and cell biomass 660 nm ranging from 12-144 hours was assessed in relation to yield of thermolabile protease enzyme.

Catalytic activity against Casein as a substrate was evaluated subsequently every 12 h for 5 days by optimized technique (Carrie Cupp- Enyard 2008). To determine optimum temperature and pH for fermentation, the inoculated batch culture fermentation media was kept at different temperatures (15-50 C) and diverse pH buffer (pH 6-10) in a rotary shaker (120 rpm) and also in static condition. The effect of inocula of different size on the production of cold active alkaline protease such 1%, 5%, 10%, 15% and 20% were evaluated in terms of enzyme yield. To assess the impact of inoculum age, media was inoculated with different age of inoculum. The inoculums of various ages ranging from 12, 24, 48 and 72 hrs. old were used to observe the effect on enzyme protease. The effect of additional supplements (1%) in production media such as fructose, D-sorbitol, raffinose, D (+) glucose and Lactose as a carbon source; and glycine, yeast extract, peptone, tryptone, casamino acid, sodium nitrate, potassium nitrate, leucine, trisodium citrate and ammonium sulphate as a nitrogen source were evaluated in terms of enzyme yield. Alkaline protease activity was measured as per standard protocol (Carrie Cupp- Enyard, 2008).

3.1.8.7. Enzyme and protein assays

The enzyme hydrolysis was measured with casein following method explained with some amendments of (Hema and Shiny, 2012). Purified enzyme molecules of 1 ml were mixed with 1 ml casein substrate at concentration of 2 % in 50 mM Tris–HCl and kept for 10 min at 30°C. 2 ml 0.4 M trichloroacetic acid was used to precipitate the undigested proteins in the reaction mixture. Centrifugation for 5 min at 10,000g was conducted to remove the undigested protein that was not catalyzed by thermolabile protease molecules and clear supernatant of 1 ml was deactivated with sodium hydroxide 1 ml of 1N and alkaline reagents of 5 ml incubated with 1 ml of Folin phenol reagent solution at 30 °C for 15 min and absorbance was measured at 660 nm. Catalytic activity of one unit is described as the biocatalysts that hydrolyzed 1 µg monomers of polypeptide chain correspondent to tyrosine/min. as described in the standard assay circumstances (Holt *et al.*, 1994; Sevinc and Demirkan, 2011).

$$\textbf{Unit/ml} = \frac{\mu\text{Moles of tyrosine x reaction vol}}{\text{Sample vol x reaction time x vol assay}}$$

3.1.8.8. Protein estimation

The method of Lowry *et al.*, (1951) was used for the estimation of protein taking BSA (bovine serum albumin) as standard. Four solutions were prepared as following.

Solution A

Na_2CO_3	1.0 g
NaOH (0.1N)	0.4 g
NaK tartarate	1.0 g
Distilled water	100 ml

Solution B

$CuSO_4.5H_2O$	0.5 g
Distilled H_2O	100 ml

Solution C

Solution A	25 ml
Solution B (fresh)	0.5ml

Solution D

Folin phenol and distilled 1:1 water

Procedure

From 10 mmol stock solution of BSA, different dilutions ranging from 10 to 100 µmol were prepared in 1ml of distilled water. Freshly prepared solution C (1 ml) was added in each test tube and kept at 20 °C for approximately 10 minutes. About 0.1ml of solution D was kept at 37 °C for about half an hour. The O.D recorded at 650 nm was plotted to calculate the slope for standard curve. The same procedure was performed for protein estimation of samples.

Calculations

Standard curve was prepared using BSA as standard and concentration of entire protein in the sample collected from the fermentation production medium investigated by the following formula:

$$\textbf{Protein (mg/ml)} = \frac{\text{Optical density of sample}}{\text{Optical density of standard}} \times \frac{\text{Concentration of standard}}{\text{ml of sample used}}$$

3.1.8.9. Determination of specific activity (Unit/mg) of crude enzyme

The specific catalytic effectiveness of crude extract (mg/ml/min/mg) was assessed by dividing the hydrolytic activity of crude extract by its protein content (U/mg)

3.1.8.10. Data analyses

The results from triplicate experiments for production of enzymes were analyzed for their statistical significance using ANOVA.

3.1.9. Purification and characterization of alkaline and cold active protease from psychrotropic bacterium *Stenotrophomonas sp.* PAK-01 for detergent formulation

3.1.9.1. Material and methods

The experimental work was performed at department of chemical engineering Texas A & M University, Texas USA, 2018. All the media components and chemicals were purchased form Sigma-Aldrich and VWR (College station, USA) and were of analytical grade.

3.1.9.2. Microorganisms

The *Stenotrophomonas sp.* Pak1 was extracted from the soil samples obtained from the Northeast Pakistan. The isolated micro-organism was upheld in agar containing slants at 25 ° C. The agar slant contained 0.4% yeast extract, 1% malt extract and 2% agar.

3.1.9.3. Production of thermolabile crude enzyme

The spore suspension of inoculum was inoculated using Erlenmeyer flasks (250 ml) containing 50 ml of the fermentation medium described by (Porto *et al.* 1996), The production media which have the following ingredients (w/v) such as 1% yeast extract, 1% tryptone, 1% glucose, 0.5% KH2PO4 and 0.5 ml of 100mg metal ions concentration including calcium chloride, iron sulfate, zinc sulfate, and manganese chloride was inoculated with 5% inoculum and cultivated for almost 4 days at 25 °C in shaking flask fermentation incubator. The supernatant, fractionated by ammonium sulfate precipitation (30 to 80%), was dialyzed against 50mM Tris–Hcl, pH 8, for 24 h to remove residual ammonium sulfate. The dialyzed sample was subjected to Superdex 200 PG 16/60 and DEAE Sepharose Chromatograph SOURCEQ 10/10.

3.1.9.4. Purification of thermolabile alkaline protease

Proteins/crude enzyme from the centrifuged crude supernatant of *Stenotrophomonas sp*, PAK01 MG662181 were extracted with 80% (NH4)2SO4 partial purification of thermolabile enzyme conducted to overnight at 4 °C with continuous stirring. The precipitous of proteins produced was gathered again by centrifuging at 20,000xg for 4 °C of half an hour using a Beckman ultracentrifuge and suspended over in a minimum amount of 20 mM Tris-aminomethane buffer, pH 8.0.

After almost fermentation of production for 4 days the crude thermolabile enzyme was subjected to centrifugation for clear supernatant at 4 °C and stored in -80°C for further analysis. (NH4)2SO4 was added to supernatant at 30% and was further treated with up to 90% concentration of ammonium salt. Precipitated thermolabile protein after centrifugation at 4 °Cwas resuspend in buffer of tris-HCl and was taken in dialyzing bag and placed in buffer of phosphate salts at low temperature of 4 °C for almost 1 day against three changes of the phosphate buffer. The dissolved proteins were applied to an ion exchange chromatography (IEX: SOURCEQ 10/10) column equilibrated and the proteins on the basis of charges was eluted by using buffer of tris-HCl with 8 pH. Fractions collected was analyzed for proteolytic activity and those with the high catalytic activity of protease enzyme was selected and subject for further purification on the bases of protein molecules sizes (GFC: Superdex 200 PG 16/60) gel chromatography to get the homogenous mixture of thermolabile protein molecules. Following to the technique of (Bradford, 1976) exploiting bovine serum albumin as a standard for protein determination and also for the purpose of specific activity in (mg/ml).

3.1.9.5. Enzyme and protein assays

Catalytic hydrolysis of polypeptide chain was measured with casein following process explained with some amendments of (Chen *et al.* 2002). Purified enzyme molecules of 1 ml were mixed with 1 ml casein substrate at concentration of 2 % in 50 mM Tris–aminomethane and kept for 15 min at 25°C. 2 ml 0.4 M TCA was used to precipitate the undigested proteins in the reaction mixture. Centrifugation for 5 min at 10,000g was conducted to remove the undigested protein that was not catalyzed by thermolabile protease molecules and clear supernatant of 1 ml was deactivated with sodium hydroxide 1 ml of 1N and alkaline reagents of 5 ml incubated with 1 ml of Folin phenol reagent solution at 30 °C for 15 min and absorbance was measured at 660 nm.

3.1.9.6. SDS electrophoresis

150 ul of each fraction was precipitated by Chloroform/Methanol method. Precipitated proteins dissolved in 40 ul of SDS sample buffer and heated for 15 min at 65 °C. 30 ul of sample was loaded on gel. Gel tainted with Silver following the procedure of Blum *et al.*, (1987).

3.1.9.7. Characterization of the alkaline protease

3.1.9.7.1. Temperature influence on thermolabile protease molecules hydrolysis and stability

The optimal temperature for this enzyme against casein substrate of the thermolabile protease molecules was observed at different temperature ranging from 10 to 50°C in Tris-HCl buffer for the best temperature at which the catalytic activity of protease was maximum in the reaction mixture for 30 minutes. Enzymatic relative activity was computed as percentage of the highest hydrolysis. Thermal stability of purified hydrolytic protease molecules was determined by incubating from low to high temperature such as from 20 to 80 °C in Tris-aminomethane buffer of 9 pH for 240 minutes. Residual hydrolytic catalysis was experimented as percentage of the starting reaction activity.

3.1.9.7.2. Effect of pH and various metal ions, surfactants and oxidants on purified enzymatic hydrolysis and stability

Hydrolytic activity of the alakline tolerant purified protease molecules against the substrate was determined against diverse innumerable pH such as phosphate buffer (pH 6–7), Tris-aminomethane acid buffer (pH 8–9), glycine sodium hydroxide buffer (pH 10–11), sodium bicarbonate-sodium hydroxide buffer (pH 12) by incubating in the production culture at 25 °C. Alkaline molecules of protease from the PAK01 was preincubated in assorted buffers before of the residual catalysis was researched for thermolabile protease for 1 h at 25 °C. Significances of distinctive metal ions on the purified molecules hydrolysis residual activity were assessed by incubating Calcium chloride (CaCl2), magnesium chloride (MgCl2), iron sulfate (FeSO4), manganese chloride (MgCl2), iron chloride (FeCl3), copper sulfate (CuSO4), barium chloride (BaCl2) and nickel sulfate (NiSO4) in the reaction mixture at the concentration of 5, 10, and 15 millimolar. Hydrolysis of substrate without ions in the reaction mixture by protease purified molecules was fixed 100% (control treatment).

The hydrolytic residual activity of the purified protease was determined by the pre-incubation of purified enzyme against different surfactants such as Tween-20, 80, Triton X-100 and oxidants such as SDS and hydrogen peroxide (H_2O_2). Hydrolytic residual proteolytic activity was measured after the experiment was conducted on the

alkaline stable purified protease was kept in the reaction mixture with with each of the substance at a final concentration of 0.5–5% at 25 °C for 24, 48, and 72 h of incubation.

3.1.9.7.3. Inhibitors and organic solvents effect on the activity and stability of protease

The effects of b-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), phenyl methane sulfonylfluoride (PMSF), dithiothreitol (DTT) were evaluated by preincubating PAK01 thermolabile protease enzyme in reaction mixture of each 5 to 15 mM concentration of inhibitors for half an hour at 25 °C before to the residual catalytic activity was experimented. Control reaction mixture without inhibitors was considered as 100% enzymatic activity.

In the reaction mixture the protease residual hydrolytic activity and stability of the thermolabile protease enzyme was incubated for 1 day at 25 °C with organic solvents at concentration of 5 and 10% was researched by modification in method of (Ogino *et al.*,2000). Standard technique was used for determination of residual activity of protease. Relative activity (%) was evaluated relative to reaction without the addition of organic solvents, which was taken as 100 %.

3.1.9.7.4. Km, Vmax, Kcat and Activation energy E_a studies using the purified alkophilic proteaes enzyme

The purified thermolabile enzyme Km and Vmax values have been recorded by means of estimating the activity with numerous concentrations of casein substrate (1gram/ 100microlitre). The kinetic constants have been estimated by employing the Lineweaver–Burk plot. On the basis of Michaelis-Menten theory, the enzyme hydrolyzed reaction was analyzed. PAK01 thermolabile protease molecules activities were determined by utilizing casein substrate and Lineweaver burk graphs were created by conducting an experiment of having fixed concentration of purified thermolabile protease against different casein concentrations. At different temperature like 4, 15 and 25 the Kcat and Kcat/Km values were determined to observe the superlative catalytic efficiency of thermolabile protease at cold environment. Arrhenius plot was acquired from ln(k_d) versus 1/T to conclude the activation energy. The turnover number or the catalytic constant of enzyme was computed by dividing the Vmax value by E(t).

$$K_{cat} = V_{max}/E(t),$$

Vmax is the maximal reaction of catalytic velocity

E(t) is the enzyme quantity in moles present in reaction mixture

On behalf of irreversible thermal denaturation of thermolabile purified protease derived from *Stenotrophomonas sp,* PAK01 the thermodynamic parameters were projected by kepting the enzyme at different temperatures ranging from 30 °C to 90 °C in 50 mM Tris- HCl buffer (pH 9). Enzyme activity was assayed according to the standard assay conditions. Data were analyzed as according to the method of the (Siddiqui *et al.,* 1999) and incorporated to the first order plots. Thermostability of purified thermolabile lipase were considered by reorganizing the absolute velocity equation according by Eyring and Stearn (1939).

$$k_d = (k_B T/h)\, e^{(-\Delta H^*/RT)}\, e^{(\Delta S^*/R)}$$

Reaction velocity constant for the first-order dissociation constant (k_d) measured from a logarithmic plot of percent of residual hydrolytic activity/time, according to equation

$$\ln \%\ \text{residual activity} = -k_d t$$

To measured activation energy for thermal inactivation ($E_{a(d)}$), from equation of ln(k_d) versus 1/T (temperature of incubation) an Arrhenius plot was estimated. The line slope was determined from the following equation:

$$\ln(k_d) = -E_{a(d)}/RT$$

3.1.10. *Stenotrophomonas sp. Pak1* thermolabile alkaline protease as a laundry additive

3.1.10.1. Stability of Thermolabile protease in commercial Detergents

Thermolabile purified protease in 5% detergents was pre-incubated against (Tide, Gain, Purex, Xtra, Tween-80, SDS and Triton X-100 obtained from commercial market of College station, Texas USA) at 25°C and at 30 °C and percent residual activity was examined against thermolabile protease control after 30 minutes and 1 hour of incubation. In the next experiment subsequent research was conducted in which, molecules stability was considered in various concentration such as 3 to 20 percent of purex and xtra following incubation at 25 °C for 1 hour and 30 minutes and also cold active protease molecules stability at 25 °C, over a phase of 24 hrs in 5 percent of purex and Xtra detergents.

3.1.10.2. Oxidizing and bleach result on thermolabile protease molecules

Thermolabile protease stability was investigated under the consequence of hydrogen peroxide at range of such as 5 to 20% and sodium hypochlorite from 50 to 500 mg/l of available chlorine and the percent residual activity was assayed after 60 minutes at 25°C.

3.1.10.3. Washing analysis of thermolabile organic solvent stable protease

The staining of new pieces of cotton cloth with blood plasma and egg yolk was conducted which was then utilized to assess the washing efficiency and to validate potential of alkaline protease as a bio detergent additive. The endogenous proteases enzyme present in commercial washing detergent were obtained from local markets and was then inactivated of these enzymes containing detergents was performed by heating diluted detergents at 70 °C for 1 hour before the addition of purified tested enzymes. After that, the blemished fabric cotton was shaking incubated at 250 rpm with different washing treatments for 1 hour at 25 °C in 1-liter beakers. The beakers were already containing a total quantity of 100 mL of four (04) washing treatment; tap water, detergent (in tap water), detergent with buffer and the detergent added with cold active alkaline protease (500 U/mL). Afterwards, the treated cotton fabric was taken out. Rinsing was done with distilled water, dried and then visually checked to record the stain removal capability of protease enzyme. On the other hand, the blood-stained cloth piece untreated was considered as a control.

Section # 2

3.2. Isolation and identification of extracellular cold active lipase from psychrotrophic *Pseudomonas peli* PAK03

3.2.1. Sampling and screening of thermolabile lipase secreting bacteria

In direction to isolate psychrotolerant bacteria, soil and water samples were amassed from vicinity of Rakaposhi glacier, northern Baltistan, Pakistan during 2017. *Pseudomonas peli* PAK03 was isolated in laboratory of Applied microbiology and biotechnology, International Islamic University Islamabad from the soil samples gathered from Gilgit Baltistan Pakistan. Samples of soil from distant sites was diluted serially and cultivated on nutrient agar plates for screening of lipase producing bacteria at pH 9 at 20 °C for 24 hr. Out of twenty strains, five bacterial stains were observed to have the potential to grow at low temperature and alkaline pH. Quantitative screening of the selected five bacterial strains was experimented in submerged fermentation for 48 hrs at 20°C in alkaline conditions at pH 9. After incubation bacterial strain that have the potential to catalyze maximum substrate of triacylglycerol was selected and stored at -20°C for further analysis. Further processing was done in the chemical engineering department of Taxas A&M university, USA

3.2.2. Isolation of genomic DNA

Selected bacterial strains were identified by 16S rRNA nucleotide sequencing technique. Isolation of genomic Bacteria DNA was done by Quick-DNA™ Fungal/Bacterial Miniprep Kit.

3.2.2.1. Amplification and nucleotide sequencing of 16S ribosomal RNA gene

Genomic DNA of selected bacteria extracted from pure fresh culture of 24 hours and nearly the complete stretch of PCR amplification of 16S rRNA of complete fragment of 1500bps was experimented. Forward and reverse primers used for elongation were 5′-AGAGTTTGATCATGGCTCAGA- 3′ and 5′-GTTACCTTGTTACGACTT-3′- equivalent to 8 to 28 and 1493 to 1510 and are segments of 16S ribosomal RNA genome of *E. coli* and so are valuable for extension of 16S ribosomal RNA genomic DNA from diverse classes of bacteria.

50 µl of reaction mixture for each PCR comprised: purified genomic DNA of 20 ng, Taq DNA polymerase for elongation at a concentration of 2.5 Units, 10 X

concentrated 5 μl volume of Taq polymerase buffer (100 mM and 500 mM of Tris-HCl and KCl at pH of 8), forward primer and reverse primer of 27F to 1492R each universal primer of 10 pmoles concentration and dNTP of 200μM and magnesium chloride at quantity of 2 mM was utilized. PCR reaction comprises initial step of denaturation for 3 minutes at 95°C, trailed by final denaturation at 94°C for 25 runs for 60 seconds, annealing temperature at 55°C of amplification of primers for 60 seconds and last amplification step of elongation/extension at 72°C for 60 seconds. Final elongation/extension for 5 minutes at 72°C was experimented. Sample of 5 μl of the positive amplified result of gene was then examined by electrophoresis technique comprised of 1 percent agarose gel with DNA visualize dye ethidium bromide and then envisioned underneath Gel doc or UV transilluminator. The PCR amplified product was than purified for further analysis using the DNA Clean & Concentrator™-5 and stored in -20°C.

3.2.2.2. Construction and analysis of Phylogeny

16S ribosomal RNA amplified nucleotide of particular hydrolytic protease yielding psychrotrophic bacteria was sequenced. To acquire the 1500 bps complete nucleotide sequence of the cleansed amplicon of 16S ribosomal RNA was then referred to the Laboratory of MCLAB California, USA. The attained genomic mapping was examined using NCBI software (BLAST). Sequences were matched and aligned with other 16S ribosomal RNA sequences added in the GenBank database of NCBI (Altschul, *et al.*, 1997) and for the documentation of bacteria software of Ribosomal Database Project II was analyzed. CLUSTAL W was utilized for the BLAST sequences aligned (Thompson, *et al.*, 1997). Alignment of the BLAST nucleotide sequences was then physically checked and modified. Pairwise evolutionary gaps for the relationship between microorganisms were processed exploiting the Cantor and Jukes calculation applied in the MEGA7 database and a tree of phylogenetic was created by the technique of joining neighbor software accessible online (Tamura, *et al.,* 2007). PAK-03 *Pseudomonas peli* nucleotide sequences was used for making phylogenetic pyramid. A complete of 100 bootstrapped replications of different values were tested to conclude a measure for every node on the consensus tree diagram. 16S rRNA nucleotide sequences were submitted to NCBI Gene bank using Submission Portal, online software by NCBI and accession numbers were assigned to the submitted partial sequences.

3.2.3. Molecular cloning, expression and characterization of organic solvent tolerant alkaline lipase gene from *pseudomonas peli* PAK03 (MH338242)

3.2.3.1. Cloning of the thermolabile alkaline lipase gene

Nucleotide genomic DNA was extracted from cells of *pseudomonas peli* PAK3 strain utilizing a Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). PCR specific primers were designed utilizing the nucleotide sequence of LipS gene, which translating the solvent-tolerant alkaline lipase of *Pseudomonas mandelii* JR-1 and available in GeneBank, LipS gene (GenBank ID JQ071496). The ylip genomic DNA was amplified from *Pseudomonas peli* PAK3 by thermal cycle of polymerase chain reaction (PCR). The genomic DNA of interest was amplified utilizing the Forward primers FLG: 5 GCGCATCCATATGTTTGTTTAACTTTAAGAAGGAG**ATGTCGCAAGGTTC TGCCACG** 3 (Nde1 position highlighted and the N- terminal fragment of LipS gene in bold-face style) and primers used for reverse amplification was RLG: 5 GCGCATCCTCGAG**TTACACGCCAGCCCCTTTCAATC** 3 (Xho1 locate highlighted and the section of C-terminal of LipS gene in bold-face style) were designed for the amplification of the entire open reading frame (ORF) ylip gene.

Digested PCR fragment of ylip gene was cleaned from other impurities using kit of DNA Clean and Concentrator ™ (Zymo Research) and inserted between the Nde1and Xho1sites of the pET28 DNA vector separated from *E. coli* using Zyppy™ Plasmid Miniprep kit (Zymo Research) creating recombinant plasmid pET28-yLip using for expression. The resulting recombinant pET28 bacterial plasmid was transformed into chemically competent recombinant cells of *E. coli* BL21 (DE3) by utilizing kit of (Zymo Research) Mix & Go Transformation & Buffer Set. Amino acid sequence linker and His_6 came from pET28 vector positioned on N terminus of ylip. Recombinant cells were confirmed by nucleotide sequencing.

3.2.3.2. Sequence analysis of nucleotide

Search for homology nucleotide sequences was completed by BLAST software (http: //www.ncbi.nlm.nih.gov/BLAST/). Multiple alignment of Numerous sequences of nucleotides were by online software of ClustalW programe **(http://www.ebi.ac.uk/Tools/msa/clustalw2/).**

3.2.3.3. Expression and purification Analysis of YLip

Bacterial plasmid pET28 bacterial plasmid comprising the complete open reading frame of yLip nucleotide sequence was transmuted into cells of competent *E. coli* BL21 (DE3). A particular colony of recombinant cells of BL21 that was grown on kanamycin agar plate was preferred for further instantaneous growth at 37 °C for 12 hours, trailed by inoculation of recombinant cells into a 0.2 Liter kanamycin nutrient broth. At the mid- exponential period when the OD_{600nm} is reached to 0.6-0.8 the temperature of the incubation was reduced to 15 °C. Afterward the addition of molecular reagent such as 0.1 mM concentration of IPTG, the cells of recombinant BL21 for an additional 16 hours duration were grown in submerged fermentation. Bacterial BL21 cells were collected through centrifugation at 10000 x g for 10 minutes. After centrifugation pellet of bacteriological cell was resuspended in binding buffer reaction (Tris-HCl 20 mM, NaCl 0.1 M and 5% glycerol at pH 8.0), followed by disruption of recombinant cells through sonication method at 4 °C. Supernatant was collected from the cell debris after centrifugation at 15000 x g for 20 minutes and the compound concentration of imidazole was corrected to 5mM for purification of crude enzyme through nickel-chelated affinity chromatography procedure. 1-mL HisTrap column was used to purified yLip. yLip enzyme bound to column was eluted operating a step-gradient (binding buffer contains 100–500mM imidazole).

Dialysis buffer was utilized to remove Imidazole with (pH 10, 25 mM KCl, 6% glycerol). AKTA Explorer system utilized the Anion-exchange chromatography purification procedure set with a 1 mL HiTrap Q- Sepharose FF column fixedness with dialysis buffer and KCl with linear gradient was used upto 25 to 1,000 mM concentration. Lipase activity exhibiting fractions were gathered. Purification stages at 4 °C were performed. The purified enzymes were kept at -80°C for further analysis.

3.2.3.4. Thermolabile recombinant lipase assay

Lipase hydrolytic assay was experimented exploiting 0.1 mM substrate of *p*-nitrophenyl palmitate (pNPP) in reaction mixture (100 mM NaCl, 100 mM Tris–HCl buffer, 0.5% Triton X-100). The liberation of p-nitrophenol (p-NP) was analyzed spectrophotometrically at 410 nm. One unit/ml of enzyme was stated 1 µmol p-NP min^{-1} from *p*-nitrophenyl palmitate at 25 °C.

3.2.4. Production of extracellular thermolabile alkaline lipase from psychrotrophic *pseudomonas peli* strain PAK3 (MH338242)

3.2.4.1. Production of thermolabile lipase enzyme

Production factors was targeted to analyze the significance of a specific physiochemical factor at a time and standardized conditions demonstrating it later before optimizing the subsequent parameter. Hydrolytic lipase efficacy was analyzed to estimate highest yield production after each step. *Pseudomonas peli* was cultivated in trybutyrin nutrient broth reaction mixture (yeast extract (5 g/500 ml), peptone (15 g/ 500ml), $CaCl_2$ 0.01 %, NaCl (2.5 g/500ml) and trybutyrin (2 ml/500ml) (Joseph *et al.*, 2006). Inoculum preparation was done by kept the inoculated nutritional media in a refrigerating shaker incubator at 150 rpm at 18 °C. Inoculum age and size for the maximum production of enzyme was optimized by the production media was inoculated with different age from 6 to 48 hours and size of the inoculum among 2.5% to 15.0%. Underneath above optimized factors, highest catalytic activity against incubation time was investigated for the maximum production of thermolabile lipase by 100 ml of tributyrin broth was infected with 5 ml inoculum and incubated at 20 °C in shaking incubator. Samples of crude enzyme were withdrawn at interims of 12 hours up to the total time of 120 hours and afterward centrifugation at 15,000 rpm for ten minutes at 4 °C, analyzed supernatant for catalytic activity and estimation of protein. To investigate the consequence of the temperature on thermolabile lipase hydrolytic activity, TB inoculated with spore suspension of bacteria were kept at temperature varying from 4-30 °C. Effect of initial pH on thermolabile lipase optimum production was investigated by the TB media were regulated to pH ranging from 7–10 with different buffers (Gupta *et al.*, 2008).

To research the consequence of diverse sources of nitrogen, TB nutrient media was utilized as a basal medium in which 1% of each nitrogen source was supplemented to optimize for the maximum production of cold active alkaline lipase. The optimized pH, temperature and time interim were utilized in every phase for standardizing the influence of supplementary carbon sources. To examine the consequence of distinctive carbon sources, basal media were augmented with 1% of each carbon sources such as Maltose, glucose, Lactose and Sucrose. Distinctive substrates for the thermolabile lipase production was experimented by the supplement

of Tween 20 and olive oil to broth basal media by substituting the trybutyrin as control.

1 % of NaCl, $MgCl_2$ and KCl were supplemented to basal media to investigate the influence of salts on the secretion of thermolabile lipase in submerged fermentation by exchanging $CaCl_2$ as control. To examine the consequence of NaCl, $CaCl_2$ was not supplemented in the basal TB media, however, for KCl, $CaCl_2$ and $MgCl_2$, NaCl was obligatory subsequently it is present in the basal fermentation media. All experimentations were evaluated for optimum culture conditions and composition of the medium were accomplished by shake flask culture fermentation.

3.2.4.2. Lipolytic assay

Lipolytic activity was analyzed by exploiting substrate of *p*-nitrophenyl palmitate (Ertuğrul *et al.*, 2007). Two substrate solutions with reaction mixture A (pNPP with concentration of 0.03 gm was dissolved in 10 ml of isopropanol) and B (gum Arabic 100 mg and Triton X-100 of 0.4 ml was suspended in 90 ml of potassium phosphate buffer of 50 mM concentration, pH 8) was prepared following stirring until completely dissolved. 0.9 ml of the *p*-nitrophenyl palmitate substrate sample from the reaction mixture was incubated with 0.1 ml of enzyme solution at 25 °C for 15 min and the liberated para-nitrophenol quantity at wavelength of $\lambda = 410$ nm the absorbance was measured.

p- nitrophenol extinction coefficient (ε) under the defined circumstances measured at frequency of $\lambda = 410$ nm wavelength of standard reaction mixtures of pNP (0.01 to 0.1 μmol/ml) ($\varepsilon 410 = 14.653$ L/mol/cm). Appropriate controls were prepared for each test. Lipolytic activity in Unit/ml was quantified as 1 nmol of p-nitrophenol catalyzed per minute under the standard assay conditions. Suspended concentration of protein was measured according to the procedure of Lowery *et al.*, (1951) exploiting bovine serum albumin (BSA) as a standard.

3.2.5. Purification and Characterization of solvent stable thermolabile lipase from Psychrotrophic *Pseudomonas peli* MG687270

3.2.5.1. Microorganisms

Pseudomonas peli sp. PAK03 was isolated from soil of Rakaposhi glacier, in northmost autonomous territory of Gilgit Baltistan, Pakistan The isolated micro-organism was upheld in agar containing slants at 25 ° C. The agar slant contained 0.4% yeast extract, 1% malt extract and 2% agar.

3.2.5.2. Production of thermolabile lipase enzyme

The *Pseudomonas peli* sp. PAK03 inoculum was inoculated using Erlenmeyer flasks (250 ml) containing 50 ml of the fermentation medium described by (Porto *et al.*, 1996), The production media which have the following ingredients (w/v) such as 1% yeast extract, 1% tryptone, 1% glucose, 0.5% KH2PO4, tributyrin 1% as inducer and 0.5 ml of 100mg metal ions concentration including calcium chloride, iron sulfate, zinc sulfate, and manganese chloride was inoculated with 5% inoculum and cultivated for almost 4 days at 25°C in shaking flask fermentation incubator. The supernatant, fractionated by ammonium sulfate precipitation (30 to 80%), was dialyzed against 50mM Tris–Hcl, pH 8, for 24 h to remove residual ammonium sulfate. The dialyzed sample was subjected to Superdex 200 PG 16/60 and DEAE Sepharose Chromatograph SOURCEQ 10/10.

3.2.5.3. Purification of thermolabile alkaline protease

Proteins from the centrifuged crude supernatant of *Pseudomonas peli* PAK03 MG687270 were extracted with 80% (NH4)2SO4 partial purification of thermolabile enzyme conducted to overnight at 4°C with continuous stirring. The precipitous of proteins produced was gathered again by centrifuging at 20,000xg for 4°C of half an hour using a Beckman ultracentrifuge and suspended over in a minimum amount of 20 mM Tris-aminomethane buffer, pH 8.0. After almost fermentation of production for 4 days the crude thermolabile enzyme was subjected to centrifugation for clear supernatant at 4 °C and stored in -80°C for further analysis. (NH4)2SO4 was added to supernatant at 30% and was further treated with up to 90% concentration of ammonium salt.

Precipitated thermolabile protein after centrifugation at 4 °Cwas resuspend in buffer of tris-HCl and was taken in dialyzing bag and placed in buffer of phosphate salts at low temperature of 4 °C for almost 1 day against three changes of the

phosphate buffer. The dissolved proteins were applied to an ion exchange chromatography (IEX: SOURCEQ 10/10) column equilibrated and the proteins on the basis of charges was eluted by using buffer of tris-HCl with 8 pH. Fractions collected was analyzed for lipase activity and those with the high catalytic activity was selected and subject for further purification on the bases of molecules sizes gel chromatography to get the homogenous mixture of thermolabile protein molecules. Following to the technique of Lowry *et al.,* (1951) exploiting bovine serum albumin as a standard for protein determination and also for the purpose of specific activity in (mg/ml).

3.2.5.4. Characterization of Purified Alkaline Lipase

3.2.5.4.1. Temperature influence on thermolabile lipase hydrolysis and stability

The hydrolysis of the ester bonds against p-nitrophenyl palmitate substrate of the thermolabile purified lipase enzyme activity was observed at different temperature ranging from 4 to 60 °C with interval of 10 °C in Tris-HCl buffer of 9 pH for the best optimum temperature at which the catalytic lipase activity was maximum using standard assay conditions. Enzymatic catalysis was computed as percentage of the highest hydrolysis. Temperature influence on the stability of purified hydrolytic lipase enzyme was determined by pre-incubation from low to high temperature such as from 4 to 75 °C in 50mM Tris HCl buffer of pH 9 for 240 minutes. Residual hydrolytic activity was experimented as percentage of the starting reaction according to the standard assay conditions.

3.2.5.4.2. Effect of pH on purified enzymatic hydrolysis and stability

Lipolytic activity of the alkaline stable lipase against the p-nitrophenyl palmitate substrate was determined at different innumerable pH such as phosphate buffer (pH 6-7), Tris-aminomethane acid buffer (pH 7-9), glycine sodium hydroxide buffer (pH 9-10), sodium bicarbonate-sodium hydroxide buffer (pH 11-12) by incubation in the production culture at 25 °C. To evaluate thermal stability of purified enzyme against different pH conditions, alkaline molecules of lipase from the PAK03 was preincubated in various buffers varying from 6-12 pH before of the residual catalytic activity was researched for thermolabile lipase for 1 h at 25 °C.

3.2.5.4.3. Effect of various metal ions on purified enzymatic hydrolysis and stability

Significant effect of distinctive metal ions on the purified lipase enzyme hydrolysis were assessed by incubating Calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), potassium chloride (KCl), manganese chloride ($MnCl_2$), iron chloride ($FeCl_3$), manganese sulfate ($MnSO_4$.), sodium chloride (NaCl), zinc sulfate ($ZnSO_4$) and EDTA in the reaction mixture at the strength of 1mM. Relative activity was calculated by regarding of substrate without ions in the reaction mixture by lipase purified molecules was fixed 100% (control).

3.2.5.4.4. organic solvents effect on the activity and stability of lipase

In the reaction mixture the hydrolytic activity of the thermolabile lipase enzyme was incubated for 1 hour at 25 °C with wide-ranging effect of organic solvents such as acetone, acetonitrile, DMSO, ethanol, propanol, hexane and methanol at concentration of 30 % was researched by modification in method of (Ogino *et al.*,2000). Standard assay technique was used for determination of relative activity of lipase against each orgain solvents. Residual activity was estimated regarding the control which was considered as 100% without addition of any organic solvents.

3.2.5.4.5. Km, Vmax, Kcat and Activation energy $E_{a(d)}$ studies using the purified alkophilic lipase enzyme

Effect of different concentration of substrate on the reaction rates of the purified lipase was experimented with p-NPP among the p-nitro phenyl esters. The purified thermolabile lipase catalyzed reaction against p-nitrophenyl palmitate follows the Michaelis Menten Theory. K_m and V_{max} values was recorded by means of analyzing the lipase activity with numerous concentrations of p-nitrophenyl palmitate substrate (0.1-2 mg/ml) soluble in 50 ml of isopropyl alcohol. The kinetic constants have been estimated by employing the Lineweaver–Burk plot. The turnover number or the catalytic constant of enzyme was computed by dividing the V_{max} value by E(t) (i.e., the enzyme quantity in moles present in reaction mixture).

$$K_{cat} = {}^{V_{max}}/_{E(t)}$$

Vmax is the maximal reaction of catalytic velocity

E(t) is the enzyme quantity in moles present in reaction mixture

On behalf of irreversible thermal denaturation of thermolabile purified lipase derived from *Pseudomonas peli PAK03* the thermodynamic parameters were projected by kepting the enzyme at different temperatures ranging from 30 °C to 90 °C in 50 mM Tris- HCl buffer (pH 9). Enzyme activity was assayed according to the standard assay conditions. Data were analyzed as according to the method of the (Siddiqui *et al.*, 1999) and incorporated to the first order plots. Thermostability of purified thermolabile lipase were considered by reorganizing the absolute velocity equation according by Eyring and Stearn (1939).

$$\mathbf{k_d = (k_B T/h)\, e^{(-\Delta H^*/RT)}\, e^{(\Delta S^*/R)}}$$

Reaction velocity constant for the first-order dissociation constant (k_d) measured from a logarithmic plot of percent of residual hydrolytic activity/time, according to equation

$$\mathbf{\ln \%\ residual\ activity = -k_d t}$$

To measured activation energy for thermal inactivation ($E_{a(d)}$), from equation of $\ln(k_d)$ versus 1/T (temperature of incubation) an Arrhenius plot was estimated. The line slope was determined from the following equation:

$$\mathbf{\ln(k_d) = -E_{a(d)}/RT}$$

3.2.6. Alkaline Lipase from Psychrotrophic *Pseudomonas peli* for Detergent Additive Capability

3.2.6.1. Microorganisms, Chemicals, and Reagents

Pseudomonas peli PAK01 secreted thermolabile lipase enzyme and extracted from the northern area of Gilgit Baltistan, Pakistan and deposited in NCBI as *Pseudomonas peli PAK01* under accession number of MG687270, preserved as glycerol stock at -20°C.

Chemical Compounds, and Reagents. Compatibility analyses of alkaline thermolabile lipase with nonionic surfactant and marketable detergents of USA, explicitly Tide, Gain, Arm & Hammer, purex, xtra, persil and sun and stability analysis toward anionic surfactants sodium dodecyl sulphate (SDS) was scrutinized in the present research. *p*-Nitrophenyl palmitate (*p*-NPP) was from Sigma, USA.

3.2.6.2. Lipase Production and Purification

Alkaline thermolabile lipase secreted from psychrotrophic *pseudomonas peli* cultivation in submerged fermentation practice and was purified through diverse purification phases particularly ammonium sulphate precipitation (80%), DEAE-Sepharose and Sephadex chromatography technique. Highest catalytic activity with pH of 9 was observed and vigorous below wide-ranging temperature of 20–60 °C (Chauhan and V. K. Garlapati, 2013).

3.2.6.3. Thermolabile lipase assay

Lipase hydrolytic assay was experimented exploiting 0.1 mM substrate of *p*-nitrophenyl palmitate (pNPP) in reaction mixture (100 mM NaCl, 100 mM Tris–HCl buffer, 0.5% Triton X-100) (Garlapati and R. Banerjee, 2010). The liberation of p-nitrophenol (p-NP) was analyzed spectrophotometrically at 410 nm. One unit/ml of enzyme was stated 1 µmol p-NP min^{-1} from *p*-nitrophenyl palmitate at 25°C.

3.2.6.4. Alkaline Lipase Compatibility with anionic Surfactants and Detergents

Relative catalytic activity in term of percent was analyzed by the addition of diverse surfactant and detergents at concentration of 8 mg/ml with the purified alkaline lipase in the reaction mixture for compatibility determination. Endogenous lipases from any other microorganism existing in these commercial detergents were deactivated by heating the diluted detergents at 80 °C for 60 minutes before to the addition of alkaline enzyme formulation. To evaluate residual stability, an aliquot taken from lipase enzyme (unit/ml) solutoin was kept with equal volume of detergent

reaction mixture at concentration of 8 mg/mL in 0.1 M of Tris-aminomethane buffer 8 pH for 60 minutes at 25°C. Relative hydrolytic activity of respectively each sample was estimated and associated with the treatment without detergent known as control and was defined as the enzyme catalytic activity deprived of any commercial detergent, incubated under the similar conditions, and was taken as 100%.

3.2.6.5. Alkaline Lipase Compatibility with Oxidizing compounds

Compatibility of alkaline lipase in the existance of oxidizing compounds was determined in Tris-amino methane buffer of 0.1 M at pH 8.0 containing with a concertation of 0.5–2% of sodium hypochlorite (Na2O2), NaClO2, sodium perborate (NaBO3) and hydrogen peroxide (H2O2) for 60 minutes at low temperature of 25 °C, after which relative catalytic assay was assessed and associated with treatment deprived of the oxidizing agents. Relative hydrolytic activity of control was described as the reaction mixture deprived of oxidizing compounds and was kept under the same circumstances and was taken as 100%.

3.2.6.6. Olive Oil muddied Cotton Cloth Formulation

Removing of oil sources from cotton cloth supposed to be make soiled was conducted by boiling in chloroform solvent for 4 hours and the experiment was repeated thrice. Soiling of defatted cotton material was carried out with the help of micropipette by spotting olive oil pigment in benzene reaction mixture solution (500 μL, 100 mg/mL).

3.2.6.7. Formulation of Washing solution

Washing solutions of 4 different kinds of cleaning reaction mixture formulation and its composition for preparation 100 ml of corresponding washing solution. Formulation solution comprises of different treatments such as surfactant, buffer and Alkaline lipase solution, washing solution was prepared by taking the commercial detergent solutions and measured buffer of 8 pH in 200 ml Erlenmeyer flask. Pretreating of the contents of the flask was done before addition of the alkaline lipase solution with condition of 25 °C for 10 min with in washing reaction solution. Buffer with reaction mixture (0.1 Molar of Tris-amino methane of pH 9) and lipase enzyme (B-L), buffer and detergent (0.5%) (B-D) and only buffer solution were also formulated in the similar way (B). Washing formulation solution with the final volume was adjusted to 200 mL with purified dislilled water. Selection for the best washing procedure conditions, initially 200 ml of four washing solution were

conducted. Subsequently after selecting of 1 best washing procedure condition through substituting one variables in formulation at a time approach and the selected best optimized condition was used for the subsequent selection of next process condition by exploiting the (Table 3.2) different proportion for the detergent solutions formulations.

Table 3.2: washing solutions and its ingredients for formulating 500ml of cleaning solution

	Volume (mL)			
Components	**B[a]**	**B[a]+L[b]**	**B[a]+D[c]**	**B[a]+D[c]+L[b]**
Tris-aminomethane	80	80	80	80
Detergents	-	-	100	50
Lipase (100U/ml)	-	20	-	20
Distilled water	120	100	20	-

[a]Buffer; [b]lipase; [c]detergent

3.2.6.8. Washing analysis and olive oil determination

The Washing process was conducted through the soiled cloth was dipped in particular washing solutions by shaking the flask contents at 25 °C for half an hour at 120 rpm. Subsequently after shaking for half an hour, soiled cloth was washed with 100 ml dislilled water at 37 °C for 10 min and dried with air. Selection for the best washing circumstances, washing procedure was conducted with the different temperatures and different time intervals exploiting different concentrations of detergent and lipase catalytic activities. Extraction procedure of olive oil was implemented by exploiting petroleum ether organic solvent from the particular washed cotton cloth. Residual olive oil weight was determined following after complete disappearance of petroleum ether from the resulted extract.

Following equation used for the % of oil removal analysis.

$$\textbf{Residual (\%)} = \frac{(W_i - W_r)}{\textbf{Wr}} \times 100$$

Where,

W_i is weight of whole olive oil before cleaning process in mg

W_r is weight of whole olive oil cleaning process in mg.

CHAPTER. 4

4. RESULTS

Section # 1

4.1. Isolation and identification of extracellular thermolabile protease producing psychrotrophic *Stenotrophomonas sp.* PAK01

Glaciers in Karakorum Range of mountains (Pakistan) are archives of psychrotrophic bacteria. Bacterial alkaline protease is usually produced during starvation and sporulation other than stress of pH and temperature etc. Five facultatively psychrotrophic bacteria were isolated from glacial samples. A thermolabile alkaline protease was purified and characterized from one of the glacial isolates identified as *Stenotrophomonas sp.* PAK01.

4.1.1. Sampling

Soil, water and ice samples were obtained from glaciers (Juglot, Jutial and Rakaposhi) in Northern Areas of Pakistan (Table 4.1). On average, glaciers were found at 35´36 and 74´27 at globe with average physical parameters as: height, 3000 m; Atmospheric pressure, ~790mb; Temperature range, -3 to 18°C; and pH 5.5 to 7.0 in the month of July 2017.

4.1.2. Isolation of bacteria from samples obtained from glaciers

Out of 20 samples, a total of 5 isolates were found capable of growing at low temperature (2-37°C). The glacial isolates include PAK01, PAK02, PAK03, PAK10 and PAKP1 isolated from different samples of water and soil, respectively.

4.1.3. Psychrotrophic Alkaline protease producing bacteria

Total thirty prominent bacterial colonies were screened from soil and water of Juglot, Jutial and Rakaposhi glacier, producing cold-active extracellular protease. Isolated psychrotrophic bacterial colonies were streaked onto casein agar petri dishes with reaction mixture (yeast extract (1%), NaCl (0.5%), agar (2.0%), and casein (1%) at pH-8 Tris-Hcl buffer. Kept the inoculated plates at 18±2 °C for 2 days. Proteolysis of casein substrate observed by the application of 30% trichloroacetic acid on the exterior surface of nutrient agar. A transparent halo across the inoculum growth was measured as being a positive reaction. Depending upon larger hydrolysis zone on 1 % casein agar plates incubated at 18±2 °C for 48 hours, four isolates were subjected to production of enzyme in the fermentation broth media as well as for 16S r RNA

analysis. One isolate was observed to have the potential for maximum enzyme (Unit/ml) production.

4.1.4. Identification of potential isolate and phylogenetic analysis

The universal primers (forward primer 27F′ and reverse primer 1492R′) for the amplification of 16S rRNA were able to amplify the region giving 1465 base pair fragment of the predicted size in all of the isolated strains. On the basis of 16S rRNA gene alignment with BLAST search from the GenBank database at NCBI (MIDILABS Inc., USA) that showed 99% homology with *Planococcus sp.*, *Exiguobacterium sp.*, *Pseudomonas aeruginosa* and *Stenotrophomonas sp* in the existing NCBI database. The phylogenetic tree of four of the psychrotrophic isolates was constructed using software MEGA7 (Tamura *et al.*, 2007). The 16S rRNA gene sequence of four psychrotrophic bacterial strains *Planococcus sp.*, *Exiguobacterium sp.*, *Pseudomonas aeruginosa* and *Stenotrophomonas sp* were submitted to NCBI GenBank under accession numbers MG757351, MG662179, MG679513 and MG662181 (Table 4.2).

Table 4.1: Sampling Data Sheet

Sr.#	Location	Transect name	Latitude	Longitude	Temp (°C)	Pressure	pH	Height
Glacial Isolates								
Rakaposhi Glacier								
			GPS Coordinates					
1.1	Water	Lake water	36°14'36.8	074°26'57.6	6	795	6	2823
1.2	Glacier	Glacier ice	36°14'35.6	074°26'58.1	-1	790	6.5	2868
1.3	Ice	Lake ice	36°14'35.6	074°26'57.7	0	794	6	2823
1.4	Lake head	Stationary lake water	36°14'34.2	074°26'58.3	6	796	6	2816
1.5	Soil	Deep soil	36°14'34.0	074°26'57.2	18	790	6.5	2821
1.6	Pond	Stagnant water	36°14'34.8	074°26'56.7	15	793	6	2841
Juglot Glacier								
2.1	Water	Lake water	35°41'06.2	074°37'26.2	4	795	6	1983
2.2	Soil	Surface soil	35°41'06.5	074°37'26.2	15	790	6.5	1988
Jutial Glacier								
3.1	Ice	Lake ice	35°5427.6	074°19'84.1	-1	784	5.5	1511
3.2	Water	Lake Water	35°5427.3	074°19'84.4	6	785	6	1510
3.3	Soil	Deep soil	35°5426.7	074°19'83.7	0	784	6	1510

Table 4.2: Identity and accession numbers of selected strains

Isolate	Identified Microorganisms	Accession Number
PAK01	*Stenotrophomonas sp.*	**MG662181**
PAK02	*Exiguobacterium sp.*	**MG662179**
PAK03	*Pseudomonas peli*	**MG687270**
PAK10	*Planococcus sp.*	**MG757351**
PAKP1	*Pseudomonas aeruginosa*	**MG679513**

4.1.5. Phylogenetic studies of glacial strains

4.1.5.1. *Stenotrophomonas sp.* PAK01

Stenotrophomonas sp. PAK01 (MG662181) presented highest nucleotide sequence resemblance through Nucleotide Blast exploration with *Stenotrophomonas maltophilia* strain (KP858919) with 98% sequence coverage with (e-value 0.0). Evaluation of Maximum parsimony was accomplished with 1000 bootstrap repeats beside values presented by nodes. 5 evolutionary steps represented by calibration bar (Figure 4.1).

4.1.5.2. *Exiguobacterium sp.* PAK02

Exiguobacterium sp. PAK02 (MG662179) presented highest nucleotide sequence resemblance through Nucleotide Blast exploration with *Exiguobacterium undae* (JX122621) and *Exiguobacterium sibiricum* (KM817276) with 99% and 98% sequence coverage with (e-value 0.0). Evaluation of Maximum parsimony was accomplished with 1000 bootstrap repeats beside values presented by nodes. 5 evolutionary steps represented by calibration bar (Figure 4.2).

4.1.5.3. *Planococcus sp.* PAK10

Planococcus sp. (MG757351) presented highest nucleotide sequence resemblance through Nucleotide Blast exploration with *Planococcus* sp (GU217715) with 99% sequence coverage with (e-value 0.0). Evaluation of Maximum parsimony was accomplished with 1000 bootstrap repeats beside values presented by nodes. 5 evolutionary steps represented by calibration bar (Figure 4.3).

4.1.5.4. *Pseudomonas aeruginosa* PAKP1

Pseudomonas aeruginosa (MG679513) presented highest nucleotide sequence resemblance through Nucleotide Blast exploration with *Pseudomonas aeruginosa* (MF144458) with 99% sequence coverage with (e-value 0.0). Evaluation of Maximum parsimony was accomplished with 1000 bootstrap repeats beside values presented by nodes. 5 evolutionary steps represented by calibration bar (Figure 4.4).

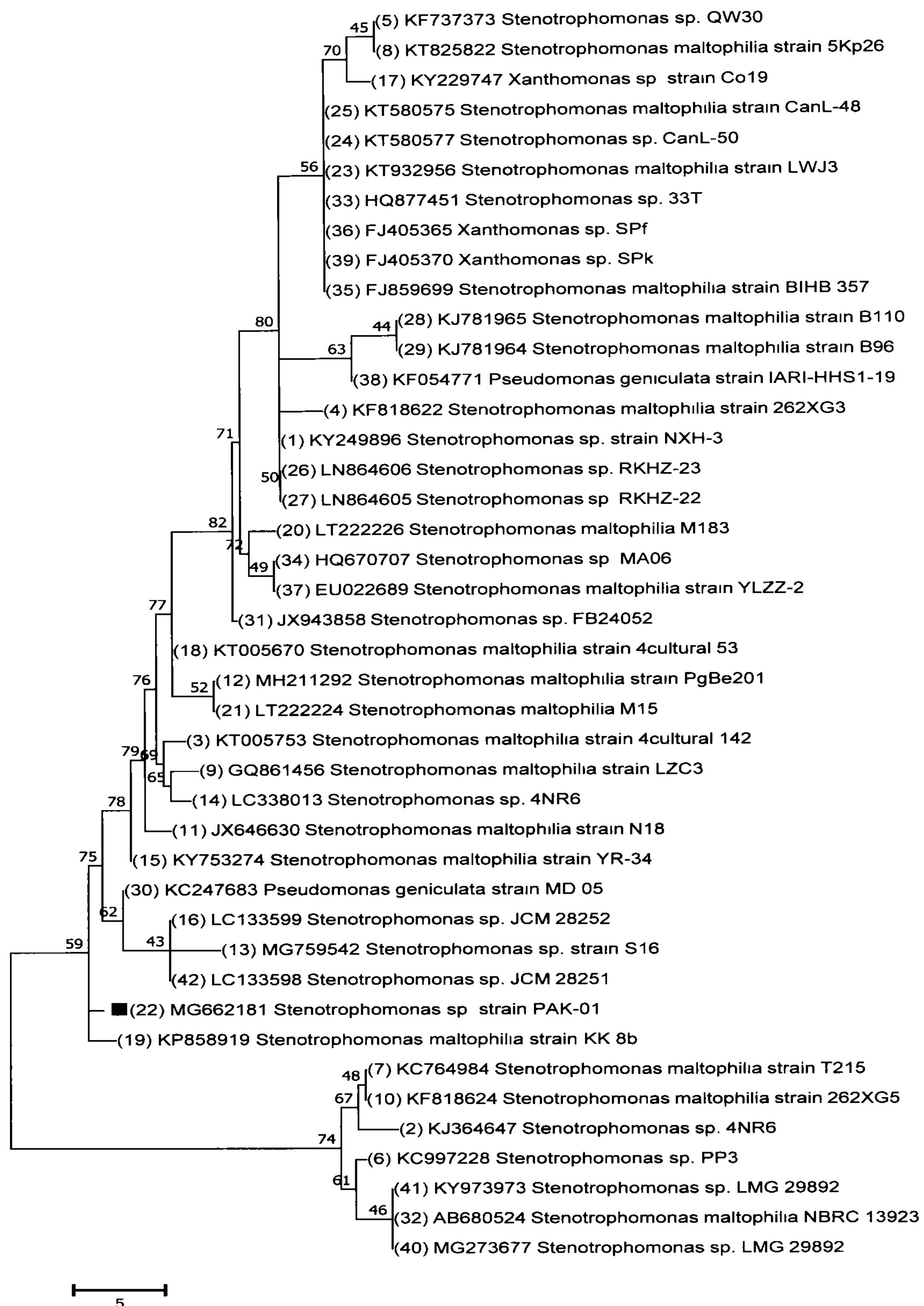


Figure 4.1: Phylogenetic tree of *Stenotrophomonas sp.* PAK01

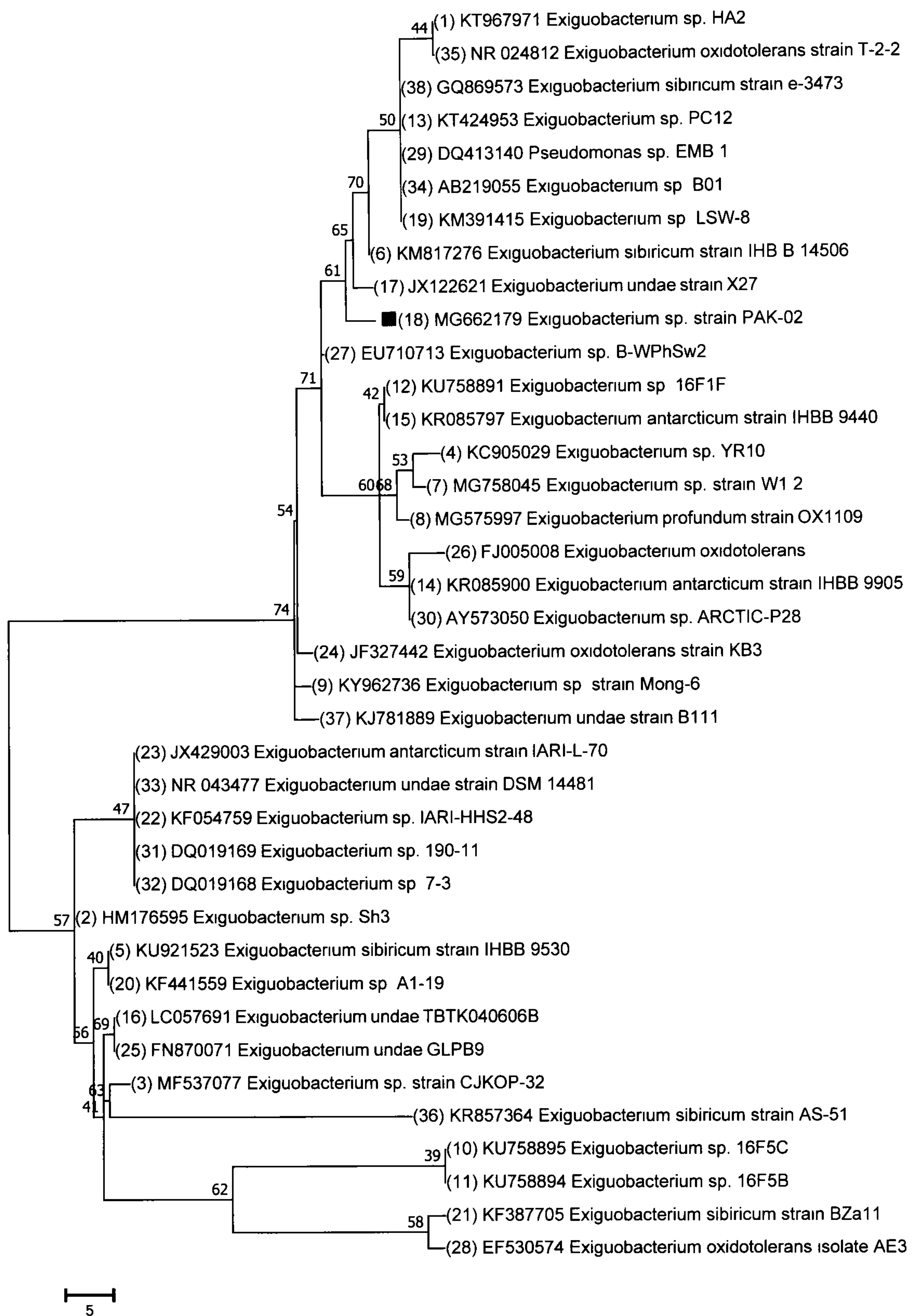


Figure 4.2: Phylogenetic tree of *Exiguobacterium sp.* PAK02

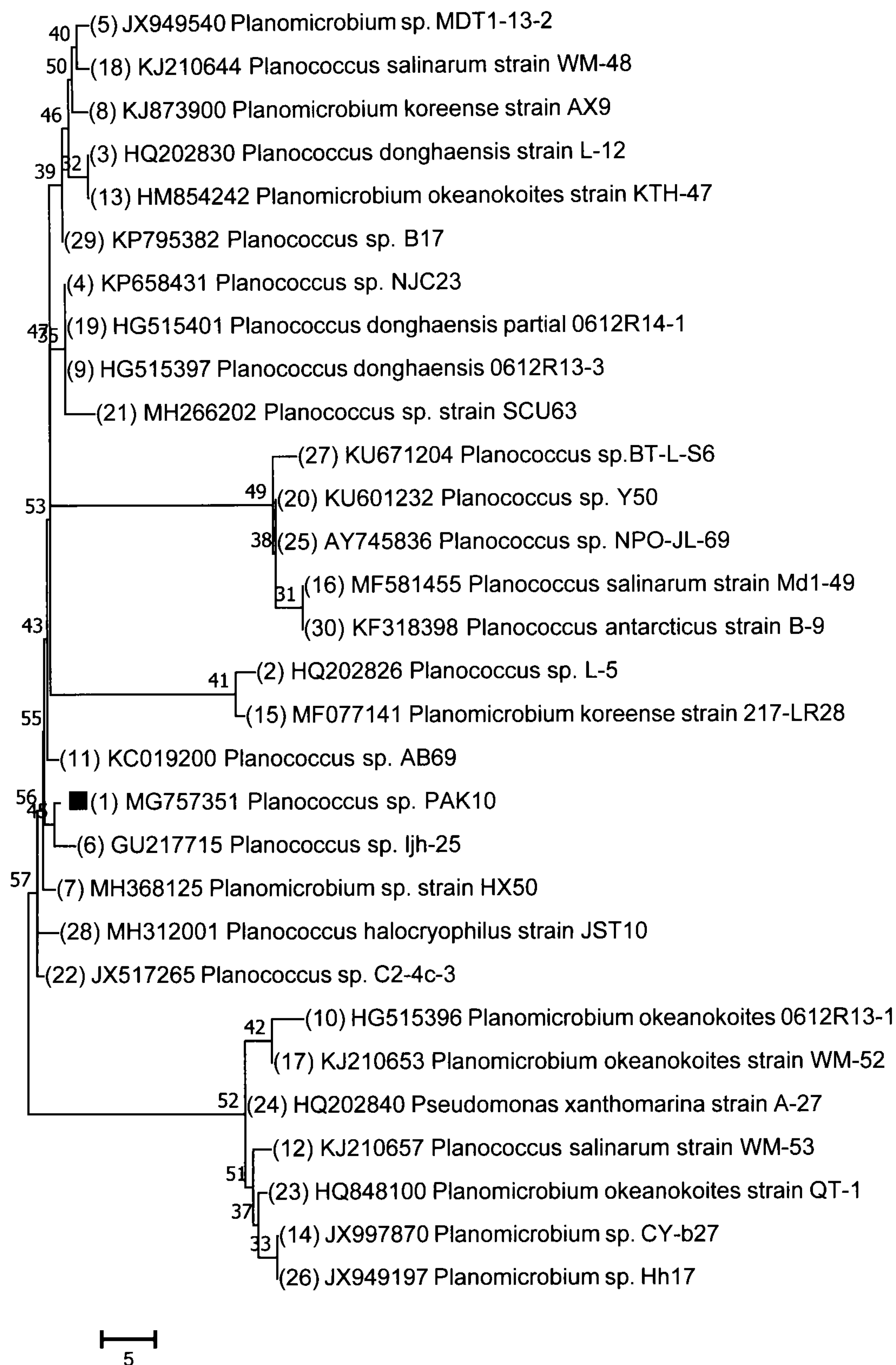


Figure 4.3: Phylogenetic tree of *Planococcus sp.* (MG757351)

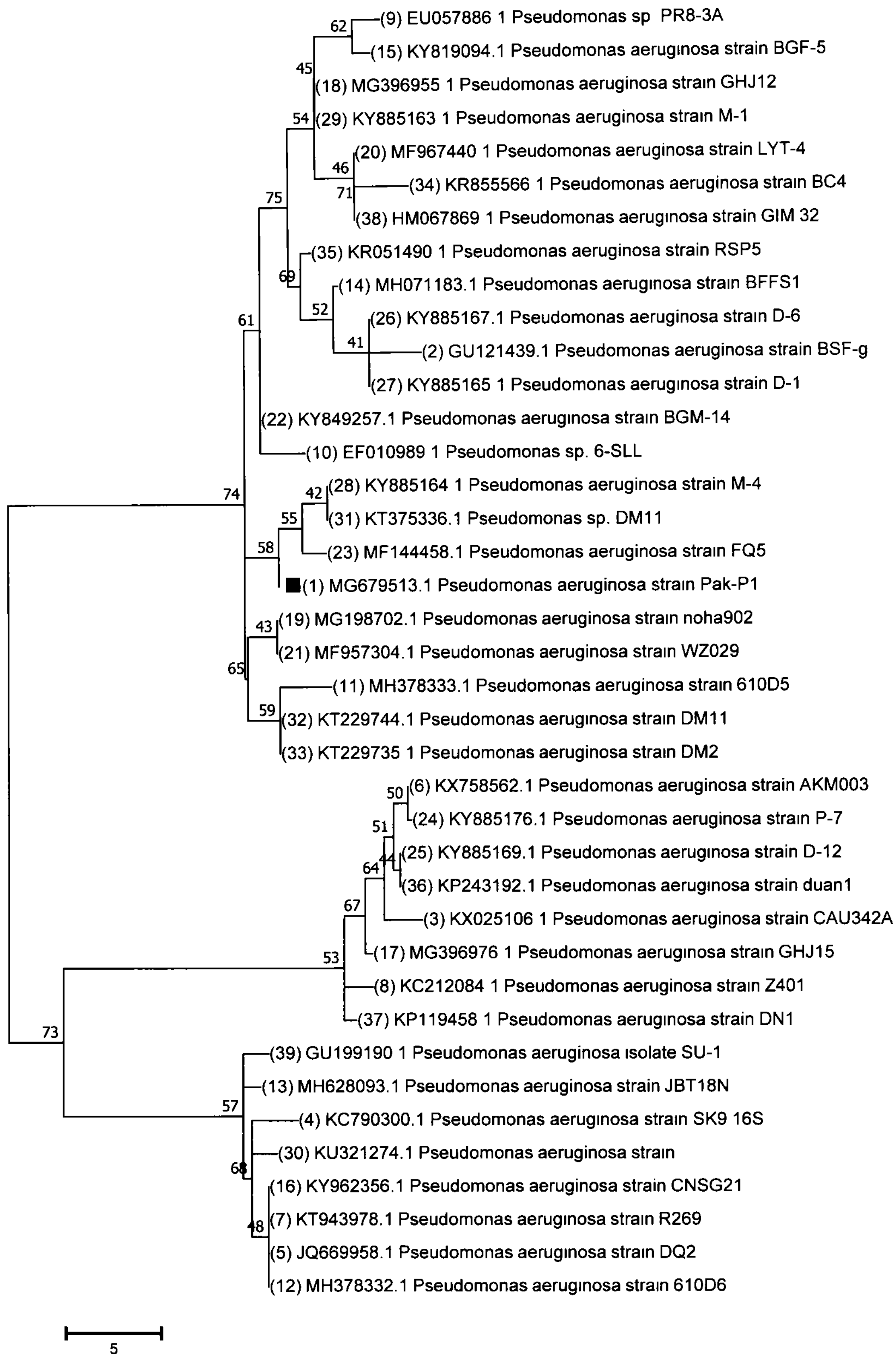


Figure 4.4: Phylogenetic tree *Pseudomonas aeruginosa* (MG679513)

4.2. Molecular Cloning and Expression in *Escherichia coli* BL21(λDE3)

4.2.1. Protease nucleotide sequence and proteomic study

Serine protease digestion of linear chain of polypeptide residues resulted in short amino acid monomers of peptides sequences and purified domain of protein was analyzed through the mass spectrometry evaluation. The resulted short peptide sequences analysis was implemented by searching for identical monomers of peptides residues sequences that were already existing in protein databases. After mass spectrometric analysis of the obtained short chain of peptide monomers we observed resemblance with the trypsin of *Stenotrophomonas* peptidase S8 family (WP_032977471) and Pr2 protein (AEL88836).

The post-translational modification of protein encrypted by the whole genome sequence of the gene was resulted in to serine protease and was submitted in NCBI with the accession numeral of (MH375859 GenBank deposited number). Amplified nucleotide size of the protease gene of *Stenotrophomonas sp.* PAK03 observed was 1740 bp (Figure 4.5) and exhibited a 96 % resemblance to the pr2 nucleotide sequence encoding trypsin of *S. maltophilia* strain and accessible in GenBank database (JF317278). 1740 bp nucleotide sequence of the serine protease gene was translated into amino acid sequence and was observed a polypeptide chain of 578 monomers of amino acid with molecular weight of 58 kDa. Protein comprise Numerous domains discovered in serine peptidase belonging to family of peptidase protein S8 domain in the position of 172 to 448 and proprotein-processing peptidases of the bacteria that digest C-terminal domain to combined basic monomers of amino acids and was examined between the protein sequence at position of 501 and 567 aa (Figure 4.6) (http://www.ncbi.nlm.nih.gov/protein).

Resulted sequence of polypeptide amino acid observed was exhibited 96 % resemblance to the Pr2 sequence of peptidase protein (AEL88836). Amino acid arrangement of the conserved domain observed has been very related for both protein sequences. It was observed that the catalytic triad in these aligned protein sequences are in the identical place as for Histidine 237 and Aspartate 177, whereas in case of Serine residue, has been observed located at AA position at 409 in the Pr2 protein sequence and in the situation of PrY protein sequence, it was observed changed to region of amino acid at 410 in the protein chain. Maximum variation in the considered

protein domains occurred partially in the active hydrolytic region amongst the amino acid chain at 340 and 405.

From the analysis it was observed that the PrY protein domain designated existence of the serine peptidase enzyme S8_S53 protein domain (serine peptidase at S8 and S53 families, sequencing analysis showed in between AA region from 170 to 443), thus recommending a resemblance to the serine peptidase family S8 and subfamilies containing S8A (subtilisin), S53 (Sedolisin) and S8B (Kexin). From the analysis that serine peptidase domain such as S8-S13 was observed in the Pr2 sequence at AA region from 170-442. Peptidases families of S8 and uncharacterized subdivision 13 domain observed in the protein sequence of the Pr2 of *Stenotrophomonas maltophilia* arrangement in between position of 170 to 442 (Figure 4)

Protein sequence for the S8-53 serine peptidase superfamily was observed in Pr2 (AEL88834) wherever a compatibility of 90% was exhibited with the studied PrY peptidase enzyme.

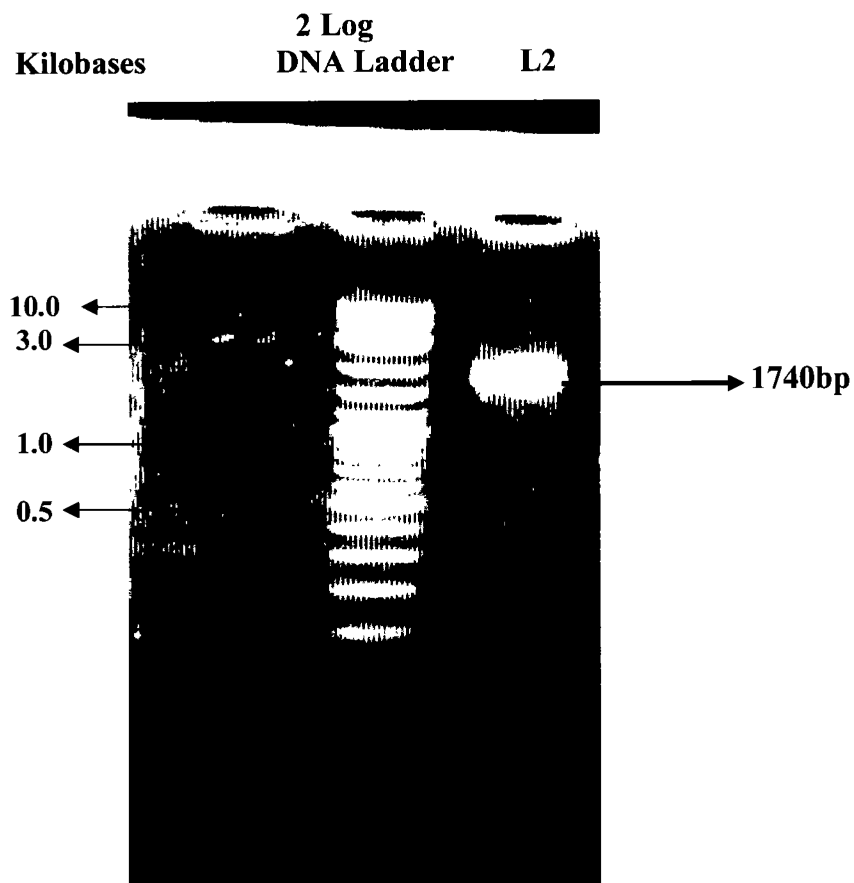


Figure 4.5. Representative agarose gel electrophoresis image of YLIP gene PCR products. **L1**: 2-Log DNA Ladder, **L2**: YLip gene of 1740 bp

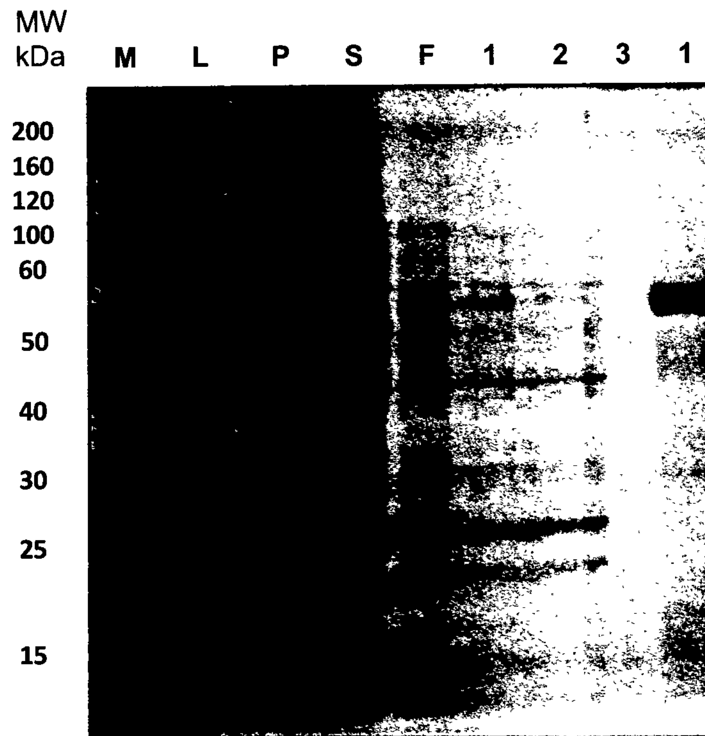


Figure 4.7: SDS gel after nickel chelate affinity chromatography. The fusion protein was expressed in *E. coli* strain BL21. A lysate (L, lane 2) was made in Cell Lytic B and separated by centrifugation into a pellet (P, lane 3) and a supernatant (S, lane 4) fraction. The supernatant was applied Affinity Gel column and the flow through (F, lane 5), wash (1-3, lanes 6-8) and elution (1, lanes 9) fractions were collected HisTrap column, respectively.

```
BAQ55742      MSQVTQPRVRRVWVVLGASVLSSLLLATPALAGDVQLSGLQSAQTHQRFIVKFRDGSAPV  60
PrY           MSQVTQPRVRRVWVVLGASVLSSLLLATPALAGDVQLSGLQSAPTHQRFIVKYRDGSAPV  60
Pr2           MSQVTQPRVRRVWVVLGASVLSSLLLATPALAGDVQLSGLQSAPTHQRFIVKYRDGSAPV  60
S8            MSQVTQPRVRRVWVVLGASVLSSLLLATPALAGDVQLSGLQSAPTHQRFIVKYRDGSAPV  60
              *******************************************  ********:*******

BAQ55742      ANTTALASSLKTAAAGIASSQGRALGLQQVRRLAVGSTVVRTDRALDQAESELLMRKLAA  120
PrY           ANTTALASSLKTAAAGIASSQGRALGLQQVRRLAVGSTVVRTDRALDQAESELLMRKLAA  120
Pr2           ANTTALASSLKTAAAGIASSQGRALGLQQVRRLAVGSTVVRTDRALDQAESELLMRKLAA  120
S8            ANTTALASSLKTAAAGIASSQGRALGLQQVRRLAVGSTVVRTDRALDQAESELLMRKLAA  120
              ************************************************************

BAQ55742      DPNVEYVDVDQIMRATLTPNDTRFSEQWGFGTSNASINVRPAWDKATGTGVVVAVIDTGI  180
PrY           DPNVEYVEVDQIMRATLTPNDTRFSEQWGFGTSNASINVRPAWDKATGTGVVVAVIDTGI  180
Pr2           DPNVEYVEVDQIMRATLTPNDTRFSEQWGFGTSNASINVRPAWDKATGTGVVVAVIDTGI  180
S8            DPNVEYVEVDQIMRATLTPNDTRFSEQWGFGTSNASINVRPAWDKATGTGVVVAVIDTGI  180
              *******:****************************************************

BAQ55742      TNHADLNANILPGYDFISDAAMARDGGGRDSNPNDEGDWYGDNECQAGYPGSNSSWHGTH  240
PrY           TNHADLNANILPGYDFISDAAMARDGGGRDSNPNDEGDWYGANECGSGIPASNSSWHGTH  240
Pr2           TNHADLNANILPGYDFISDAAMARDGGGRDSNPNDEGDWYGANECGSGIPASNSSWHGTH  240
S8            TNHADLNANILPGYDFISDAAMARDGGGRDSNPNDEGDWYGANECGSGIPASNSSWHGTH  240
              ***************************************** *** :* *.*********

BAQ55742      VAGTVAAVTNNSTGVAGTAFNAKVVPVRVLGKCGGYTSDIADAIVWASGGTVSGVPANAN  300
PrY           VAGTVAAVTNNSTGVAGTAFNAKVVPVRVLGKCGGYTSDIADAIVWASGGTVSGVPANAN  300
Pr2           VAGTVAAVTNNSTGVAGTAFNAKVVPVRVLGKCGGYTSDIADAIVWASGGTVSGVPANAN  300
S8            VAGTVAAVTNNSTGVAGTAFNAKVVPVRVLGKCGGYTSDIADAIVWASGGTVSGVPANAN  300
              ************************************************************

BAQ55742      PAEVINLSLGGGGSCSTTYQNAINGAVGRGTTVVVAAGNSKHQRILVGTGELPERDRGGG  360
PrY           PAEVINMSLGGGGSCSSTYQNAINGAVGRGTTVVVAAGNSNTNVSSAVPANCPNVIAVAA  360
Pr2           PAEVINMSLGGGGSCSSTYQNAINGAVGRGTTVVVAAGNSNTNVSSAVPANCPNVIAVAA  360
S8            PAEVINMSLGGGGSCSTTYQNAINGAVGRGTTVVVAAGNSNTNVSSAVPANCPNVIAVAA  360
              ******:*********:***********************: :   .  .: *:    ..

BAQ55742      DHLGRRTCQLLQLRHRHRYLRTG-------PEHPV--HPQQRHHHPGAAPVTRPTNGTMS  410
PrY           TT---------SAGARASFSNYGTGIDISAPGQSILSTLNSGTTTPGS-ASYASYNGTMS  410
Pr2           TT---------SAGARASFSNYGTGIDISAPGQSILSTLNSGTTTPGS-ASYASYNGTSM  409
S8            TT---------SAGARASFSNYGTGIDISAPGQSILSTLNSGTTTPGS-ASYASYNGTMS  410
                         .   *  : . *       * : :    :.    **:       *****

BAQ55742      AAPHVAGVVALMQSVAPSPLSPAQVESIIKSTARPLPGACSGGCGAGIIDADAAVTAAIN  471
PrY           AAPHVAGVVALMQSVAPSPLSPAQVESIIKSTARPLPGACSGGCGAGIIDADAAVTAAIN  470
Pr2           AAPHVAGVVALMQSVAPSPLSPAQVESIIKSTARPLPGACSGGCGAGIIDADAAVTAAIN  470
S8            AAPHVAGVVALMQSVAPSPLSPAQVESIIKSTARPLPGACSGGCGAGIIDADAAVTAAIN  470
              ************************************************************

BAQ55742      GTTPNPGGTVLQNNVPVTGLGAASGASLSYTVNVPAGSTQLRVAISGGSGDADLYLRQGS  531
PrY           GTTPNPGGTVLQNNVPVTGLGAASGASLSYTVNVPAGSTQLRVAISGGSGDADLYLRQGS  530
Pr2           GTTPNPGGTVLQNNVPVTGLGAASGASLSYTVNVPAGSTQLRVAISGGSGDADLYLRQGS  530
S8            GTTPNPGGTVLQNNVPVTGLGAASGASLSYTVNVPAGSTQLRVAISGGSGDADLYLRQGS  530
              ************************************************************

BAQ55742      APTDTVYTCRPYLSGNNETCTVNSPTAGTWYVRVKAYSTFSGVTVNAQY  580
PrY           APTDTVYTCRPYLSGNNETCTVNSPTAGTWYVRVKAYSTFSGVTLNAQY  579
Pr2           APTDTVYTCRPYLSGNNETCTVNSPTAGTWYVRVKAYSTFSGVTLNAQY  579
S8            APTDTVYTCRPYLSGNNETCTVNSPTAGTWYVRVKAYSTFSGVTVNAQY  579
              ********************************************:****
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Figure 4.6: Amino acid sequence alignment of enzyme PrY with alkaline proteases from Pr2 [*Stenotrophomonas maltophilia*] GenBank: AEL88836, peptidase S8 [*Stenotrophomonas*] NCBI Reference Sequence: WP_032977471, serine protease [*Stenotrophomonas maltophilia*] GenBank: BAQ55742. Asterisk (*) means that the residues in that column are identical in all sequences in the alignment. (:) means that conserved substitutions have been observed. (.) means that semi-conserved

substitutions are observed. PrY possesses a catalytic triad consisting of Asp 177 (yellow), His 237 (yellow) and Ser 410 (yellow).

4.2.2. Expression and purification of protease gene PrY

The protease nucleotide sequence was cloned into the pET28a plasmid multiple cloning region of T7 promoter with N-terminal His_6 residues. Subsequently, *E. coli* BL21 (DE3) was made into recombinant cell. PrY proteolytic gene was expressed in the existence of 1 mM IPTG. Intracellular proteins were collected through ultrasonication of recombinant cells of BL21 (DE3)/pET28a /PrY, Recombinant PrY gene protease protein was expressed as a soluble fraction in DE3 and purified by nickel chelate chromatography (Figure 4.7) followed another technique of sepharose column chromatography (Table 4.3). As shown in (Figure 4.8, lane Q) PrY exhibited as a 58 KDa mass of protein on Coomassie blue SDS tainted gel.

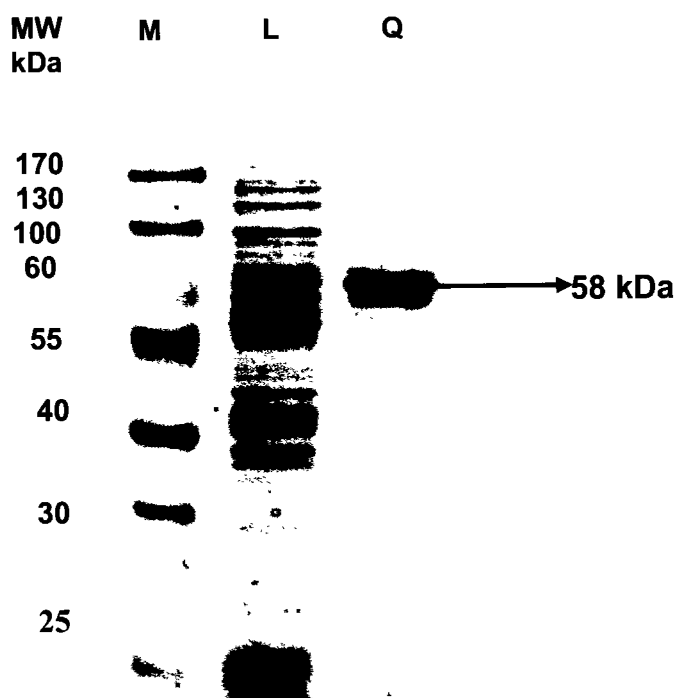

Figure 4.8: Expression and Purification of Pry. SDS gel after anion-exchange column chromatography. M, molecular weight marker. L, cell lysate. Q, eluted fraction from Q- Sepharose column (13.5mL). The molecular weight of Pry was calculated to be 58 kDa.

Table 4.3: Purification of Pry from BL21 (DE3).

Purification steps	Total protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purity (%) Fold
Cell lysate	110	9763	88.5	100	36
Nickel-chelate column	32	6265	197.26	64	79.28
Q-Sepharose column	19	4653	245	48	100

4.3. Production optimization of extracellular thermolabile alkaline protease from *Stenotrophomonas sp.*

4.3.1. Psychrotrophic protease producing bacteria

Determination of quantitative measurement of the proteolytic catalytic analysis revealed that the four wild bacterial isolates have the potential that produce thermolabile proteases with varying concentrations. As segment of the preliminary selection criteria, proteolysis for separately of the 5 wild selected strains were measured through fermentation production cultures for 72 hours at 20°C at with Tris-Hcl buffer at pH 9.

From the results it was showed that *Planococcus sp.*, *Exiguobacterium sp.*, *Pseudomonas aeruginosa sp.* and *Pseudomonas peli* secreted a hydrolytic activity of 162 U/mg, 195 U/mg, 234 U/mg and 332 U/mg crude protease enzyme however *Stenotrophomonas* sp. showed significantly higher protease activity ($p<0.0001$) in supernatant (463 U/mg) (Figure 4.9); therefore, it was selected for further study.

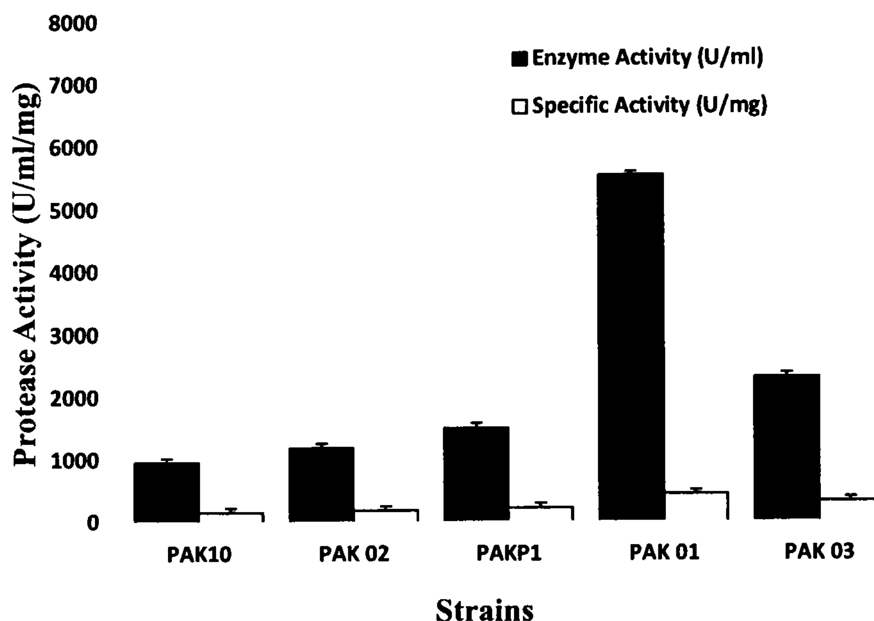

Figure 4.9: Screening for alkaline protease activity in supernatant of bacterial cells. All values are represented as mean ± sd of three replications.

4.3.2. Tyrosine Standard Curve

To formulate the standard curve 2 mg/ml of Tyrosine standard solution with reaction mixture (Na_2CO_3 with concentration of 0.5M, 50 mM of potassium phosphate buffer, pH 7 and attenuated 1N Folin reagent) and were experimented. Prerequisite quantity of potassium phosphate buffer and Tyrosine were mixed in every experiment tube excluding blank. Subsequently 2.5 ml Na_2CO_3 was added to reaction mixture in individually tube involving blank. Following 500µl Folin reagent chemical was used and the reaction mixture was mixed instantaneously and sustained for 30 minutes at 25 °C. Conclusively, OD was analyzed at 660nm exploiting spectrophotometer and the tyrosine curve was mapped (Figure 4.10).

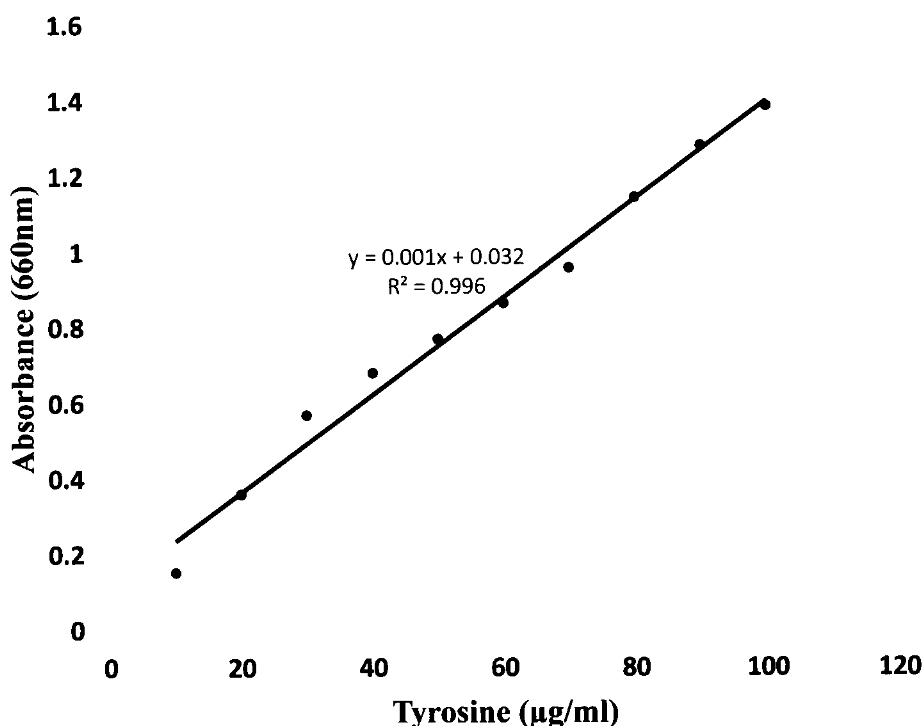

Figure 4.10: Tyrosine (µg/ml) standard curve

4.3.3. Optimization production of cold active alkaline protease

Eight diverse media were used for inoculum development in batch culture fermentation procedure, medium # 8 (Saba *et al.*, 2012) contains 15g/500ml soymeal, 22.5g/500ml crude protein, 3g/500ml crude fat, 9.5g/500ml carbohydrate, 4g/500ml NaCl, 0.5g/500ml sodium glutamate, 2.5g/500ml yeast extract, 0.75g/500ml KH2PO4 and 0.15g/500ml MgSO4 at pH 9 (Tris-Hcl buffer) was found best stimulator for protease enzyme as compared to other production fermentation reaction mixture. After isolation and screening, the shake flask batch culture experiment was conducted for 120 hours for detection of maximal enzyme production. For to check the effect of culture media on enzyme production through the production time course for 3 days, the pH of the culture filtrate remained more than 8 with increased and decreased growth of the test organism.

Of the 6 media investigated, medium no 8 was found most suitable for the production of the active alkaline protease with enzyme activity 4516.43 U/ml at pH 8, 150 rpm and 18°C after 72 hours of incubation. As the medium contains very least amount of salts per liter, is the best cost efficient medium at lab scale as well as for commercial scale enzyme production. Therefore, it was used as the basal culture medium for production in the following studies.

Alkaline protease production by strain PAK01 began after 48 h and reached maximum at 96 h (573 U/mg) in logarithmic phase at 20 °C ($P<0.0001$) (Figure 4.11). However, the cell growth was maximal at 120 h (Figure 4.12) of incubation which indicate that the production of enzyme was independent to cell growth. Optimum temperature was found to be 25°C, over 96 h of incubation ($P<0.0001$) (Figure 4.13). However, progressively deterioration was observed in catalytic activity because of the proliferation in incubation temperature and it was observed that the enzyme activity is nearly inhibited at 50°C.

Highest catalytic activity value for protease enzyme was observed by incubation of production medium with Tris-Hcl buffer at pH 9 and was significant proliferation whereas correlating with 7 pH. High alkaline condition at and above 10 pH decreased proteolysis. Though, from the results it was observed that protease was found stable at alkaline circumstances. pH conditions for microbial fermentation strongly influence several biocatalytic reactions and transport of chemical substance across the cell membrane (Figure 4.14) it was well-defined that proteolysis was

highest at pH 9 (P<0.0001) (502 Units/mg). Thermolabile protease secreted outstanding to its potential alkaline nature appears to exist of substantial importance towards industrial applications for example tannery (leather processing), sewage treatment and as an additive ingredient in detergent formulations.

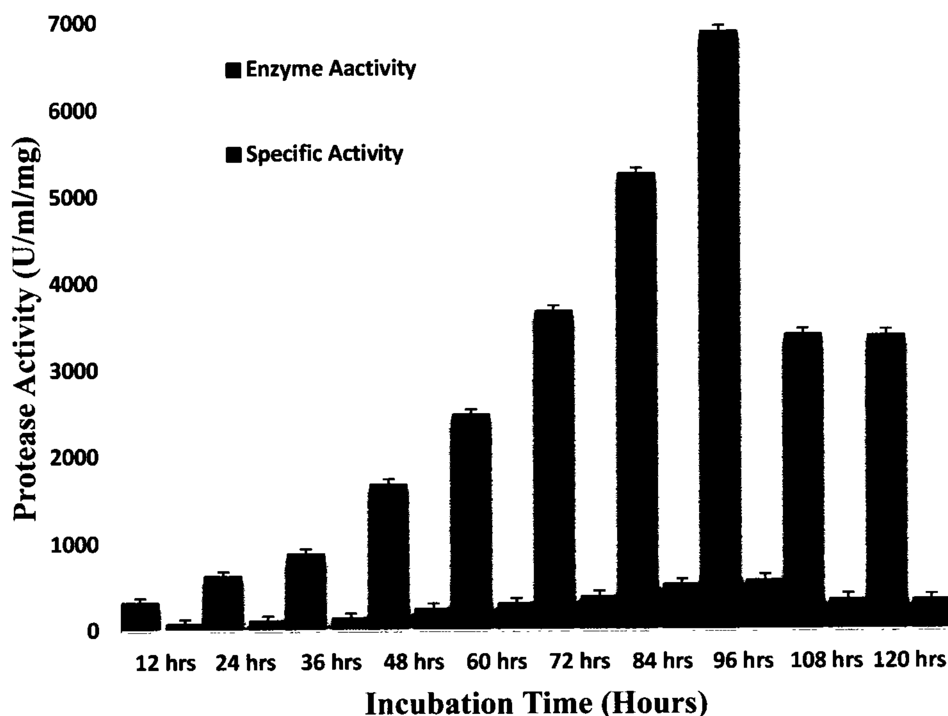

Figure 4.11: Effect of Incubation period on the production of alkaline protease at 20°C. All values are represented as mean ± sd of three replications.

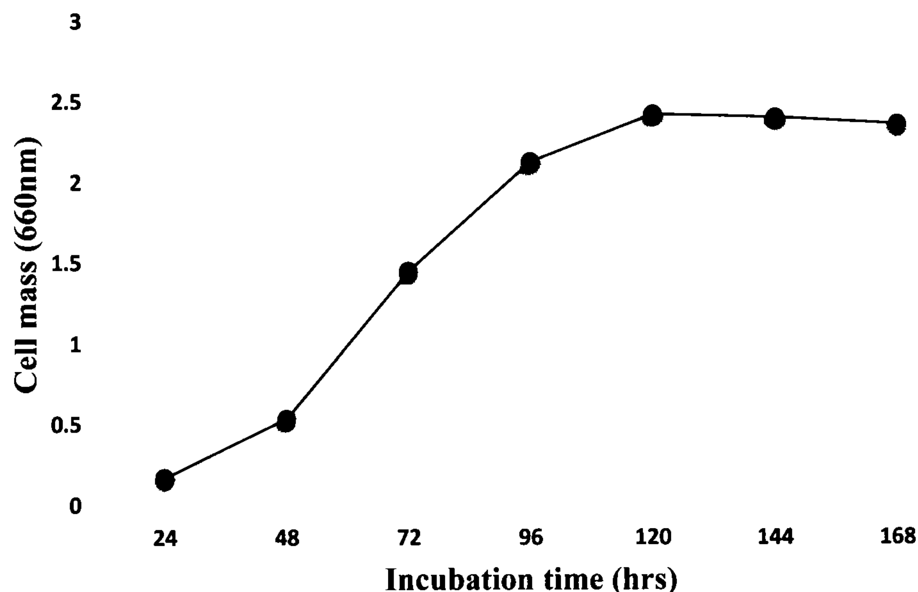

Figure 4.12. Effect of incubation period on growth by *Stenotrophomonas sp.* Pak1 at 20 °C

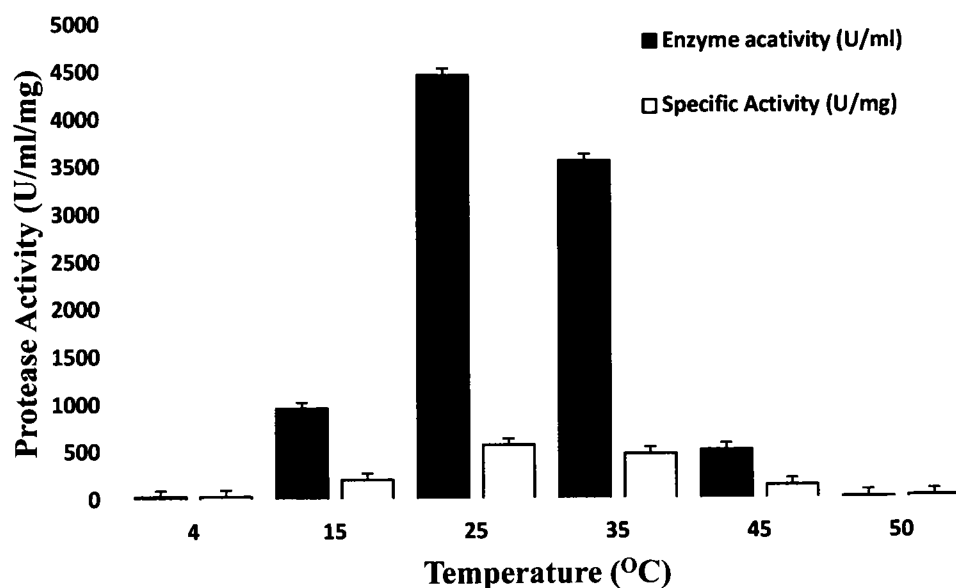

Figure 4.13: Effect of Different growth temperature on production of alkaline protease. All values are represented as mean ± sd of three replications.

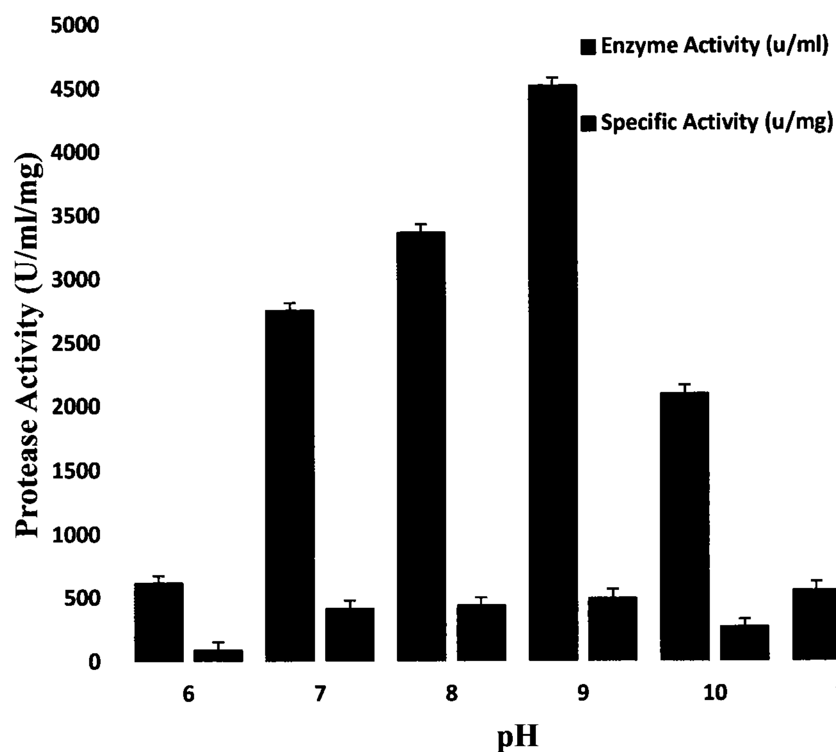

Figure 4.14. Effect of pH on enzyme production by PAK01 after 96 h of incubation at 25 °C. All values are represented as mean ± sd of three replications.

Exploitation of best sources of carbon and nitrogen are essential for highest production of protease enzyme, because these can considerably diminution the cost. Therefore, utilization of various carbon, organic, inorganic and amino acid nitrogen sources were evaluated. Among the various nitrogen sources checked and supplementary added at 1% strength to the fermentation reaction, it was observed that the organic source of nitrogen, optimum production was observed with yeast extract (516 U/mg) ($P<0.0001$) (Figure 4.15), followed by tryptone, peptone and casamino acid. In case of inorganic nitrogen source like sodium nitrate, potassium nitrate and trisodium citrate, maximum production was experimented with ammonium sulfate ($P<0.0001$) (478 U/mg), showed stimulatory effect on cold active alkaline protease production (Figure 4.16).

Among the different carbon sources utilized for protease production, maximum protease activity of (486 U/ mg) was found when glucose was supplemented into the medium followed by sorbitol and fructose ($P<0.0001$) (Figure 4.17). Similar findings were reported by (Gomma *et al.*, 1990) and (Sen and Satyanarayana, 1993) where the maximum protease production was observed when the carbon source was maintained at glucose and starch respectively. Recent interpretations are in good coordination with previous analyses showing highest protease production observed when glucose was used as carbon source in the culture broth (Pastor *et al.*, 2001).

The inoculum was added to the production medium in varying concentration of 1%, 5%, 10%, 15% and 20%. Among the inoculum size, enzyme activity was observed at 5% was (548 U/mg) showed significant stimulatory effect on protease production ($P<0.0001$) (Figure 4.18). It is quite evident from the results that maximum activity (543 U/ml) (Figure 4.19) was observed after 96 hours of incubation ($P<0.0001$) when 24 hours old grown broth culture of *Stenotrophomonas sp.* was used as an inoculum for production of protease.

Heavy metals existent in environments accomplish significant role in the cell biomass. Proteolytic hydrolysis observed was stimulated by $CaCl_2$ (544 U/mg) and $MgCl_2$ (442 U/mg) which showed stimulatory effect on protease production. The other heavy metals such as $HgCl_2$, $FeCl_3$ and $ZnSO_4$ have no considerable influence although maintain approximately more than 20% of enzyme production ($P<0.0001$) (Figure 4.20).

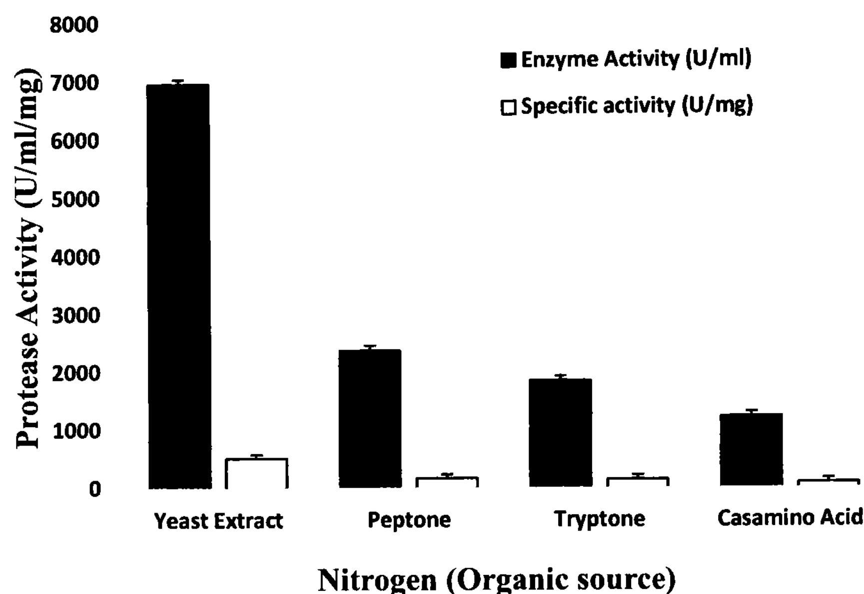

Figure 4.15: Effect of Nitrogen (organic) source on Alkaline protease production by *Stenotrophomonas sp.* (PAK01). All values are represented as mean ± sd of three replications.

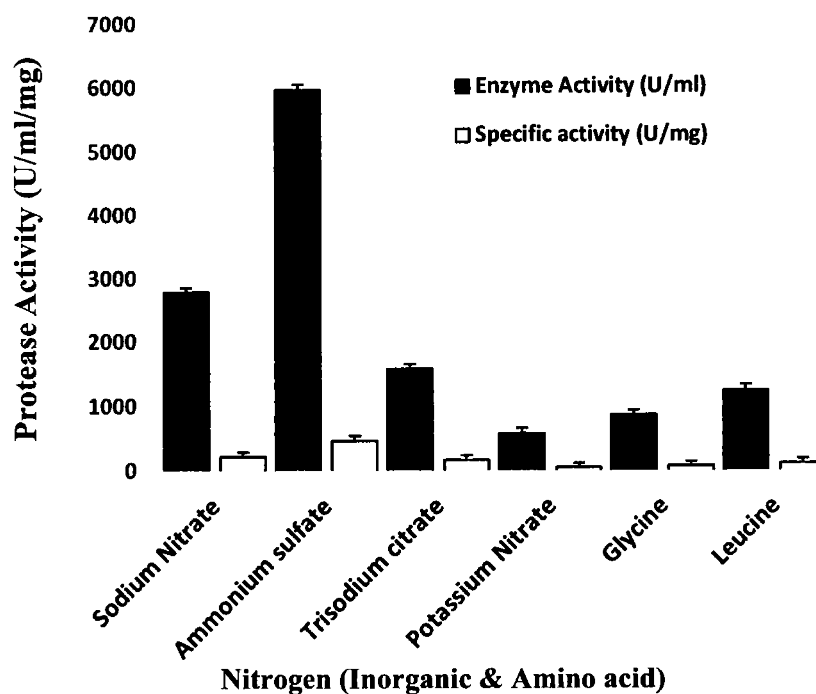

Figure 4.16: Effect of Nitrogen (Inorganic and Amino acid) Source on Alkaline protease production by *Stenotrophomonas sp.* (PAK01). All values are represented as mean ± sd of three replications.

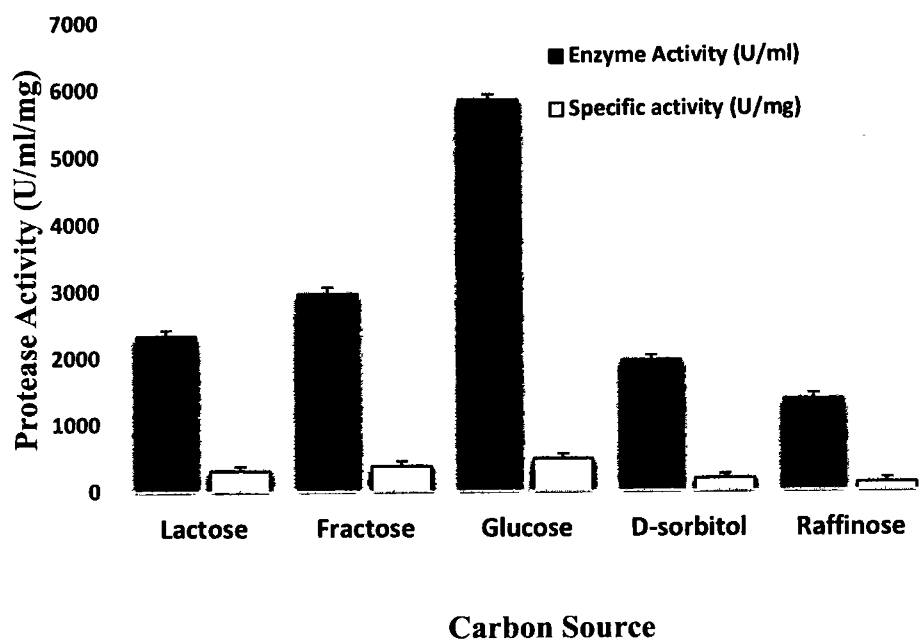

Figure 4.17: Effect of Different Carbon source on Alkaline protease production by *Stenotrophomonas sp.* (PAK01). All values are represented as mean ± sd of three replications.

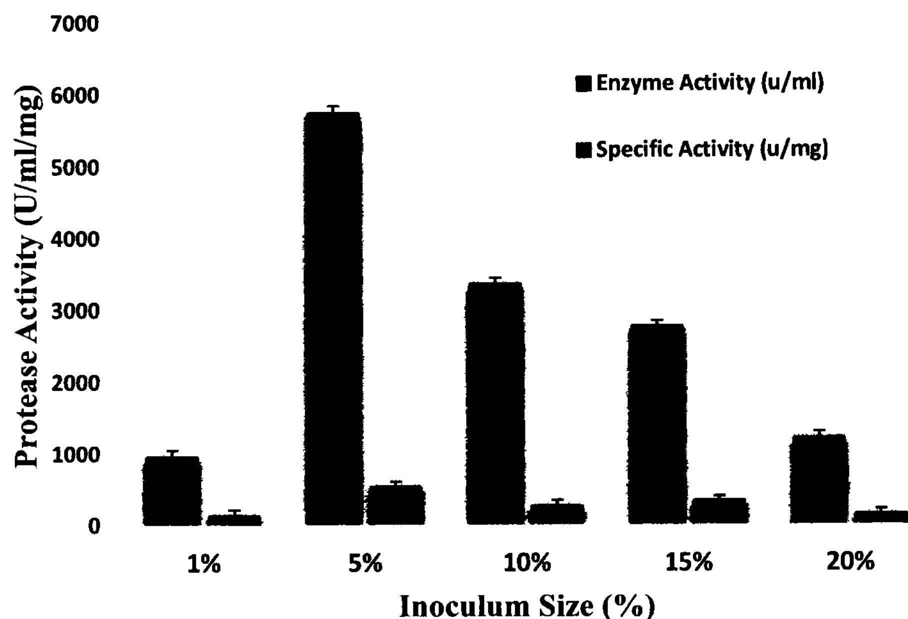

Figure 4.18: Effect of diverse size of inoculum on the production of alkaline protease by *Stenotrophomonas sp.* (PAK01). All values are represented as mean ± sd of three replications.

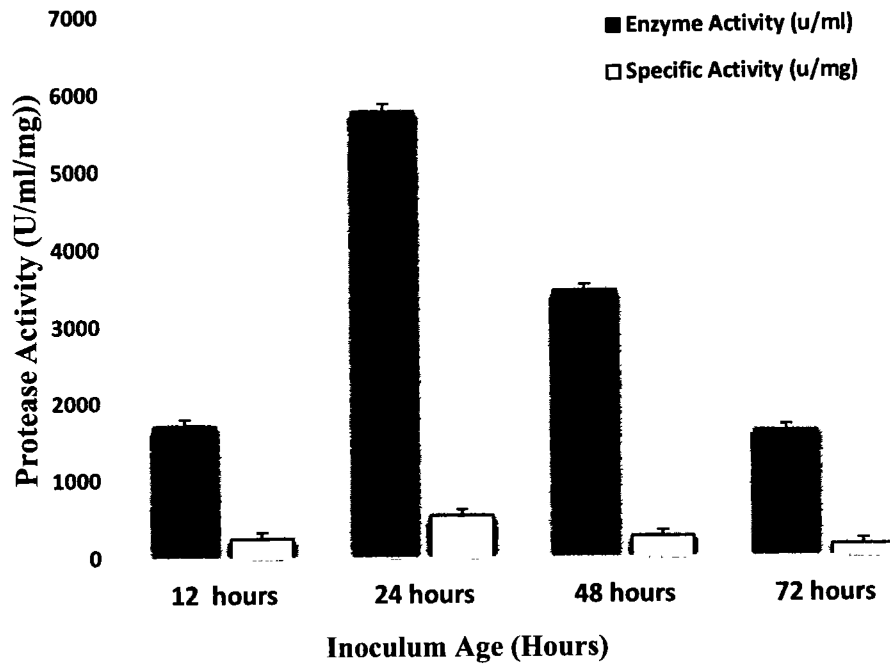

Figure 4.19: Effect of age of inoculum on the production of alkaline protease by *Stenotrophomonas sp.* (PAK01). All values are represented as mean ± sd of three replications.

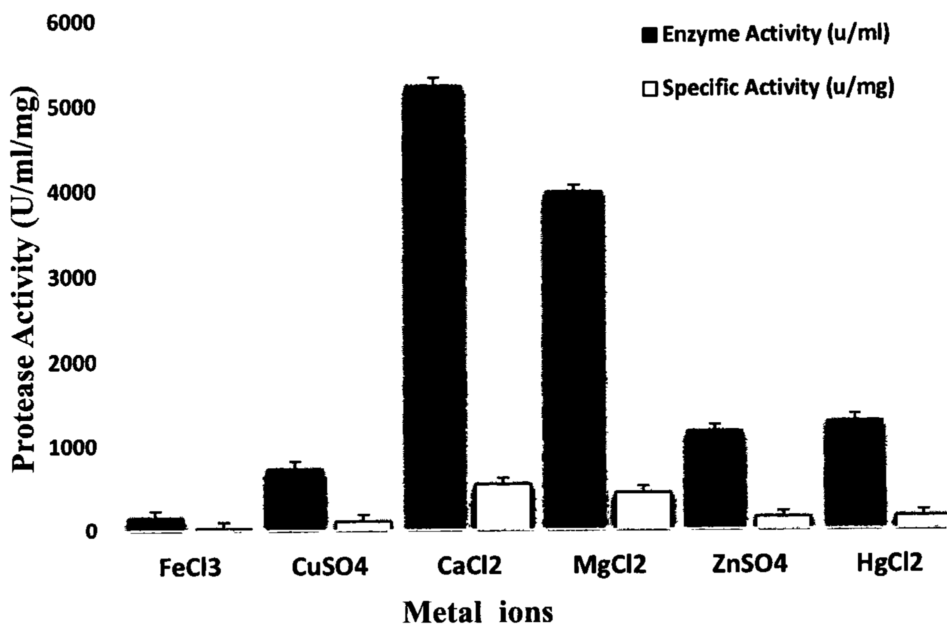

Figure 4.20: Effect of various metal ions on the production of alkaline protease by *Stenotrophomonas sp.* (PAK01). All values are represented as mean ± sd of three replications.

4.4. Characterization of alkaline and cold active protease enzyme from psychrotropic bacterium *Stenotrophomonas sp.* PAK-01 for detergent formulation

4.4.1. Enzyme purification

Highest enzymatic activity for *Stenotrophomonas sp* PAK01 discovered approximately after 96 hours of inoculation. Centrifugation of the bacterial culture PAK01 was carried out at low temperature (4°C) and 15,000*g* directed at 15 minutes, and the 800 mL crude enzyme supernatant was exploited for further purification steps. Cold active alkaline protease enzyme purification was performed with the crude enzyme supernatant of the *Stenotrophomonas* sp., PAK01 culture broth. Alkalophilic detergent stable protease biocatalyst secreted from *Stenotrophomonas sp.,* PAK01 purification has been conducted by selective 80% $(NH_4)_2SO_4$ precipitation then followed (IEX: SOURCEQ 10/10) DEAE-Cellulose Ion exchange and Superdex 200 PG 16/60 column phases. purification technique outcomes have been summarized in Table 4.4. All purification procedures were accomplished at 4 °C. Crude enzyme preparation of eight hundred milliliters (800 ml) was exposed to ammonium sulfate precipitation at concentration of 80% through gentle continuous stir. Next the saturated ammonium sulfate suspension was kept at low temperature 4°C for overnight incubation. Subsequently after centrifugation at 20,000*g* for 15 minutes, the collected precipitate protein has been suspended in a least quantity of 20 millimolar Tris-HCl buffer via pH 8.0. Maximum specific activity of protein was accomplished at 80% $(NH_4)_2SO_4$ proteins amino acid precipitation. This 80% $(NH_4)_2SO_4$ proteins precipitation step reduced protein content residues via approximately 38% besides subsequently, the specific activity (U/mg) observed was improved nearby twofold. The active alkalophilic enzyme fraction concentrated near 15 mL has been loaded on DEAE-Cellulose Ion exchange chromatography equilibrated previously via 20 millimolar Tris-HCl buffer by pH 8. Active fractions were gotten via sodium chloride $(NaCl_2)$ gradient at concentration by 0.3 Molar elution (0.1-1 M). Elution of protein was carried out at 60 mL/h flow rate and fractions of 5-mL were collected. Protease activity of proteins with 2 peaks (peaks A and B) were separated by DEAE-Cellulose Ion exchange chromatography (IEX: SOURCEQ 10/10) (Figure 4.21).

The results suggest that PAK01 secreted protein perhaps is probably an alkalophilic protease biocatalyst (cathodic movement in acidic solution states). The pooled fractions from (A) peak of the previous step has been loaded to a Superdex 200 PG 16/60 gel column then equilibrated through 20 millimolar Tris-HCl buffer of 8 pH. Next the Superdex 200 PG 16/60 gel column followed rinsed with the identical buffer reaction solution, subsequently then elution has been accomplished through a linear gradient of sodium chloride ($NaCl_2$) via 0 to 0.5 Molar concentration. So that fractions mixture thus exhibited maximum hydrolytic activity of alkalophilic enzyme has been concentrated in a particular single peak which has been eluted via sodium chloride ($NaCl_2$) gradient at concentration of 300 mM. Fractions showed highest activity were collected, pooled and concentrated through Amicon centriprep- 30K concentrator (Sigma-Aldrich). Hydrolytic activity of both the points was assayed and the fraction with high catalytic activity was subjected to Gel Filtration Chromatography (GFC: Superdex 200 PG 16/60) (Figure 4.22) and the purified thermolabile fractions with high enzymatic catalysis from both the peaks was eluted and subjected on SDS-PAGE analysis (Figure 4.23). Under silver staining protease protein showed purity with an expected 58 KDa molecular mass respectively. Purified monomer proteins were observed from the protein analysis. Specific activity of 1171 U/mg with purification fold of 55.9 and yield recovery of 20.43% was observed from the overall purification procedure.

Table 4.4: Purification of the cold-adaptive alkaliphilic protease from *Stenotrophomonas* sp. PAK-01.

Purification steps	Total Activity (U)	Total protein (mg)	Specific Activity (U/mg)	Purification fold	Yield (%)
Crude Extract	29825	1421.7	20.98	1	100
Ammonium Sulfate Precipitation	24233	534.6	44.58	2.124	81.25
Dialysis	20124	127.6	157.76	7.517	67.47
Ion Exchange Chromatography (IEX: SOURCEQ 10/10)	9828.8	16.3	612.99	29.22	32.95
Gel Filtration Chromatography (GFC: Superdex 200 PG 16/60)	6093.3	5.2	1171.79	55.853	20.43

- All steps were carried out at 4°C
- One unit of activity (U) was defined as the amount of the enzyme releasing 1µg of tyrosine per 1 min at 25°C
- Protein was eluted in a linear gradient between 0 and 0.5 M NaCl with 20 mM Tris-HCl buffer (pH 8.5) desalting was carried out by dialyzing against 10 mMTris-HCl buffers (pH8.0)
- Protein was eluted with 10 mM Tris-HCl buffer (pH8.0)

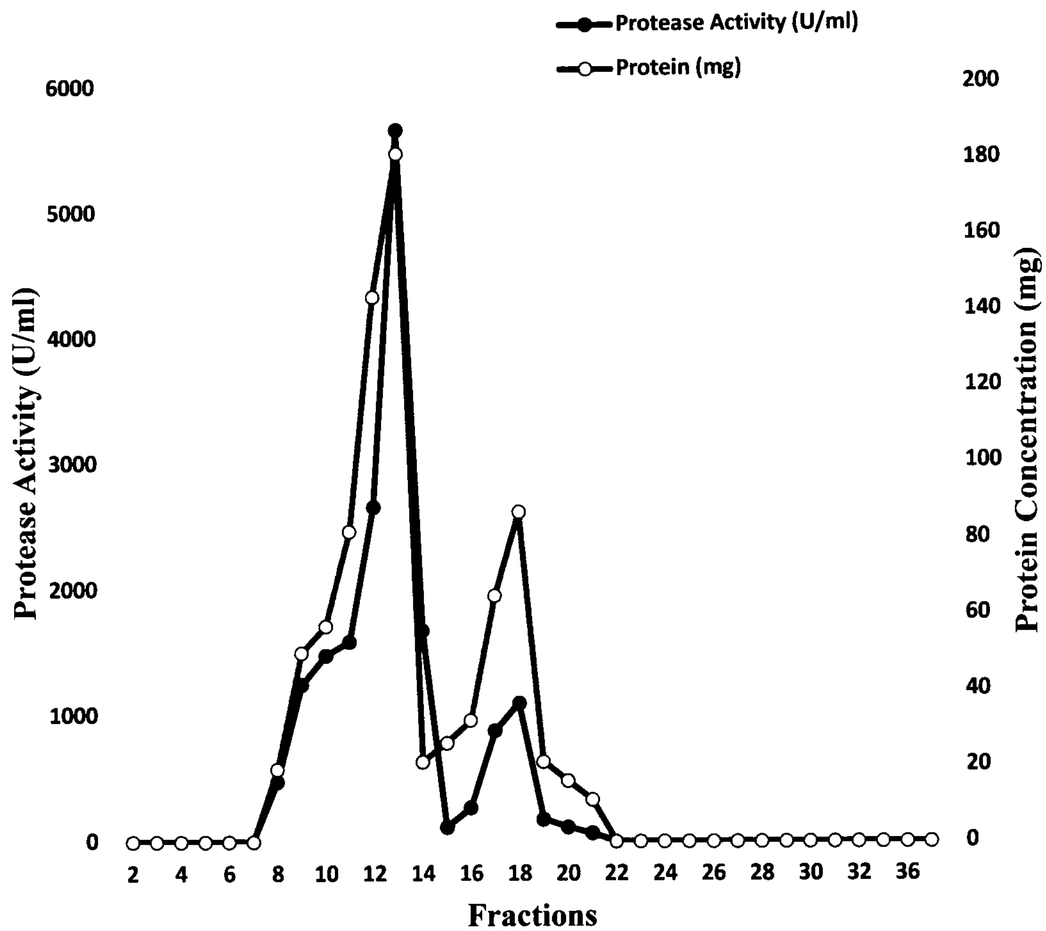

Figure 4.21: Chromatograph of alkaline protease on Ion exchange chromatography (IEX: SOURCEQ 10/10) column

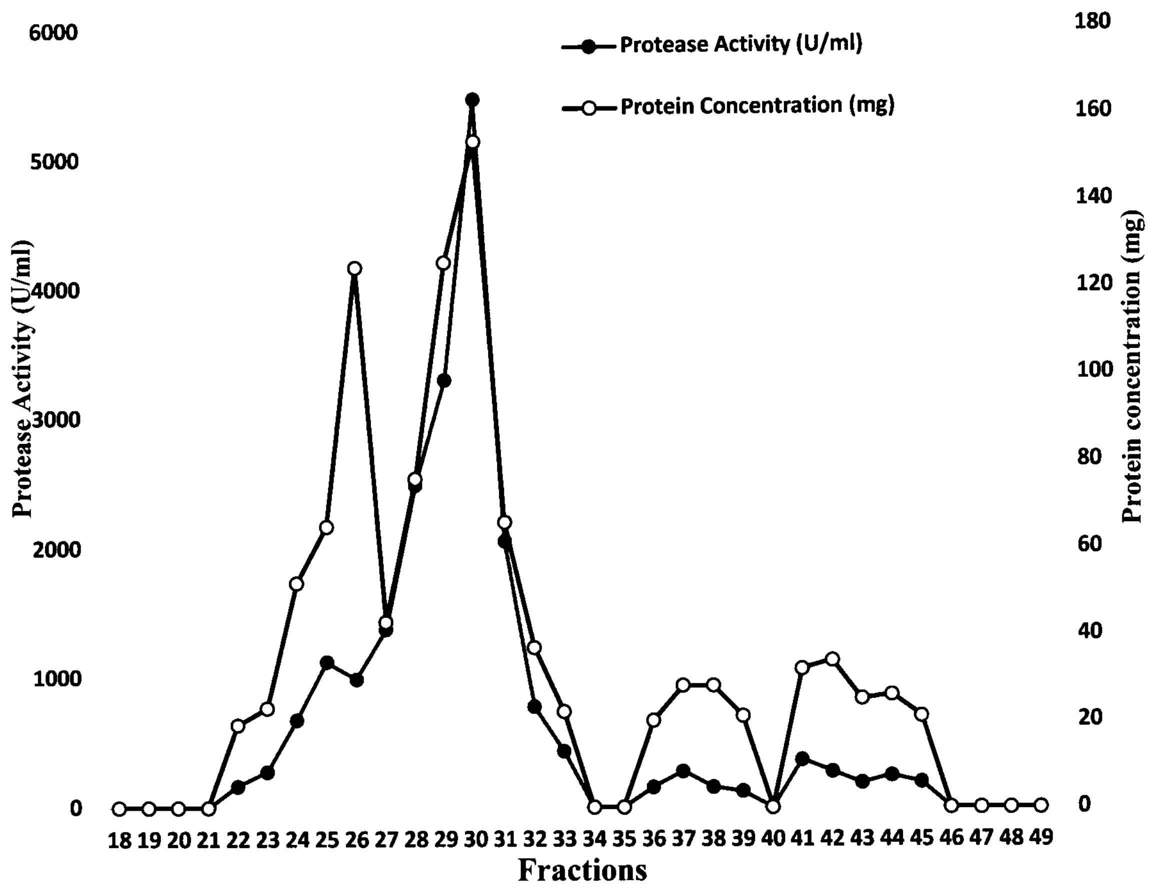

Figure 4.22: Chromatograph of alkaline protease on Gel Filtration Chromatography colum (GFC: Superdex 200 PG 16/60).

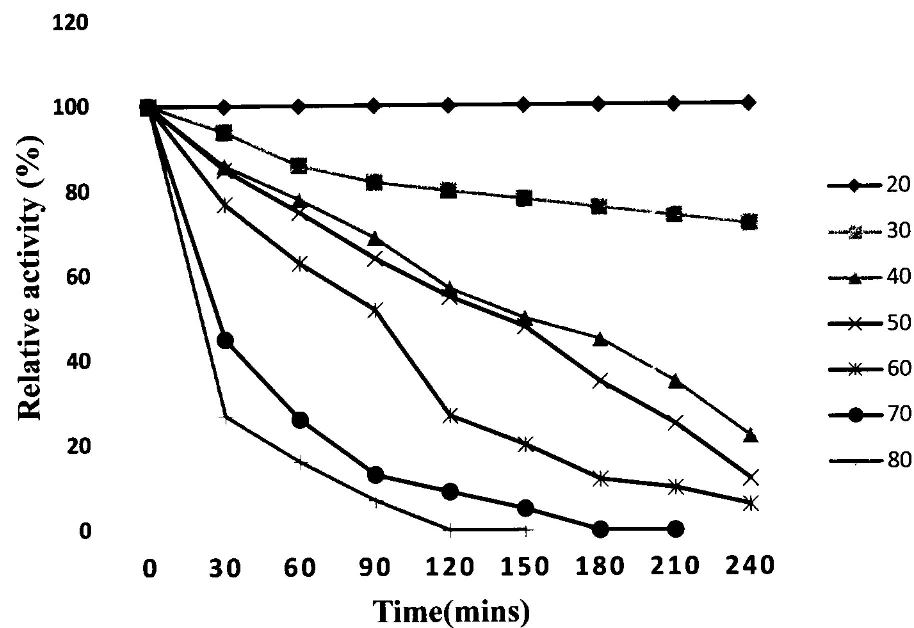

Figure 4.25: Effect of temperature on the stability of the protease produced by *Stenotrophomonas* sp.

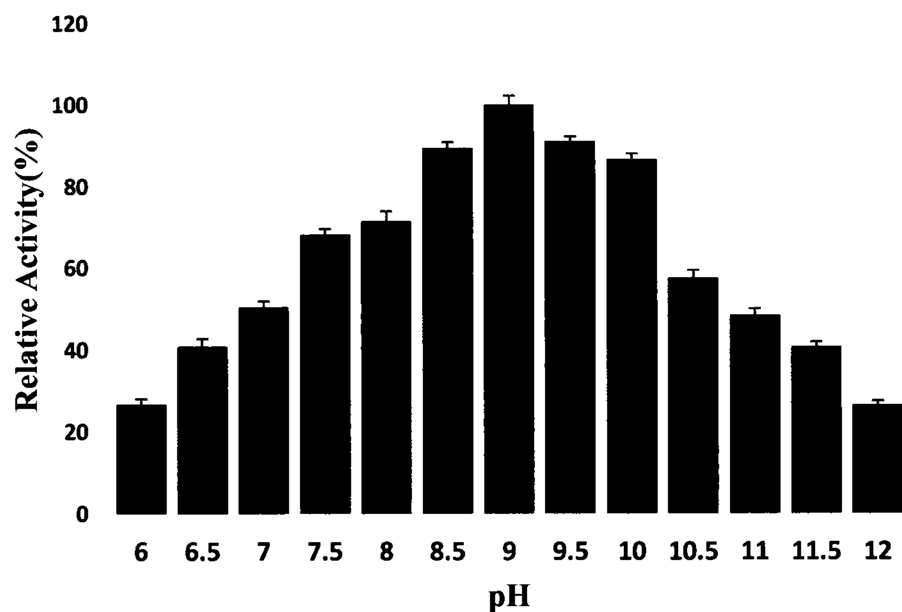

Figure 4.26: Enzyme activity and stability of purified cold-active protease from *Stenotrophomonas sp*, at various pH at 25 °C. The relative activity (%) was calculated relative to the case of reaction at which maximum activity was taken as 100 %. All values are represented as mean ± sd of three replications.

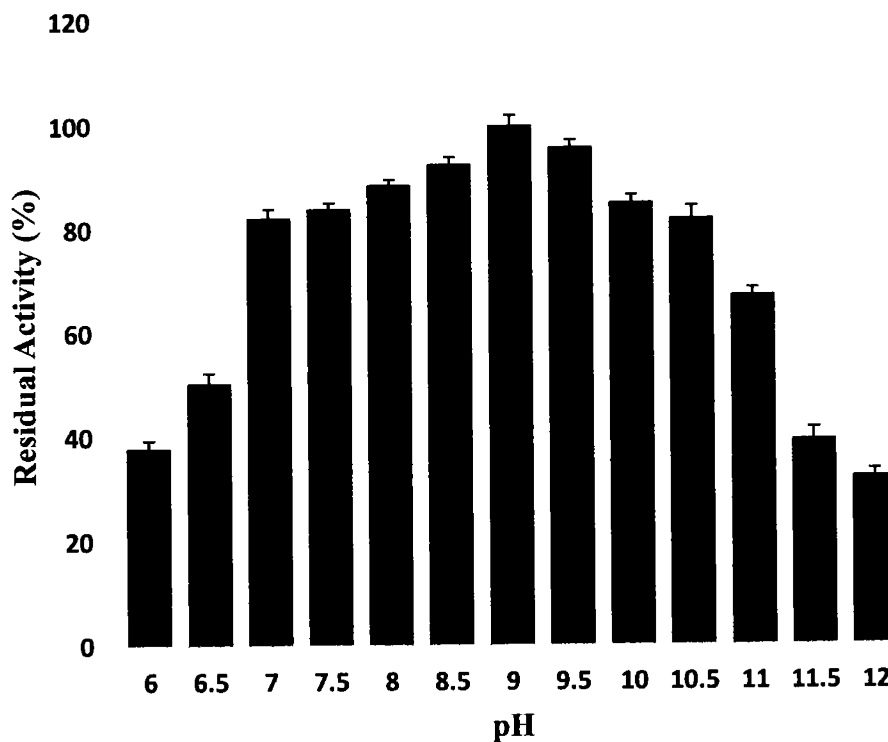

Figure 4.27: Enzyme stabilities of purified cold-active protease from *Stenotrophomonas sp*, at various pH at 25 °C. The relative activity (%) was analyzed relative to the instance of reaction at which greatest activity was taken as 100 %. All values are represented as mean ± sd of three replications.

4.4.3. Metal ions effect on hydrolytic protease activity

As summarized in Figure 4.28 different concentrations of metal ions such as 5, 10- and 15-mM consequence was experimented in the reaction mixture. Calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$) and potassium chloride (KCL) were observed have a stimulatory consequence on hydrolysis of protease and was observed having good hydrolysis of peptide bonds by the binding of the ions on the active catalytic triad of the protease enzyme.

In the presence of Manganese chloride ($MnCl_2$) and nickel sulfate Ni^{2+} in the reaction mixture having stimulating influence and approximately no influence on the enzyme activity have been found for Manganese chloride Mn^{2+} and nickel sulfate Ni^{2+}, respectively, at 5- and 10-mM concentrations, however 15 mM concentration was observed to have constraining effect on the hydrolytic activity of enzyme.

Hydrolytic activity observed was not significantly influenced by 5 mM $FeCl_3$, although by increasing gradually the concentration of $FeCl_3$, the residual activity for the enzyme observed was complitley inbibited. The negative consequences of Fe^{2+} and Cu^{2+} were progressively increased with concentration rise.

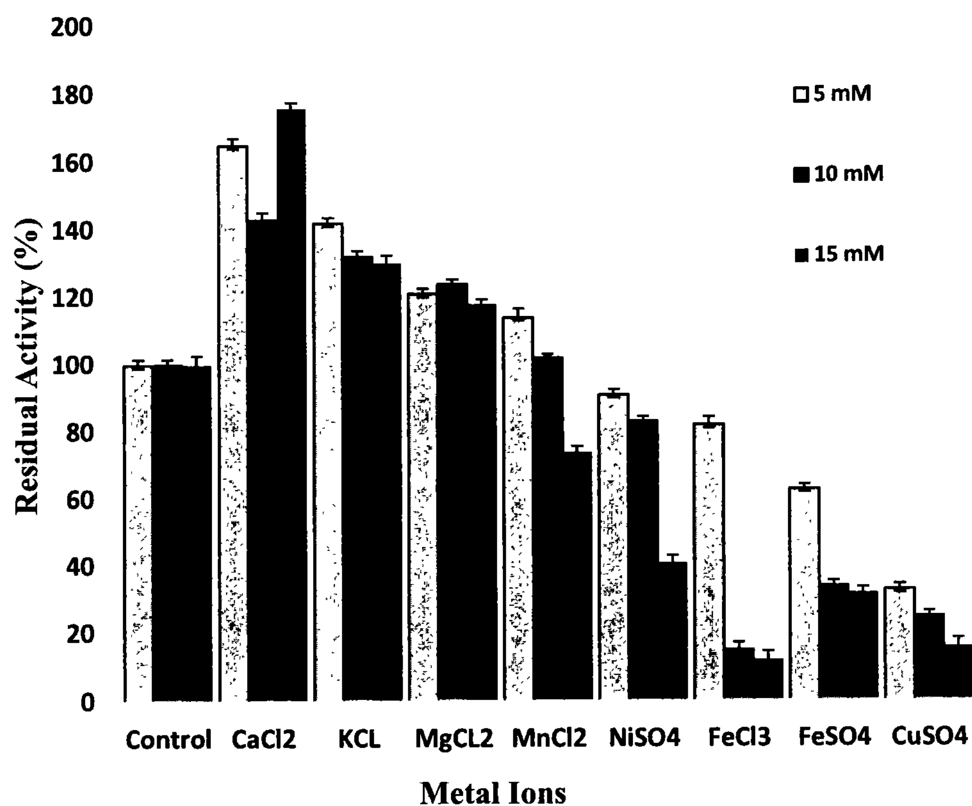

Figure 4.28: Effects of metal ions on the activity of the protease produced by *Stenotrophomonas sp.* All values are represented as mean ± sd of three replications.

4.4.4. Surfactants and oxidants effect on activity of protease hydrolysis

Protease enzyme catalytic activity against various surfactants showed great stability as shown in (Figure 4.29). Different Surfactant of Tween-20, Tween-80, and Triton X-100 of 5% concentration with purified thermolabile alkaline protease molecules in the reaction mixture showed stability of protease enzyme and exhibited stimulatory effect on residual activity which was observed retained among 84 to 112% effect of each surfactant (Tween-20, Tween-80, and Triton X-100) on enzyme activity after 12 to 36 hours of incubation at 25 °C.

PAK03 proteaes enzyme molecules still retained 87 and 79% original residual activity, respectively, even after subsequently incubation of thermolabile hydrolytic protease with 0.5% SDS and 1% H_2O_2 at 25 °C for 1 hour (Figure 4.30).

The stability of *Stenotrophomonas sp.* protease enzyme regarding oxidants and surfactants permit this enzyme as an active ingredient in detergents formulation.

4.4.5. Inhibitors effect on activity of Protease enzyme

Among different inhibitors, the purified thermolabile protease was moderately inhibited by 2-mercaptoethanol at 15 mM concentration and the hydrolytic actitivty of enzyme against protein substrate was affected by DTT and EDTA with a residual activity of 57% for both the inhibitors at concentration of 15 mM as shown in (Figure 4.31).

These results suggest that PAK03 may not be a metalloprotease, but only uses certain cations as stabilizers (Haddar *et al.*, 2009; Jellouli *et al.*, 2009). However, PMSF completely constrained the enzyme activity with a residual activity of 9% at final concentration of 15 mM and was observed from the results that serine residue in the enzyme catalytic site was sulfonated by the PMSF in the operational position and no hydrolytic activity was observed.

The strong inhibition by PMSF suggests that PAK03 could be a serine protease (Beg and Gupta, 2003).

Figure 4.29: Enzyme activity and stability of purified cold-active protease from *Stenotrophomonas sp.* at various surfactants at 25 °C. The relative activity (%) was calculated relative to the case of reaction at which premier activity was taken as 100 %. All values are represented as mean ± sd of three replications.

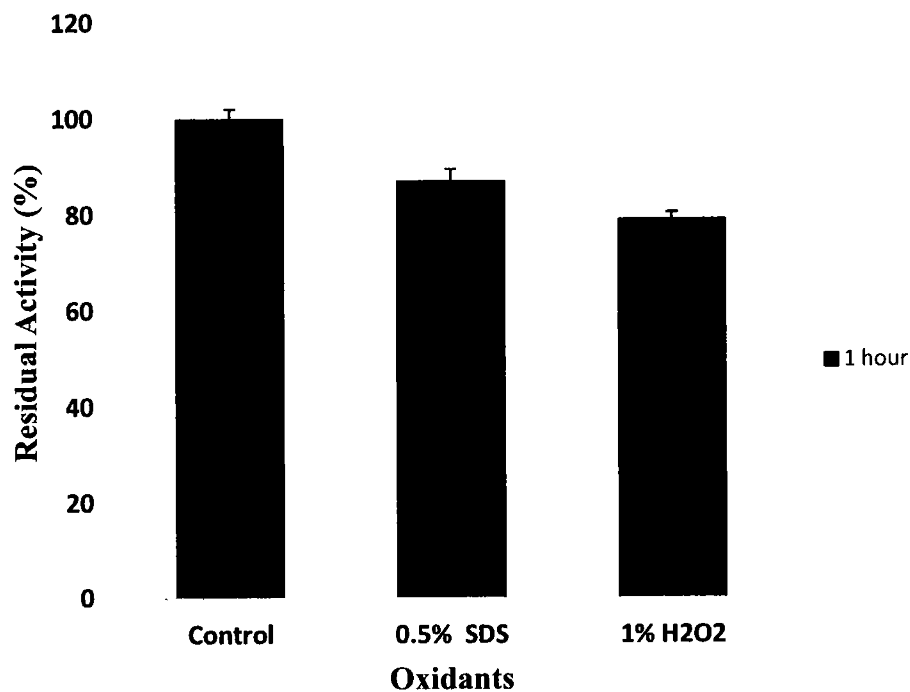

Figure 4.30: Effects of oxidants on the activity and stability of *Stenotrophomonas sp.* Pak03. All values are represented as mean ± sd of three replications.

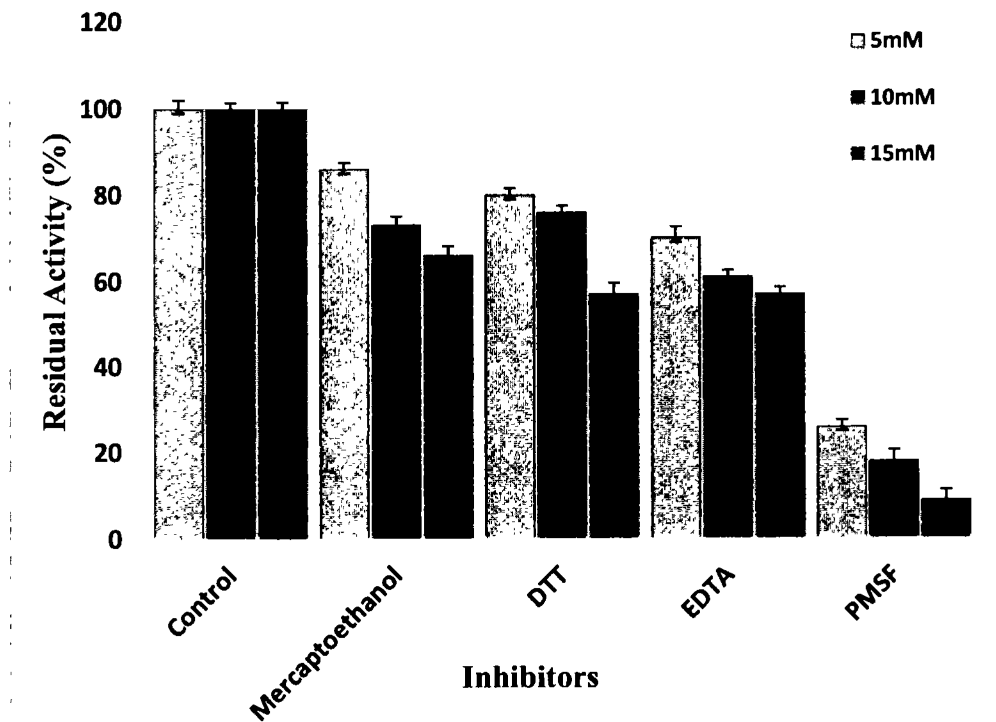

Figure 4.31: Effect of inhibitors on protease activity. Enzyme activity measured in the absence of any inhibitor was taken as 100%. The remaining protease activity was measured after pre-incubation of enzyme with each inhibitor at 25 °C for 30 min. All values are represented as mean ± sd of three replications.

4.4.6. Organic solvents effect on protease activity and stability

Numerous organic solvents consequence at 5 and 10% strength on the catalytic reaction of alkaline protease are concise in (Figure 4.32). Nevertheless, the enzyme hydrolysis of the peptide bond catalyzed by protease might be withdrawn for peptide bond formation because of the certain circumstances, such as the organic solvents addition in the reaction solution.

The organic solvents effect of 5 and 10% on alkaline protease stability was examined by pre-incubating at 25°C beside with the shaking for 1 hour. Results reveals that the protease shows good stability in the presence of acetone, ethanol, isopropanol and methanol, while acetonitrile, butanol, hexane, 2-methylpropan and ethyl acetate constrained the enzyme activity and stability to a greater extent.

From the results, it is therefore reasonable to conclude that the influence of the organic solvents on the enzyme stability totally relies on the organic solvent's nature.

The good stability of protease in the occurrence of acetone, ethanol, isopropanol and methanol might be elucidated by the hydrophobic interaction's enhancement within the protease molecules. This situation could result a desirable conformational alteration for the interaction among the substrate and active site of the enzyme domain.

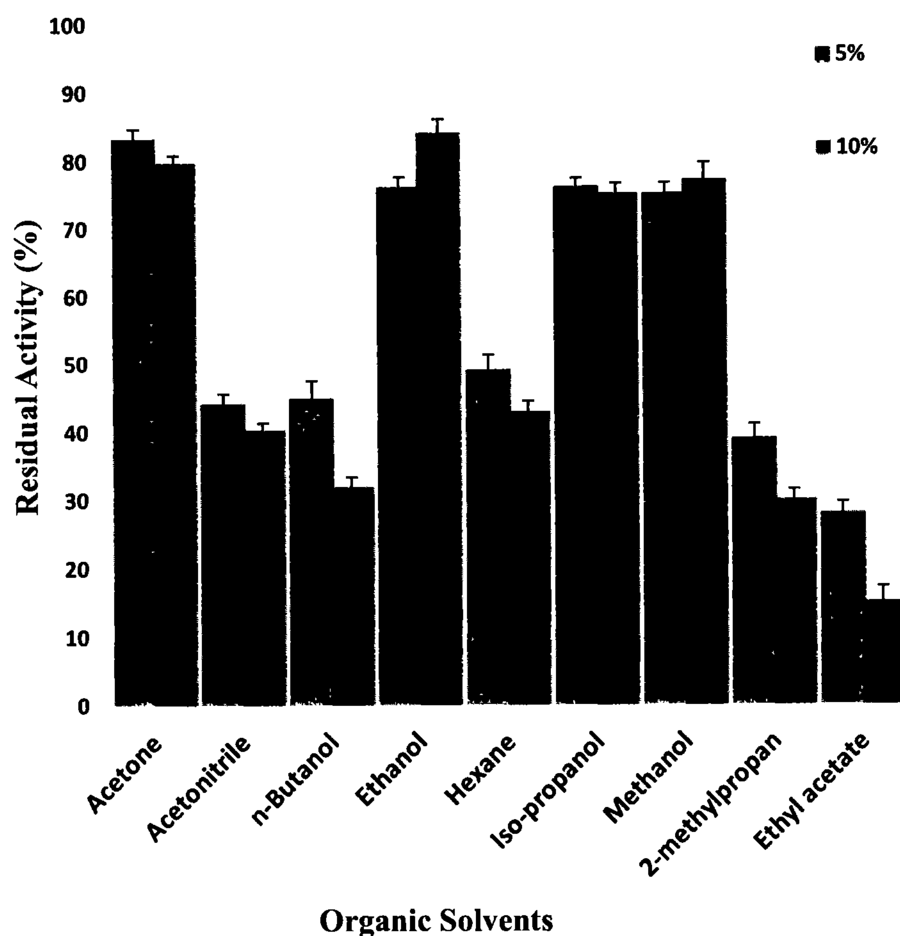

Figure 4.32: The enzyme was incubated at pH 9.0 and 25°C in the presence of 5 and 10% water-soluble organic solvents or alcohols. The stability of the protease was determined by incubating the enzyme in the presence of organic solvents at 25 ° C with shaking for 24 h. Residual activity was measured at pH 6.0 and 25 °C. The non-incubated enzyme was considered as control (100%). All values are represented as mean ± sd of three replications.

4.4.7. Determination of kinetic parameters, Activation Energy and thermal denaturation of protease enzyme

Alkaline protease was allowed to undergo hydrolysis of casein (1%) at low temperature such as 4°C and 15°C and 25°C for 15 minutes (Figure 4.33, 4.34 and 4.35). Whilst it was problematic to evaluate casein molecular mass in molar concentration and consequently percentile concentration remained used in this research. As predictable from the thermolability of protease molecules activity, the catalytic efficiency was observed for 25 °C as compared to 4 and 15 °C which are considered as cold enough to hindered catalytic efficiency of purified protease enzyme.

In Figure 4.34 values of thermodynamic parameters for alkaline protease was calculated at distinctive temperatures ranging from 30 to 90 °C. Ea(P) calculated for protease enzyme form *Stenotrophomonas sp PAK01* was 37.4 kJ/mol (Figure 4.37). Values of K_m and K_{cat} of catalytic efficiency at pH 9 Tris-Hcl and temperatures at 4, 15 and 25 °C are presented in (Table 4.5). The lower Ea(P) suggested that lesser energy was required by alkaline hydrolytic protease enzyme to reach the transition state of tyrosine formation.

Enzyme thermal stability has been experimental in temperatures varying from (30 to 90 °C) for 80 min. Outcome for thermal denaturation are presented in (Table. 4.6). Log (ln % Residual Activity) of the thermolabile enzyme are displayed in (Figure 4.36). Dissociation constant k_d for the thermal denaturation of extracellular thermolabile proteas are respectively exhibited in (Table 4.7) and (Figure 4.37).

Thermolabile enzyme activity has been showed stability at 30 °C temperature subsequently after 80 min of incubation period exhibiting maximum activity of enzyme (79 U/ml/min). Thermolabile enzyme activity has been progressively decrease above 30 °C. Cold active enzyme has been observed volatile and denature at 80 °C ubsequently after 70 min of incubation presenting the minimum protease enzyme activity (6 U/ml/min).

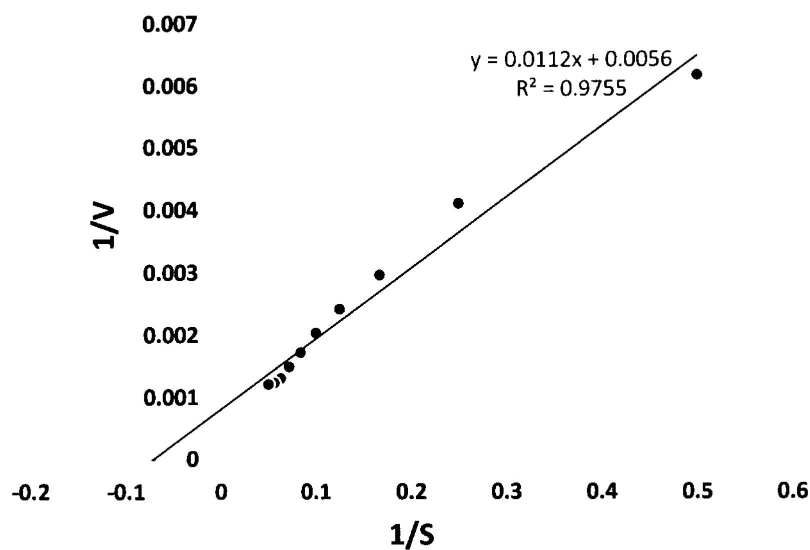

Figure 4.33: Determination of Kinetic parameters of protease enzyme at 4°C by line Weaver-Burk Plot

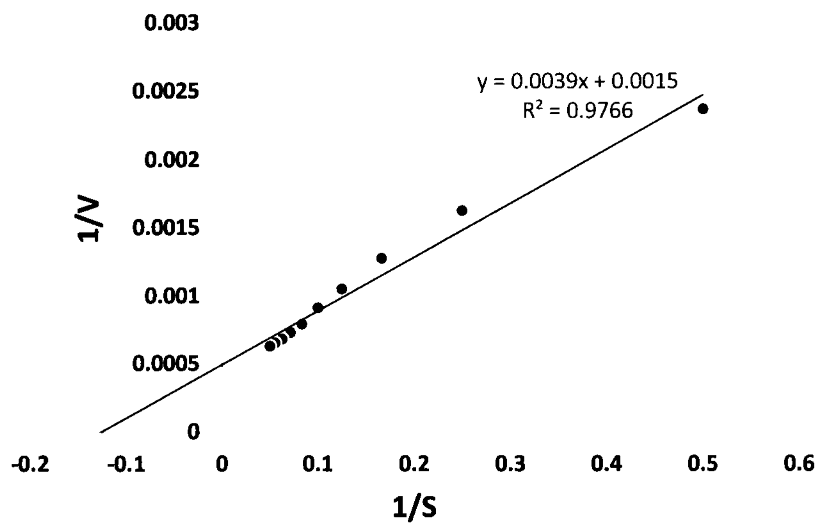

Figure 4.34: Determination of Kinetic parameters of protease enzyme at 15°C by line Weaver-Burk Plot

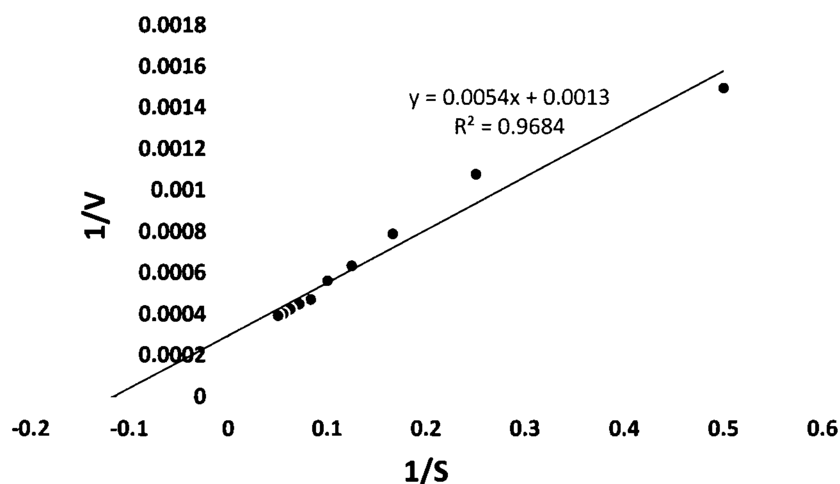

Figure 4.35: Determination of Kinetic parameters of protease enzyme at 25 ° C by line Weaver-Burk Plot

Table 4.5: Values of K_m and K_{cat} of enzymatic catalyzed reaction at pH 9.0 and different temperatures.

Temperature (°C)	K_{cat} (min^{-1})	K_m (mg^{-1})	K_{cat}/K_m (min^{-1} mg^{-1})
4	166.38	2	83
15	306.11	2.6	118
25	632.57	4.2	150.61

Table: 4.6 Thermal denaturation of extracellular cold active protease enzyme

Temperature °C	10	20	30	40	50	60	70	80
30	96	93	91	88	86	84	82	79
40	89	86	84	81	79	72	70	67
50	77	74	72	70	67	64	57	50
60	70	66	64	62	60	58	55	48
70	54	50	48	44	32	27	22	18
80	38	34	27	21	17	10	6	0
90	21	17	13	5	0	0	0	0

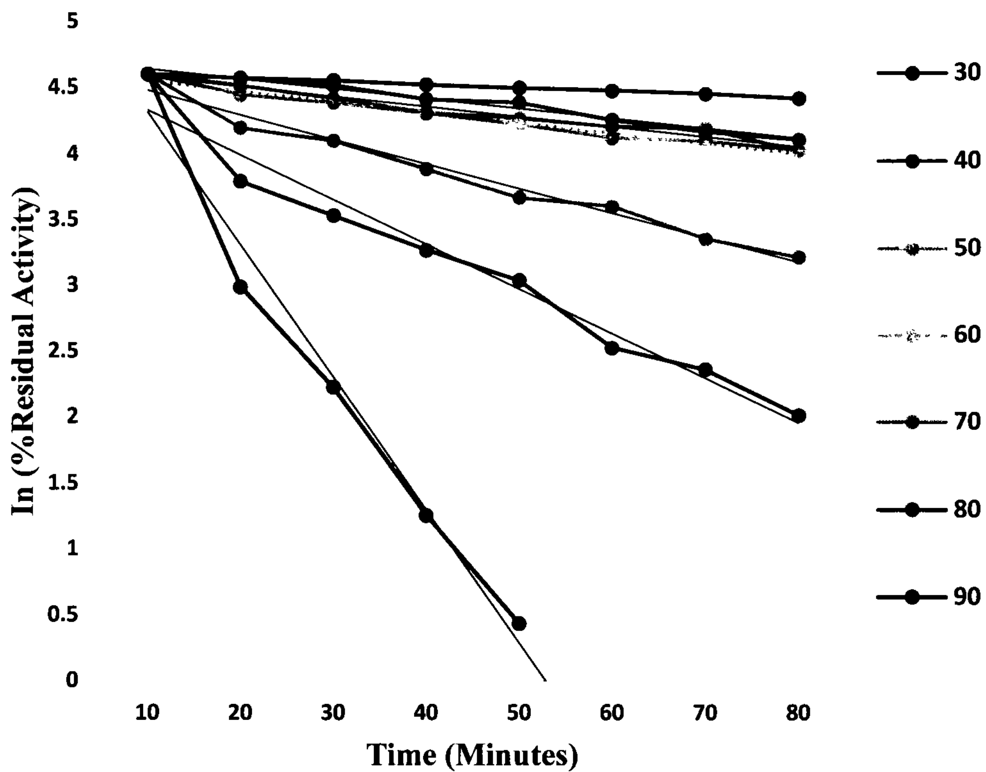

Figure 4.36: ln (% Residual Activity) of extracellular purified protease enzyme

Table: 4.7 k_d value of extracellular lipase enzyme

Temperature °C	K	k_d
30	303	0.0268
40	313	0.0417
50	323	0.0361
60	333	0.0411
70	343	0.0594
80	353	0.0919
90	364	0.2365

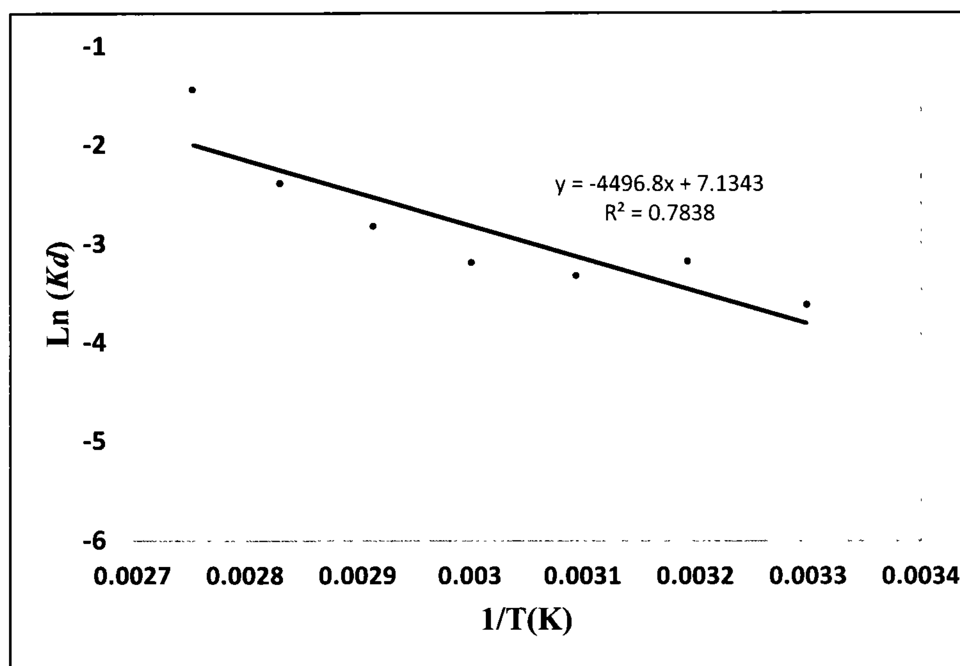

Figure 4.37: Arrhenius plot for the determination of activation energy ($E_{a(P)}$).

4.5. *Stenotrophomonas sp.* PAK01 thermolabile alkaline protease as a laundry additive

4.5.1. Stability of Protease in Detergents/Surfactants

From the outcomes of the present experimentation it was observed that in the existence of purex and xtra, thermolabile protease molecules presented remarkable stability after an incubation of half an hour (Figure 4.38). As compared to control 85% of the residual activity in hydrolysis was observed for purex and in case of xtra 82% (percent) activity has been retained in hydrolysis reaction after incubation of 60 minutes at 25 °C.

Results from incubation of enzyme up to 60 minutes with detergent indicates that the hydrolysis of substrate by thermolabile protease enzyme activity was retained up to 48% residual activity in the existence of Gain detergent and 8% activity has been retained with Tide whereas the hydrolytic activity has been slightly decreasing due to Triton X-100, SDS, Tween-80 at 25 °C. Protease enzymes observed was not influenced by Non-ionic detergents and showed stability against Triton X-100, Tween-80 and SDS but hydrolytic activity was decreased in the present finding is unknown. Enzymes compatibility with different concentrations of (3 to 20% of 15mg/ml) of the selected detergents (xtra and purex) after incubation of 90 minutes at 25 °C has been demonstrated in (Figure 4.39).

Hydrolytic activity of the thermolabile protease molecules was stable in purex at concentration of 3% (v/v) and 6% (v/v) for xtra, respectively. Finding of the present study indicates that by increasing the concentration of selected detergents upto 20% (v/v), the residual hydrolytic activity has been observed for xtra detergent with a residual activity of 7% and with purex 24 % activity has been retained and this might be due to hydrolytic protease enzyme structure denaturation of at higher strength of detergents.

Stenotrophomonas sp secreted protease enzyme stability profile in 24 hrs in 5% (v/v of 15 mg/ml) of xtra and purex revealed 87 % and 89 % residual hydrolytic activity after incubation for 4 hours (Figure 4.40). Nevertheless, thermolabile enzyme molecules showed stability against purex and xtra and has been observed that the activity retained of 51 and 46 % residual hydrolytic activity of alkaline protease at 24 hours of incubation with purex and xtra detergents.

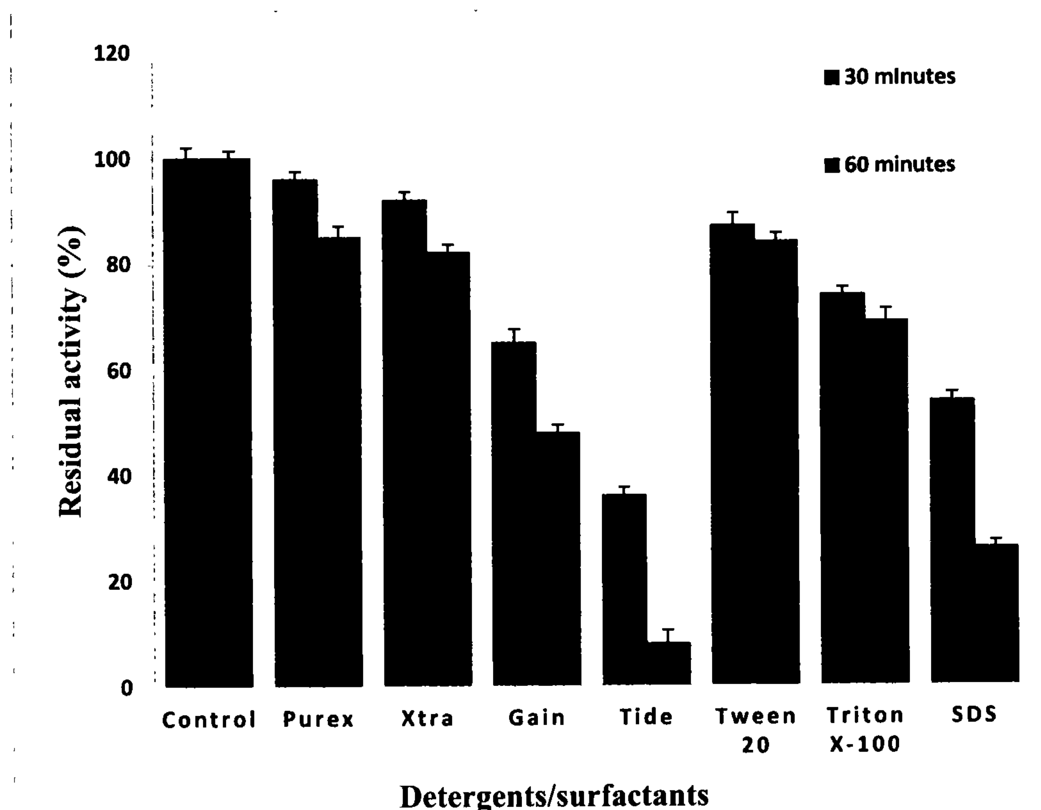

Figure 4.38: Stability of protease of *Stenotrophomonas* sp. in various local detergents/surfactants (5% of 10 mg/ml) after 30 minutes and 1 hr incubation at 25°C. Protease activity measured using casein (2.5% in 50 m*M* Tris HCl, *p*H 9.0) at 30°C after 15 min of incubation. Relative activity (%) is calculated against enzyme control incubated in buffer (100% activity = 4621 U/ml). All values are represented as mean ± sd of three replications.

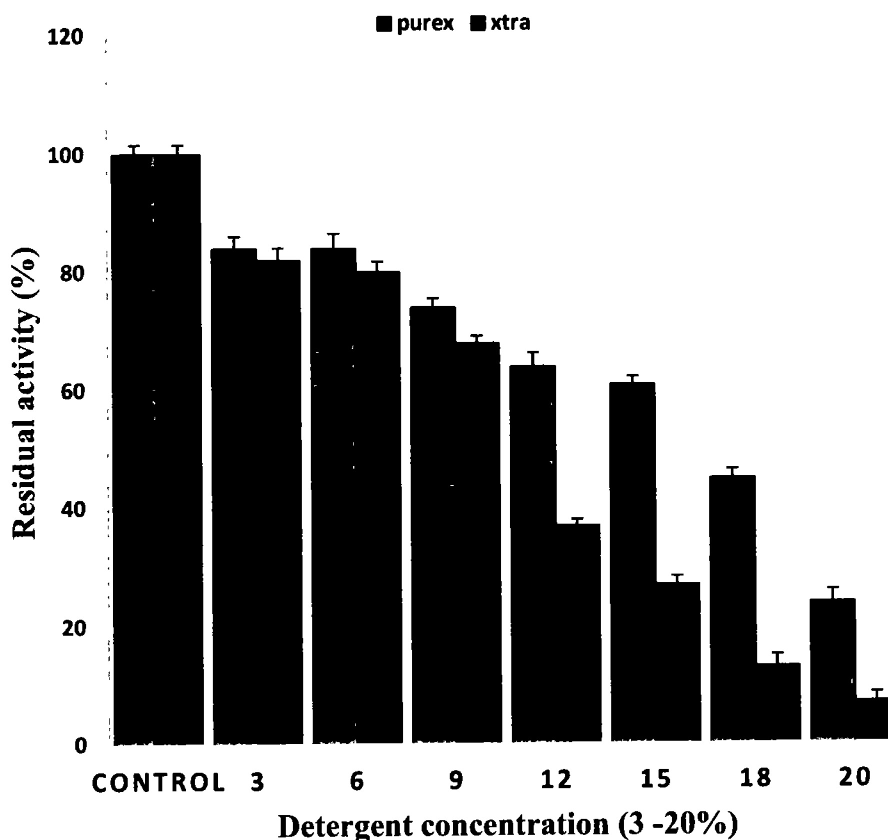

Figure 4.39: Stability of protease of *Stenotrophomonas sp.*in different concentrations (3-20% of 15 mg/ml) of purex and xtra after 90 min incubation at 25 °C. Protease activity measured using casein (1% in 50 m*M* Tris HCl, *p*H 9.0) at 25 °C after 5 min of incubation. Relative activity (%) is calculated against enzyme control incubated in buffer (100% activity=3675 u/ml). All values are represented as mean ± sd of three replications.

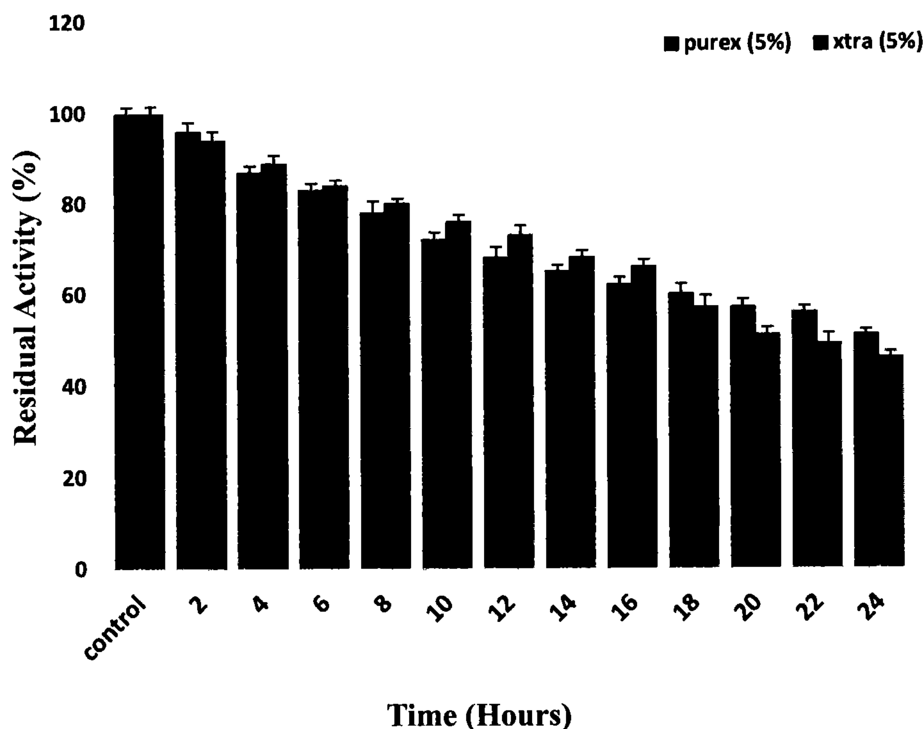

Figure 4.40: Stability of *Stenotrophomonas sp.* protease after incubation at 25°C over a period of 24 hour in (5% of 15 mg/ml). Protease activity measured using casein (1% in 50 m*M* Tris HCl, *p*H 9.0) after 15 min of incubation at 25 °C. Relative activity (%) is calculated against enzyme control incubated in buffer (100% activity = 3767 U/ml). All values are represented as mean ± sd of three replications.

4.5.2. Bleach and Oxidizing Agent on thermolabile protease

The residual hydrolytic activity of thermolabile protease enzyme has been obtained by preincubation with in sodium hypochlorite ranging from 50 to 500 mg/l for 60 minutes at 25 °C. The cold active purified protease showed stability against bleaching agent and retained 86, 83, 76% of the residual hydrolytic activity in concentration of such as 50 to 200 mg/l, respectively. Though, 64 % of the proteolytic activity was retained even at greater strength of 500 mg/liter of sodium hypochlorite (Figure 4.41).

However, up to 15% hydrogen peroxide (H_2O_2) the protease enzyme retained only 6 % of its initial activity for 1 hour of incubation at 25°C and was completely inhibited at 20% H_2O_2 (Figure 4.42).

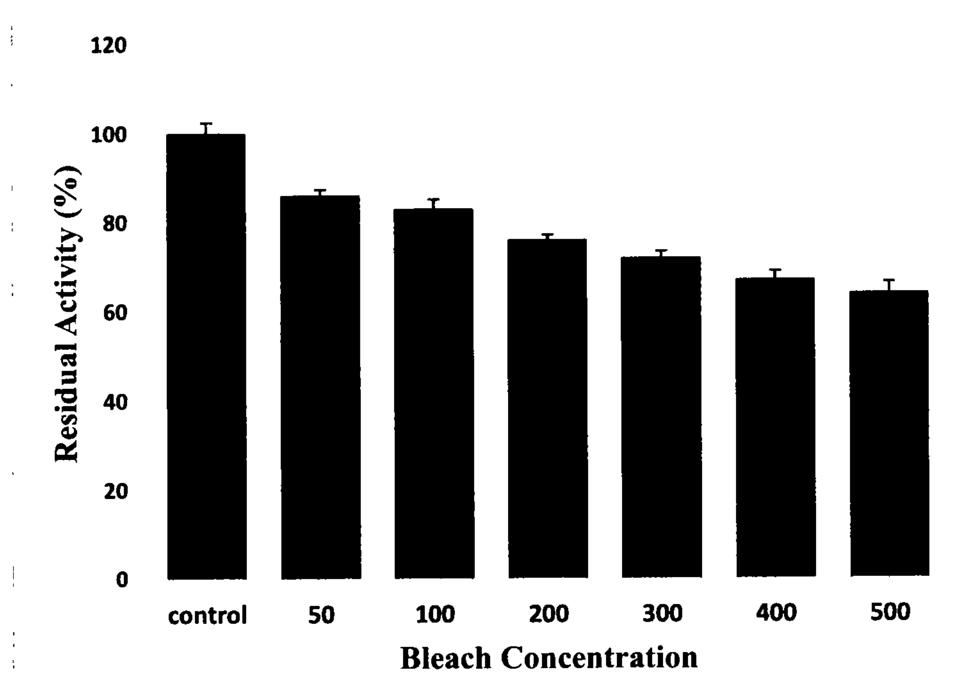

Figure 4.41: Stability of protease of *Stenotrophomonas sp.* in various concentrations of sodium hypochlorite (50-500 mg/l of available chlorine) after 1-hour incubation at 25 °C. All values are represented as mean ± sd of three replications.

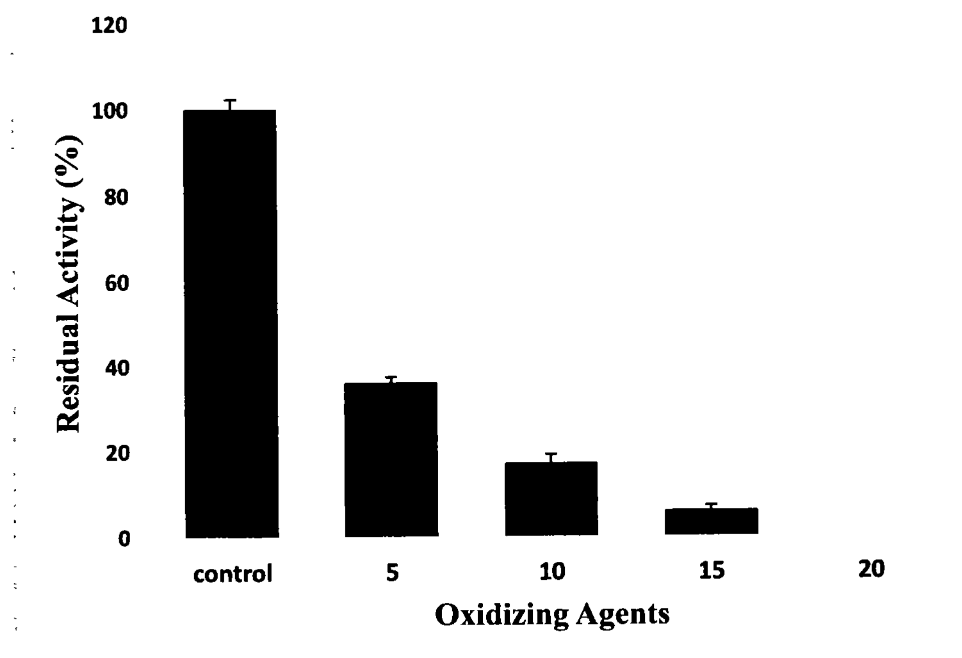

Figure 4.42: Stability of protease of *Stenotrophomonas sp.* in various concentrations of hydrogen peroxide (5-20%) after 1-hour incubation at 25 °C. All values are represented as mean ± sd of three replications.

4.5.3. Thermolabile protease enzyme effect on proteinous pigment removal

The results from the present study showed that at distinctive temperature from 15, 25 and 35 °C with 100 U/ml enzymes and (1% of 15 mg/ml) of purex. Thermolabile protease enzyme has been examined for removing blood plasma pigment from test cotton fabric.

At very low temperature such as 15 °C it took very long 2 hours to remove pigments of blood plasma whereas hydrolytic activity of thermolabile protease molecules are active to hydrolyze the peptide bonds of blood protein in 30 minutes and 1 hour of incubation at given environment of 25 and 35 °C and in the absence of hydrolytic purified protease molecules, the time acquired for hydrolysis to remove oil and proteinous dirt was very slow.

In subsequent experiment when water was substituted with Tris HCl of 9 pH the proteinous dirt hydrolysis was in 15-20 min at 25 °C with 100 U/ml enzyme and 1% of 15 mg/ml purex (Figure 4.43).

The thermolabile protease enzyme molecules was also competent in hydrolyzing peptide bonds of protein of egg yolk from test cloth at 25 °C with active enzyme of 100 U/ml and (1% of 15 mg/ml) of purex which required 15 min to utterly remove strains (Figure 4.44).

Stenotrophomonas sp. PAK01, with its stability in hypochlorite and purex and wash performenace application, seems to be a potential cold active alkaline protease for dynamic ingredients of detergent formulation for washing purpose.

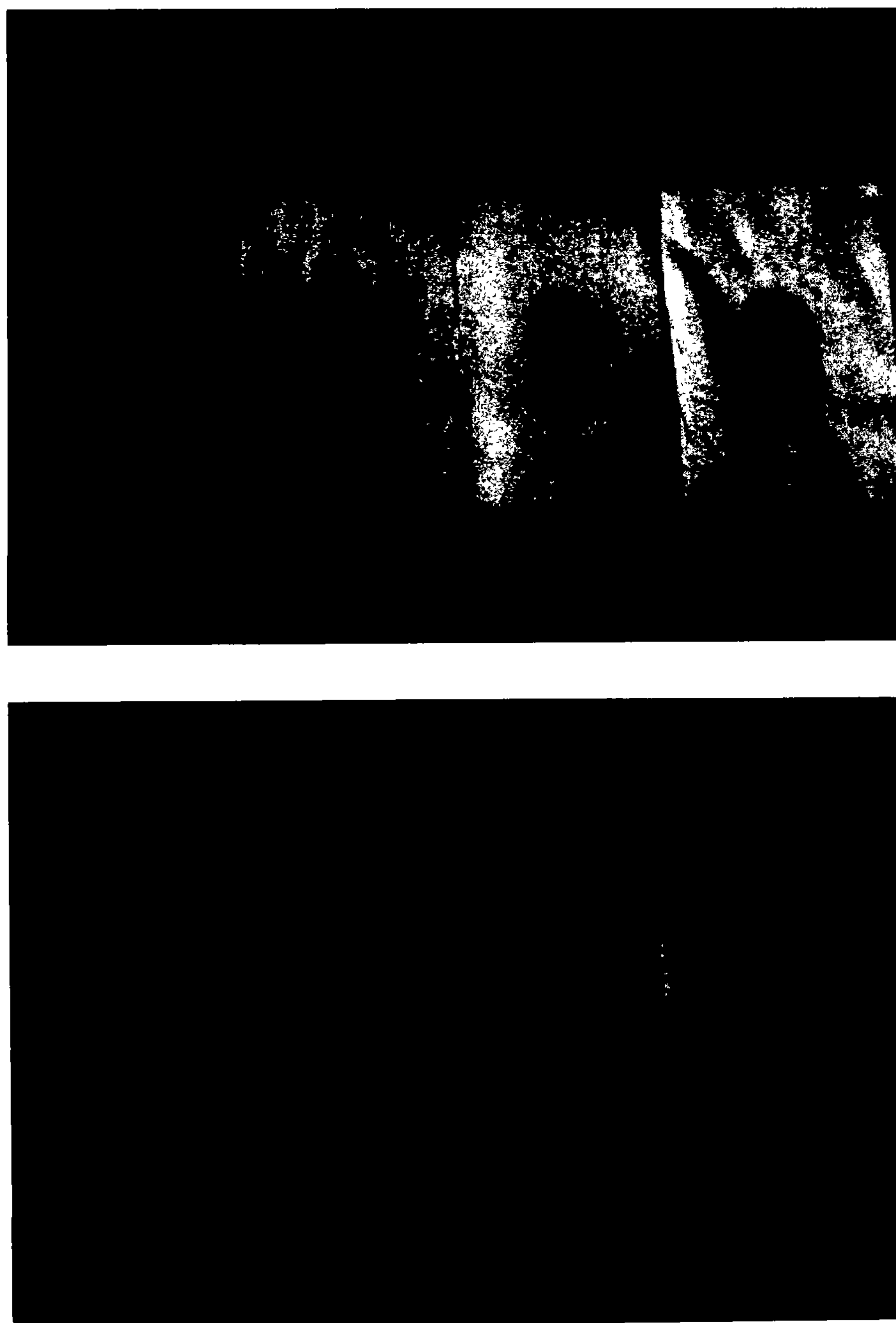

Figure 4.43: Washing performance analysis test of alkaline protease from *Stenotrophomonas sp*. Effect of *Stenotrophomonas sp.* alkaline protease on bloodstain removal at 25°C: a) water and buffer only, b) detergent (5%) in buffer, c) enzyme (500 U/ml) in buffer, *p*H 9.0), and d) detergent (5%) + enzyme (500 U/ml) in buffer. I: untreated cloths (control) and II: treated cloths

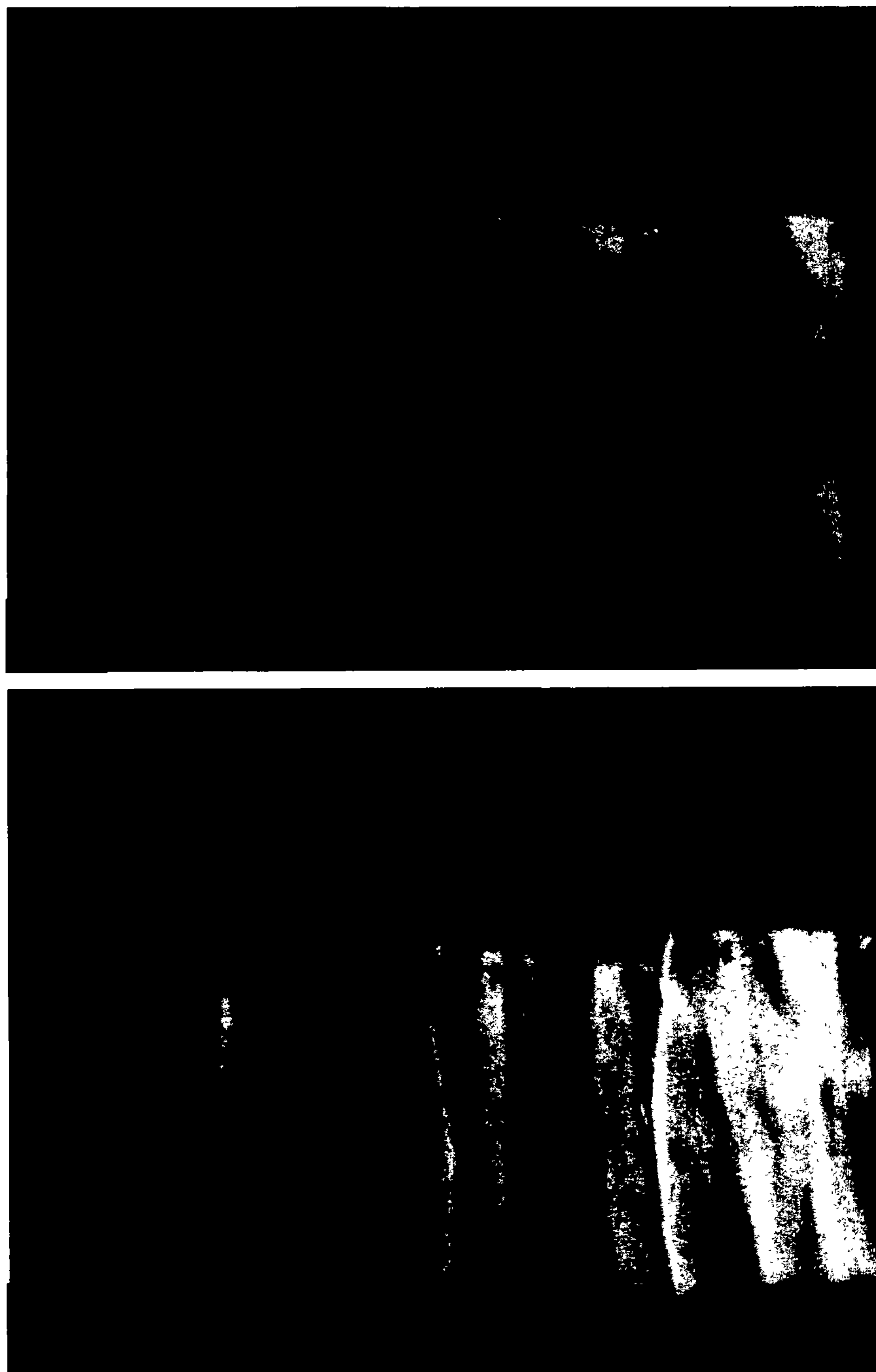

Figure 4.44: Washing performance analysis test of alkaline protease from *Stenotrophomonas sp.* Effect of *Stenotrophomonas sp.* alkaline protease on egg yolk removal at 25°C: a) water and buffer only, b) detergent (5%) in buffer, c) enzyme (500 U/ml) in buffer, *p*H 9.0), and d) detergent (5%) + enzyme (500 U/ml) in buffer. I: untreated cloths (control) and II: treated cloths

Section #: 2

4.6. Production of Extracellular Psychrotrophic Alkaline Lipase from *Pseudomonas peli* strain PAK3

4.6.1. Isolation and phylogenetic analysis

Total twenty diverse psychrotrophic alkaline bacterial strains from soil of Rakaposhi glacier were screened, producing thermolabile lipase enzyme. Based upon greater zone of hydrolysis on trybutyrin agar plate at 20 °C, five hydrolytic isolates were investigated for enzyme production in lipase stimulating broth media. Depending upon maximum catalytic enzyme production (1523 U/ml) one potential psychrotrophic isolate, entitled as PAK03 was chosen for advance studies.

Isolated thermolabile alkaline lipase producing psychrotrophic bacterium was recognized on the foundation of 16S rRNA alignment with BLAST database search from the GenBank database at NCBI (MIDILABS Inc., USA). The phylogenetic tree was constructed using software MEGA 7 (Tamura *et al.*, 2007). Final sequence of PAK03 was submitted to GenBank having an accession no.MG687270. Psychrotrophic organism grew healthy in the pH range of 7-11 and temperature range of 10-37 °C but did not have the potential to grow beyond 40°C.

4.6.2. Phylogenetic evaluation of glacial strain

4.6.2.1. *Pseudomonas peli* PAK03

Pseudomonas peli (MG687270) presented highest nucleotide sequence resemblance through Nucleotide Blast exploration with *pseudomonas anguilliseptica* (AF439803) and *pseudomonas cuatrocienegasensis* (JN64459) with 99% sequence coverage with (e-value 0.0). Evaluation of Maximum parsimony was accomplished with 1000 bootstrap repeats beside values presented by nodes. 5 evolutionary steps represented by calibration bar (Figure 4.45)

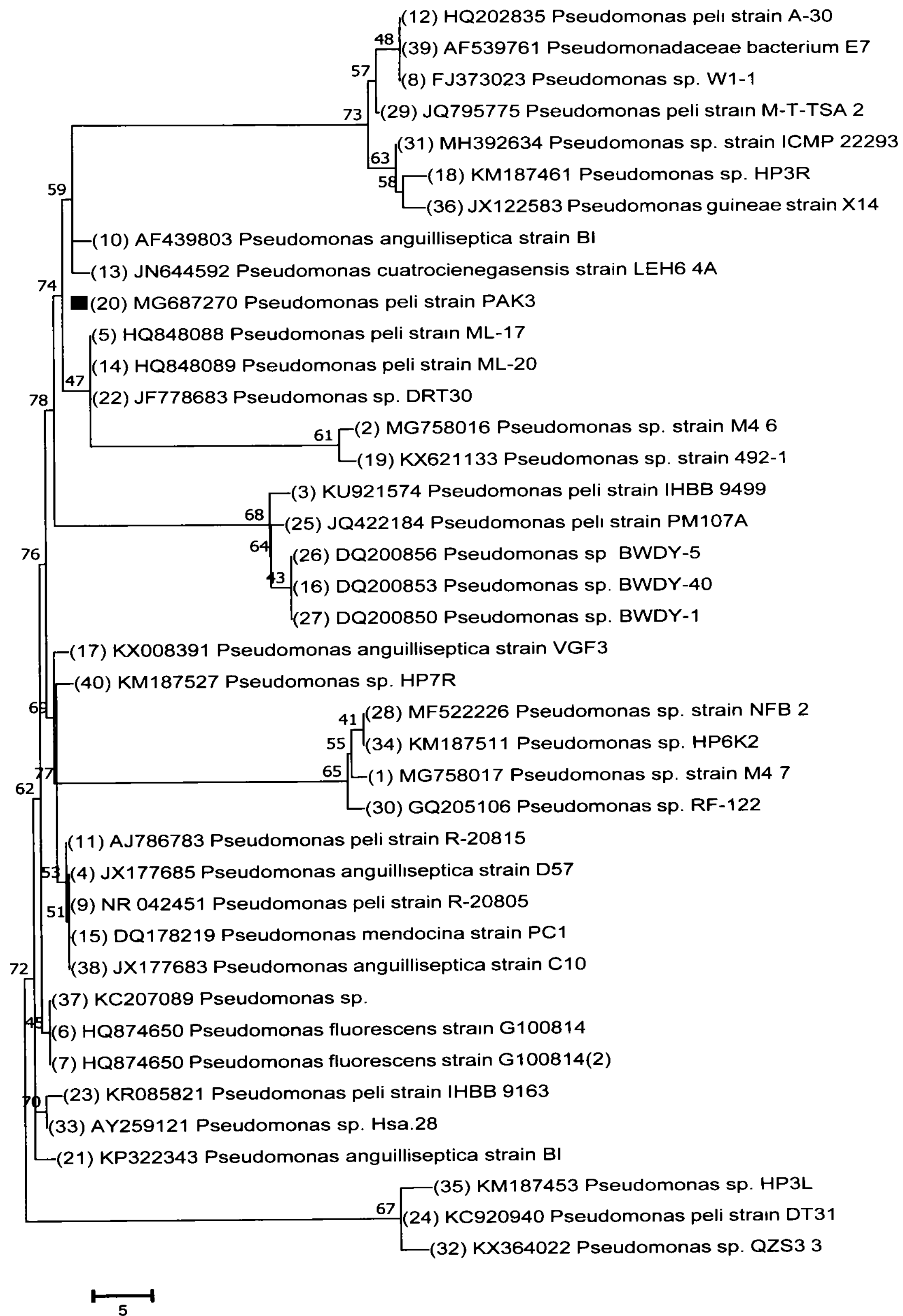

Figure 4.45: Phylogenetic tree of *Pseudomonas* peli PAK03

4.7. Molecular Cloning and Expression in *Escherichia coli* of *Pseudomonas peli* lipase gene

4.7.1. Cloning and sequence of YLip gene analysis

For cloning of the YLip gene encoding solvent resistant alkaline protein, information of nucleotide sequence from *Pseudomonas mandelii* JR-1 was used, which is associated closely to *Pseudomonas peli*, and the specific primers was designed based on the region of the nucleotide noncoding sequences covering the encoding thermolabile lipase gene of *Pseudomonas mandelii* JR-1.

Product of PCR estimated was 891bp (Figure 4.46) YLip gene from the psychrotrophic *Pseudomonas peli* PAK03 was amplified and the amplified genome was inserted into a pET28 bacterial vector. Protein translated from the YLip gene comprised a Gly-His-Ser-Gln-Gly sequence (Figure 4.48), a distinguishing motif of the protein family belonging to serine lipase (Gly-X-Ser-X-Gly) (Cygler *et al.*, 1993).

Clones gave protein of molecular mass of 32 kDa has been analyzed. From the multiple sequence alignment, it was characterized that 96 % identity of the YLip sequence at the level of the amino acid was observed identical to organic solvent stable protein encoding from LipS gene, from *Pseudomonas mandelii* JR-1 (Figure 4.48).

Q3KIU1, YLip, rPFL and LipS genes resulted was 296 matching residues of amino acid. Lip9 gene amplified from *Pseudomonas aeruginosa* exhibited sequence identity of 44% but demonstrated a conserved motif of serine lipase family (Figure 4.48). Residues of 2 Asp as a calcium binding motif were observed (Figure 4.48).

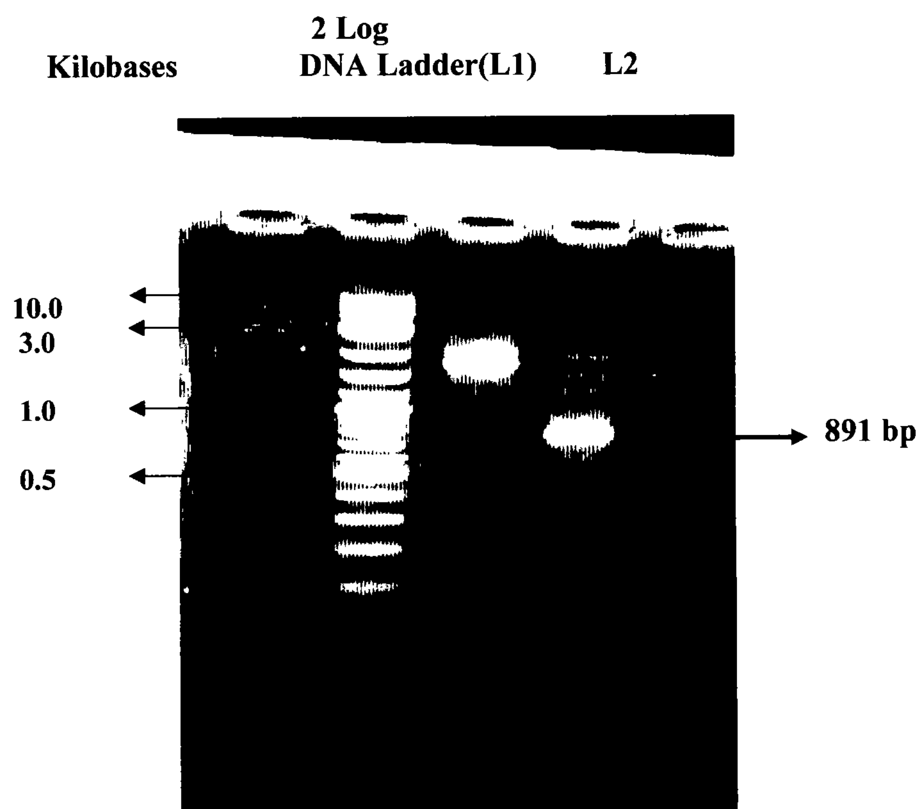

Figure 4.46: Representative agarose gel electrophoresis image of YLIP gene PCR products. **L1**: 2-Log DNA Ladder, **L2**: YLip gene of 891 bp

4.7.2. Examining the expression and purification of the cloned lipase gene YLip

YLip gene complete nucleotide sequence was inserted in a pET28a vector with His_6 residues on N-terminal position. YLip recombinant protein was effectively expressed in *E. coli* BL21 strain as a soluble protein and nickel chelate affinity chromatography was used for purification analysis trailed by Q- Sepharose column chromatography procedure. (Table 4.8). Though, YLip large fraction did not attached to the resins of nickel and observed 5% of quite low yield (Figure 4.47). Technique to recover the YLip yield from purification methods is being sought. Profiles of elution from purification steps such as nickel chelate and Q-Sepharose columns chromatography held pace with the activity of lipase (data not shown). YLip protein showed as a 32-kDa protein on a Coomassie Blue tainted SDS gel analysis (Figure 4.47B, lane Q). Sequence (VNLIGHSQGSLTAR) of peptide was analyzed by the MALDI-TOF MS from the 32-kDa protein band correspondingly matched the LipS sequence (underlined in Fig. 1).

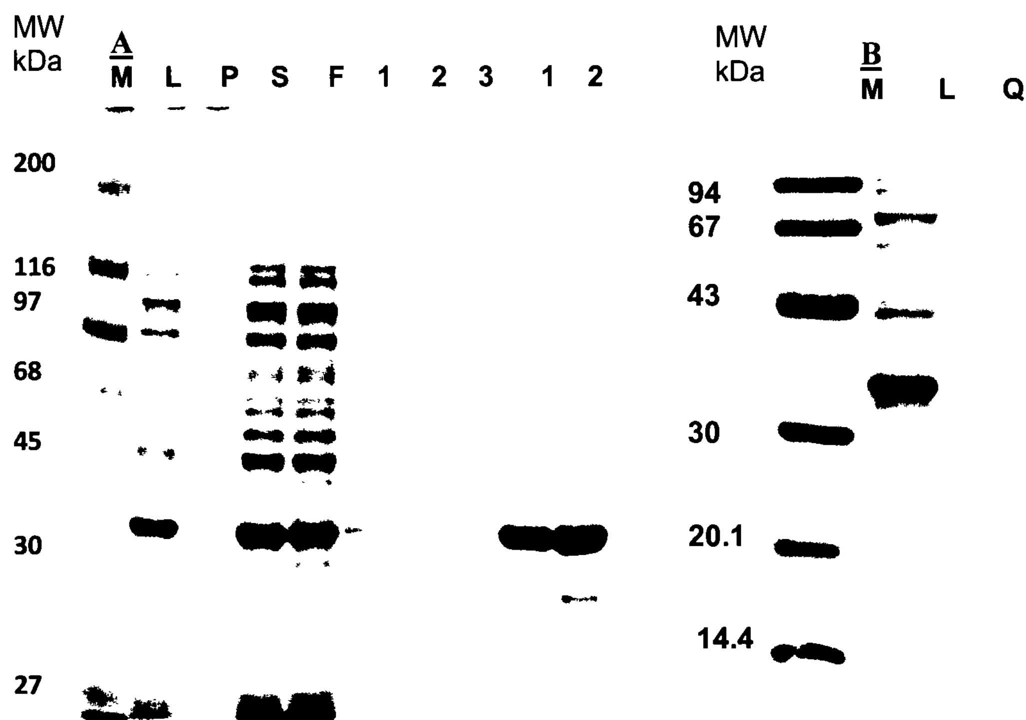

Figure 4.47: Expression and Purification of YLip.

A, SDS gel after nickel chelate affinity chromatography. The fusion protein was expressed in *E. coli* strain BL21. A lysate (L, lane 2) was made in Cell Lytic B and separated by centrifugation into a pellet (P, lane 3) and a supernatant (S, lane 4) fraction. The supernatant was applied Affinity Gel column and the flow through (F, lane 5), wash (1-3, lanes 6-8) and elution (1-4, lanes 9-10) fractions were collected. **B**, SDS gel after anion-exchange column chromatography. M, molecular weight marker. L, cell lysate. Q, eluted fraction from Q- Sepharose column (13.5mL). The molecular weight of YLip was calculated to be 32 kDa.

Table 4.8. Purification of YLip from BL21 (DE3).

Purification steps	Total protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purity (%) Fold
Cell lysate	86.32	7763	89.93	100	35.83
Nickel-chelate column	2.26	436	192.92	5.62	76.86
Q-Sepharose column	0.86	216	251	2.8	100

```
Lip9            MKKKSLLPLGLAIGLASLAASPLIQASTYTQTKYPIVLAHGMLGFDNILGVDYWFGIPSA  60
rPFL            -------------------------MSQELATRYPLVLVPGMLGFVRLLLYPYWYGIIPA  35
Q3KIU1          -------------------------MSQDSATRYPLVLVPGMLGFIRLVLYPYWYGIIKA  35
Ylip            --------------------------SQGSATRYPLVLVPGMLGFIRLVLYPYWYGIISA  34
LipS            -------------------------MSQGSATRYPLVLVPGMLGFIRLVLYPYWYGIISA  35
                                          *    *:**:**. ***** .::   **:**  *

Lip9            LRRDGAQVYVTEVSQLDTSEVRGEQLLQQVEEIVALSGQPKVNLIGHSHGGPTIRYVAAV  120
rPFL            LRRGGAQVIAVQVSPLNSSEVRGEQLLAQIQRIMAETGAARVNLIGHSQGALTARYAAAR  95
Q3KIU1          LRRGGATVIAVQVSPLNSTEVRGEQLLTRIDEILRETGAAKVNLFGHSQGSLTARYAAAK  95
Ylip            LRRGGATVFAVQVSPLNSSEVRGEQLLARIEEILRETGAEKVNLIGHSQGSLTARYAAAK  94
LipS            LRRGGATVFAVQVSPLNSSEVRGEQLLARIEEILRETGAEKVNLIGHSQGSLTARYAAAK  95
                ***.** * ..:** *:::******** :::.*:  :*  :***:***:*. * **.**

Lip9            RPDLIASATSVGAPHKGSDTADFLRQIPP-GSAGEAILSGLVNSLGALISFLSSGSTGTQ  179
rPFL            RPDWVASVTSVAGPNHGSELADYLQRHSPAHSLRGRVLSLLLRGISSLMRLLETGYRGPK  155
Q3KIU1          RPDLVASVTSVAGPNHGSELADYLAKYYPADSAKGRILEALLRSVGWLMALLETGYHGPK  155
Ylip            RPDRVASVTSVAGPNHGSELADYLEKHYPADTFKGRVLSVLLRWIGALMSLLETGYRGPK  154
LipS            RPDRVASVTSVAGPNHGSELADYLEKHYPADTFKGRVLSVLLRWIGALMSLLETGYRGPK  155
                *** :**.***..*::**: **:* :  *  :    :*. *:. :. *: :*.:*  * :

Lip9            NSL---GSLESLNSEGAARFNAKYPQGIPTSACGEGAYKVNGVSYYSWSGSSPLTNFLDP  236
rPFL            QPVDIHASHQSLTTEGVALFNRQYPQGLPQEWGGQGPAQVDGVHYYSWSGILQPGKTNRG  215
Q3KIU1          LPVDIHASHHSLTTEGVALFNQLYPQGLPQTWGGHGPEEVNGVRYYSWSGTLQPGKTDRG  215
Ylip            LPVDIPASHHSLTTQGVALFNQRYPQGLPETWGGHGPEEVNGVRYYSWSGTLQPGNTDRG  214
LipS            LPVDIPASHHSLTTQGVALFNQRYPQGLPETWGGHGPEEVNGVRYYSWSGTLQPGNTDRG  215
                  :   .* .**.::*.* **  ****:*    *.*  :*:** ******     :

Lip9            SDAFLGASS-------LTFKNGTANDGLVGTCSSHLGMVIRDNYRMNHLDEVNQVFGLTS  289
rPFL            RNLFDGTNRSCRLFARTFVREAGQCDGMVGRYSSHLGQVIGDDYPLDHFDIVNQSLGLVG  275
Q3KIU1          GNLFDGTNRSCRLFAKTFVREPGQCDGMVGRYSSHLGTVIGDDYPMDHFDIVNQSLGLVG  275
Ylip            GNRFDGTNRSCRLFARTFVRETGHCDGMVGRYSSHLGTVIGDEYPMDHFDIVNQSLGLVG  274
LipS            GNRFDGTNRSCRLFARTFVRETGHCDGMVGRYSSHLGTVIGDEYPMDHFDIVNQSLGLVG  275
                 : * *:.          .::    **:**  ***** ** *:* ::*:* *** :**..

Lip9            LFETSPVSVYRQHANRLKNASL  311
rPFL            K-GAEPIRLFVEHARRLKAAGL  296
Q3KIU1          K-GADPVRLFVEHAARLKAAGV  296
Ylip            K-GADPVRLFVEHAARLKGAGV  295
LipS            K-GADPVRLFVEHAARLKGAGV  296
                   :.*: :: :** *** *.:
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Figure 4.48. Multiple Sequence Alignment for YLip (*Pseudomonas peli* PAK3), LipS (*P. mandelii* JR-1), Q3KIU1 (*P. fluorescens* Pf0-1), rPFL (*P. fluorescens* JCM5963), and Lip9 (*P. aeruginosa* LST-03). GXSXG motif (yellow). LipS possesses a catalytic triad consisting of Ser^{83} (yellow), Asp^{241} (yellow), and His^{263} (yellow) residues. Calcium binding motif (_).

4.7.3. Thermal stability of purified lipase enzyme

Study of thermal stability showed that yLip maintained above 50% of its catalytic activity from 4–50 °C (Figure 4.49). 44 % of its residual hydrolytic activity observed was retained after 1 hour of incubation at 60 °C (Figure 4.49).

At low temperatures the activity observed was maintained, when thermolabile enzymes mostly show amino acid structure flexibility at the expense of its protein stability with a decreased number of intramolecular non-covalent bonds taking place within the protein molecules such as ionic interactions, hydro-phobic relations and hydrogen bonds, comparative to their counterparts of higher-temperature (Joseph *et al.*, 2008, Feller, 2003).

YLip encoding protein was observed distinctive in retaining high thermal stability as compared to an enzyme resulting from a psychrotrophic bacterium. Comparably, thermolabile esterase enzyme from *Psychrobacter cryohalolentis* was observed to sustain nearby 60% of its hydrolytic activity after for 1 hour of incubation at 80 °C (Novototskaya *et al.*, 2012).

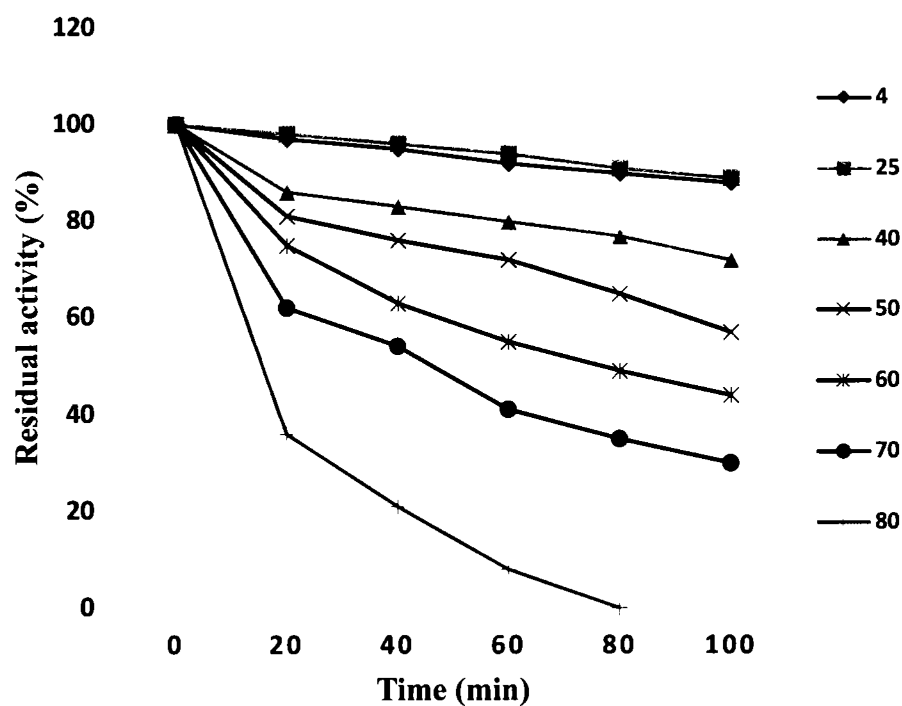


Figure 4.49: Aliquots of yLip were pre-incubated in reaction buffer (100 mM Tris-Cl, 100 mM NaCl, 0.3%, pH 9) at 4, 10, 20, 40, 50, 60, 70, and 80 ^{0} C for the indicated durations. Lipase activity was measured in a reaction buffer at 25 ^{0}C for 10 min with 0.5 mM PNPP. Prior to incubation, the activity of LipS at 25 ^{0}C was 100%.

4.7.4. Organic solvents, metal ions and detergents effect on recombinant lipase

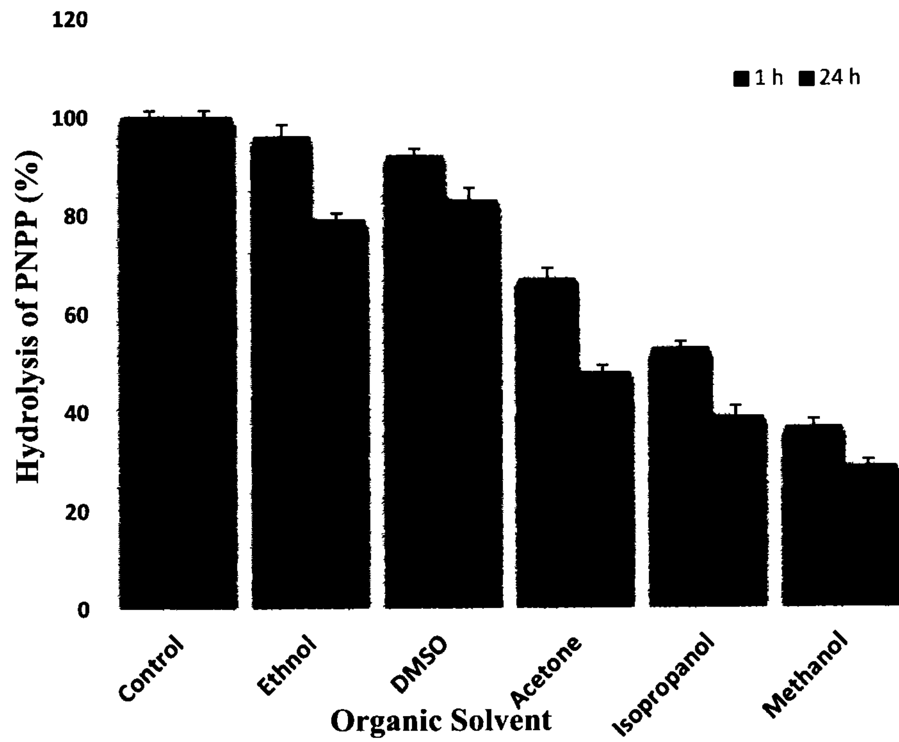
YLip encoding lipase catalytic activity was estimated in the existence of numerous organic solvents (Figure 4.50A.). YLip observed was stable in the existence of toward acetone and isopropanol. To our wonder, lipase catalytic activity was observed to be stimulated by ethanol with 96% and DMSO with 92% after incubation for 1hour. Moreover, YLip hydrolytic lipase activity at an elevated level observed for DMSO and ethanol was retained 83% and 79% for 24 hours of incubation of reaction mixture.

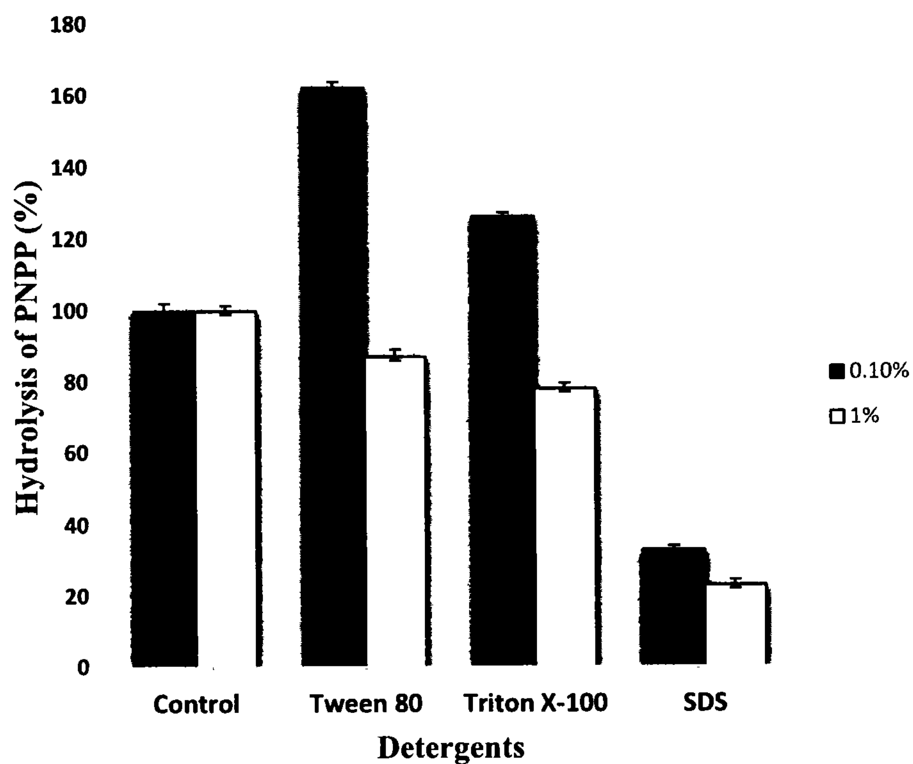
In contrast, YLip catalytic activity was observed decreased to 50% in aqueous buffer solution. In methanol YLip lipase catalytic activity retained of about 30% residual activity has been experimented when associated to control. With this concern, YLip was alike to the organic solvent resistant thermolabile lipase from *Pseudomonas mandelii* (Junsung *et al.*, 2013) in relations of relative stability and stimulated catalytic hydrolysis in the existence of different organic solvents.

Different detergents effect on the hydrolytic activity of YLip were experimented, as present in (Figure 4.50B). 0.1% detergents strength was observed overall more effective as compared to concentration of 1.0%.

Detergent concentration at 0.1%, thermolabile lipase activity was stimulated and increased by 62% in the existence of Tween 80 and in the presence of Triton X-100 with 26% of activity as compared to control, but it was constrained 67% by effect of SDS. In an earlier experiment, some substances of the surface-active surfactants were observed to stabilize the interfacial area between molecules, facilitating the approach of the different substrate to the enzyme active site (Karadzic *et al.*, 2006).

Nucleotide sequence analysis proposed that two residues of Asp as calcium-binding domain of amino acid encoding from YLip gene (Figure 4.48). Maximum catalytic activity for yLip was experiential in the attendance of $CaCl_2$ with a 43% stimulation of activity (Figure 4.50C). Consequences of KCL and $MgCl_2$ were minimal, but the presence of $CuSO_4$ and EDTA decreased enzymatic activity significantly.


120
100
80
60
40
20
0
Hydrolysis of PNPP (%)
1 h
24 h
Control
Ethnol
DMSO
Acetone
Isopropanol
Methanol
Organic Solvent


180
160
140
120
100
80
60
40
20
0
Hydrolysis of PNPP (%)
0.10%
1%
Control
Tween 80
Triton X-100
SDS
Detergents

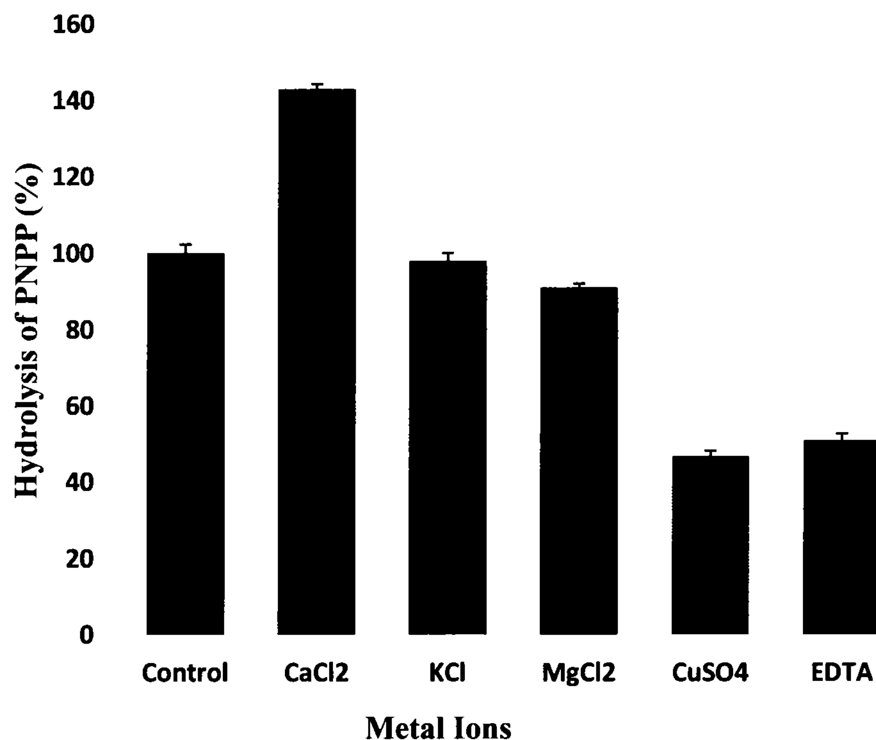


Figure 4.50: Effects of Organic Solvents, Detergents, and Metal Ions. **(A).** The effects of organic solvents were measured after incubation of yLip for 1 h and 24 h in a reaction buffer containing 30% (w/v) of each organic solvent at 25 °C for 10 min with 1 mM PNPP. **(B).** The effect of detergents was measured in the same reaction buffer containing 0.1% (w/v) and 1.0% (w/v) of detergent at 25 °C for 10 min with 1 mM PNPP. **(C).** The effects of metal ions were measured in the same reaction buffer containing 5mM of each metal ion at 25 °C for 10 min with 1 mM PNPP. All values are represented as mean ± sd of three replications.

4.8. Production of extracellular thermolabile alkaline lipase from *pseudomonas peli* strain PAK03 (MH338242)

4.8.1. Production of thermolabile alkaline lipase

Determination of quantitative analysis of the lipolytic activity revealed that five wild bacterial isolates have the potential to secrete thermolabile proteases with varying concentrations. For the production of alkaline lipase 5 glacial were screened utilizing standard assay conditions. Because of segment of the preliminary selection criteria, lipase activity was observed for separately of the 5 wild selected strains through fermentation production in batch cultures for 72 hours at 20 °C and Tris-Hcl buffer at pH 9.

From the results it was showed *Planococcus sp.*, *Exiguobacterium sp.*, *Pseudomonas aeruginosa sp.*, *Stenotrophomonas sp.*, generated a protease hydrolytic activity of 162 U/mg, 195 U/mg, 234 U/mg and 332 U/mg, and however *Pseudomonas peli* showed significantly higher protease activity ($p<0.0001$) in supernatant (511.24 U/mg) (Figure 4.49); therefore, it was selected for further study.

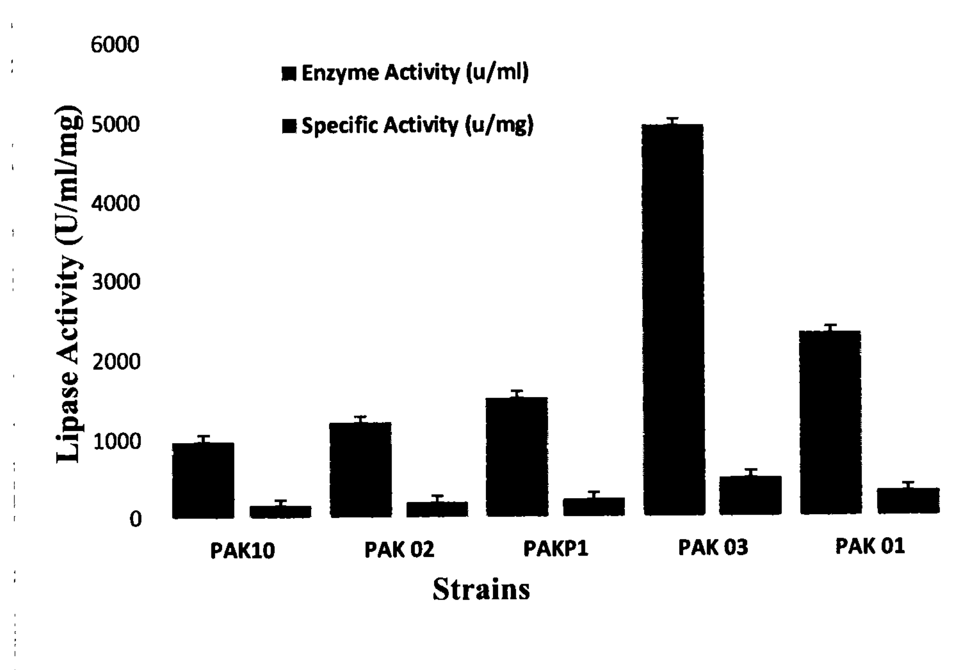


Figure 4.51: Screening for alkaline lipase activity in supernatant of bacterial cells. All values are represented as mean ± sd of three replications.

4.8.2. Optimization of lipase production

Supernatant of production culture media inoculated for 30 hours with 7% inoculum provided highest catalytic production of thermolabile lipase. From the results it was quite apparent that maximum specific activity of enzyme observed was (677 U/mg) when 30 hours old growth broth culture of *pseudomonas peli* was inoculated as an inoculum for lipase production. when the inoculum age of 6, 12, 18, 24, 36, 42 and 48 hours were inoculated the fermentation media, specific activity observed was 293, 327, 453, 489, 716, 493 and 263 U/mg experimented respectively (Figure 4.50).

In this experiment the basal fermentation medium was inoculated with distinctive concentration of 1, 5, 7, 10, 15 and 20 % inoculum size at pH 9. Products indicated that the maximum specific activity was witnessed (591 U/ml) in case of 7% inoculum size whereas the effect of different inoculum size 1, 5, 10, 15, and 20% on lipase production efficiency of isolate PAK03 were observed to be specific activities of 280, 473, 504, 336 and 172 U/mg, respectively (Figure 4.51).

To optimize the incubation period the submerged fermentation process was observed uninterruptedly for 144 hrs in lipase stimulating production media and the growth pattern was also observed for 144 hrs in basal media for pH 9 at 25 °C. After 96 hours incubation, thermolabile lipase production rise gradually and highest activity was observed as (518 U/mg) (Figure 4.52). Though, cell growth observed was maximal at 120 h of incubation which indicate that the production of enzyme was independent to cell growth (Figure 4.53).

Optimum temperature for growth and lipase production was determined by *pseudomonas peli,* the selected potential strain was cultivated in the production fermentation medium at numerous temperatures; 4, 10, 15, 20, 25, 30, 40 and 50°C. After the production culture was inoculated with the above optimized inoculum age and size and incubated at different temperatures, at 15°C the thermolabile lipase production was more or less identical as 25 °C which was observed as (569 U/mg) suggesting that the 15 °C cold temperature is appropriate for lipase enzyme production (Figure 4.54). Nevertheless, secretion of lipase at 4 °C was extremely truncated (21 U/mg). This indicates that enzyme was thermolabile lipase.

Extracellular lipase production influence by pH factor was considered and observed that lipase was grow in wide assortment of pH 7 to 10 but the specific

catalytic activity observed was highest at pH 9 by 641 U/mg, and it was a significant proliferation while associating with specific activity of pH 7 at 434 U/mg. The lipase production was declined after pH 10. Though, thermolabile lipase enzyme was observed tolerant toward the alkaline condition and was found appropriate for production of lipase (Figure 4.55).

Best carbon and nitrogen sources consumption are essential for lipase production at maximum level, as these can considerably diminish the expense. Consequently, exploitation of numerous carbon source, mineral salts, organic and inorganic source of nitrogen sources were estimated. The effect of supplement of source of organic and inorganic nitrogen in a batch culture fermentation system is shown in (Figure 4.56). Peptone, yeast extract and ammonium nitrate at 1% concentration additional source of nitrogen augmented the thermolabile lipase secretion from *pseudomonas peli* up to 509 U/mg, 467 U/mg and 420 U/mg in every instance. Conversely, the addition of Beef extract, Magnesium nitrate, citric acid and sodium citrate was observed do not have any significantly stimulating effect on the secretion of thermolabile lipase from PAK03 in fermentation reaction.

Comparable to conclusions in the above experiment, limited descriptions are existing on peptone as the suitable source of the carbon (Saxena *et al.*, 2003) and stimulation in secretion of lipase when ammonium nitrate was provided as inorganic source of nitrogen to the microorganism in basal culture medium (Gopinath *et al.*, 2003). Main aspect for the lipase hydrolytic activity expression was described as the source of carbon, subsequently lipases enzymes are inducible (Gupta *et al.*, 2004). Usually these enzymes stimulated in the existence of a triacylglycerol substrate for example lubricant, grease or any further inducer, such as Tweens, fatty acids, hydrolysable esters, triacylglycerols, glycerol bile and salts (Sharma *et al.*, 2009).

Diverse sources of the carbon were experienced for their potential capability to provision of production of lipase enzyme. Results from these observations designate that *pseudomonas peli* was potentially proficient to use the carbon sources for the stimulation of lipase enzyme molecules in supernatant, however highest growth was achieved using as carbon source tributyrin substrate (Figure 4.57). Tributyrin supplementation as a substrate of triacylglycerols in the fermentation media was observed to stimulate the thermolabile production of lipase and the catalysis was investigated as 433 U/mg activity.

Tributyrin might have a proper stimulator for the lipase enzyme production in fermentation reaction mixture. Carbon source such as glucose and sucrose were observed hydrolytic activity of 409 U/ml and 340 U/ml however maltose, lactose, tween 20 and olive oil did not have much stimulatory effect on the cold active alkaline lipase catalysis.

Mineral salts such as calcium ions $CaCl_2$ indicated inducing effect with the catalytic activity of 3766 U/ml (656 U/mg) on thermolabile lipase production though sodium $NaCl_2$ with 1176 U/ml (423 U/mg) and magnesium $MgCl_2$ with 1212 U/ml (467U/mg) was observed to have reasonable effect on stimulation of lipase as shown in (Figure 4.58). However, potassium ions, ferric chloride and Zinc sulfate revealed inhibitory influence on the lipase catalytic activity.

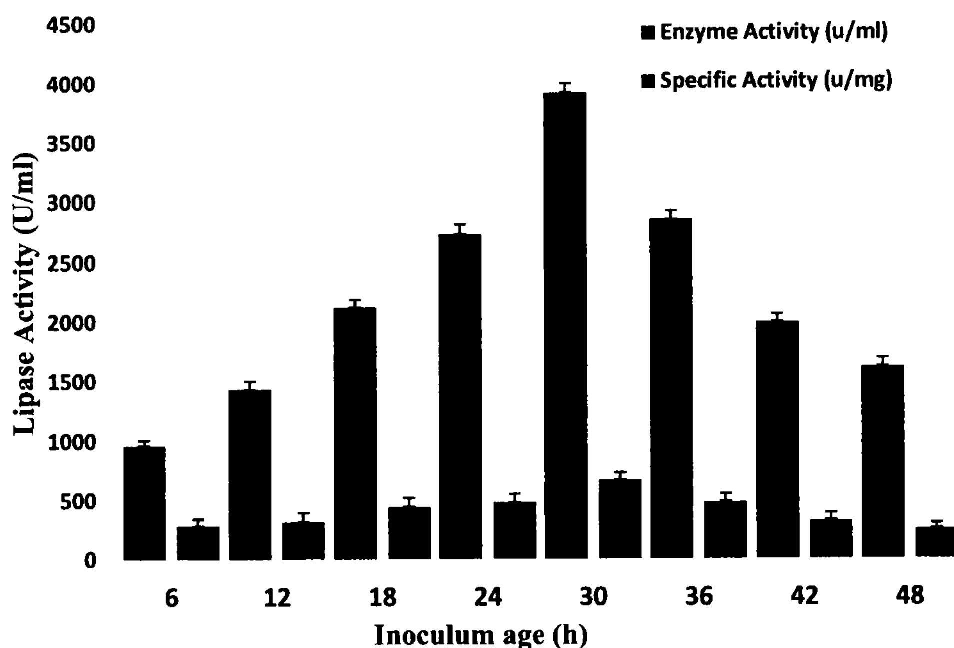


Figure 4.52: Effect of inoculum age on the production of lipase from *pseudomonas peli*. Inoculum size for this experiment was 5% v/v. All values are represented as mean ± sd of three replications.

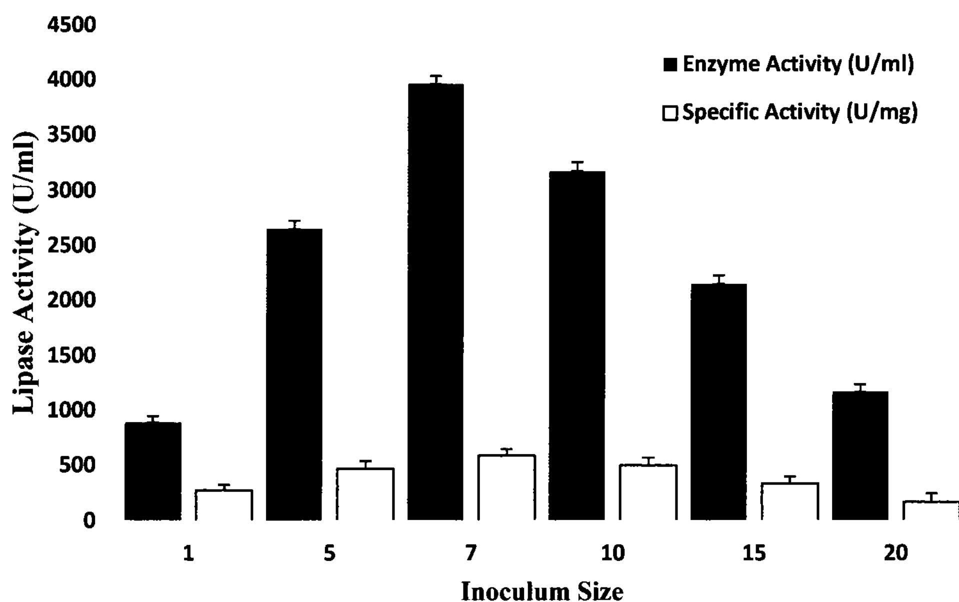


Figure 4.53. Effect of inoculum size on production of lipase from *pseudomonas peli*. Inoculum age for this experiment was 30 h at temperature 20 °C and pH 9.0. All values are represented as mean ± sd of three replications.

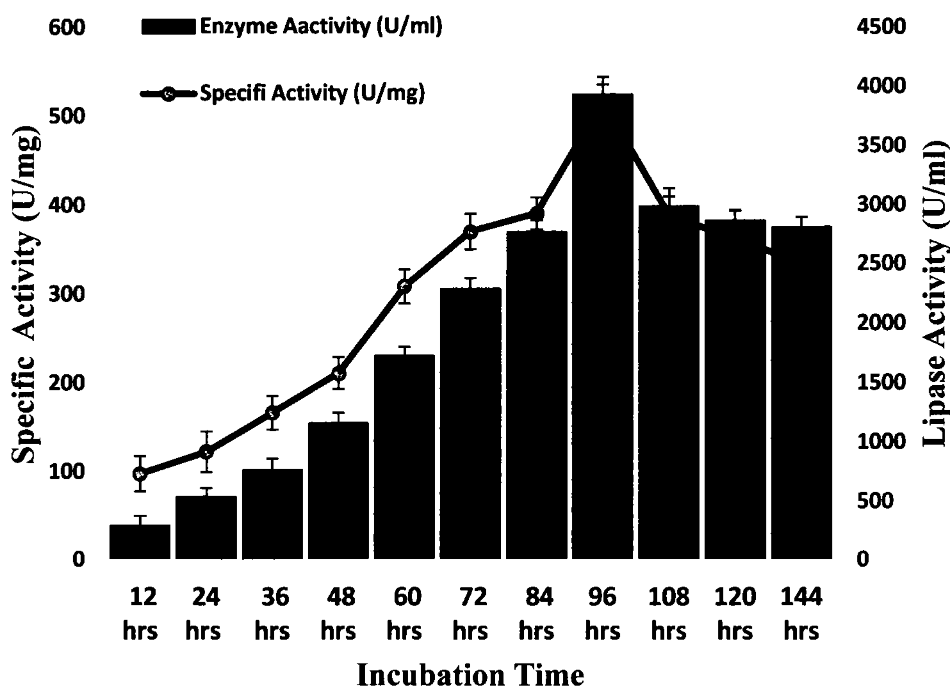


Figure 4.54: Effect of Incubation period on the enzyme production by *pseudomonas peli* PAK03. All values are represented as mean ± sd of three replications.

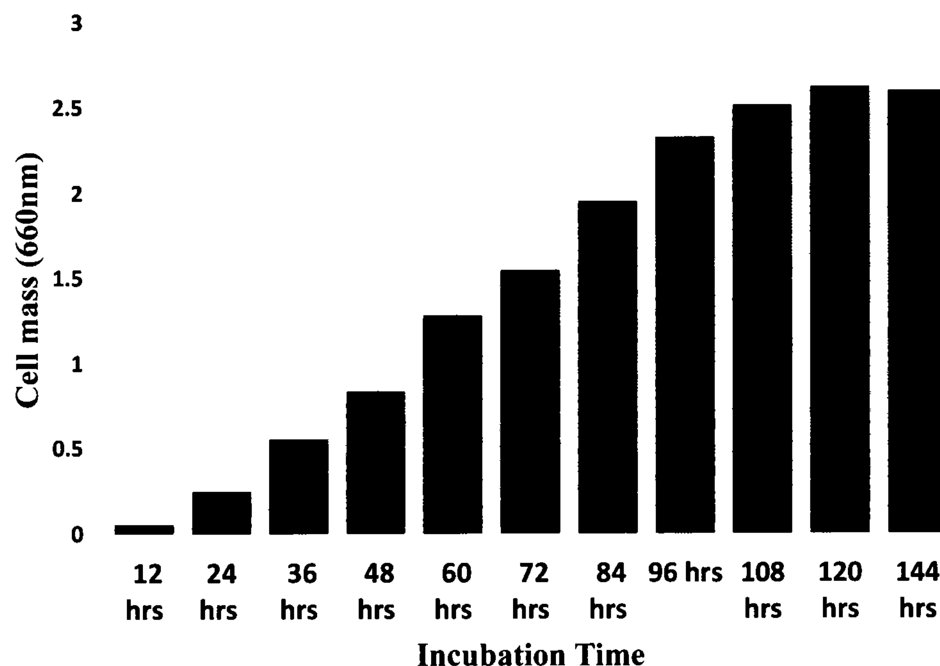


Figure 4.55: Effect of Incubation period on the growth by *pseudomonas peli* PAK03

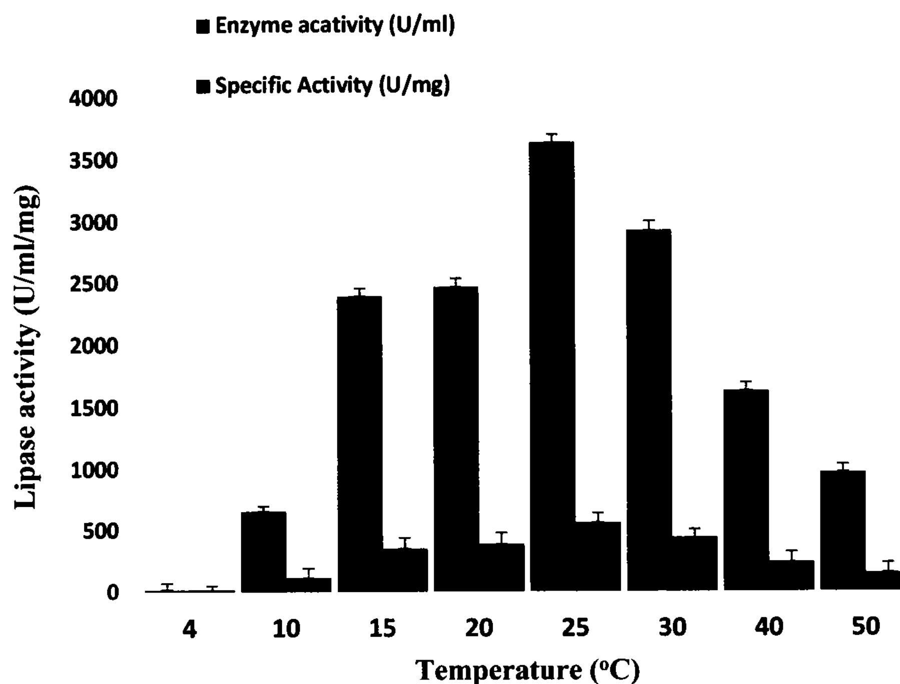


Figure 4.56: Effect of distinctive Temperature on the production of alkaline Lipase by *pseudomonas peli* PAK03. All values are represented as mean ± sd of three replications.

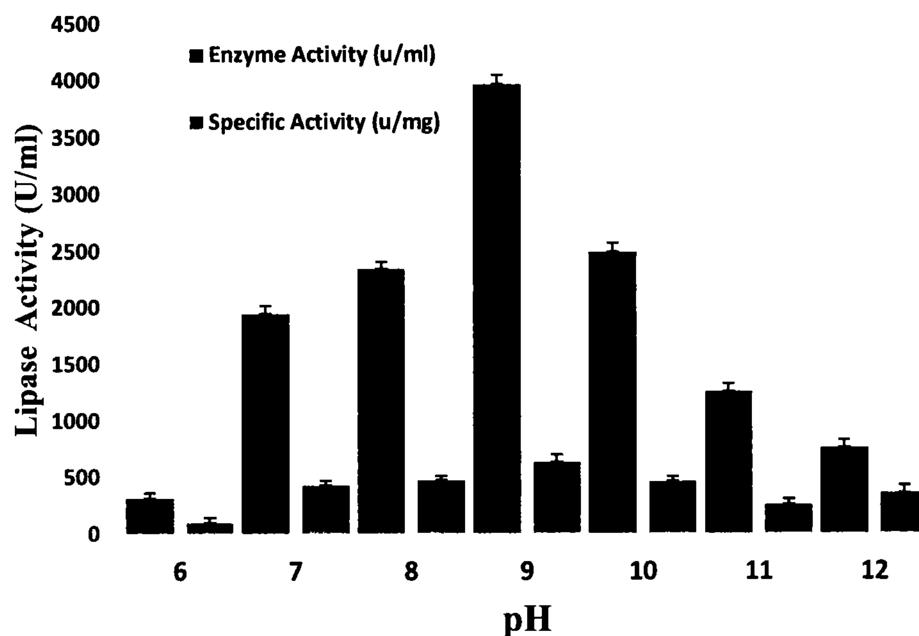


Figure 4.57: Effect of distinctive pH on the production of alkaline Lipase by psychrotrophic *pseudomonas peli* PAK03. All values are represented as mean ± sd of three replications.

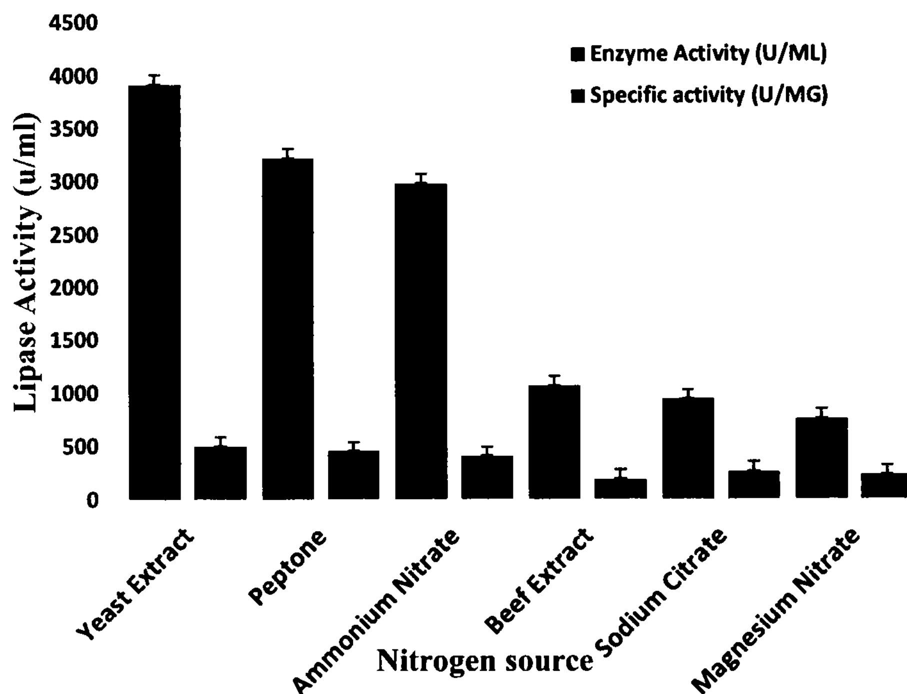


Figure 4.58: Effect of different organic and inorganic nitrogen source on lipase production by *pseudomonas peli*. All values are represented as mean ± sd of three replications.

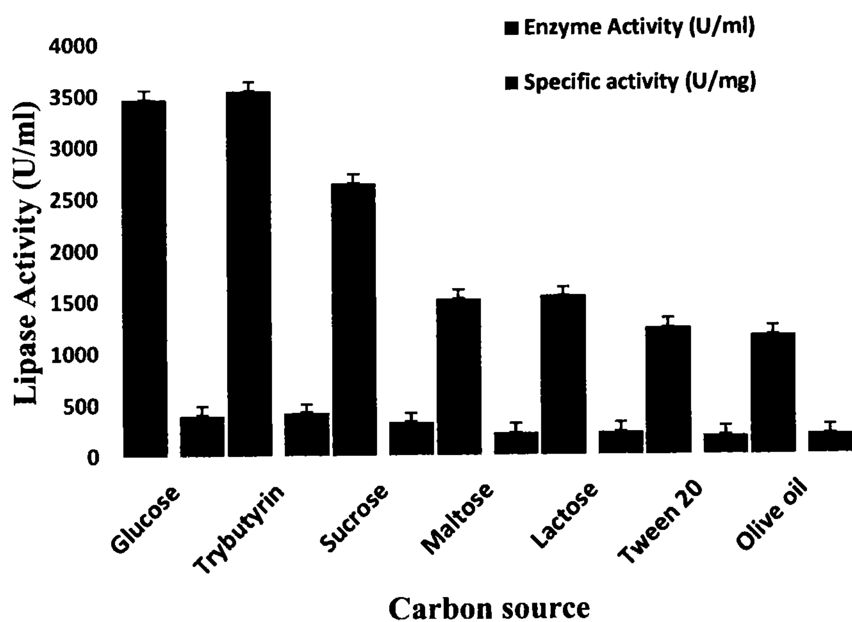


Figure 4.59: Effect of different carbon source on lipase production by *pseudomonas peli* PAK-03. All values are represented as mean ± sd of three replications.

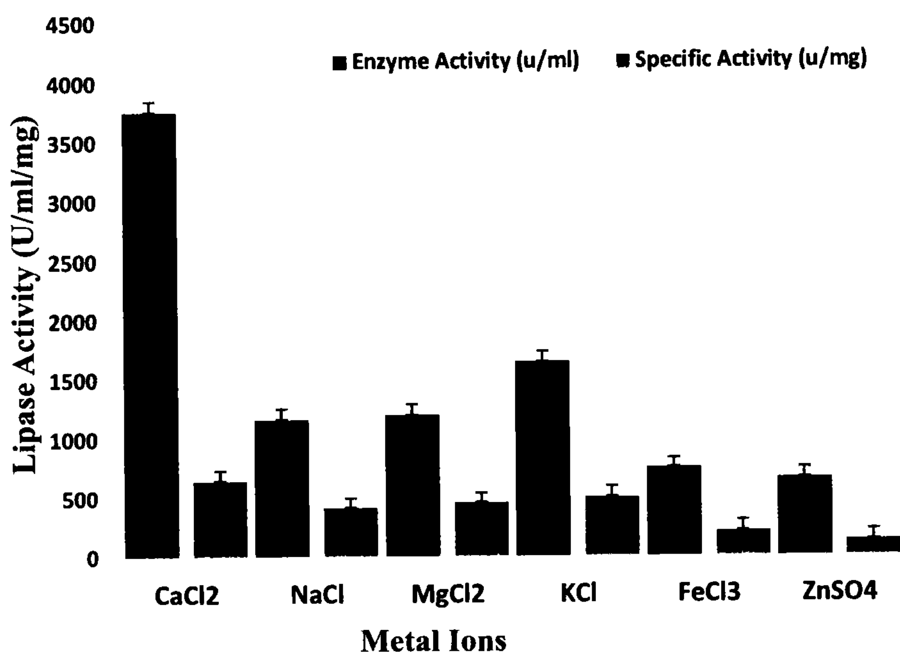


Figure 4.60: Effect of different Mineral Salts on lipase production by *pseudomonas peli* PAK-03. All values are represented as mean ± sd of three replications.

4.9. Purification and Characterization of thermolabile lipase from Psychrotrophic *Pseudomonas peli* MG687270

4.9.1. Purification of cold-active alkaliphilic Lipase

In this experiment purified cold-active lipase produced by PAK03 in the fermentation production medium was subjected to a different technique of purification. PAK03 a psychrotolerant microorganism, grew very fast in the fermentation medium and maximum extracellular lipase enzyme production has experimented subsequently after 96 hours in the stationary growth phase of bacteria. The supernatant obtained after centrifugation was cleaned and exploited as the extracellular crude lipase enzyme. The cold active alkaliphilic lipase enzyme was purified with distinguishing purification techniques that are concise in (Table 4.9). Precipitated $(NH_4)_2SO_4$ partially purified protein was obtained were subjected to dialysis for further removal of excess of salts and impurities. The dialyzed extract enzyme was first subjected onto an Ion exchange chromatography technique (IEX SOURCEQ 10/10) pre-equilibrated with Tris–HCl buffer at the concentration of 50 mM, pH 8. Elution of unbound proteins was carried out with the equilibration buffer and 5 ml fractions were collected at a flow frequency of 60 mL/hour. The column was washed with the equilibration buffer and then elution with a linear gradient of 0.1–1.0 Molar concentration of sodium chloride $NaCl_2$ was exploited and the two peaks of cold-active purified proteins were eluted showed in (Figure 4.61). Fractions collected were analyzed for protein assay by measuring their absorbance at 280 nm wavelength and enzyme activity was analyzed by standard assay conditions and those with the enzymatically active protein fractions from DEAE-Cellulose Ion exchange chromatography were selected, pooled and subject for further purification at 18ml/hour onto gel chromatography Superdex 200 PG 16/60 equilibrated with 50 mM Tris–HCl at pH 8. After loading the sample, the column was rinsed with equilibration buffer and eluted with a linear gradient from 0.1 to 0.5 Molar $NaCl_2$ in the uniform buffer to get the homogenous mixture of cold-active lipase enzyme molecules (Bae *et al.*, 2014).

Hydrolytic activity of both the points from ion exchange chromatography technique was assayed and the fractions with high catalytic activity were pooled and

subjected to Gel Filtration Chromatography (GFC: Superdex 200 PG 16/60) and the active fraction of this step is presented in (Figure 4.62). After these purification stages, lipase was purified to clear homogeneity as estimated by SDS– PAGE analysis (Sharma *et al.*, 2017). The molecular weight of the purified lipase protein was assessed to be approximately 32,000 ± Da (Angkawidjaja and Kanaya, 2006) after silver staining SDS– PAGE analysis respectively and lipase enzyme was observed single polypeptide enzyme as SDS-PAGE illustrated (Figure 4.63). Under silver staining lipase protein showed purity with an expected 32 KDa molecular mass respectively. Most of the lipases secreted from the *Pseudomonas* genus have been observed in the range of 30–62 kDa molecular mass (Arpigny and Jaeger, 1999) such as, *Pseudomonas pseudoalcaligenes* F-111 with 32 kDa (Lin et al., 1996), *P monteilii* TKU009 with 44 kDa (Wang *et al.*, 2009), *Pseudomonas aureginosa* with 54 kDa (Karadzic *et al.*, 2006). Purified monomer proteins were observed from the protein analysis. The specific activity of 1381.22 U/mg with purification fold of 68.42 and yield recovery of 20.52% was observed from the overall purification procedure analysis.

Table 4.9. Purification of extracellular lipase from *Pseudomonas peli* PAK03.

Purification steps	Enzyme Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude extract	27600	1367.3	20.186	1.0	100
Ammonium sulfate precipitation	19199	441.6	43.48	2.154	69.56
Dialysis	16264	97.7	166.47	8.247	58.928
Ion exchange chromatography (IEX: SOURCEQ 10/10)	8789	13.8	676.08	33.493	31.84
Gel Filtration Chromatography (GFC: Superdex 200 PG 16/60)	5663	4.1	1381.22	68.42	20.52

- All steps were carried out at 4°C
- One unit of activity(U) was defined as the amount of the enzyme releasing 1μg of p-Nitrophenol per 1 min at 25°C
- Protein was eluted in a linear gradient between 0 and 0.5 M NaCl with 20 mM Tris-HCl buffer (pH8.5), desalting was carried out by dialyzing against 10 mMTris-HCl buffers (pH8.0)
- Protein was eluted with 10mM Tris-HCl Buffer (pH 8.0)

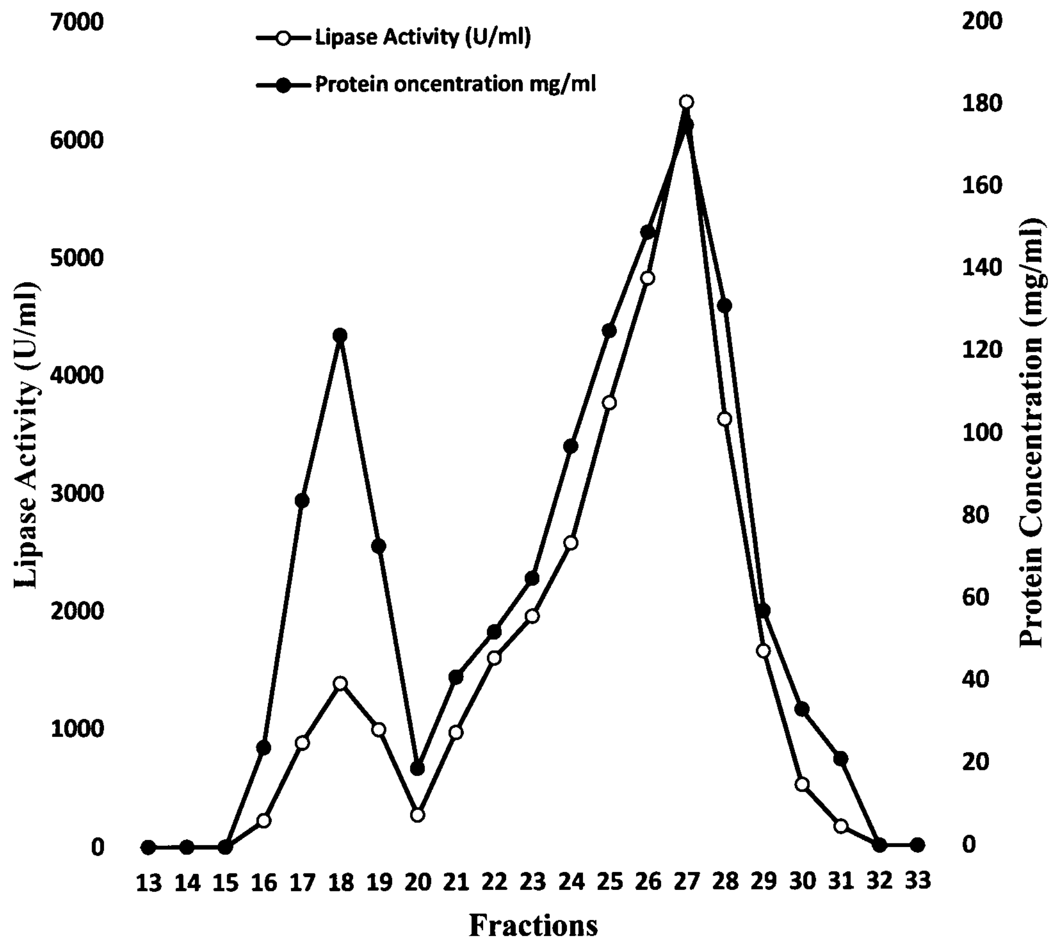


Figure 4.61: Protein and enzyme activity profile of fractions of ion exchange chromatography (IEX: SOURCEQ 10/10) of the dialyzed lipase of *Pseudomonas peli* sp.

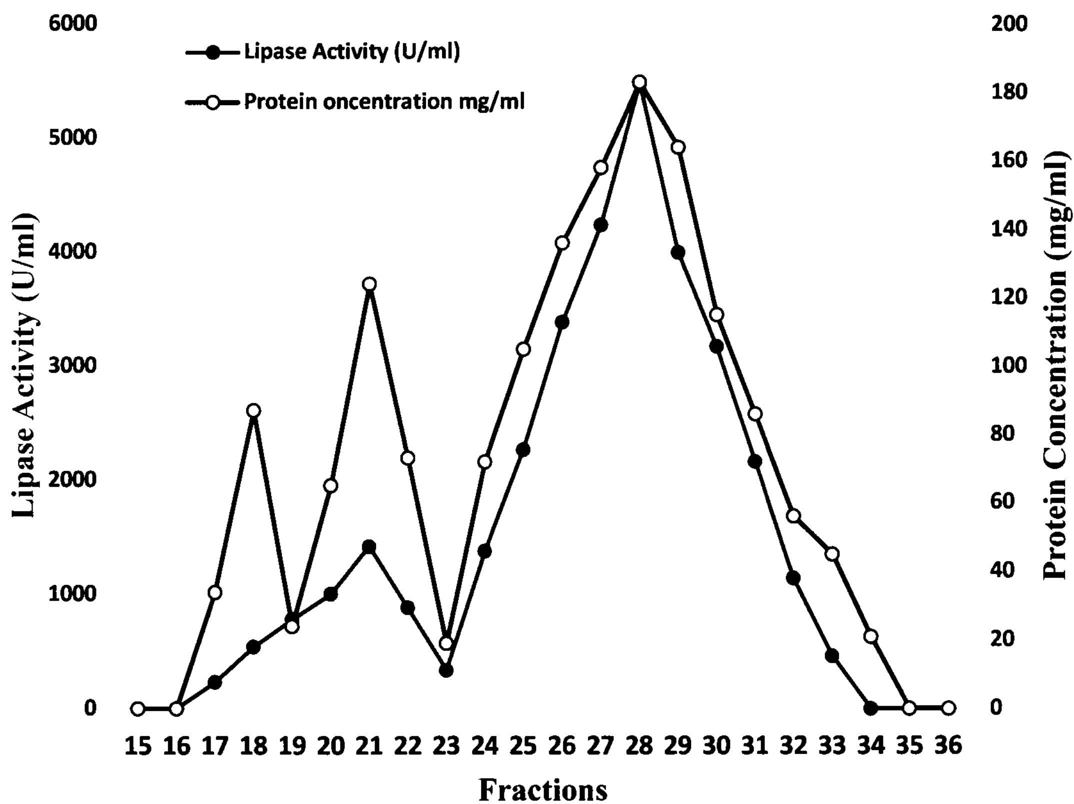


Figure 4.62: Chromatograph of pooled fractions of IEX: SourceQ of alkaline lipase on Gel Filtration Chromatography colum (GFC: Superdex 200 PG 16/60).

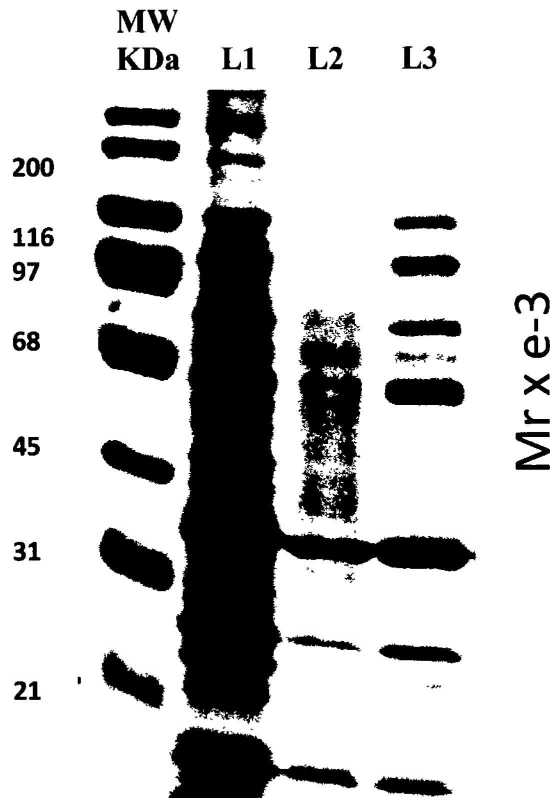


Figure 4.63: 150 ul of each fraction was precipitated by Chloroform/Methanol method. Precipitated proteins dissolved in 40 ul of SDS sample buffer and heated for 15 min at 65C. 30 ul of sample was loaded on gel. Gel stained with Silver according to Blum *et al.*, (1987). L1(Crude supernatant) and L2 & L3 (Purified protease enzyme).

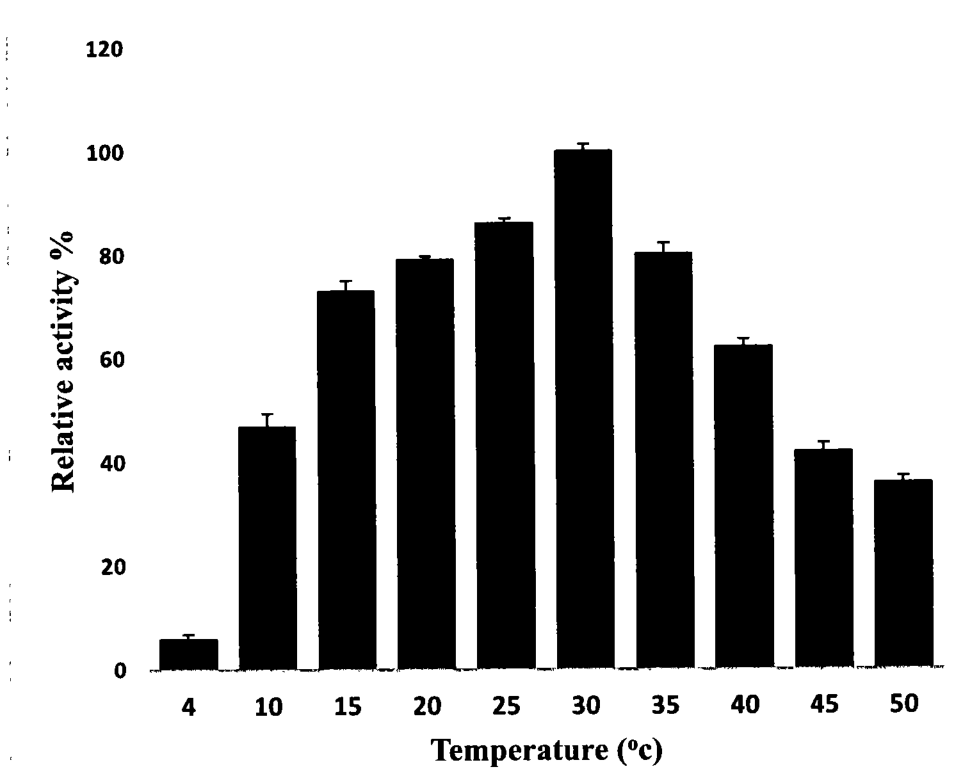


Figure 4.64: Effect of temperature on the activity of the extracellular lipase from *Pseudomonas peli*. Lipase activities were monitored at the indicated reaction temperatures, in 50 mM Tris-HCl buffer, pH 8.0, using p-nitrophenyl palmitate as substrate. The maximum activity at 30 °C was taken as 100%. All values are represented as mean ± sd of three replications.

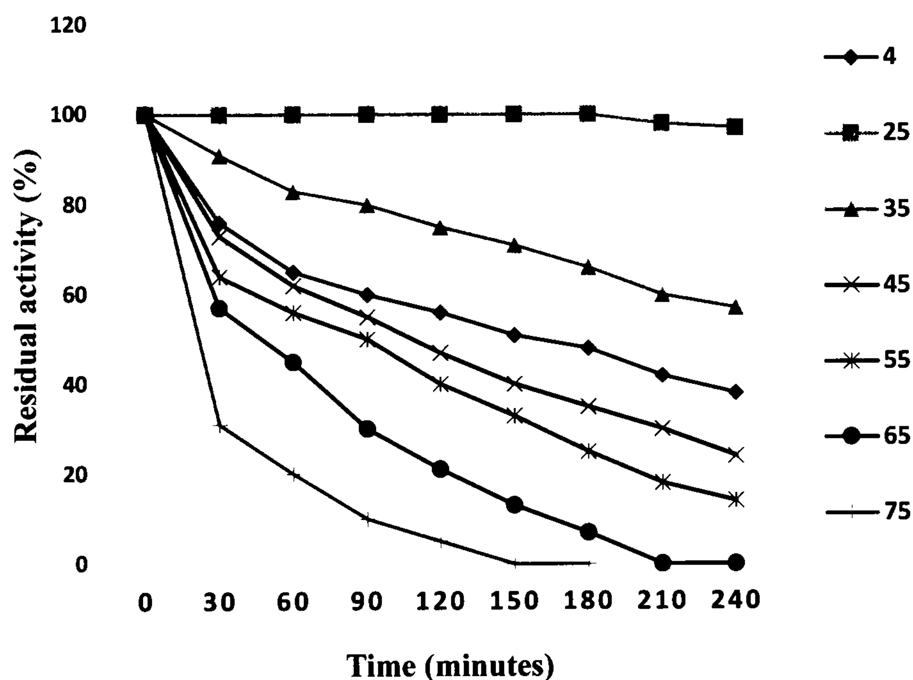


Figure 4.65: Effect of the temperature on the stability of the extracellular lipase from *Pseudomonas peli*. The thermal stability of PAK03 lipase was investigated by pre-incubating the lipase at different temperatures (25, 35, 45, 55, 65vand 75 °C for 240 min and 4 °C). Residual lipase activity after incubation at each temperature was assayed. Lipase activity at 25 °C, pH 8.0, without any prior incubation was taken as 100%.

4.9.2. Temperature influence on the extracellular lipase enzyme hydrolytic activity and stability

Lipolytic assay was assessed in the wide-ranging temperature from 4 to 60 °C, and the highest catalytic Unit/ml value experimented was at 30 °C (Figure 4.64). To investigate the thermostability properties of the purified hydrolytic enzyme, preincubated the enzyme at high temperature and the residual hydrolytic activity was investigated. Thermolability properties have been observed due to stability of cold active lipase enzyme at low temperature circumstances and has been observed from the results that enzyme exhinited relatively high activity from 15 to 35 °C

Residual activity of 47% of hydrolytic activity was experimented at 10 °C and was completely inhibited at 4 °C. Comparable to our thermolabile lipase producing microorganism, some of the gram-negative lipase producing mesophilic or psychrotrophic was observed to have highest lipolytic activity above 40 °C than the growth at optimal temperatures circumstances of their source bacteria (Angkawidjaja and Kanaya, 2006). Numerous psychrotolerant *Pseudomonas peli* PAK03 secreting thermolabile enzyme was experimented with the highest catalysis of substrate from 20 to 45 °C (Alquati *et al.*, 2002; Zhang and Zeng, 2008).

Against 50 °C *Pseudomonas peli* secreting thermolabile lipolytic enzyme with the residual activity of 32% ($p<0.0001$). Consequently, exceeding from 45 °C negatively influenced the lipolytic activity and was observed that the thermolabile lipase do not showed activity at high temperature. Thermolabile enzyme exhibited maximum hydrolytic activity at temperatures lower then 40 °C, and approximately half of its lipolytic hydrolysis was constrained at temperatures of 45 °C. Nonetheless, after preincubation at 50 °C its denatured lipase enzyme molecules and no catalytic hydrolysis was observed.

Thermostability analysis of the enzyme was experimented at different temperature from 4 to 75°C. Thermolabile enzyme demonstrated stability at low temperature 4 °C and after the incubation of reaction mixture at 240 minutes observed retained 38% of the residual activity (Figure 4.65). Half of its activity was inhibited at 55 °C and preserved 50% of its residual preliminary activity by incubation of the reaction mixture at 90 min and the thermolabile enzymes was supposed to have stability toward high temperature

From the present observation it was observed that the thermolabile extracellular lipase secreted from *Pseudomonas peli* PAK03 has been showed higher stability as compared to other psychrophiles secreted lipase enzyme previously investigated by (Zhang and Zeng, 2008, Lee *et al.*, 2003, Yuan *et al.*, 2010, Joseph *et al.*, 2006, Yadav *et al.*, 2011 and Cai *et al.*, 2009). Thus, is a key property particularly for biocatalysts intended to be utilized for prolonged periods in industrial applications.

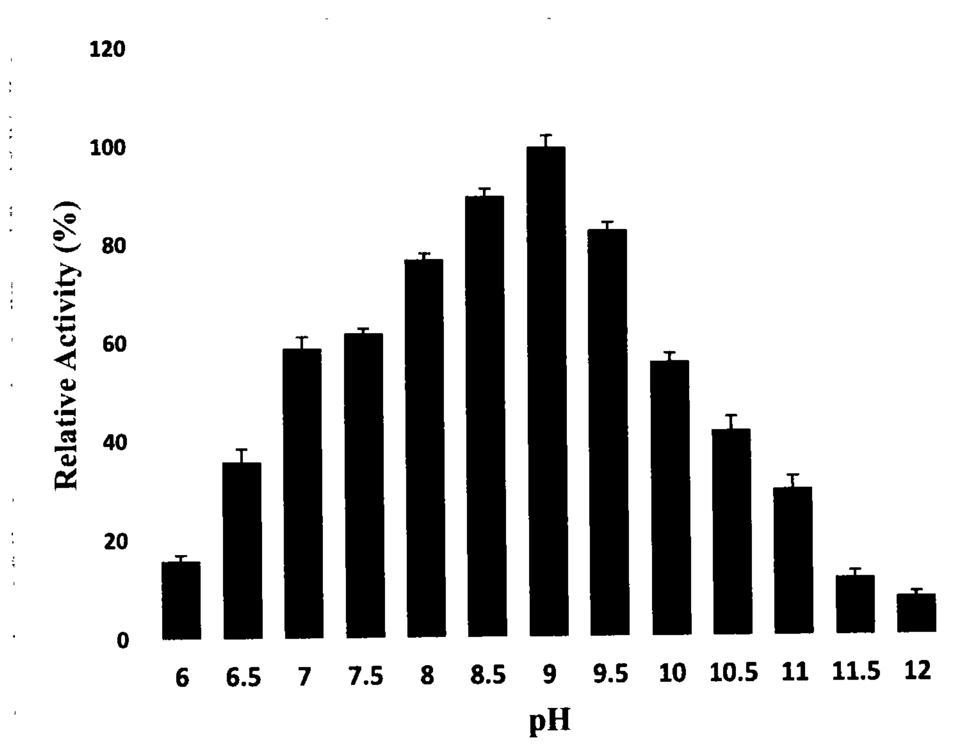


Figure 4.66: Effect of pH on the activity of the extracellular lipase from *Pseudomonas Peli.* The lipase activity was assayed in buffers of different pH values at 30 °C, using p-nitrophenyl palmitate as substrate. The buffer systems were potassium phosphate buffer (pH 5.0–7.0), Tris-HCl buffer (pH 7.0–9.0), and glycine-NaOH buffer (pH 9.0–12.0). The maximum activity at pH 8.0 was taken as 100%. All values are represented as mean ± sd of three replications.

4.9.3. Effect of pH on the activity and stability of the extracellular lipase

pH is a significant consideration influencing the activity of lipase hydrolysis toward its p nitrophenyl palmitate substrate due to its consequence on the 3-dimensional arraignments of the protein structure in the reaction mixture. The alkaline stable catalytic activity of lipase enzyme was examined in the pH choice of 6 to 12, and the highest Unit/ml value was detected at pH 9 (Tris-aminomethane buffer) ($P<0.01$) (Figure 4.66).

Residual activity of 56%, 30% and 8% of its hydrolytic activity was retained at high alkaline conditions of pH 10, 11 and 12, correspondingly. The hydrolytic activity in the acidic environments observed significant diminution in lipolytic hydrolysis was detected. For instance, incubation at acidic condition against 6 pH, 16% of the residual activity has been retained.

In order to research the pH stability, the activity has been investigated for 60 minutes against different pH range such as 6–12 pH at 25 °C, and the retained residual activity (%) in the reaction mixture was analyzed (Figure 4.67).

The highest activity was observed toward pH 9 by the purified enzyme inhibiting 12 % with residual activity of 82%. Half of the residual activity was inhibited against 7 pH and has been observed that the enzyme gradually decrease the activity in acidic conditions. Furthermore, lipolytic activity suddenly vanished maximum of its activity, with retained residual activity of 59 % and 26 % toward preincubation with 10 pH following 11 pH, respectively.

From the present research disclosed that lipolytic enzyme was observed to be stable in alkaline conditions as compared to lipases secreted from other psychrophiles studied in the previously exploration of (Joseph *et al.*, 2008; Rajan and Nair, 2011; Ülker *et al.*, 2011), and greatly stable to diverse pH deviations.

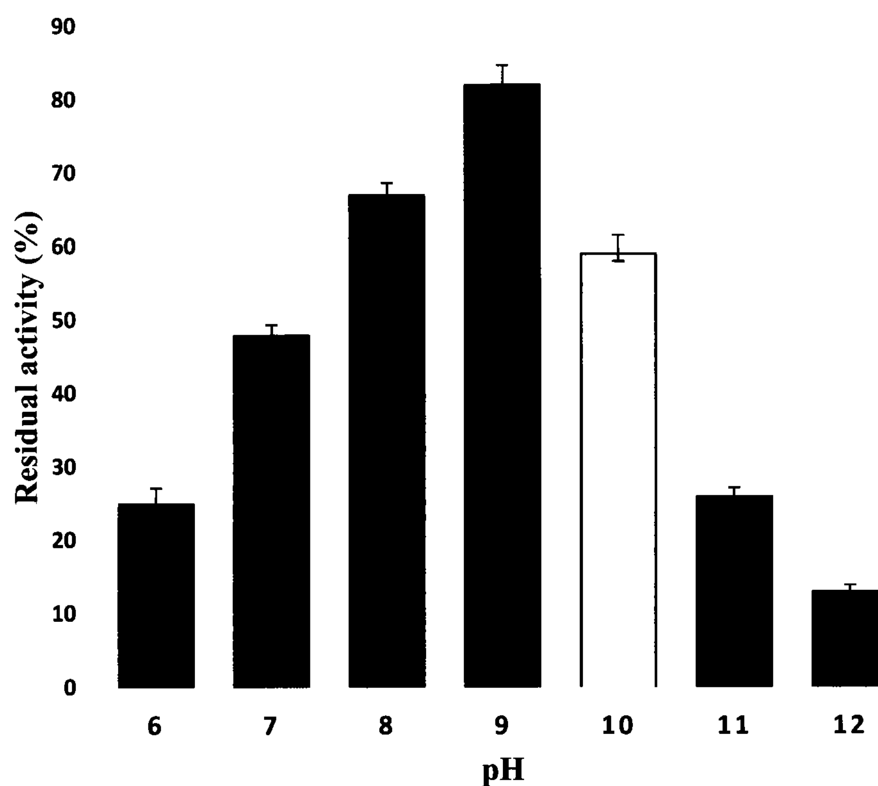


Figure 4.67: Effect of pH on the stability of the extracellular lipase from *Pseudomonas Peli*. The pH stability of alkaline lipase was investigated by pre-incubating the lipase at different pH (6.0–12.0 at 1-unit intervals) values for 60 min. Residual lipase activity after incubation at each pH was assayed. Lipase activity at 30 °C, pH 9.0, without any prior incubation was taken as 100%. All values are represented as mean ± sd of three replications.

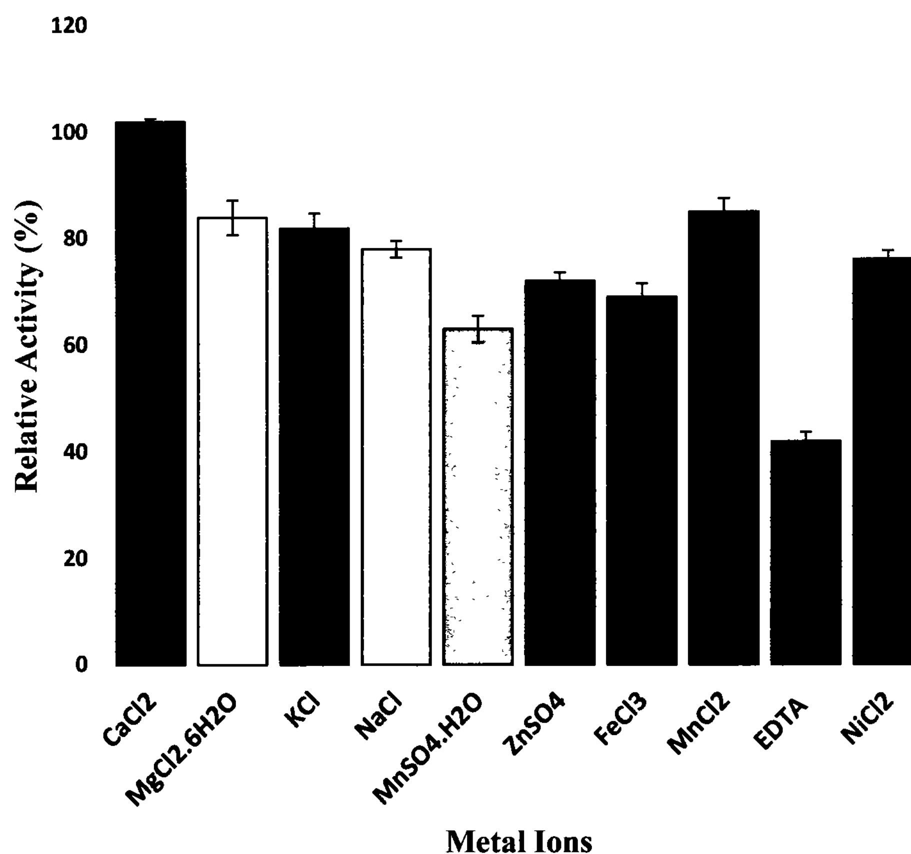


Figure 4.68: Effect of various metal ions on extracellular lipase activity from *Pseudomonas peli*. Lipase activity assay was performed at 30 °C, pH 9.0, in the presence of 1 mM various metal ions. Activity of control (no metal ions in the mixture) was taken as 100%. All values are represented as mean ± sd of three replications.

4.9.4. Metal ions effect on the thermolabile lipase enzyme activity and stability

Metal ions are considered to be responsible on behalf of the preservation of the stability and hydrolytic activity of thermolabile lipases enzyme (Ahmed *et al.*, 2010). Consequently, to investigate out whether the diverse metal ions stimulate or inhibit the residual activity (%), the consequences of $CaCl_2$, $MgCl_2$, KCl, NaCl, $MnSO_4$, $ZnSO_4$, $FeCl_3$, $MnCl_2$ and EDTA (effective as metal chelator) at 1 mM concentration on hydrolytic activity have been investigated and the consequences are summarized in (Figure 4.68).

Highest activity has been observed by incubation with CaCl2 with 2% stimulation in activity as compared to control ($P<0.0001$). Though, slightly inhibition in hydrolytic activity was observed in the existence of $MgCl_2.6H_2O$, KCl, NaCl, $MnSO_4$. H_2O, $ZnSO_4$, $MnCl_2$ in the reaction condition as more than 72% of the residual activity was retained by the enzyme.

$MnSO_4$. H_2O, and $FeCl_3$ was observed inhibited the hydrolytic activity by 37 % and 31% residual activity as for the other *Pseudomonas* lipases however to a smaller extent (Alquati *et al.*, 2002, Zhang and Zeng, 2008 and Rashid *et al.*, 2001). Incubation of alkaline enzyme toward EDTA inhibited half of its maximum hydrolytic activity, recommending thermolabile alkaline enzyme was a metalloprotein.

From the above research it is concluded that lipase secreted from *Pseudomonas peli* PAK03 showed stability regarding diverse metal ions. Similarly, the residual activity of alkaline lipase as treated with $NiCl_2$ exhibited a discriminating characteristic associated to other psychrophilic *Pseudomonas* secreted lipases, subsequently they were frequently stimulated only by $CaCl_2$, however not in the existence of the $NiCL_2$ that showed a distinguishing characteristic as compared to other *Pseudomonas* lipases investigated by the previous finding of (Joseph *et al.*, 2008).

4.9.5. Effects of different organic solvents on activity and stability of enzyme

Stability of enzyme towards organic solvent is a desired characteristic particularly in biocatalytic reactions such as transesterification and esterification (Gupta *et al.*, 2004). In direction to research the consequence of diverse sources of organic solvents on lipolysis such as acetone, n-hexane, ethanol acetonitrile DMSO,

2-propanol and methanol were experimented against at 30% concentration. DMSO stimulated the hydrolytic activity with residual activity of 83% (Figure 4.69).

The retained residual activity was about 34%, 69%, and 50% of catalytic activity has been examined following in the existence with ethanol, acetone, and methanol in the reaction solution (P<0.001). Inhibited residual activity was observed against Acetonitrile, n-hexane and 2-propanol.

From the present observation there are some conceivable justifications for the stimulating effects of organic solvents on hydrolytic activity of thermolabile lipase enzyme that the solvent might increase enzyme activity without of causing any denaturation by modifying oil and water interface. Furthermore, solvents may create modificational changes in the 3-dimensional protein structure or may stop lipase disaggregation into its integral parts (Ahmed *et al.*, 2010). In dissimilarity diverse solvents displaying negative influence on the hydrolytic activity might distort its functional catalytic triad on the active site of the enzyme, thus consequently preventing the triacylglycerol substances from efficiently accessing to the active site of the enzyme (Peng *et al.*, 2010).

Greater stability of enzyme against DMSO was a distinguishing characteristic compared to the other lipases secreted from psychrotrophic bacteria such as *Pseudomonas* sp. B11-1 (Joseph *et al.*, 2008). Lipase enzyme from PAK03 might have potential usage in organic synthesis of chemical composite and chiral resolution effects that involve the usage of DMSO as solvents in the biochemical reactions.

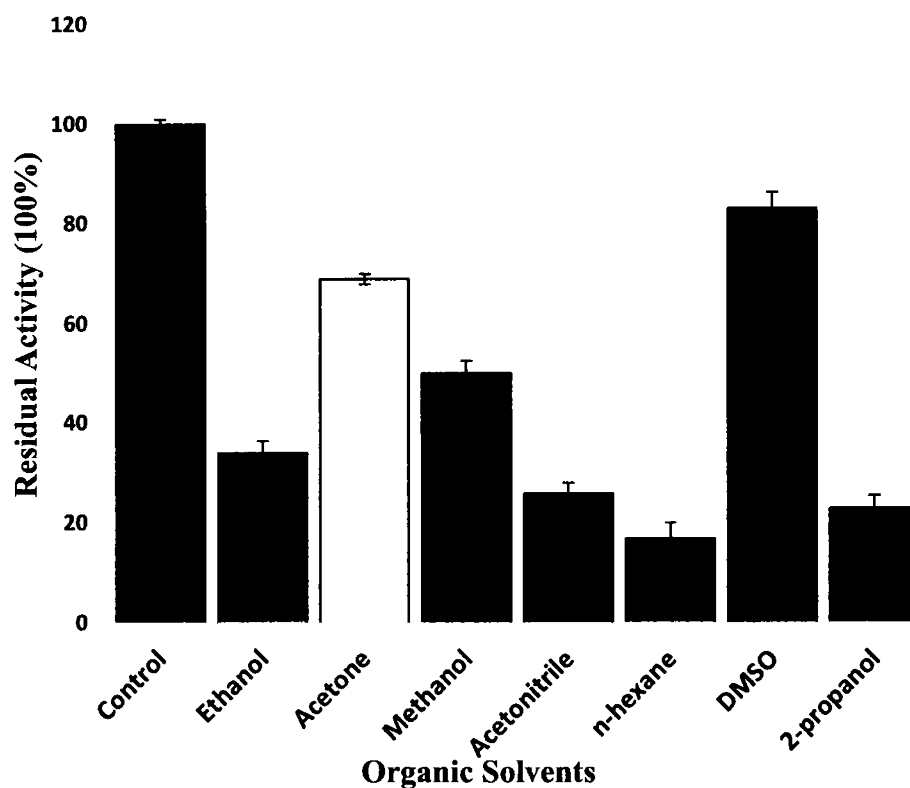


Figure 4.69: Effect of various organic solvents on extracellular lipase activity from *Pseudomonas peli.* Lipase activity assay was performed at 30 °C, pH 9.0, in the presence of 30% (v/v) various organic solvents. Activity of control (no organic solvents in the mixture) was taken as 100%. All values are represented as mean ± sd of three replications.

4.9.6. Determination of Km, Vmax, Kcat and Activation energy Ea(d) for the purified Lipase

Km was assessed from the double reciprocal Plot following of Lineweaver Burk equation for p-NPP as 4-Nitrophenyl palmitate (Figure 4.70). Lipase activity was progressively increased from (0.1 to 2 mg/ml) concentration of p-Nitrophenyl palmitate and show maximum enzyme activity (2500 U/ml/min) at 2 mg/ml concentration of olive oil. The Km value was determined to be 1.75 mg/ml for substrate respectively. Reciprocal of the intercept was conducted for the determination of Reaction rate Vmax.

The low apparent Km designates that the thermolabile lipase has greater affinity for p- NPP triacylglycerol substrate. The turnover number, k_{cat} and kinetic efficiency were calculated as 47.04 min^{-1} and 69.18 $min^{-1}mg^{-1}ml^{-1}$, respectively.

4.9.7. Thermal denaturation of lipase enzyme

Enzyme thermal stability has been experimental in temperatures varying from (30 to 80 °C) for 80 min. Outcome for thermal denaturation are presented in (Table.4.10). Log (ln % Residual Activity) of the thermolabile enzyme are displayed in (Figure 4.36). Dissociation constant k_d for the thermal denaturation of extracellular thermolabile proteas are respectively exhibited in (Table 4.11) and (Figure 4.71).

Thermolabile enzyme activity has been showed stability at 30 °C temperature subsequently after 80 min of incubation period exhibiting maximum activity of enzyme (76 U/ml/min). Thermolabile enzyme activity has been progressively decrease above 30 °C. Cold active enzyme has been observed volatile and denature at 80 °C ubsequently after 80 min of incubation presenting the minimum protease enzyme activity (6 U/ml/min).

The values of thermodynamic parameters for alkaline lipase was calculated at diverse wide- ranging temperatures from 30 to 80 °C. $E_{a(P)}$ calculated for protease enzyme form *pseudomonas peli* was 40.17 KJ/mol (Figure 4.72). The lower $E_{a(P)}$ suggested that lesser energy was required by alkaline hydrolytic lipase enzyme to reach the transition state of *p* nitrophenol formation.

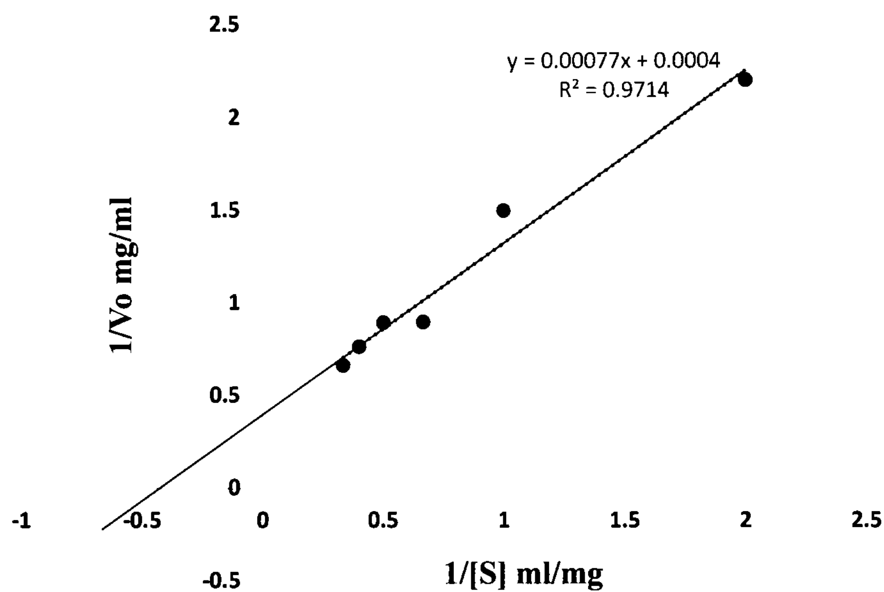


Figure 4.70: Determination of Kinetic parameters of purifiied extracellular lipase enzyme by line Weaver-Burk Plot

Table 4.10: Thermal denaturation of extracellular lipase enzyme

Temperature (°C)	10	20	30	40	50	60	70	80
30	96	93	91	88	86	82	80	76
40	89	86	84	81	79	72	70	67
50	77	74	72	70	67	60	55	49
60	70	66	64	62	57	48	42	39
70	54	50	48	40	32	26	22	14
80	38	34	32	30	20	12	6	0

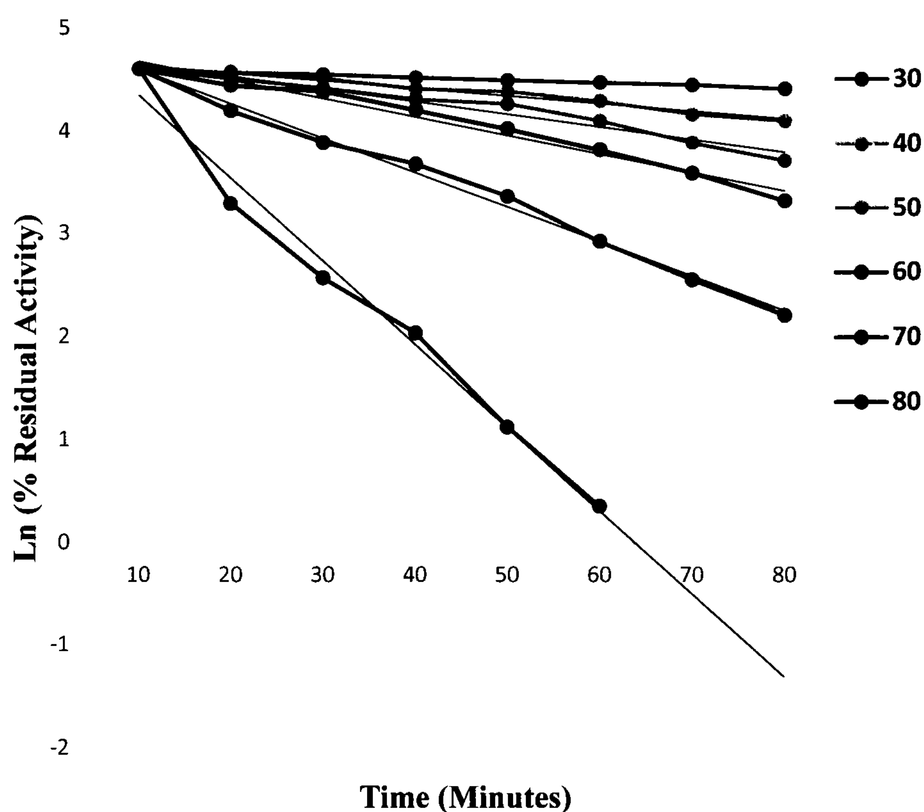


Figure. 4.71: ln (% Residual Activity) of extracellular lipase enzyme

Table. 4.11 k_d value of extracellular lipase enzyme

Temperature °C	K	*Kd*
30	303	0.0323
40	313	0.0417
50	323	0.0361
60	333	0.0411
70	343	0.0594
80	353	0.1395

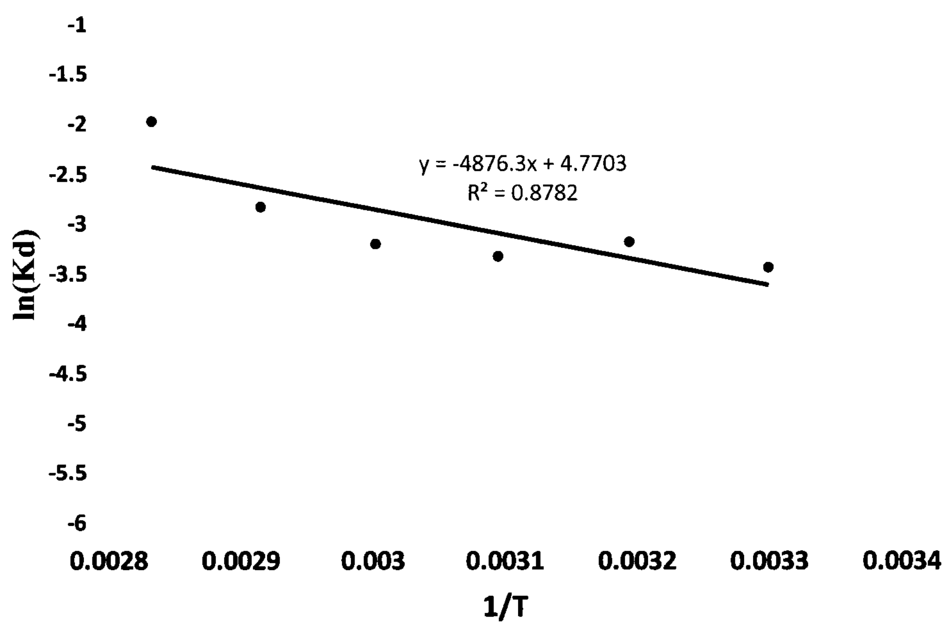


Figure 4.72: Arrhenius plot for the determination of activation energy ($E_{a(P)}$).

4.10. Alkaline Lipase from Psychrotrophic *Pseudomonas peli* PAK03 for Detergent Additive Capability

4.10.1. Lipase compatibility with commercial detergents and surfactants

The *Pseudomonas peli* lipase showed exceptional compatibility and stability in existence of sodium, ammonium, sulfonate and ethoxylates, alkoxylates and cocamide surfactants as well as in laundry detergents. Alkaline lipase from the experiment presented decent lipolytic activity in existence of Tween 80 and SDS through the same findings were also resulted for surfactant tolerant alkaline lipases from *Rhizopus* sp. and *Aspergillus* sp. (Derewenda *et al.*, 1994 and Saisubramanian *et al.*, 2006).

For effectual usage under extreme circumstances in the laundry detergent industry, alkaline enzyme required be companionable and stable amongst all frequently used as the additive in the detergent formulation constituents (Kamini *et al.*, 2000).

Amongst innumerable detergents was tested, better enhanced hydrolytic activity of lipase was observed from the present experiment with Gain and alkaline hydrolytic lipase revealed 86% residual activity after 1 hour of incubation with SDS (Figure 4.73).

Nonetheless, the lipolysis of *Ralstonia pickettii* (Hemachander and Puvanakrishnan, 2000) and *Aspergillus carneus* has been suppressed in the existence of SDS (Saxena *et al.*, 2003) although lipolytic activity was encouraged in situation of *H. lanuginosa* secreted lipase (Omar *et al.*, 1987).

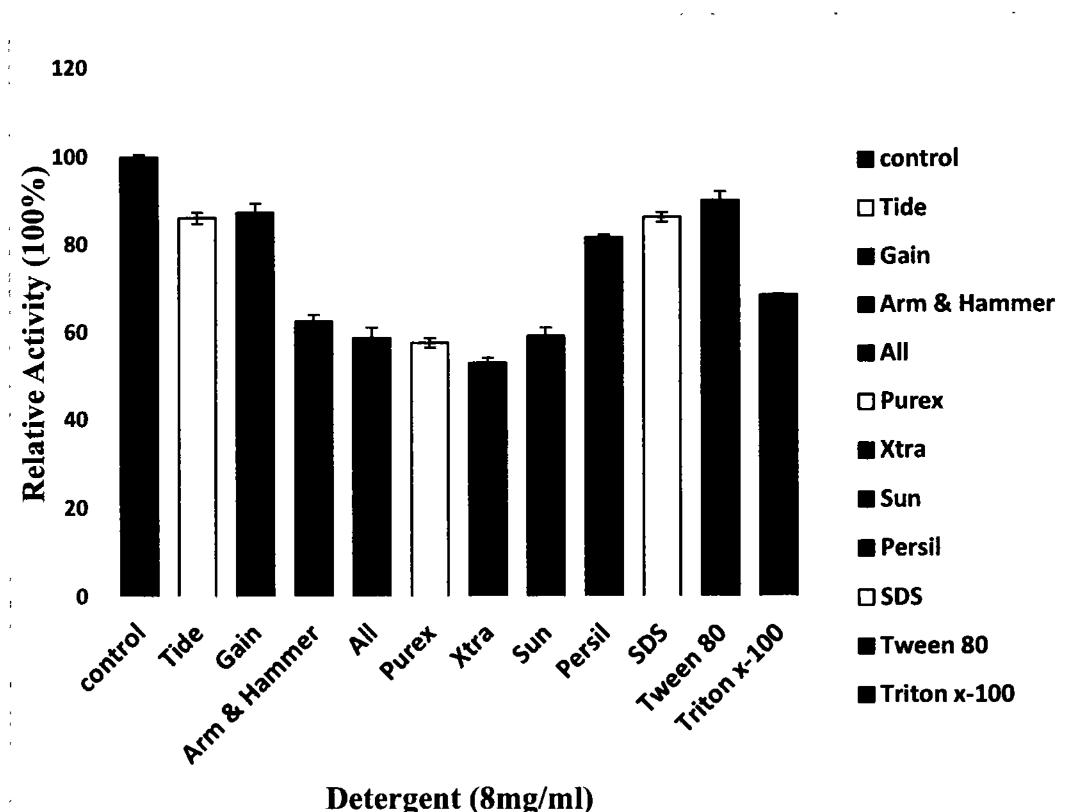


Figure 4.73: Compatibility of *Pseudomonas peli* lipase with surfactants and detergents. For the control, lipase was incubated with buffer devoid of surfactants and detergents and its activity was taken as 100%. All values are represented as mean ± sd of three replications.

4.10.2. Compatibility of alkaline lipase with oxidizing compounds

Hydrolytic activity (unit/ml) of alkaline lipase with oxidizing agents was correspondingly tested in occurrence of sodium perborate, sodium peroxide, hydrogen peroxide and with sodium hypochlorite.

Results exhibited was greatly stable regarding oxidizing compound at 1.5% strength for 1h at 25 °C and 83% of residual activity was preserved at 2.0% H_2O_2 concentration, by rising in concentration of sodium perborate, sodium hypochlorite and sodium peroxide from 1.0 to 2.0% though activity was progressively decreased (Figure 4.74).

Astonishingly, from the results it has been observed that the hydrolytic activity of lipase molecules exhibited suitable stability regarding strong oxidizing agents specifically hypochlorite (70.5% lipolysis at 1% strength) related toward the relative hydrolytic activity of Lipolase, showed 57% inhibited residual activity after preincubation with 60 minutes from the observations presented by (Rathi *et al.,* 2001).

As it was concluded from the observations, that in presence of oxidizing agents' higher stability for the alkaline lipase make this hydrolytic enzyme catalysis that be able to be integrated as a constituent of the detergent additive interpretation for improved results.

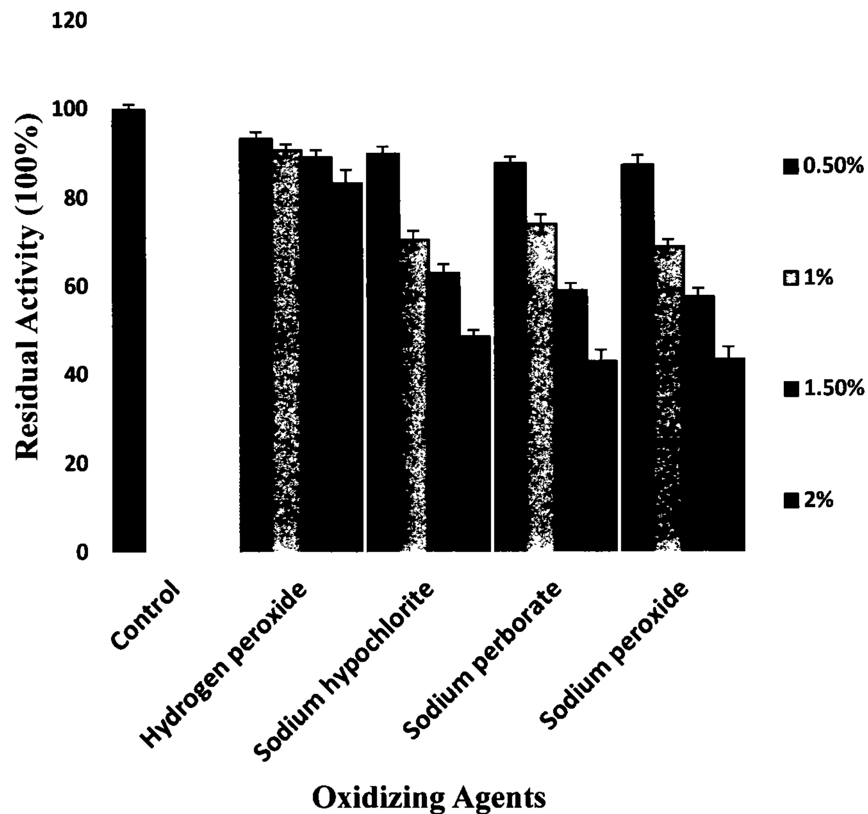


Figure 4.74: Compatibility of *pseudomonas peli* alkaline lipase with oxidizing agents. For the control, lipase was incubated with buffer alone without oxidizing agent and its activity was taken as 100%. All values are represented as mean ± sd of three replications.

4.10.3. Washing detergent effect and its different concentration on oil removal

Distinctive laundry detergents influence on eradication of oil was demonstrated in (Figure 4.75). Detergents with concentration of 0.5%, higher removal of oil 58% relative activity was observed with Gain detergent between all other commercial detergents and was selected for further analysis. Lipase has been more applicable and compatible with nonionics as compared to anionics which contribute to the inhibition of greater hydrolytic activity by anionic surfactants (Flipsen *et al.*, 1998, Sajna *et al.*, 2013).

Surfactant concentration alongside with alkaline lipase and oil elimination relationship has been displayed in (Figure 4.75). Treatment such as oil removal with Buffer, Gain and Lipase (B+D+L) solution mixture has been experimented to be higher than with Buffer and Gain (BD) solution without lipase enzyme, which resulted the benefit of lipase inclusion as an additive in detergent formulation.

Present results suggested that the usage of psychrotrophic *pseudomonas peli* thermolabile alkaline lipase with 0.6% Gain detergent progresses the removal of oil pigments from sullied fabric by 41% for Buffer and Gain solution reaction mixture (B+D) to 57% improvement for Buffer, Gain and Lipase solution reaction mixture (B+D+L) exploiting 60 U of lipase at 25 °C for half an hour of exploiting the lipase capability to use as an additive of detergent formulation (Figure 4.76).

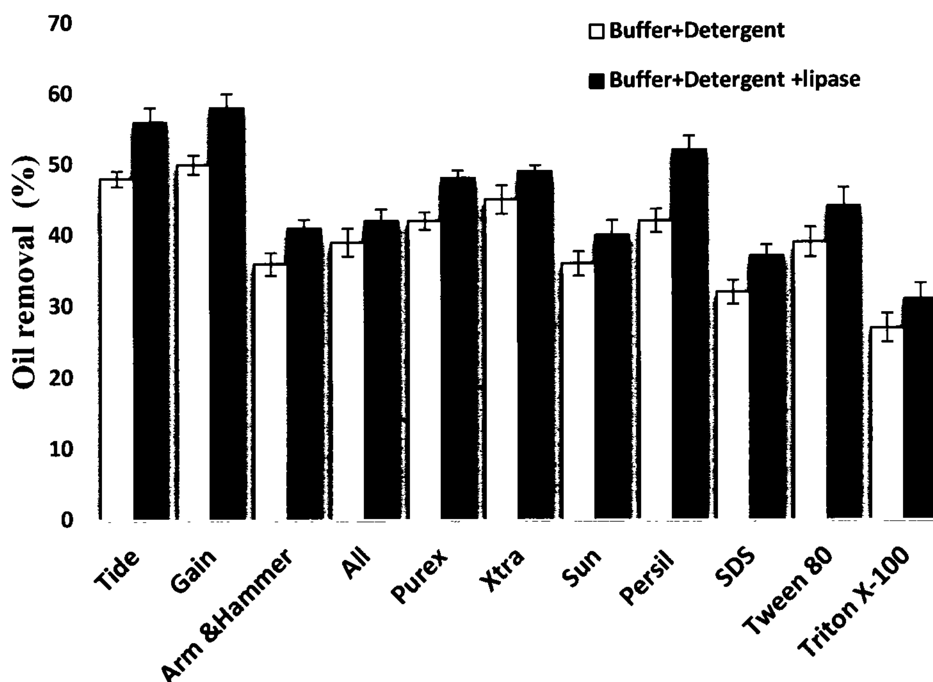


Figure 4.75: Effect of lipase on removal of olive oil from cotton fabric with various detergents. All values are represented as mean ± sd of three replications.

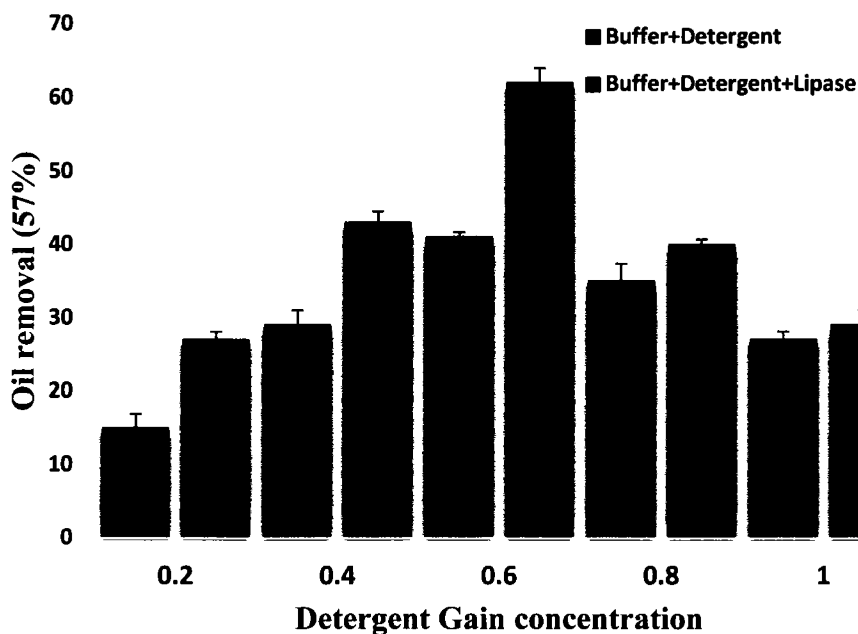


Figure 4.76: Effect of detergent and its concentration on oil removal (%) (experimental conditions: lipase amount 60 U; washing temperature 25 °C; Washing time 30 min). All values are represented as mean ± sd of three replications.

4.10.4. Use of different concentration effect of lipase on oil removal

Lipase (Unit/ml) of different concentration and removing of oil comprising materials association was demonstrated in (Figure 4.77). It was observed from the results that in both cases, with lipase concentration the oil removal rises till accomplishing the stability situation at a strength above than 60 units/ml.

Equilibrium state attainment after detergent stable lipase enzyme concentration depend on the initial rate of catalysis of ester bonds of triglycerides-based lipase on the interface region amongst unsolvable triacylglyceride and lipase aqueous solution. Surface area of a particular quantity of olive oil was examined to be constant afterward a certain concentration of hydrolytic lipase with which interface observe is saturated.

As shown in Figure 5, an improvement with the addition of the lipase was observed 34% to 69% without the detergent and with existence of the detergent. Results observed for the PAK03 lipase was enhanced in combination with marketable detergent which has been observed in situation of *Pseudozyma* (Sajna *et al.*, 2013) and *Pseudomonas aeruginosa* lipases has been reported by (Grbavcic *et al.*, 2011).

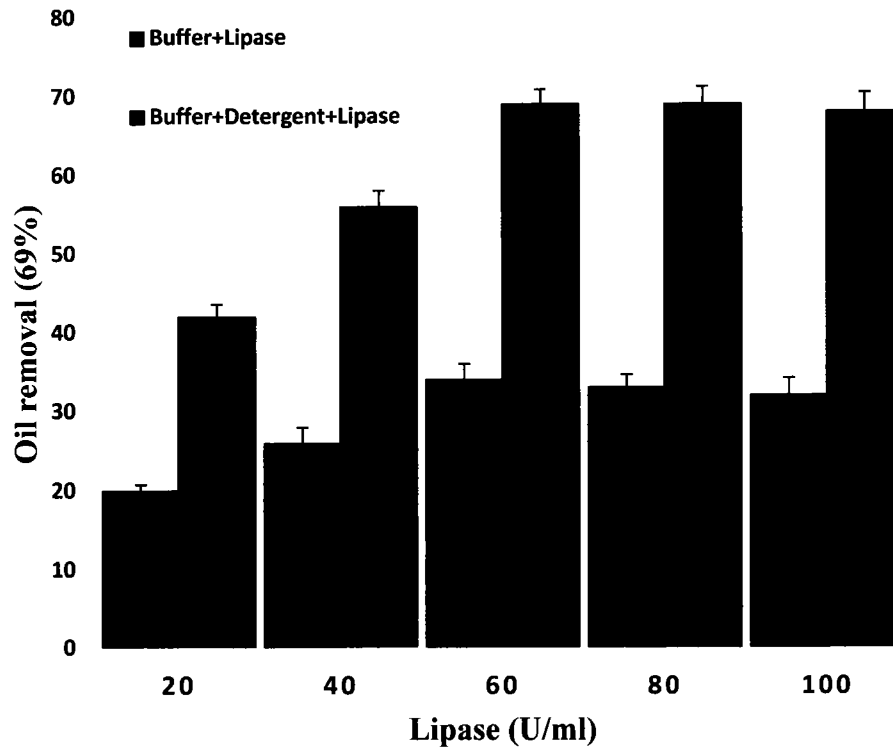


Figure 4.77: Effect of lipase amount on oil removal (experimental conditions: detergent concentration 0.5%; washing temperature 25 °C; washing time 30 min). All values are represented as mean ± sd of three replications.

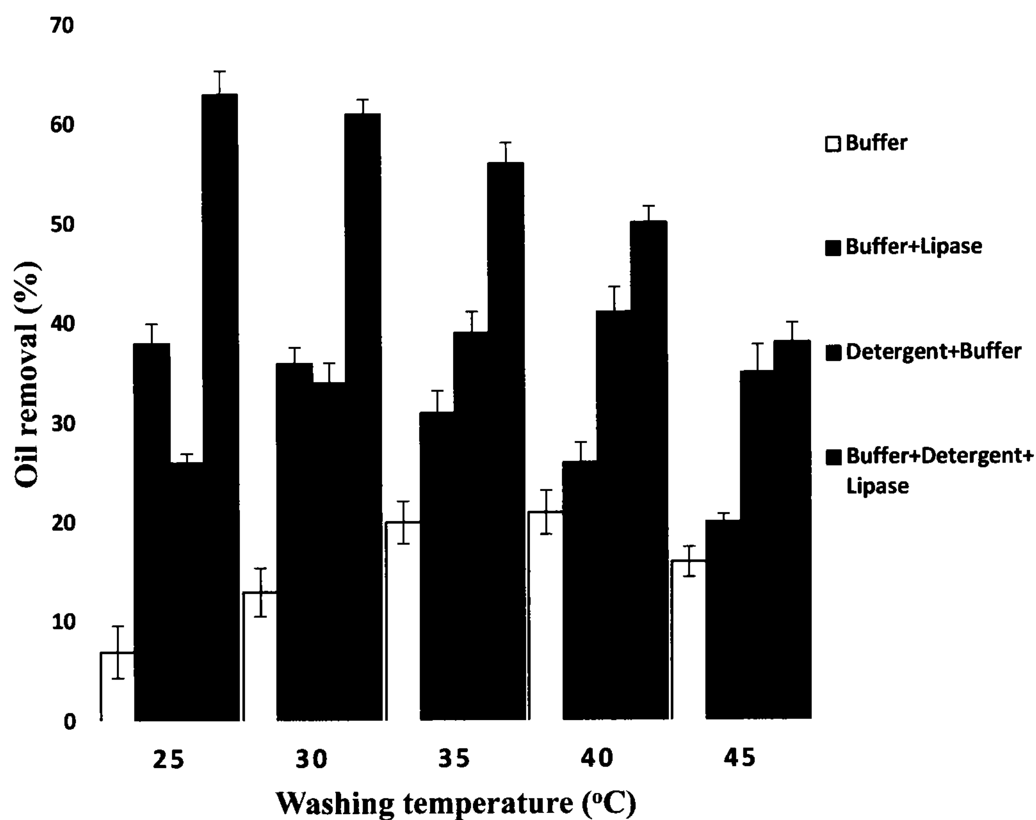


Figure 4.78: Effect of washing temperature on oil removal (experimental conditions: detergent concentration 0.5%; lipase amount 60 U; washing time 30 min). All values are represented as mean ± sd of three replications.

4.10.5. Different Washing temperature effect on removal of oil

At distinctive temperatures the results of triacylglycerol based such as oil stain removal with and without of lipase was presented in (Figure 4.78). A maximum oil elimination was observed by the hydrolysis of triglycerides in the experiment of Buffer with Lipase solution (B-L) washing mixture at 25°C.

In the situation of Buffer-Detergent (B-L) solution mixture, higher washing temperature was resulted is a requirement for enhanced oil exclusion Horchani *et al.*, 2009 and Romdhane *et al.*, 2010. It was noticed that higher oil removal was experimented with Buffer-Detergent-Lipase (B-D-L) solution reaction correlated with other washing solution mixture at several temperature contribution of lipase for higher oil removal observed was at 25°C.

4.10.6. Different Washing time effect on removal of oil

Washing interval consequence on triacylglycerol oil stain removal was represented in (Figure 4.79). It has been detected from the results that on longer extensive washing time, barely with Buffer and Lipase (B-L) solution functioned properly for removal of oil from muddied fabric. The solution of Buffer with Detergent (B-D) used for oil removal from the results, was observed to be constant subsequently after 20 min.

From the outcomes it has been observed that exploitation of the solution comprising Buffer with Detergent along with Lipase (B-D-L) solution encouraged percent residual activity of oil removal up to 71% of the outcomes using 40 min washing cycle. It has also been witnessed that the significant contribution of lipase regarding hydrolysis of oil removal observed with longer washing period.

Fabric washing assessment of biosurfactant from the *Pseudozyma* sp. secreted lipase revealed that stain removal experiment has been enhanced with increased in the washing duration (Sajna *et al.*, 2013). Similarly, the enhancement with increased washing duration also has been observed for *Pseudomonas aeruginosa* segregated lipase by the Grbavcic *et al.*, in study of enzyme biodetergent analysis (Grbavcic *et al.*, 2011).

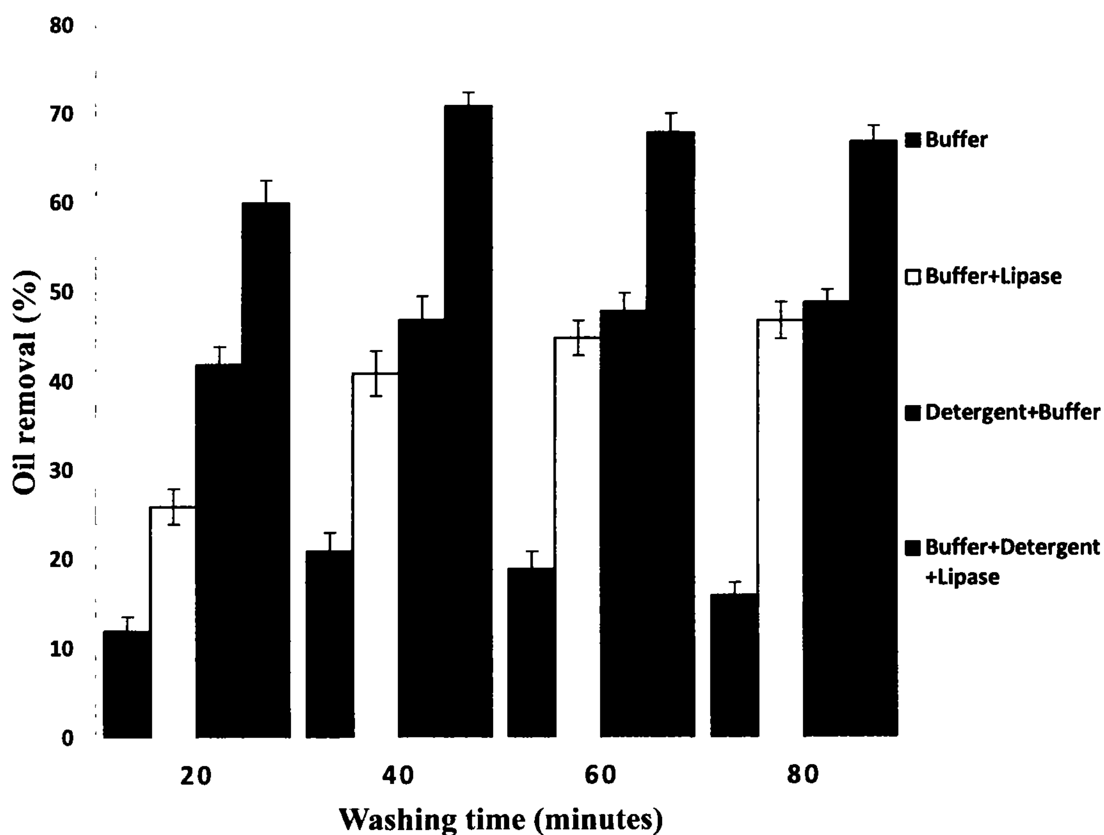


Figure 4.79: Effect of washing time on oil removal (experimental conditions: detergent concentration 0.5%, lipase amount 60U; Washing temperature 25 °C). All values are represented as mean ± sd of three replications.

CHAPTER. 5

5. DISCUSSION

Out of distinctive aspects influencing environment of microorganisms, one of the most important cause is temperature that influences most of the biochemical responses. Thereby psychrotrophic microorganisms have established diverse adaptive approaches to survive in extreme environmental circumstances (Margesin *et al.*, 2002). Psychrotrophs have the potential to produce enzymes which have properties of low energies activation and at low temperatures extraordinary activities (Morita *et al.*, 1997) deliberating significant evolution regarding energy reserves. Thermolabile enzymes are of profitable importance because they can be utilized in purposes at cold temperature and there is scientific attentiveness in the association among thermal stability of enzymes and protein structure. Biotechnologically psychrophiles are important candidates especially due to their enzymes active at lower than usual temperature. Thermolabile enzymes from psychrophiles have adaptive features in their structure, like, Reduced hydrophobicity, Fewer salt links, Increased number of interactions between the enzyme and the Solvent Reduced aromatic residues. (Moncheva *et al.*, 2001). Cold ecosystems are biological laboratories for monitoring *in situ* interactions between dynamics and environments of microbial biodiversity, and to analysis impact of climate and geography change on the structure of entrapped species, observe their preservation and survival mechanisms and (Hoffman *et al.*, 1997). Isolates from glacial ice of Karakorum Range of mountains (0.75 million years old) demonstrated their physiology like facultative psychrophiles or obligate. The proteins like alkaline protease and lipase produced by these stressed bacteria function optimally at same conditions with higher specific activity.

Severe environmental circumstances are very usual on earth. In these areas the microbial diversity is considered of particular concern since the prokaryotes are adapted to these circumstances and thermolabile enzymes from psychrotrophic microbes with industrial significance might be acquired from them. Cold temperature is of the particularly common among extreme environments, in equally both of the natural and synthetic situations. More than 90% of the marine environments with about 80% of the biosphere having the cold temperature inferior than 5°C (Brenchley, 1996 and Margesin and Schinner, 1994). Relatively less consideration was paid reasonably to extremophiles growing at cold habitat, conceivably as a consequence of

the difficulty in handling these bacteria and slow growth rate (Morita, 1975). Psychrophiles and psychrotrophs are the cold-adapted microorganisms and are existing in nearly all these adobes and secrete thermolabile enzymes known as psychrozymes. Amongst of the industrial treating enzymes three of the main group of these enzymes are proteases, amylases and lipases, enzyme such as protease solitary account more than 60% of the entire global sale of protease and are largely originated from source of microorganisms only. Purpose of protease enzyme in the bio detergents, leather as well as for baking, pharmaceutical industries, dairy products, debittering of enzyme (protein) hydrolysate and manufacture of soy products (Dube *et al.*, 2001; Kalisz, 1988; Outtrup and Boyce, 1990; Rao *et al.*, 1998 and Kumar and Takagi, 1999).

5.1. Site of sampling and its importance

The Karakorum, Himalayas and Hindu Kush are three mightiest mountain ranges of the world that diverge from a "junction point" (36′27N and 74′52 E) near Gilgit, Pakistan. The Gojal and Hunza valleys are located at the base of Karakorum Range along Karakorum Highway (KKH) and Indus River beneath, with famous glaciers like Rakaposhi (36°14'368′, 74°26'576″), Jutial (35°54'276″, 74°19'841) and Juglot (35°41'06′, 74°37'26″) near Khunjerab Pass on Sost border of Pakistan and China. We collected samples from the above mentioned three glaciers and the parameters we considered during sampling e.g. physical state and condition of samples, geographic location pH, temperature, pressure and height and dissolved oxygen correlate with Christner, (2002) who adapted the strategies of sampling from frozen terrestrial environments analogs for Mars and Europa.

5.2. Isolation and identification of *Stenotrophomonas sp.* isolate

In the existing research total thirty prominent colonies of bacteriological strains were investigated from soil and water of Juglot, Jutial and Rakaposhi glacier, producing cold-active hydrolytic extracellular protease and screened on casein agar dishes with reaction mixture (peptone at concentration of 0.1%, NaCl with 0.5%, agar strength of 2.0%, and finally casein at concentration of 1% at pH-8 (Tris-HCL buffer). Bacteriological strains containing nutrient agar plates were kept in low temperature incubator at 18±2 °C for almost 2 days. Enzymatic hydrolysis of casein substrate was examined by the function of 30% TCA concentration on the nutrient

agar exterior top. Enzymatic hydrolyzed zone of hydrolysis nearby the microbial contagious growth was observed as a constructive catalytic reaction.

Depending upon larger hydrolysis zone on 1 % casein agar plates incubated at 18±2 °C for 48 hours, four isolates out of thirty produced zone of hydrolysis at low temperature indicating best source of thermolabile protease production, and these four strains as hyper-protease producer was used for further studies. The zone of hydrolysis was also performed on 10 percent skimmed milk agar plate (Hawumba *et al.*, 2002, Olajuyigbe and Ajele, 2005, Ramesh *et al.*, 2007, Gupta *et al.*, 2008), marine agar media with 1 percent skim milk agar plate (Kumar *et al.*, 2004), casein agar plates (Zeng *et al.*, 2003 and Vidyasagar *et al.*, 2007), nutrient agar with 1 percent casein (Naidu and Devi, 2005).

(Kuddus and Ramteke, 2011) isolated psychrotolerant bacterium *S. maltophilia* that have the potential to secrete an extracellular thermolabile alkaline protease enzyme was extracted from bacteriological type of culture collection, Chandigarh and the psychrotrophic selected strain was streaking on petri dishes containing skim milk agar with the reaction mixture of (skim milk powder 100(g/l), peptone 5(g/l) and agar 15(g/l) and incubated at temperature of 4°C.

Saba *et al.*, 2012 isolated *Stenotrophomonas sp.* from samples of soil gathered from elevated altitudes of glacier situated in Sonamarg, ganderbal district region of Kashmir, india. *Stenotrophomonas sp* was observed to have the maximum catalytic activity amongst 25 isolated microbial strains and was preferred for maximum thermolabile protease production on the foundation of clear zone of hydrolysis on substrate such as skimmed milk agar petri plat analysis.

23 bacteriological strains were measured for qualitative and quantitative (U/ml) protease catalytic analysis were observed. Maximum amount of hydrolytic activity exhibiting strain was observed. Using casein agar medium catalytic activities of all the selected bacteriological strains were analyzed and observed for a diameter of clear zone of hydrolysis. For qualitative test of protease casein agar was the best method. Bacteriological *Streptococcus* sp. strains 1 and 2 showed high proteolytic activity was observed among 23 selected strains (Ahmed *et al.*, 2013).

Gupta *et al.*, (2005) isolated wild strains of bacteria and analyzed for catalytic activity using petri dishes containing skim milk agar used as substrate and was observed that the *Streptomycin* sp. CD3 have the potential to hydrolyze the maximum

substrate between the isolated strains. 70 fungal selected isolates strains were qualitatively test by Chekireb *et al.*, (2009) who experimented these strains for their competencies to yield zone of hydrolysis around the fungal growth in gelatin ager substrate by means of the formation with clearing zones.

The isolated bacteriological strain on molecular source of 16s ribosomal RNA sequence in the present study was identified. Blast analysis of the 70 S bases of molecular sequence explore from database of the NCBI GenBank exhibited 99% nucleotide sequence homology with *Planococcus sp.*, *Exiguobacterium sp.*, *Pseudomonas aeruginosa* and *Stenotrophomonas sp* in the existing NCBI database. The phylogenetic tree of four of the psychrotrophic isolates was constructed using software MEGA7 (Tamura *et al.*, 2007) (Figure 4.1, 4.2, 4.3 and 4.4). The 16S rRNA gene sequence of four psychrotrophic bacterial strains *Planococcus sp.*, *Exiguobacterium sp.*, *Pseudomonas aeruginosa* and *Stenotrophomonas sp* were submitted to NCBI GenBank under accession numbers MG757351, MG662179, MG679513 and MG662181. (Saba *et al.,* 2012) identified strain on the basis of 16s rRNA. Nucleotide sequence of 1.447 kilobases ribosomal RNA attained from IIIM-ST045 presented 99% gene sequence homology to the consequent *Stenotrophomonas sp.* strain nucleotide sequences.

For bacterial strain identification 16S ribosomal RNA complete gene with 1523 base pair from the chromosomal DNA was amplified and sequenced. Using program software of ClustalW data base (Saitou and Nei, 1987; Kimura, 1980 and Thompson *et al.*, 1994) multiple gene sequence alignment was executed and consigned in NCBI GenBank. *Exiguobacterium artemiae* (99.5%) with maximum homology was analyzed. Isolate gene sequence was aligned and matched with earlier published species gene sequences from *Exiguobacterium* genus (Ramesh and Yadav, 2007). Details on *Exiguobacterium sp.* presented psychrotrophy, for example *E. undae, E. antarticum E and E. sibiricum* (Rodrigues *et al.*, 2006). (Rosales and Sowinski, 2011) reported 16S rDNA gene of 1500 base pair molecular weight evaluating the phylogenetic analysis of the selected strains. Nine isolates matching genus *Pseudomonas,* 97-99% nucleotide identity to the family of Gammaproteobacteria and four selected microbes identical to the Flavobacterium with 97 to 98% genome identity. Thermolabile protease producing psychrotrophic bacteria from Arctic and Antarctic territories was earlier reported by (Brambilla *et al.*,

2001; Groudieva *et al.*, 2004, Olivera *et al.*, 2007; Mannisto and Haggblom, 2006, Liu *et al.*, 2009).

5.3. Optimization production of cold active alkaline protease

Two media were used for inoculum development in batch culture, medium PG Nyc 1 contains NaCl, yeast extract and casein as 0.5% with peptone and gelatin as 0.3% at pH 8 was found best stimulator for protease enzyme, and medium no. 2 (Ohta *et al.*, 1995) contain KH2PO4, peptone, casein and gelatin as 0.1 percent and yeast extract as 0.5% at pH 8 did not activated and developed satisfactory seed culture for protease production.

After isolation and screening, the shake flask batch culture experiment was conducted for 120 hours for detection of maximal production of enzyme. For to check the consequence of culture media on production of enzyme through the production time course for 3 days, the pH of the culture filtrate remained more than 8 with increased and decreased growth of the test organism. Of the 8 diverse media investigated, medium no 8 was found the best recipe of nutrients and salts for the maximum production of the active alkaline protease with enzyme activity 4616.43 U/ml at pH 8, 150 rpm and 18 °C after 72 hours of incubation. As the medium contains very least amount of salts per liter, is the best cost effective medium at lab scale as well as for commercial scale enzyme production. Therefore, it was used as the basal culture medium for production in the following studies.

The cell mass at 660nm and enzyme catalytic activity were observed for 120 hours in thermolabile protease stimulating production nutrient media at 18 °C at pH 8 buffer. Thermolabile enzymatic activity increased progressively and the activity was highest at 2786 units at 96 hours incubation. Production of cold active protease was growth independent (Figure 4.11 and 4.12). Comparable to several catalytic enzymes, it is likewise excreted mostly at the late logarithmic phase (Kuddus and Ramteke, 2008 and Dube *et al.*, 2001).

Temperature is considered to be critical factor that has to be diverse and controlled from amongst different microorganisms and is most important factor affecting the enzyme production. however, studies by (Kuddus and Ramteke, 2011) exposed that a relationship endured among enzyme production and energy breakdown in *Stenotrophomonas maltophilia,* which was controlled by and oxygen uptake and temperature.

The basal medium (Containing g/l, soymeal 15, Crude protein 45, Crude fate 6, carbohydrate 19, NaCl 8, Sodium glutamate 1, Yeast extract 5, $KH2PO4$ 1.5 and $MgSO4$ 0.3) inoculated with 5% of 24 hours old seed culture at pH 8 and incubated at different temperature ranging from 4 to 45 °C at 150 rpm for 96 hours. The maximum cold active alkaline protease was produced (6886 U/ml) with specific activity (573 U/mg) at 25 °C after 96 hrs of incubation ($P<0.0001$). After 96 hours at lower temperature 4 and 15 °C marked decrease in enzyme production was observed and at elevated temperature like 35 and 45 °C showed no enhanced stimulatory effect on enzyme production. So, the optimized temperature for enzyme stimulation was found to be 25 °C increasing or decreasing the temperature led to the decrease in enzyme production (Figure 4.13). At higher temperature 35 and 45 °C enzyme production decrease as 3065 and 621 unit/ml of the total original enzyme production respectively. While at lower temperature 4 and 15 °C it decreases up to 36 and 964 unit/ml respectively.

These results clearly indicate that by increasing the temperature from lower to higher, enzyme production increased up to its optimum temperature 25 °C, but further increase in temperature more than 25 °C, the production decreased. The protein contents also increased and decreased with increase or decrease in enzyme activity.

(Kuddus and Ramteke, 2011) studied about novel psychrotrophic *Stenotrophomonas maltophilia*. Psychrotrophic *S. maltophilia* was isolated from soil samples collected from different sites of Gangotri glacier, India that have the potential to excreted thermolabile protease with 56.2 U/ml at 20°C and a buffer of 9.0 pH after 5 days incubation in submerged fermentation condition. (Saba *et al.*, 2012) identified *stenotrophomonas sp.* on the basis 16S ribosomal RNA and was able to grow satisfactory within a temperature of 4 to 37°C though, exhibited best growth at 15°C. Selected strain categorized as a psychrotroph according to the Morita *et al.*, (1997) explains them as psychrotrophs as they have the capability to grow at 0°C and best growth temperatures from 15–25°C. Related consequences by Helmke and Weyland (1991), who found thermolabile proteases enzyme had the best surviving temperatures from 10 to 20 °C in contrast with from mesophilic bacteria. Temperature effects enzyme secretion by altering the cell membrane physical conditions (Rahman *et al.*, 2005)

Inorganic and organic sources of nitrogen are metabolized into different nucleic acids, amino acids and cell wall constituents. 15.6 percent nitrogen comprises by catalysis of alkaline protease and its stimulation are dependent on carbon and nitrogen source (Kole *et al.*, 1988).

Protease potential uses for progress of economical approaches for maximum hydrolysis with objective of decreasing the overall expense of industrial practices, the use of solid state, submerged and biphasic system using cheaper industrial in a substitute in accomplishing maximum yields. After the experimentation of various nitrogen sources in basal salt medium, it was found that the specific and suitable nitrogen has great effect on enzyme production and yeast extract and ammonium sulfate gave good observation with yield of enzyme activities as (6965 U/ml) and (5976 Unit per ml) ($P<0.0001$).Whereas for tryptone, peptone and casamino acid observed satisfactory results with specific activities as 1853, 2376 and 1227 U/ml protein, and only 3-15 percent production was found with diverse sources of organic nitrogen.

Most of the organic sources of nitrogen. stimulated the enzyme secretion. Results of present study are in contrast with the previous finding of (Do-Nascimento and Martins, 2004) observed in *Bacillus sp.* and (Banerjee *et al.*, 1999) observed in *bacillus brevis* found repressed enzyme production by organic nitrogen sources. Sources of inorganic nitrogen did not produce maximum secretion than organic. Inorganic and amino acid sources of nitrogen such as sodium nitrate, potassium nitrate, trisodium citrate, glycine and leucine with specific activities as 271, 65, 216, 95 and 187 U/mg. (Gouda, 2006) observed opposite results and found sodium and potassium nitrate as the best source. (Kaur *et al.*, 2001) found sharp decrease in production by ammonium sulfate in contrast to our results. Consequences of present research are in complete accordance with the previous finding of (Ferrero *et al.*, 1996) found increased protease production with ammonium and nitrate. (Saba *et al.*, 2012) experimented consequences of source of different nitrogen such as inorganic, organic and low-cost nitrogen sources on thermolabile alkaline protease by *Stenotrophomonas sp.* Urea with 38.9 U/mg, ammonium chloride 62.2 U/mg and ammonium sulfate 57.9 U/mg in contrast to our results. Related conclusions were observed by (Saba *et al.*, 2012) and found that organic source such as yeast extract was observed to have stimulated effect on the production of thermolabile alkaline protease. Beef extract

increased protease production (Akcan and Uyar, 2011). (Mukherjee *et al.*, 2008) described that beef extract stimulated protease secretion by *B. subtilis* DM-04. (Yang *et al.*, 2000) observed that *B. subtilis* Y-108 secreted enzyme was suppressed by some of the nitrogen sources. (Nascimento and Martins, 2004) observed maximum activity was obtained with Ammonium nitrate as nitrogen source. Safey and Abdul-Raouf, (2004) observed ammonium sulfate as best nitrogen source for extracellular enzyme secretion.

In the present experiment different carbon supplements were added in the protease production broth. The results indicate that the maximum enzyme production was enhanced by the addition of carbon enriched supplements like glucose, fructose, maltose, sucrose, and lactose. Among these supplements, maximum enzyme production (5833 U/ml) were obtained when glucose was used as carbon supplement. These results are related with previous studies showing highest protease production when glucose is used as a carbon source in the protease production broth (Pastor *et al.*, 2001; Santhi, 2014). Similar findings were reported by (Sen & Satyanarayana, 1993; Pastor *et al.*, 2001; Santhi, 2014), where glucose and starch gave maximum protease production, respectively. (Badhe *et al.*, 2016) observed that the maximum protease activity was obtained when glucose was used as a supplement in the basal medium for enzyme production. Starch, sucrose, and lactose substantiated that protease production from *Bacillus sp* was maximum. (Naidu and Devi, 2005). Protease secretion was optimum in the existence of glucose, dextrin and starch observed for selected *Bacillus coagulans* and *licheniformis* (Asokan and Jayanthi, 2010). Carbon source such as lactose was observed best that stimulated the protease secretion from the isolated strain of *Bacillus subtilis* (El-Safey and AbdulRaouf, 2004). Optimum carbon source for hydrolytic activity of protease was observed as glucose for isolated 4 *Bacillus* species isolates trailed by fructose, cellulose, maltose, sucrose and starch (Boominadhan *et al.*, 2009).

Though, from the research work it was observed that that soured of glucose considerably inhibited fabrication of protease enzyme (Puri *et al.*, 2002 and Fukushima *et al.*, 1989). (Sevinc and Demirkan, 2011) was experimented that lower cell mass and maximum production of protease was observed by the supplementation of starch in the basal medium, but HadjAli *et al.*, (2007) reported the results that was in contrast to the present results. Cellobiose, Maltose, and starch was observed to

have inhibitory effect on secretion of protease enzyme (Shafee *et al.*, 2005), however starch as carbon supplement was experimented to have stimulatory effect on hydrolytic catalysis of substrate in Bacillus species (Johnvesly and Naik, 2001). (Ash *et al.*, 2018) studied different conditions optimization for enzyme excretion. Many physical factors were deliberated to optimize the maximum yield of alkaline protease. The encouraging circumstances for the maximum secretion of enzyme was achieved when fermentation medium incubation for 36 h was supplemented with 1% glucose in the basal medium. Different culture media are used by various microorganisms (Sen *et al.*, 1993). Carbon sources influence protease production. Lack of glucose in media resulted in dramatic decrease in enzyme production (Gajju *et al.*, 1996; Sonnleitner, 1983). Including the numerous sources of carbon, glucose was observed to have stimulate the maximum catalytic activity of protease (Dorcas and Pindi, 2016)

The significant feature of alkalophilic microorganism is their sturdy dependency on the pH for growth of the cell mass and hydrolytic activity. pH of fermentation culture also intensely influences different catalytic reactions and transportation of numerous constituents through the bacterial cell membrane (Moon and Parulekar, 1991). pH of the fermentation culture and the transportation of numerous constituents through the bacterial cell membrane raising the alkaline protease yields (Kumar *et al.*, 2004).

In the present research the consequence of the initial pH on the fermentation culture medium to their catalytic activity was studied in pH range 6.0 to 12.0. The initial pH was adjusted with NaOH or HCl. The enzyme was produced over pH range 6.0 to 12.0 with highest catalytic activity of 4516 U/ml at pH 9.0. The enzyme synthesized in acidic as well as in alkaline pH but maximally produced at alkaline pH. As the S*tenotrophomonas sp.* PAK01 growth range is also in between 6 to 12 pH and 4 to 45 °C temperature, so the enzyme production at pH range and growth pattern at temperature range of the isolate indicate that it is alkalophilic and psychrotrophic organism which produced thermolabile alkaline hydrolytic enzyme. Present observations are similar to the results of (Kuddus and Ramteke, 2011) studied that the enzymatic processes were strongly effects of pH of culture and passage of substances through the cell membrane. Enzyme hydrolytic catalysis was highest with 62.2 Unit at pH 9 buffer after 5 days of incubation at 20°C. During the growth of microorganism pH change observed also affects product stability in the medium. Observation are in

covenant with the finding of (Ash *et al.*, 2018) who isolate *P. aeruginosa* was studied wide ranging pH from 4 to 13 different buffer. Best suited pH for *P. aeruginosa* was 9.0. The pH homeostatic mechanism following the Donnan equilibrium theory which was recently established for alkaliphilic bacteria (Tsujii, 2002) suggests a plausible explanation to support this observation of medium pH effect on enzyme production. Results obtained in this experiment suggested that hydrolytic activity was delicate to pH below at 6. Highest catalysis was obtained at basic condition of pH 9 buffer attainment of 682 unit/mg and activity was progressively reduced to 86.7% at 10 pH and 10% at 12 pH. The above explanations agree with the result of El-Hawary and Ibrahim, 1992, they determined best pH should encounter the requirements of the protein encoding gene and bacteria are sensitive to pH whilst utilized for the secretion of protease enzyme. Thangam and Rajkumar, (2002) observed catalytic activity in basic pH conditions of protease from *Alcaligenes faecalis*. Best protease activity (U/ml) observed at 9 pH and was in harmony of protease excreted from *Fusarium culmorum* recounted by Anja and other strain *Aspergillus terreus* testified by Syamal (Anja *et al.*, 2002 and Rashbehari *et al.*, 2003). Consequently, these properties showed that this enzyme is an alkaline tolerant protease. pH studies results suggested a wide-ranging pH catalytic activity (Zhu *et al.*, 2009). Similar results were observed for other protease secreting strains. *Bacillus subtilis* excreting protease was highest at 9 (Almas *et al.*, 2009). Studies related with optimum pH 9.0 for protease production in *P. aeruginosa* (Gupta and Khare 2007). Catalytic activity was best at pH 9 to 12 in *Bacillus patagoniensis* strain (Olivera *et al.*, 2007).

Distinctive divalent metal ions are mandatory in the submerged fermentation reaction highest catalytic activity (Kumar and Takagi, 1999). Present study observation showed that the metal ions has significant effect on enzyme production and heavy metals existing in environment have a significant role on growth. Enzyme assay (Unit/ml) was enhanced by $CaCl_2$ (5231U) and $MgCl_2$ (3984U) and showed stimulatory effect on protease production. The results observed for other heavy metals such as $HgCl_2$, $FeCl_3$, $CuSO_4$ and $ZnSO_2$ have 1276, 155, 735 and 1167 unit/m/ protein showed no stimulation on activity but retain more than 20% of hydrolytic activity ($P<0.0001$) (Figure 16). Nilegaonkar *et al.*, 2007) obtained maximum production in the medium by the addition of distinctive divalent salts such as, iron magnesium, calcium ions and potassium. Janssen *et al.*, 1994) experimented decrease

in protease catalysis from *Thermus sp.* Calcium showed stabilizing consequence on activity and assumed that phosphates and chelators decreasing calcium availability, so they customized eliminate chelators, inorganic phosphate strength decreased, and calcium concentration was boosted without growth rate reduction. The results of present study are in understanding with finding of (Kuddus and Ramteke, 2011) who studied cold active alkaline protease was enhanced by Copper (126.8%) and Chromium (134.6%) while Cobalt (43.5%) worse production. 50% of enzyme catalytic activity retained by heavy metals e.g. Hg, Cd and Zn. Calcium and Magnesium ions joined was investigated and activity was very low. Adding of Li, Ba, Mn caused in low activity. Ca and Mg together was superior than tested metal ions (Sevinc and Demirkan, 2011). It has been reported that Calcium and Magnesium ions were affected on hydrolytic activity (Kalaiarasi and Sunitha 2009 and Feng *et al.*, 2001;). Inhibitory influence of Cu and Li on activity (Adinarayana *et al.*, 2003). The impact of diverse metal salts on activity was assessed and demonstrated. (Ash *et al.*, 2018) studied protease production significantly enhanced by adding cations such as Cu and Zn compared to the control which are in contrast to the results of this experiment. Nevertheless, examined ions instigated reduction of the enzymatic catalysis, at high strength. These results support the earlier findings (Mcconn *et al.*, 1964) who observed metal ions such as Zn enhancing the activity of protease in *Bacillus subtilis*, and diverse ions Magnesium and Zinc stimulated hydrolytic catalysis of protease from Bacillus licheniformis Vigneshwaran *et al.*, (2010). Calcium, Magnesium and Manganese have reported increase and stability of protease production. They are described to proliferation of enzyme thermal stability. The present studies revealed increase in the hydrolysis of protease on addition with calcium chloride (Dorcas and Pindi, 2016)

The level of inoculum was correspondingly significant parameter for the secretion of alkaline protease. Effect of various inoculum levels (1, 5, 10, 15 and 20%) were used to study on protease production. The enzyme production was found in all inoculum sizes with a little difference however the maximum production was obtained at 5% inoculum with (5754 unit/ml) protein activity. Whereas in other inoculum sizes it maintained in range of 30 to 50% production level. The production of enzyme decreased due to the gradually decline of nutrients in the fermentation reaction due to the increase of inoculum level. Furthermore, in an unabsorbed from

surplus liquid present was observed to have an additional diffusional obstruction along with that of the imposed by the solid nature constituent and to a diminution in production of enzymes and growth of microorganisms (Nutan *et al.*, 2002). In the results of this study, inoculum size 5% is suitable and it proliferated actively in young culture to increase the thick mass due to presence of rich nutrients and after exhaustion of nutrients the culture immediately converted to spore forms because isolate start to produce enzyme with the onset of sporulation. So, in initial incubation period no production occurred, but after 60 hours of incubation protease start to be produced by isolate. Distinctive observations were reported by researchers, *Bacillus sp* with 1% inoculated size (Mehrotra *et al.*, 1999), for *Bacillus lichenniformis 5%* inoculated size (Mabrouk *et al.*, 1999) and for *Bacillus sp.* 10% inoculated size (Jasvir *et al.*,1999). (Dorcas and Pindi, 2016) studied effect of inoculum size was investigated by culturing the isolate in fermentation reaction and inoculating in varied inoculum size viz., 1%, 2%, 5%, 10%, and 15% and the enzyme activity was noted. The results are supported by the finding of (Dorcas and Pindi, 2016) who observed that with 5% inoculum size, maximum protease production was recorded in the study. This result is in accordance with the result reported on 5% inoculums size for optimum protease production by Bacillus subtilis (Abusham *et al.*, 2009). Inoculum size creates a balance with the available materials that enhance the protease production.

Inoculum size for highest enzyme activity was investigated by 1 to 5% in batch fermentation reaction. 2% inoculum was highest for activity and rise in size caused deterioration in activity of enzyme (Mushtaq *et al.*,2014). These results of the present finding were in harmony of Niyonzima and More (2013) observed 2% size of *A. terrus* strain in submerged fermentation reaction was the highest for catalytic analysis unit/ml. Radha *et al.*, (2011) also described 4% size of *Aspergillus strain* in submerged fermentation reaction and was observed maximum secretion of alkaline protease. The same results were observed by (Ozdenefe *et al.*, 2017) and find that catalytic value 73.14 Unit/mL for 5% was observed to be suitable for maximum secretion of alkaline protease by *Bacillus subtilis.* Parallel to the results of present experiment, the optimum inoculum amount was determined as 5% by (Genckal and Tari, 2006). Chauhan and Gupta (2004) experimented 2% amount of spore suspension and the results showed maximum secretion of protease enzymes in fermentation

medium. 2-5% spore suspension size was observed optimum for *Bacillus* strains which back-ups the results of the present finding by (Genckal and Tari, 2006, Chauhan and Gupta 2004).

Results of the present experiment indicates the inoculum of 24 hours old is highly supporting the protease production with yield as (5765 unit/ml) protein activity. However, the inoculum of 30 and 40 hours also showed good enzyme production, but it takes long time to harvest the product, and inoculum of ages 6, 12, 18 and 48 hours showed the production but not to the level of 24 hours old inoculum. Kaur *et al.*, 2001 and Do-Nascimento & Martins, 2004 used overnight grown seed culture, Razak *et al.*, 1994 used 18 hours old inoculum and Prakash *et al.*, 2005 used 24 hours old inoculum for production of protease enzyme. For optimization of the fermentation conditions inoculum age is an important parameter. With 4 hours catalytic activity 73.14 U/mL was observed to be the best for protease production (Ozdenefe *et al.*, 2017). Chauhan and Gupta (2004), observed highest catalysis using 3 h old inoculum.

5.4. Purification and Characterization of thermolabile alkaline protease

Psychrotrophic micro-organisms producing thermolabile organic solvent tolerant protease was purified from diverse bases. (Khairullin *et al.*, 2009, Saeki *et al.*, 1994 and Dube *et al.*, 2001) utilized the ultrafiltration technique for the concentration of thermolabile protease acquired from the batch culture fermentation process, acetone precipitation (Tariq *et al.*, 2011 and Turkiewicz *et al.*, 2003), ammonium sulfate (Zeng *et al.*, 2003, Zhu *et al.*, 2009, Kuddud *et al.*, 2008 and Alam *et al.*, 2005). A few methods involve (Chen *et al.*, 2007) use of PEG and (XiangSheng 2011) experimented lyophilization technique. After enzyme concentration, purification is accomplished by particular practice or by coalescing two distinctive approaches. In most circumstances separates polar and ions particles based on their affinity to the ion exchanger is a method of choice. Carboxy methyl and diethyl amino ethyl group comprehending substances are utilized to which molecules can be eluted what is more get adsorbed moreover by pH change of the eluent buffer. For purification technique affinity method is also efficacious technique. Gel filtration and hydrophobic interaction technique was utilized significantly for thermolabile enzyme refinement either at an initial to intermediate stage. For filtration tenacity Topopearl, Superose, Sephacyl and Superdex gels are frequently used.

The inoculum was carried out using Erlenmeyer flasks (250 ml) containing 50 ml of the fermentation medium. The production media which have the following ingredients (w/v) such as 1% yeast extract, 1% tryptone, 1% glucose, 0.5% KH2PO4 and 0.5 ml of 100mg metal ions concentration including calcium chloride, iron sulfate, zinc sulfate, and manganese chloride was inoculated with 5% inoculum and cultivated for almost 4 days at 25 °C in shaking flask fermentation incubator. The supernatant, fractionated by ammonium sulfate precipitation (30 to 80%), was dialyzed against 50mM Tris–Hcl, pH 8, for 24 h to remove residual ammonium sulfate. The dialyzed sample was subjected to Superdex 200 PG 16/60 and DEAE Sepharose Chromatograph SOURCEQ 10/10. Proteins from the supernatant were precipitated with 80% ammonium sulfate overnight at 4°C with constant stirring. The precipitate formed was recovered by centrifuging at 25,000g and 4°C for 30 min using a Beckman ultracentrifuge and resuspended in a minimum amount of 20 mM Tris-HCl buffer, pH 8.0. After almost fermentation of production for 4 days the crude thermolabile enzyme was subjected to centrifugation for clear supernatant at 4°C and stored in -80°C for further analysis. (NH4)2SO4 was added to supernatant at 30% and was further treated with up to 90% concentration of ammonium salt. Precipitated thermolabile protein after centrifugation at 4°Cwas resuspend in buffer of tris-HCl and was taken in dialyzing bag and placed in buffer of phosphate salts at low temperature of 4°C for almost 1 day against three changes of the phosphate buffer. The dissolved proteins were applied to an ion exchange chromatography (IEX: SOURCEQ 10/10) column equilibrated and the proteins on the basis of charges was eluted by using buffer of tris-HCl with 8 pH. Fractions collected was analyzed for proteolytic activity and those with the high catalytic activity was selected and subject for further purification on the bases of molecules sizes gel chromatography to get the homogenous mixture of thermolabile protein molecules. bovine serum albumin was used as a standard for protein determination and also for the purpose of specific activity in (mg/ml). *Stenotrophomonas* sp. a psychrotolerant microorganism when cultivated in the fermentation medium it was witnessed that in the bacterial stationary growth curve the highest thermolabile protease molecules was experimental subsequently in 96 hours.

The thermolabile alkaline protease molecules was purified with distinctive purification practice is concise in Table 4.4. Precipitated $(NH_4)_2SO_4$ partially purified molecules was obtained were subjected to dialysis for further removal of excess of salts and impurities. The dialyzed extract enzyme was first subjected onto an Ion exchange chromatography technique (IEX SOURCEQ 10/10) and the two peaks of thermolabile purified proteins was eluted showed in Figure 4.21. Hydrolytic activity of both the points was assayed and the fraction with high catalytic activity was subjected to Gel Filtration Chromatography (GFC: Superdex 200 PG 16/60) (Figure 4.22) and the purified thermolabile fractions with high enzymatic catalysis from both the peaks was eluted and subjected on SDS-PAGE analysis (Figure 4.23). Under silver staining protease protein showed purity with an expected 58 KDa molecular mass respectively. Purified monomer proteins were observed from the protein analysis. Specific activity of 1171 U/mg with purification fold of 55.9 and yield recovery of 20.43% was observed from the overall purification procedure. The results of present study are in complete agreement/accordance with the previous finding of finding of (Kuddus and Ramteke, 2011) experimented partially purification of enzyme by particular method of DEAE-cellulose chromatography technique and observed about 55-fold of purification with the catalytic activity of 20964 U/mg for purified enzyme and transferred as a particular band in SDS-PAGE analysis signifying purified enzyme was uniform. SDS-PAGE analysis, of the purified enzyme from *Stenotrophomonas sp.* is about 75000 Da.

(Zambare *et al.*, 2011) used innumerable chromatographic procedures to concentrated protease from *P. aeruginosa* and determined its molecular weight. Saba *et al.*, 2012 experimented the moderately concentrated hydrolytic enzyme from PAK01 is harmonized and observed 55.0 kDa by SDS-PAGE analysis, Cold active alkaline protease is maximally operational at 20 °C produced from *Stenotrophomonas maltophilia* and the enzyme activity is preserved after reiterated cycles of freez thaw process (Kuddus and Ramteke, 2011). M1 aminopeptidase produced form the marine psychrophilic bacterium *Colwellia psychrerythraea* strain revealed maximum hydrolysis at 19 °C (Huston *et al.*, 2004). Zhu *et al.*, 2009 reported 35 °C as a maximum temperature for hydrolytic enzyme by *Penicillium chrysogenum* and about 10 to 15 °C inferior than naturally utilized as an industrial protease. Same results were obtained by (Kumar, 2002) with single serine alkaline protease having 28 KDa with

26.2% recovery, 36.6-fold purification by $(NH_4)_2SO_4$, gel filtration and ion exchange chromatography. (Shimogaki *et al.*, 1991) experimented purification of and obtained 8-fold and 52% recovery with 42 KDa. The same results were also obtained by (Saba *et al.*, 2012) experimented extracellular protease was partially and observed purification fold of 18.45 with a 41.2 U/mg catalytic activity. Molecular mass protease observed 55.0 kDa. SDS-PAGE analysis, of the purified enzyme from *Stenotrophomonas sp.* is about 55.0 kDa (Saba *et al.*, 2012). Nearly related molecular mass was observed for earlier characterized purified enzyme (Thangam and Rajkumar 2002). Crude enzyme after $(NH_4)_2SO_4$ process was functional on DEAE-Sepharose column chromatograph technique. After this process fractions showed highest catalytic activity was run on column of Sephadex-G100. Subsequently purified protease molecules were processed on SDS– PAGE analysis and was observed with 41000 Da. Enzyme was 103.2-fold purified and with recovery of 9.8% (Zhu *et al.*, 2009).

Thermolabile protease enzyme was delicate to high temperature and was observed decreased in the hydrolysis of reaction mixture when incubated beyond 40 °C (Figure 4.24). Maximum hydrolytic activity was observed for 25 °C and 93 and 87% of the hydrolytic catalysis of substrate was observed for 20 and 30 °C. 54% of the activity was retained by thermolabile molecules by incubating the reaction mixture at 40°C and the activity was gradually decreased and are measured as truncated thermostability when the thermolabile purified enzymes was exposed to temperature beyond 40 °C respectively. The optimal temperature observed for the thermolabile hydrolytic enzyme was 25 °C. It was observed from the experiment that purified thermolabile enzyme was active to hydrolyze the peptide bonds at temperature among 15 to 35°C. Half of its activity was lost at 60°C and preserved 50% of its preliminary activity by incubation of the reaction mixture at half an hour and the thermolabile enzymes was perceived to have lability toward high temperature. Its concluded from the temperature results that enzymes from PAK01 protease molecules was thermolabile hydrolytic enzyme (Figure 4.25). some thermolabile protease exhibiting highest catalytic activity at temperature of 20–25°C (Zeng *et al.*, 2004). Results of present observation are in accordance with the previous finding of (Zhu *et al.*, 2009) find that the high hydrolytic activity was observed at temperature from 15 to 35°C and 0°C the residual activity was 41%, similar with the properties of

thermolabile enzyme. 90% of catalytic activity was inhibited at 60°C at 5 min after incubation. At 20 °C 100% of the hydrolytic activity was observed classified as cold stable protease enzyme (Morita, 1975). Catalytic activity was improved at 4 to 20 °C afterward it was observed to be deteriorated. catalytic enzyme activity was observed 85% at temperature amongst 4 to 20°C and was observed stable at 20°C (Kuddus and Ramteke, 2011). Thermolabile enzyme with these properties have fascinating functions in biotechnological practices. Present study agrees with the preceding finding of (Zeng *et al.*, 2003) studied that purified enzyme showed highest activity at 40°C with residual activity of 60% was restrained at 25°C. catalytic activity preserved 100%, 83% and 62% after 1 day of incubation at 10°, 20° and 30°C. After 30°C, activity was gradually deactivated.

(Saba *et al.*, 2012) experimented maximum hydrolytic activity was investigated among 10 to 40 °C, though, highest catalysis was observed at 15°C and are classified as a cold stable enzyme. 90% of the hydrolytic activity was retained after incubation at 10 to 20°C.Stability of enzyme was observed at temperatures from 4°C to 80°C. properties of thermostability was observed, catalytic activity was retained 100% after incubation at 50°C and activity was 70% at 60°C and inhibited completely after incubation at 80°C (Ramesh *et al.*, 2007). (Salwan and Kasana, 2013) observed catalytic activity of purified enzyme from *Acinetobacter sp.* was effective around wide-ranging of temperature from 4 to 60 °C and showed 65 % of the relative catalytic activity after incubation at 4 °C. Highest catalytic activity for thermolabile enzymes from psychrotrophic bacteria ranges from 10 to 50 °C (Harada *et al.*, 2012). Purified protease hydrolytic activity from *Colwellia psychrerythraea* observed was highest after incubation at 19 °C (Huston *et al.*, 2004) and catalytic activity from *Stenotrophomonas* presented substantial activity at 10 to 40 °C, with highest catalytic activity at 15 C (Saba *et al.*, 2012).

Between 15 and 35 °C the enzyme disclosed reasonably high activity. 41% of hydrolysis at 0 °C. *Pedobacter cryoconitis* showed maximum hydrolysis was at 40 °C. At 50 °C hydrolysis was considerably decreased, and absolute inhibition was observed at 60 °C. 28 to 79 percent of the optimum hydrolysis was observed for 20 to 35 °C (Margesin *et al.*, 2005). Salwan and (Kasana, 2013) observed peptidase from *Acinetobacter sp.* which showed functional over wide range such as 4 to 60 °C with increased hydrolysis was at 40 °C. Peptidase showed beyond than 65 % of the relative

hydrolysis at 4 °C. The enzyme activity is momentously exaggerated reaction mixture acidity or basicity and every enzyme shows maximum activity at its own optimum pH. (Kuddus *et al.*, 2011) isolated *Stenotrophomonas maltophilia* producing an alkaline protease and are dynamic at basic conditions (pH 10). *Pseudoalteromonas* sp. secreted alkaline protease has maximum enzyme activity at pH 8.0 and at pH 5.0 (31 percent) and 38 percent hydrolytic activity at strong basic condition such as pH 11 respectively (Baghel *et al.*, 2005). (Zeng *et al.*, 2003) isolated *Pseudomonas* sp. strain have the potential of producing alkaline enzyme showed wide-ranging pH contour such as pH 6 to 12 and augmented hydrolysis amongst pH 8 and 10 and at 10 pH premier residual activity (%) was observed. Wang *et al.*, 2008 studied *Pseudoalteromonas* sp. produced thermolabile protease was partly repressed by ions such as Magnesium, Calcium, Copper, Zinc, Barium, Iron, Lead and Manganese. In the presence of 2 molar NaCl molecules were unchanging for one-hour incubation, moreover, up to strength of 3 molar NaCl strong conditions, 56.5% hydrolysis of the substrate was observed.

Studies on optimum pH for activity and stability also enunciated a broad range (6-12). There found an existing a negative correlation between pH and proteolytic activity of proteases, and pH 9 was found most suitable for high activity of enzyme. The fluctuation in proteolytic activity at different pHs may be due to the effect on activity of amino acids that constitute the active site of serine protease, hence affecting the hydrolytic activity. Highest catalytic activity observed was at pH 9.0. Hydrolysis of enzyme against the casein substrate was observed maximum catalytic efficiency in the reaction mixture at 25 °C when the buffer used was of 9 pH (Figure 4.26). Alkaline tolerant protease enzyme results indicate 71 and 86% hydrolytic activity was retained when buffer used for reaction mixture was 8 and 9 pH and have the competency to persist at wide of pH range. From consistency test of pH experiment, protein molecules showed resistance at pH 6 to 10 and gradually the results are significantly declined with pH above 10. The protease retained 80 and 100 % activity at pH 7.0 and 9.0 and proteins was not sturdy enough and inhibited toward acidic conditions and was observed from the present finding that thermolabile enzyme lost half of its hydrolytic activity at 6 pH. Thermolabile alkaline molecules on overall average retained 80% of its catalytic efficiency in the reaction mixture at 25 °C of buffer used from 8 to 10 pH after preincubation for I hour (Figure 4.27). our results

are in accordance with previously finding of (Kuddus and Ramteke, 2011) observed hydrolytic activity of purified enzyme was observed active at pH 7 to 11 with highest catalytic activity towards casein was observed at pH 10. Thermolabile protease activity improved from pH 6 to 10, and 93% of the residual catalytic activity was observed after pre-incubation between at pH range of 8 to 11. Kumar, 2002 observed pH optimum with different pH buffer from 10 to12, with highest activity observed was at pH 11, further purified protease was 100 % tolerant at wide-ranging of pH 6 to 12 after 4 hours and 6-11 after 24 hours incubation. In present studies, the optimum pH 9-12 was recorded (Kumar, 2002), pH 10 (Gupta *et al.*, 2008), pH 9 (Dhandapani & Vijayaragavan, 1994) and pH 10.5 (Beg and Gupta, 2003).

Related values were also given by Johnvesly and Naik 2001, with optimum pH 11of enzyme from Bacillus sp. and also reported its high activity in the pH range 8-12. Related results were also reported by (Ramesh *et al.*, 2007) observed catalytic activity of enzyme from *Exiguobacterium sp.* was highest at pH 8.0 and preserved approximately 60% of its residual catalytic activity at pH 9 however, hydrolytic activity of protease from *Bacillus sp.* (Kumar and Bhalla, 2004) and *Shewanella strain* (Kulakova 1999) observed maximum at pH of 9. Results of present study are in accordance with the previous observation of (Salwan *et al.*, 2013) observed highest activity of enzyme after incubation at pH 9.0, comparable to catalytic activity of purified protease from *Bacillus cereus* strain (Joshi *et al.*, 2007). The proteases from *Pseudomonas strains* (Zeng *et al.*, 2003) and *Stenotrophomonas maltophilia* (Vazquez *et al.*, 2005) observed highest protease activity after incubation at pH 10 and 11 correspondingly. Properties of thermolability, alkali and stability recommended its effectiveness in detergent industry.

The effects of numerous metal ions at strengths of 5, 10, and 15 mM on catalytic activity of enzyme from PAK03 are concise in (Figure 4.28). Catalytic activity of enzyme observed having stimulatory effect after incubation with Ca^{2+}, KCL, and Mg^{2+} at each strength of 5, 10, and 15 mM was observed as 165, 143 and 176% residual activity for calcium chloride, 143, 132 and 130% residual activity for KCL and 121, 124 and 118% residual activity for magnesium chloride. Effects of KCL on alkaline enzyme remained not detected by Beg and Gupta (2003) and Hadder *et al.*, (2009), but consequences of Ca^{2+} and Mg^{2+} was described earlier by (Beg and Gupta, 2003; Dipasquale *et al.*, 2008; Haddar *et al.*, 2009).$CaCl_2$, $MgCl_2$and

KCLwere observed have a stimulatory result on hydrolysis of molecules and was found having good catalytic hydrolysis of peptide bonds by the binding of the ions on the active site of the molecules. In the presence of $MgCl_2$and $NiSO_4$in the reaction mixture resulted that protease molecules were also tolerant and augmented the catalytic reaction slightly. In reaction mixture as the concentration of $FeCl_3$, $CuSO_4$ and $FeSO_4$increased it was observed the protease hydrolysis against the substrate was entirely inhibited. Catalytic activity of Serine protease from *Bacillus sp.* observed to preserve approximately 221–237 % and 113–96.2 catalytic activities at different concentration was retained after incubation with $Fe^{2+}$and Cu^{2+} (Singh *et al.*, 2001). So Ca^{2+}, KCL, MnCl2 and Mg had a stimulatory effect on enzyme activity and was observed boosted and stabilized hydrolytic activity. highest catalytic activity is due to stimulation of catalytic site of these metal ions. Results are in accordance with the previous finding of (Zhu *et al.*, 2009) who observed the consequences of ions and EDTA. Ca, Na, Mg, K, NH4 boosted the catalytic activity inconsequentially. Cu, Co, Fe and EDTA effect observed was inhibitors on catalytic activity. Fe and EDTA observed completely inhibited the activity. Strong inhibitory effect of Cu and Co on protease enzyme activity. (Ramesh *et al.*, 2007) observed Mg and Ca ions, catalytic activity observed was boosted by 7% and 5% however the catalytic activity of enzyme after incubation with Zn ion stimulated 9% activity as compared to control condition. Consequences of ions on activity of enzyme exhibited recovered because of Ca and Mg at strength of 10 mM was observed boosted by 30%. Hydrolytic activity was improved slightly by 8% and was observed significant role in stability. Co, Cu and Zn inhibited the catalysis of enzyme, while Fe, Mn, Li, Hg, K Ag was observed no consequence on enzymatic activity (Zeng *et al.*, 2003).

Zeng *et al.*, 2003 reported that calcium and magnesium metal ions augmented 8% hydrolysis and imperative part in stability of hydrolysis by conducting the *Pseudomonas* sp. fermentation that was improved by 30 percent in the existence of calcium and magnesium at concentration of 10 mM. Among the cations tested Iron, Manganese, Potassium, Lithium, Mercury, Silver had no observable effect on enzymatic activity, while Cobalt, zinc and copper subdued the hydrolytic process (Zeng *et al.*, 2003). Protease activity from *Penicillium chrysogenum* was increased by the addition of Calcium, Sodium, Magnesium, Potassium and Ammonium, while Copper, Cobalt, Iron and EDTA inhibited the enzyme activity (Zeng *et al.*, 2003). Ps5

metalloprotease of *Pseudomonas lundensis* showed inhibitory effect on enzyme activity when incubated with Cu and Fe, while cobalt, manganese, aluminum and iron reduced molecule exploit to 32 to 14 percent (Yang *et al.*, 2010). Yang *et al.* 2010 observed inhibition by Zinc and there might be a different hydrolytic trail for contrivance of Ps5 molecules as antagonistic to former metallomolecules. Huston *et al.*, 2004 observed Zinc and Manganese inhibited ColAP molecules and Calcium was marginally inhibitory, Magnesium stimulated at 10 mM or greater concentration, that is the intensity of seawater. Thermolabile protease of psychrotrophic *Pseudoalteromonas* sp. was introverted by ethylene glycol tetra acetic urea, thiourea, phenylmethylsulfonylfluoride, ethylenediaminetetraacetic acid, dithiothreitol and sodium dodecyl sulfate had no effect on cystein inhibitor (wang *et al.*, 2008). Calcium ion somewhat improved activity experiential for other proteases (Amoozegar, 2007), although Copper ion enhanced catalytic activity as (Patel *et al.*, 2006). (Ji *et al.*, 2014) observed that metal ions have no influence on the enzyme catalytic activity and protease presented tolerance to metals. Protease exhibited significant role in bioremediation sites polluted with heavy metals.

Stability of enzyme was reduced up to 40%, 32%, 43%, 30% and 15% in acetonitrile, butanol, hexane, 2-methylpropan and ethyl acetate at 10% concentration. Acetone had good effect at 5 and 10% and enzyme retained 83 and 79% its activity. Ethanol had positive effect on the protease and its stability increased up to 76 and 84% at concentration of 5 and 10% concentration. Numerous organic solvents consequence at 5 and 10% strength on the catalytic reaction of alkaline protease are concise in (Figure 4.32). Nevertheless, the proteases utilization in the synthesis of peptide is inadequate by the instability and specificity of enzymes in the existence of organic solvents. The organic solvents effect 5 to 10% on alkaline protease stability was examined by pre-incubating at 25°C along with the shaking step for 1 hour. Figure 4.30 reveals that the protease shows good constancy in the presence of acetone, ethanol, isopropanol and methanol, while acetonitrile, butanol, hexane, 2-methylpropan and ethyl acetate disturbed the enzyme stability to a greater extent. It is therefore reasonable to conclude that the organic solvents enzyme stability totally relies on the organic solvents nature. The good stability of protease in the occurrence of acetone, ethanol, isopropanol and methanol can be elucidated by the hydrophobic interactions enhancement within the protein molecules. This situation may result in

anticipated conformational alteration for the interaction among the substrate and active site. Results display catalytic activity of the thermolabile protease toward organic solvents. Due to disulfide bonds competence might be the existence of the disulfide bonds in the catalytic traid of enzyme and was observed necessary for its catalytic activity and reports on significance of disulfide bonds for tolerance of the hydrolytic catalysis of thermolabile enzyme toward solvents (Ogino *et al.*, 2001). Thermolabile hydrolytic enzyme for fermentation reaction observed was useful in existence of solvents. Importance of this property is to decrease microbial pollution through degradation process. (Najafi *et al.*, 2005) reported that protease enzyme has the potential to hydrolyzed in the presence of different solvents. Catalytic activity was observed highest such as 80 to 90% after incubation with 20% of xylene, toluene, methanol and ethylene. 50% of catalytic activity was resulted at 10% concentration in reaction mixture excluding acetone. Wang *et al.*, 2008 observed protease activity as 85, 50, 42, 98, 115, 15, 20, 15, 20 and 50% activity in existence of 20%, methanol, toluene, ether, ethanol, acetone, acetate, acetonitrile, alcohol, isopropyl, isoamyl and remaining activity was 70, 28, 15, 90, 115, 78, 22, 2, 42 and 50% after incubation in existence of alcohol, ethanol, methanol, ether, toluene, acetone, acetonitrile, acetate, isoamyl and isopropyl. Water molecules substitute in catalytic traid of enzyme with organic solvent molecules balance the structure. Enzyme utilized in organic solvents reaction to turn balance of the reversible reaction between catalysis of peptides to complete the hydrolysis of substrate (Wang *et al.*, 2006 and Wang and Yeh, 2006).

The thermolabile protease studied in our work showed interesting results (Figure 4.27). When tween 20 was used as 5% concentration, the enzyme activity showed increased by 5% as compared to control treatment but at 12 hours incubation at 5% inhibited on 5% activity and by further incubation at 5% for 36 hours it slightly inhibited only 10 % activity. whereas tween 80 was found highly stabilizing, at 5% concentration the activity of enzyme was increased by 12% after for 12 hours of incubation, but at 24 hours incubation at 5% concentration activity was increased up to 2% and increased its activity when incubation of 36 hours at 5% concentration the activity was observed stimulated up to 6%. The present results of this study are in accordance of the finding of (Pena-Montes *et al.*, 2008) that alkaline protease of *A. nidulans* in presence of tween 80 increased up to 160%. In SDS at 1% concentration enzyme activity was 4 %, but concentration up to 3% it increased up to 239 and at 5%

it was 248% as residual activity. Triton X-100 had positive effect on protease at 5% concentration the enzyme showed 93% after 12 hrs of incubation, at 24 hours incubation at 5% the activity observed was 97% and at 36 hours of incubation at 5% concentration of triton x-100 it showed 94% residual activity. even the oxidant was also found effective, like H_2O_2 at 1% concentration with enzyme showed activity of 79% residual activity. Oxidant such as SDS at the concentration of 0.5% when incubated for 1hour protease showed 87% residual activity. Results of present study are plausible with the previous finding of (Salwan *et al.*, 2013) studied purified enzyme stability in the existence of numerous agents and 50 % relative catalytic activity was observed after incubation with Tween 20 and 80, urea and H_2O_2. Enzyme showed stability in existence of commercial surfactants and activity retained was 60 % after 2 hours incubation. Proteases from diverse bacterial species have differences in their structure constancy against distinctive commercial detergents maintaining 42–93 % residual catalytic activity of enzyme after 1hour incubation (Dodia *et al.*, 2008, Venugopal and Saramma, 2006). The results of present study are in complete plausible with the previous finding of (Gouda, 2006) who observed two proteases were found to be very stable toward SDS, triton X-100 and tween 80. (Wang *et al.*, 2008) reported in the existence of different surfactants, catalytic activity was retained 79%, 80% and 110% as compared to control. Though, underneath the same situation catalytic activity retained 100%, 100% and 121% as compared to control. At concentration of 1 mM SDS, hydrolytic catalysis of two protease protein with 42% and 0% relative activity.

In the present research the protease was partially inhibited by 2-mercaptoethanol at 15 mM concentration and the hydrolytic reaction of enzyme against protein substrate was affected by DTT and EDTA with a residual activity of 57% for both the inhibitors at concentration of 15 mM. However, PMSF completely constrained the enzyme activity with a remaining activity of 9% at final concentration of 15 mM and indicated that in the catalytic position existence of serine residue and enzyme of metalloprotease in nature. It was observed from the results that serine enzyme dynamic site was sulfonated by the PMSF in the effective position and no hydrolytic activity was observed. Catalytic activity of enzyme was completely inhibited in the existence of radical EDTA and PMSF designated existence of serine domain in the catalytic site and enzyme of metalloprotease in nature. Enzyme

hydrolytic activity was observed inhibited by even at 1 mM concentration of metalloprotease, EDTA and chelating agent and Hg, EDTA and PMSF affected activity of enzyme as 14, 16 and 8% (Alam *et al.*, 2005). Protease from *Pseudomonas* sp. strain inhibited by benzenesulfonyl fluoride hydrochloride and diisopropyl fluorophosphate designates that it was enzyme belong to group of serine and tolerate DTT at 5% intensity, signifying that in conserving hydrolytic activity disulfide bonds are not tangled. (Zeng *et al.*, 2003) studied that enzyme molecules at concentration of 4 molar was sensitive to urea, guanidine at 1 molar and SDS at 1 percent intensity, observed that in the hydrolysis mechanism hydrogen bond play a vital role. (Yang *et al.* 2010) 70% of hydrolytic activity observed for Ps5 molecule was inhibited by PMSF (10 millimolar), and also showed that molecules were resistant to at 1 millimolar concentration of EDTA. Stimulation of activity 124% at 1.0% of H2O2 on Ps5 protease demonstrated an interesting property and at concentration to 10% it retained 73% activity on increasing H2O2. 10% urea encouraged enzyme activity, but in the SDS existence of 1 percent concentration it lost the activity completely. Huston *et al.,* 2003 observed ColAP molecules was resistant to PMSF and at 10 millimolar potency the molecules were inhibited by EDTA and DTT

The results of the present observation are plausible with the results of (Zhu *et al.*, 2009) who observed that hydrolysis of substrate in the presence of (PCMB), b-mercaptoethanol and iodoacetate have no effect on the stability of enzyme. In dissimilarity, catalytic activity was intensely inhibited by PMSF and DFP after incubation with concentration of 3mM. Inhibition instigated by PMSF suggested enzyme have serine at the catalytic site of protease. The results of the present finding are in complete accordance to the previous observation of (Salwan *et al.*, 2013) who observed that the hydrolytic activity of purified protease was intensely hindered by PMSF, recommending peptidase underneath exploration is peptidase having serine at catalytic site. PMSF exhibited negative influence on catalytic activity of protease from *Bacillus cereus* (Joshi *et al.*, 2007) and *Colwellia sp.* (Wang *et al.*, 2005). The inhibitory and stimulatory consequences of diverse substances on hydrolytic activity of protease from psychrotrophic bacteria was reconsidered (Kasana, 2010).

5.5. Kinetic studies

The protease was considered for kinetic studies parameters of Vmax and Km toward substrate. purified catalytic activity of protease against different concentration of casein substrate and the hydrolytic activity information were designed as 1/V versus 1/S to get a reciprocal curve of Line Weaver-Burk double. Alkaline protease was allowed to undergo hydrolysis of casein (1%) at low temperature such as 4 °C, 15 °C and 25 °C for 15 minutes (Figure 4.33, 4.34 and 4.35). As it was problematic to calculate casein molar concentration and thus percentile concentration was used in this research. As predictable from the thermolability of purified protease activity, the highest catalytic efficiency was observed for 25 °C as compared to 4 and 15 °C which are considered as cold enough to hindered catalytic efficiency of purified protease enzyme. Thermodynamic parameters for alkaline protease was calculated at distinctive temperatures stretching from 30 to 90 °C. $Ea_{(P)}$ calculated for protease enzyme form *Stenotrophomonas sp PAK01* was 27.567 kJ/mol (Figure 4.37). Values of K_m, K_{cat} and K_{cat} / K_m of enzymatic catalyzed reaction at pH 9.0 and different temperatures are shown in table 2. The lower $Ea_{(P)}$ suggested that lesser energy was required by alkaline hydrolytic protease enzyme to reach the transition state of tyrosine formation. Arrhenius equation was studied by Irwin *et al.*, 2001 who observed exponential diminutions at low temperature in occurrences of biochemical reactions. In disparity to molecules from mesophilic, at temperatures between 0 and 30 °C these enzymes revealed 3 characteristics: a higher hydrolytic effectiveness kcat/Km, for hydrolysis of substrate optima subordinate temperature, and because of the high temperature and mediators' changes in molecules structure reduced stability (Feller *et al.*, 1996). Evaluation of Km and Kcat studied was observed by (Feller *et al.*, 1996 and Feller *et al.*, 1997) between molecules resistant to cold temperature and molecules from thermophilic and mesophilic correlative. The results of present study are in accordance with the previous finding of (Zhu *et al.*, 2009) who observed hydrolytic activity Kcat/Km of molecule was active at 35 °C. (Alam *et al.*, 2005) observed inverse proportion in case of intensification in *K*m for the molecules with temperature decrease and materializes features of weak attachment to a substrate resulting decrease in Ea. Alam *et al.*, 2005 studied true psychrophilic enzymes are invariably thermolabile and are more flexible in structure and observed increased Km for molecules of Q10 at cold temperature. *K*m and *k*cat/*K*m of molecules were 0.41

millimolar and 45/s/mM at 35°C and 54 percent hydrolysis was preserved of *k*cat and *k*cat/*K*m at 0 °C with 34.8 kJ/mol Ea (Wang *et al.*, 2008). Huston *et al.* 2004 reported ColAP molecules have the premier specific activity at 19 °C from *Colwellia psychrerythraea* strain and hydrolytic efficiency of 5.0/s/mM was for molecules at 19 °C, and immovability of 44% retained at 9 °C. There are appropriate insufficient experiments on molecules resistant to detergent/bleach (Bhosale *et al.*, 1995 and Khaparde and Singhal, 2001). An alkaline protease from *B. sphaericus* (Sing *et al.*, 1999) has disclosed steadiness with a diversity of marketable detergents at 37°C.

The results of present study are in complete accordance the previous finding of (Zhu *et al.*, 2009) who estimated thermostability of catalytic activity and observed the highest catalytic efficiency at 35°C. Highest catalytic efficiency for protease was observed at 45 °C from mesophilic *Penicillium chrysogenum* (Maria *et al.*, 2002) and best temperature for hydrolytic activity of thermolabile protease was investigated as 35 °C for FS010. The Km and Vmax observed was 0.3529 mg/ml and 294.11 g/ml/min whereas 2.03 mg/ml and 384.61 g/ml/min was observed against gelatin substrate. Casein with low Km value recommends that the binding affinity of substrate toward enzyme was highest as competed to gelatin (Salwan *et al.*, 2013).

5.6. Application of thermolabile alkaline protease

Hydrolytic reaction of the thermolabile molecules was stable in purex at intensity of 3% and 6% for xtra, correspondingly (Figure 4.39). Finding of the present study indicates inverse proportion among detergent strength and thermolabile enzymes so at 20% strength residual hydrolytic catalysis reduced in up to 7% with xtra and with purex 24 % and the investigated results may be due to hydrolytic molecules destabilization of at complex strength of commercial detergents. *Stenotrophomonas sp* secreted protease molecules stability contour in 24 hrs in 5% of xtra and purex revealed 78 and 83 percentile residual hydrolytic activity after incubation for 3 and 4 hours (Figure 4.40). Nevertheless, thermolabile molecules showed resistant against purex and xtra and observed from the present finding are 61 and 51 % residual hydrolytic activity of protease molecules at 24 hours of incubation with purex and xtra. The thermolabile protease showed resistance against bleaching active inhibitors and catalytic activity was retained 86, 83, 76% of the residual hydrolytic reaction in concentration of such as 50 to 200 mg/l, correspondingly. Though, 64 % of the hydrolysis was engaged even at greater strength 500 mg/liter of

sodium hypochlorite (Figure 4.41). However, up to 15% hydrogen peroxide (H_2O_2) the protease molecules reserved only 6 % of its preliminary activity at 25°C and was completely inhibited at 20% H_2O_2 (Figure 4.42). The present results are in accordance to finding of (Moreira *et al.*, 2002) who detected 73.5% of hydrolytic activity at pH 10 and at pH 12, 61.7% catalysis after 120 min. Almost 60% of catalysis endured after 120 min. 87% of residual hydrolysis was reserved in the existence of 10 percent peroxide and was also resistance in the occurrence of distinctive surfactants and oxidants, saponin, Tween 20, 80 and SDS after Half an hour. Stimulated activitation was observed from 6 to 17% in the existence of potassium, sodium, and magnesium ions. The productivity of *stenotrophomonas sp.* producing hydrolytic enzyme was experienced for eradicating blood pigments from experimented fabric at distinctive temperatures containing 15, 25 and 35 °C beside 100 U/ml catalytic activity of enzymes and 5% concentration of 15 mg/ml purex. It acquired 3 to 4 hours to entirely eliminate the blood tint at 15 °C however at 25 and 35°C, stains observed were detached in 1 hour and 30 min. Though, when enzyme is not present in the reaction experiment, the time acquired to remove pigment experimented was long enough. So, when buffer was replaced with Tris HCl of 9 pH, the mark removed experienced in 30 min at 25 °C alongside 100 U/ml catalytic enzyme and 5% purex (Figure 4.43). The thermolabile molecules was also competent in hydrolyzing protein of egg yolk from test cloth at 25 °C with active molecules of 500 U/ml and 30 min required to utterly remove strains (Figure 4.44). *Stenotrophomonas sp. PAK01*, with its firmness in purex, wash implementation and hypochlorite, seems to be a hypothetical cold active molecule for vigorous ingredients of formulation for washing purpose.

Protease in the present experiment presented respectable activity of hydrolysis and active stability in the manifestation of commercial Ariel trailed by different marketable detergents and catalytic activity of protease enzyme preserved 50% hydrolysis of proteinaceous pigmented stain with maximum of the detergents experienced afterward incubation at 3 h 15 °C of temperature (Saba *et al.*, 2012). Maximum catalytic activity of peroxide-based bleach active protease from *Arthrobacter ramosus* and *B. alcalophilus* in eradicating blood pigment from cotton fabric is also reported (Kanekar *et al.*, 2002). Numerous detergent active proteases were integrated as bio builders to hydrolyses and eradicate (Ito *et al.*, 1998 and Kobayashi *et al.*, 1995) proteinaceous pigmented stain from the tested clothes.

(Adinarayana and Ellaih, 2003) observed alike investigational effort was experimented for low temperature laundry reasons by numerous other researchers with hydrolytic purified protease extracted from distinctive bacterial strains. Silk and wool fabric observed highly sensitive when washing at high temperature.

Stenotrophomonas sp. thermolabile protease enzyme was considerably highly compatible and considered as not destructive against the fragility of wool beside at 15°C with marketable detergent. The Present study finding is in accordance with the previous finding of (Salwan *et al.*, 2013) who extracted molecules that are stable synergistically with the detergents and at cold environment commendably detached blood tinges. Molecules from *Bacillus sp.* (Arulmani *et al.*, 2007) and *Botrytis cinera* (Abidi *et al.*, 2008) who's observed that both the protease has the potential to remove the blood pigments at extreme environment. *Pseudomonas aeruginosa* molecules (Gupta and Khare, 2007) potentially active at cold *environment* to remove blood stain. *Acinetobacter sp.* extracted molecules effectively confiscate strains presented hypothetical for detergent productiveness. Proteases molecules resistant to detergent formulation from *Arthrobacter ramosus* and *B. alcalophilus* confiscating blood marks from fiber fabric is also reported (Kanekar *et al.*, 2002). (Ito *et al.*, 1998 and Kobayashi *et al.*, 1995) experimented molecules as bio builders into durable detergents to hydrolyse proteinaceous constituents in tainted garments.

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5.7. Cold active lipases

Microorganisms existing in extreme cold temperature are considered as psychrophiles having potential to propagate at temperature below 20°C however additional group is existing as psychrotrophic microorganisms having highest growth at temperature of 37°C in the mesophilic range and the potential to cultivate also at close to freezing temperature (Moyer and Morita, 2007). Earlier research indicates of receiving more psychrotrophic from temperate habitats similar to Antarctica than optimum temperature at 10 °C for growth (Antony *et al.*, 2016; Hatha *et al.*, 2013; Vaz *et al.*, 2011). The probabilities of attainment psychrophiles and psychrotrophic microorganisms are more observable at polar circumstances and can also be discovered in elevated mountains, ocean, alpine soils and glaciers (Maharana and Singh, 2018). Lipases functioning at low temperature covering a wide spectrum of purposes of biotechnology such as detergents formulation, food production additives, biotransformation, pollutants eradicate from soiled regions, functions in molecular biology, and expression of heterologous gene in psychrophilic microorganisms to preclude appearance of inclusion elementary bodies (Joseph *et al.*, 2008). Thermolabile lipases has numerous capabilities in positions of lower activation energy overheads and microbial impurity in the different industrialized procedures (Marshall, 1997; Alquati *et al.*, 2002). Lipases from microorganisms' have biocatalyst function as in solvents having non-aqueous properties proposal novel capacities such as thermodynamic stabilities changing in favor of synthesis, substrate specificity monitoring by solvent engineering, facilitating the utilization of substrates with hydrophobic properties and improving resistance toward high temperature of the hydrolytic enzymes (Koops *et al.,* 1999). *Pseudomonas* is observed as an extraordinary producer for thermolabile lipase (Maharana and Ray, 2013, 2014a, 2015b; Zeng *et al.*, 2004). Furthermore, other bacterial species for enhanced hydrolytic lipase production are belonged to the genera of Microbacterium, Acinetobacter, *Bacillus*, *Aeromonas Staphylococcus*, *Psychrobacter* and *Moraxell*a (Joseph *et al.*, 2008). Growth of the microorganisms depends on numerous chemical and physical aspects similar to temperature range, pH such as salinity, substrates specificity, different sources of carbon and nitrogen, amino acids and minerals salts. Production of enzyme is directly proportionate to the microorganism's growth but in most situations, enzyme production is maximum in the fermentation medium by the

microbes are an inactive form, consequentially in inferior hydrolytic activity (Maharana and Ray, 2015a). The current research focused on the optimization of thermolabile solvent stable lipase production by *pseudomonas* and cloning and expression of alkaline hydrolytic lipase, YLip gene (MH338242 GenBank ID) from *Pseudomonas peli* PAK3 *peli* isolated from Rakaposhi glacier, in northmost autonomous territory of Gilgit Baltistan. The Rakaposhi glacier situated in the Nagar region (between35°24'0N 73°48'0E) Pakistan, 5000 km^2 in zone, and largest glacier in the northern Baltistan (Nafees *et al.*, 2014). In summer temperature is 2 to 5 °C and extreme in winter.

5.8. Glacier Bacteria

The *Pseudomonas* sp. *Planococcus sp.*, *Exiguobacterium sp.*, *Pseudomonas aeruginosa* and *Stenotrophomonas sp* were isolated from different glaciers in Pakistan (Table 4.1). Among recent reports on isolation of bacteria from glacial ice cores (Abyzov, 1993; Hamilton and Lenton, 1998), *Bacills subtilis*, *Arthrobacter agilis*, and *Stenotrophomonas africae* have been documented from 5 to 20,000 years old ice cores from Greenland, China, Bolivia and Antarctica (Christner *et al.*, 2000). The frequent isolation of related genera from geographically different sampling sites (Table 4.1) suggests that these bacteria are adapted to low temperature or freezing conditions.

(Kersters *et al.*, 1996; Anzai *et al.*, 2000) Studied the diverse species of *pseudomonas* comprised about 100 species but characterization and validation by modern tools provided a long list of members by reclassifying into different genera Among recent reports on *Pseudomonas spp.*, *Pseudomonas balearica* sp *and Pseudomonas stutzeri* studied by (Bennasar *et al.*, 1996); *P. peli* and *P. borbori* observed by (Vanparys *et al.*, 2006); *Pseudomonas guineae* was investigated by (Bozal *et al.*, 2007); *P. alcaliphila* sp. nov. (Yumoto *et al.*, 2001); *Pseudomonas antarctica* specie and *Pseudomonas proteolytica* specie was studied by (Reddy *et al.*, 2004); *Pseudomonas putida* P8 (Diefenbach *et al.*, 1992); *Pseudomonas flavescens* specie (Hildebrand *et al.*, 1994); *Pseudomonas psychrophila* sp. nov. (Yumoto *et al.*, 2001) are well characterized.

5.9. Isolation and identification of *pseudomonas peli*

In order to isolate psychrotolerant bacteria, soil and water samples were collected from vicinity of Rakaposhi glacier(36°14'368′, 74°26'576″), Jutial(35°54'276″, 74°19'841) and Juglot (35°41′06′, 74°37′26″) glacier situated in the Nagar region located at (35°24'0N 73°48'0E) with and altitude of 2,688m is the largest valley type glacier in the northern Baltistan (Nafees *et al.*, 2014). *Pseudomonas peli* PAK03 was isolated in laboratory of Applied microbiology and biotechnology, International Islamic University Islamabad from the soil samples gathered from Gilgit Baltistan Pakistan. Samples of soil from distant sites was diluted serially and cultivated on nutrient agar plates for screening of lipase producing bacteria at pH 9 at 20 °C for 24 hr. Out of twenty strains, five bacterial stains were observed to have the potential to grow at low temperature and alkaline pH. Quantitative screening of the selected five bacterial strains was experimented in submerged fermentation for 48 hrs at 20 °C in alkaline conditions at pH 9. After incubation bacterial strain that have the potential to catalyze maximum substrate of triacylglycerol was selected and stored at -20°C for further analysis. Further processing was done in the chemical engineering department of Taxas A&M university, USA. Genomic DNA of selected bacteria was extracted from pure fresh culture of 24 hours and nearly the complete stretch of PCR amplification of 16S rRNA of complete fragment of 1500 bps was experimented. Forward and reverse primers used for elongation were 5′-AGAGTTTGATCATGGCTCAGA- 3′ and 5′-GTTACCTTGTTACGACTT-3′- equivalent to 8 to 28 and 1493 to 1510 and are segments of 16S ribosomal RNA genome of *E. coli* and so are valuable for extension of 16S ribosomal RNA genomic DNA from diverse classes of bacteria. Selected bacterial strains were identified on the basis 16S rRNA sequencing. Isolation of genomic Bacteria DNA was done by Quick-DNA™ Fungal/Bacterial Miniprep Kit. Sequences were matched and aligned with other 16S ribosomal RNA sequences added in the GenBank database of NCBI (Altschul, *et al.*, 1997) and for the documentation of bacteria software of Ribosomal Database Project II was analyzed. CLUSTAL W was utilized for the BLAST sequences aligned (Thompson, *et al.*, 1997). Alignment of the BLAST nucleotide sequences was then physically checked and modified. Pairwise evolutionary gaps for the relationship between microorganisms were processed exploiting the Cantor and Jukes calculation applied

in the MEGA7 database and a tree of phylogenetic was created by the technique of joining neighbor software accessible online (Tamura, *et al.*, 2007).

Final sequence of PAK03 was submitted to GenBank having an accession no.MG687270. The *Pseudomonas* specie. In the present study are facultatively psychrophilic and grew wide range of 2 to 37 °C with pH 8 on average while *Pseudomonas guineae* sp. (Bozal *et al.*, 2007) and *Pseudomonas alcaliphila* sp. nov. (Yumoto *et al.*, 2001) exhibited their grow range of -4 to 30 °C and 4 to 37 °C, respectively. (Gokbulut and Arslanoglu, 2013) isolated and identified strain of *Pseudomonas fluorescens* by 16S ribosomal RNA nucleotide sequencing method. Psychrotolerant characteristics was observed best growing temperature at 25 °C. (Borkar *et al.*, 2009) isolated *Pseudomonas aeruginosa* sp. from soil contaminated with oil and petroleum spilled soil and conserved with reaction mixture (Yeast extract, Beef extract, NaCl, Peptone, and Agar at 4°C at 9 pH). Identification was done based on 16S rDNA technique. Genome sequence modified and aligned with standard domain of Gene bank (http://www.ncbi.nih.gov) by BLAST program (Altschul *et al.*, 1997) after that microorganism permitted a genus.

Bacillus methylotrophicus PS3 was identified as by 16S ribosomalRNA with accession of LN999829 (Sharma *et al.*, 2017). (Saun *et al.*, 2014) isolated strain from soil of hot spring and identified as *Bacillus aerius* using 16s rRNA method. (Jadhav *et al.*, 2013) isolated psychrotrophic bacterial strain from an Antarctic seawater sample (Latitude S 59°40'24.6" and Longitude E 68°33'23.7") and Its lipolytic activity was screened using tributyrin agar base at 15°C and pH 7.0. *Halomonas* sp. BRI 8 was maintained on Marine Salt Medium (MSM) (Jadhav *et al.*, 2010). The 16s ribosomal RNA genome sequence studied isolate belongs to *Halomonas* (929 bp).

5.10. Cloning, expression and characterization of extracellular psychrotrophic alkaline lipase from *pseudomonas peli* strain PAK3 (MH338242)

5.10.1. Cloning and sequence of YLip gene analysis

For cloning of the yLip gene encoding solvent resistant alkaline protein, information of nucleotide sequence from *Pseudomonas mandelii* JR-1 was used, which is associated closely to *Pseudomonas peli*, and the specific primers was designed based on the region of the nucleotide noncoding sequences covering the encoding thermolabile lipase gene of *Pseudomonas mandelii* JR-1. Product of PCR estimated was 891bp yLip gene from the psychrotrophic *Pseudomonas peli* PAK03

was amplified and the amplified genome was inserted into a pET28 bacterial vector. Protein translated from the yLip gene comprised a Gly-His-Ser-Gln-Gly sequence (Figure 4.48), a distinguishing motif of the protein family belonging to serine lipase (Gly-X-Ser-X-Gly) (Cygler *et al.*, 1993). Clones gave protein of molecular mass of 32 kDa was calculated (Figure 4.47). From the multiple sequence alignment, it was characterized that 96 % identity of the yLip sequence at the level of the amino acid was observed identical to protein tolerant toward organic solvent encoding from LipS gene, from *Pseudomonas mandelii* JR-1 (Figure 4.48). Q3KIU1, yLip, rPFL and LipS genes resulted was 296 matching residues of amino acid. Lip9 gene amplified from *Pseudomonas aeruginosa* exhibited sequence identity of 44% but demonstrated a conserved motif of serine lipase family (Figure 4.48). Residues of 2 Asp as a calcium binding motif were observed.

5.10.2. Examining the expression and purification of the cloned lipase gene YLip

Bacterial plasmid pET28a vector comprising the complete open reading frame of yLip nucleotide sequence was transformed into cells of competent *E. coli* BL21 (DE3). yLip gene complete nucleotide sequence was inserted in a pET28a vector with His_6 residues on N-terminal position. yLip recombinant protein was effectively expressed in *E. coli* BL21 strain as a soluble protein and nickel chelate affinity chromatography (Figure 4.47A) was used for purification analysis trailed by Q-Sepharose column chromatography procedure. (Table 2). Though, yLip large fraction did not attached to the resins of nickel and observed 5% of quite low yield. Technique to recover the yLip yield from purification methods is being sought. Profiles of elution from purification steps such as nickel chelate and Q-Sepharose columns chromatography held pace with the activity of lipase. yLip protein showed as a 32-kDa protein on SDS gel analysis (Figure 4.47B, lane Q). Sequence (VNLIGHSQGSLTAR) of peptide was analyzed by the MALDI-(Figure 4.46B, lane Q). Sequence (VNLIGHSQGSLTAR) of peptide was analyzed by the MALDI-TOF MS from the 32-kDa protein band correspondingly matched the LipS sequence (underlined in Fig. 4.45) Kim *et al.*, 2013). A promising zone of exploration in enzymology area is to acquire radically diverse and novel hydrolytic enzyme through different molecular methodologies such as protein engineering, DNA recombinant technology, metagenomic approach and directed evolution. Primary achievements in

the secretion of heterologous proteins using *E. coli* bacteria to expressed distinctive kind of proteins. Cloning of lipase gene in *P. fragi* and sequencing of nucleotide (Kugimiya *et al.*, 1986) and (Aoyama *et al.*, 1988) experimented lipase gene cloning, nucleotide sequencing and expression of gene in to protein from *P. fragi.*

Cloning and gene expression for thermolabile lipases in mesophilic bacteria such as *E. coli* did not produce a steady incorporation of gene coding of thermolabile lipase genes within their hosts bacteria (Feller *et al.*, 1990; Feller *et al.*, 1991a). Gene cloned from the *Moraxella sp.* strain and inserted in E. coli for three lipases was reported by (Feller *et al.*, 1991a) and after the expression gene was sequenced (Feller *et al.*, 1991b). Cloning, expression of gene in E. coli and sequencing analysis of lipase coding gene *P. immobilis* and considered the structural characters (Arpigny *et al.*, 1993). (Dieckelmann *et al.*, 1998) cloned thermolabile encoding gene lip A from *P. fluorescens* strain and (Rashid *et al.*, 2001) studied C9 *Pseudomonas sp.* strain and *P. lipolyticum* was reported by (Ryu *et al.*, 2006). Gene translating Phospholipase protein C from *P. fluorescens* cloned and expressed in *E. coli* and sequenced (Preuss *et al.*, 2001) and protein expression of lipase B gene in Pichia pastoris from *C. Antarctica* (Rotticci-Mulder *et al.*, 2001). Protein expression and molecular studies of thermolabile lipase gene cloned from *P. fragi* and the recombinant lipase gene after expression kept considerable hydrolysis at cold temperature (Alquati *et al.*, 2002). Separation of lipase enzyme gene straightforwardly from ecological DNA, PCR technique was designed utilizing primers based on lipase consent (Bell *et al.*, 2002). Cloning, gene expression and characterization of enzyme molecules was carried out in Psychrobacter sp. (Kulakovaa *et al.*, 2004).

LipP gene of 837 bp encoding for a thermolabile lipase of Moritella sp. a psychrophilic bacterium collected from Antarctic area was cloned and expressed in *E. coli* and nucleotide sequenced. 278 amino acid deposits with a molecular weight of 30 Kda was observed. Nucleotide sequence exhibited sequences of pentapeptide comprising the hydrolytic active serine motif (Gly-Trp-Ser-Leu-Gly) and a preserved dipeptide His- Gly in the N-terminal side of the hydrolyric lipase enzyme. Cloned gene encoding lipase protein was cloned into pET-28a bacterial vector to create a protein with recombinant lipase and expressed in *E. coli* BL21 (Yang *et al.*, 2008). Modern development in research of lipase is the innovative and advanced lipase

development through molecular methodologies for example on the metagenomic method directed evolution and discovering natural populations (Gupta *et al.*, 2004).

Molecular biotechnology signifies a very striking technique that could be utilized to enhance lipase expression generally in the situation of isoenzymes because of the very low yield after purification procedure. Lipolase was the earliest lipase resulted by genetic engineering was announced as Novozymes in 1988 in the market. Increasing number of lipases through recombinant technique is endorsed to the advancement in the modern molecular techniques (Kademi *et al.*, 2005). Isolated novel lipase from a metagenomic archive of bacteria from Sea sediment (Hardeman and Sjoling, 2007). Microorganisms DNA was isolated and replicated into a duplicate regulator fosmid bacterial vector engendering a collection of 47,000 duplicates through 24 to 39 kb of inserts. Nucleotide from lipase catalytic reaction from the subclones were sequenced and of 978 bp of gene translating a 35.4 kDa lipase Lip1 with 54% similarity to a *Pseudomomas putida* amino acid. Active site of catalytic triad conserved regions such as Glu242 Ser148 and His272. Lipase Lip1 was overexpressed, purified and exhibited to fatty acids of hydrolyse p-nitrophenyl esters with C14 sequence size. Novel lipo1 a psychrophilic producing esterase achieved straight from the DNA recombinant technique was extricated from the stimulated slurry (Roh and Villatte, 2008). Psychrotrophic Pseudomonas mandelii lips gene encrypting a unique alkaline organic solvent resistant lipase was extracted, cloned and expressed in *E. coli.* Expressed protein was purified by standard practices and molecular mass of 32 kDa was resulted from SDS-page analysis. 80 percent of catalytic activity was observed at basic conditions and at 40 to 50°C optimum temperature for hydrolytic activity was experimented. It preserved its hydrolytic reaction in organic solvents and also observed that the catalysis was augmented in the existence of DMSO and ethanol (Kim *et al.*, 2013)

5.11. Optimization production of thermolabile alkaline lipase

Goal of the research was to isolate and identified potential pyschrotrophic with innovative characteristics and to increase maximum production of thermolabile lipase. For to check the effect of culture media on enzyme production through the production time course for 1 to 3 days, the pH of the culture filtrate remained more than 8 with increased and decreased growth of the test organism. The basal medium trbutyrin broth (TB) was found most suitable for the production of the active alkaline lipase

with enzyme activity 3976 U/ml at pH 9, 150 rpm and 18 °C after 96 hours of incubation. As the medium contains very least amount of salts per liter, therefore, it was used as the basal culture medium for production in the following studies. The TB medium with reaction mixture (peptone, yeast extract, $CaCl_2 \cdot 2H_2O$, and NaCl, and trybutyrin) inoculated with 5% of 24 hours old seed culture at pH 9 and kept at diverse temperature varying from 4 to 30 °C at 150 rpm for 96 hours. The maximum cold active alkaline lipase was produced (3643 U/ml) with specific activity (569 U/mg) at 25 °C after 96 hrs of incubation ($P<0.0001$) (Joseph *et al.*, 2011 and Gokbulut and Arslanoglu, 2013). After 96 hours at lower temperature 4 and 15 °C marked decrease in enzyme production was observed and at elevated temperature like 25 and 30 °C showed no enhanced stimulatory effect on enzyme production. So, catalytic activity observed at temperature for thermolabile enzyme at 25 °C. Increasing or decreasing the temperature controlled to the decline in enzyme secretion (Rashid *et al.*, 2001) (Figure 4.56). At higher temperature 30 °C enzyme production decrease as 2931 U/ml of the total original enzyme production respectively. While at lower temperature 4 and 15 °C it decreases up to 0 and 65 U/mg respectively. The results of present study are in complete agreement with the previous finding of (Maharana and Sing, 2018) who experimented the optimization of varios factors for thermolabile alkaline lipase production and the activators used are 1% w/v of galactose and peptone, 0.1% w/v KCl and 2.5% v/v ghee at pH 11.5 and 15°C enhances the lipase production by 4.01-fold (3.35 U/ml) using *Cryptococcus sp.* The present study successfully produced cold-active lipases with novel properties like low temperature and high pH stability, which can be used in the degradation of lipid wastes in cold regions and also can be used for detergent formulation for cold temperature washing of delicate clothes.

Extracellular lipase production influence by pH factor was considered and observed that lipase was grow in wide assortment of pH 7 to 10 but the specific and catalytic activity observed was highest at pH 9 by 641 U/mg, 3976 U/ml, and it was a significant proliferation while associating with specific activity of pH 7 at 1954 U/ml (Joseph *et al.*, 2011). Thermolabile lipase production observed was declined after pH 11. Though, alkaline pH conditions were discovered appropriate for production of lipase production (Figure 4.57). Initial pH influence of the culture conditions on the enzyme production was studied in pH range 6.0 to 12.0. The initial pH was adjusted

with NaOH or HCl. The enzyme was produced over pH range 6 to 12 with highest value at pH 9.0. Thermolabile enzyme synthesized in acidic as well as in alkaline pH but maximally produced at alkaline pH. As the *pseudomonas sp.* PAK03 growth range is also in between 6 to 12 pH and 4 to 40 °C temperature, so the enzyme production at pH range and growth pattern at temperature range of the isolate indicate that it is alkalophilic and psychrotrophic organism which produced hydrolytic alkaline hydrolytic enzyme. The results of present study are in complete accordance with the previous finding of (Jadhav *et al.*, 2013) found maximum lipase activity was observed when the isolate was grown at 25 °C and is the second report in which the optimum temperature for lipase production from *Halomonas* sp. is observed below 30 °C. Previously, optimum temperature of 28°C was reported for *Halomonas* sp. (Dang *et al.*, 2009). pH 7 was found the best pH for lipase secretion. Similar pH optimum has been reported in *Halomonas lutea* (Wang *et al.*, 2008). Maximum production of 21 U /ml was reached after 16 h of cultivation. Thus, the best lipase secretion was experimental at 25°C, pH 7.0 after 16 h in the optimized medium (Jadhav *et al.*, 2013)

The effect of supplement of source of organic and inorganic nitrogen in a batch culture fermentation system is shown in (Figure 4.58). Peptone, yeast extract and ammonium nitrate at 1% concentration additional source of nitrogen augmented the thermolabile lipase secretion from *pseudomonas peli* up to 3226 U/ml, 3923U/ml and 2989 U/ml in every instance (Saxena *et al.* 2003, Jadhav *et al.*, 2013 and Joseph *et al.*, 2011). Conversely, the addition of 1% of Beef extract, Magnesium nitrate, citric acid and sodium citrate in the basal fermentation culture was observed as 1076 U/ml, 765 U/ml and 954U/ml showed do not have any significantly stimulating effect on the secretion of thermolabile lipase. Comparable to conclusions in the above experiment, limited descriptions are existing on peptone as the suitable source of the carbon (Saxena *et al.*, 2003) and stimulation in secretion of lipase when ammonium nitrate was provided as inorganic source of nitrogen to the microorganism in basal culture medium (Gopinath *et al.*, 2003). (Gupta *et al.*, 2004) observed significant growth factor for the secretion of thermolabile lipolytic activity was described as source of carbon, primary metabolites lipases are inducible enzymes. Existence of a triacylglycerol sources such as oil or another different inducer stimulated enzyme production inproduction medium (Sharma *et al.*, 2009). In the present research diverse sources of the carbon were experienced for their potential capability to

provision of production of lipase enzyme. Results from these observations designate that *pseudomonas peli* was potentially proficient to use the carbon sources for the stimulation of lipase enzyme molecules in supernatant, however highest growth was achieved using as carbon source tributyrin substrate (Figure 4.59). Tributyrin supplementation as a substrate of triacylglycerols in the fermentation media was observed to stimulate the thermolabile production of lipase and the catalysis was investigated as 3557 U/ml activity. Tributyrin might have a proper stimulator for the lipase enzyme production in fermentation reaction mixture. Carbon source such as glucose (Joseph *et al.*, 2011) and sucrose were observed hydrolytic activity of 3477 U/ml and 2654 U/ml however maltose, lactose, tween 20 and olive oil did not have much stimulatory effect on the cold active alkaline lipase catalysis. The present study is agreement with the study of Joseph *et al.*, (2011) who observed tributyrin induced lipase production by *Microbacterium luteolum*. But there is a report of (Rapp and Backhaus, 1992; Divya and Padma, 2015) getting *Rhodotorula sp.* able to produce cold active lipase using olive oil. An increase of 6.2% with olive oil as related to initial production medium comprising tributyrin. Maximum lipase secretion observed with olive oil at 1%, and peptone at 3% concentration. This medium was used for all other experiments (Jadhav *et al.*, 2013). (Mobarak-Qamsari *et al.*, 2011) also observed that the olive oil and peptone was the optimum carbon and nitrogen source with 0.46 and 0.17 U/ml of lipase activity respectively in *Pseudomnas aeruginosa.* Similarly, Kumar and Valsa, (2007) have also experimented the maximum lipase activity from *Bacillus coagulans* in the addition of 1% olive oil and 3% peptone in the basal medium for lipase production. Other reports on peptone as the best nitrogen source for lipase production were published by Gao *et al.*, (2004) for *Serratia marcescens* and Kumar *et al.*, (2012) for *Bacillus* sp. MPTK 912. However, the best carbon sources were found to be Tween 80 and glucose respectively. (Singh and Ramana, 1998) studied highest enzyme activity from the psychrotrophic Antarctic bacteria and the activity was reported in the range of 1.7-1.9 U/ ml using carbon and nitrogen source.

Inoculum level was significant influence for the production of thermolabile alkaline protease. Effect of various inoculum levels (1, 5, 7, 10, 15 and 20%) were used to study on protease production. The enzyme production was found in all inoculum sizes with a little difference however the maximum production was

obtained at 7% inoculum with 3965 U/ml protein activity. Whereas in other inoculum sizes the results indicated 897 U/ml, 2654 U/ml, 3178 U/ml, 2154 U/ml, and 1172 U/ml was observed for 1%, 5%, 10%, 15% and 20% inoculum size. The production of enzyme decreased due to gradually deterioration of nutrients in the production fermentation mixture due to the increase of inoculum level. In the present study, inoculum size 7% is suitable and it proliferated actively in young culture to increase the thick mass due to presence of rich nutrients and after exhaustion of nutrients the culture immediately converted to spore forms because isolate start to produce enzyme with the onset of sporulation. Consequently, high spore sizes could not essentially present superior protease harvest or cell mass. Though, higher cell mass might result in the deficiency of oxygen and nutrients reduction in the media for fermentation. Results in the present experiment indicates the inoculum of 30 hours old is highly supporting the protease production with yield as 3932 U/ml protein activity. However, the inoculum of 24, 30 and 36 hours also showed good enzyme production which was observed as 2742, 2932 and 2865 U/ml, but it takes long time to harvest the product, and inoculum of ages 6, 12, 18, 24, 36, 42 and 48 hours showed the specific activity 967, 1442, 2132, 2742, 2865, 2007 and 1632 U/ml, but not to the level of 30 hours old inoculum. Small and long incubation duration affect the growth of microorganisms by influencing the metabolic activities. Physiological condition of the inoculum is crucial to the length of the lag phase. Inoculum culture use is still in the exponential phase and lag phase may not occurred and growth may begin immediately. So, for the later secretion of both prime and secondary metabolites proper measured volume transfer of inoculum is essential. Amongst the mineral ions, highest catalytic activity 3766 U/ml was observed for $CaCl_2$ showed stimulatory effect on lipase production (Joseph *et al.*, 2013) while NaCl and $MgCl_2$ have less significant effect on lipase production and was observed as 1176 and 1212 U/ml (Joseph *et al.*, 2013) as shown in (Figure 4.60). However, NaCl, $MgCl_2$ and KCl showed truncated effect on the production and the results observed was 423, 467 and 511U/mg. $FeCl_3$ and $ZnSO_4$ had showed no stimulatory effect on the lipase production and was observed as 763 and 669 U/ml protein activity. In this experiment the lipase excretion in the presence of calcium ion was more stable and this indicates a stabilizing nature of calcium on lipase enzyme.

5.12. Purification and characterization of cold active alkaline lipase

Proteins from the supernatant were precipitated with 80% ammonium sulfate overnight at 4 °C with constant stirring. The precipitate formed was recovered by centrifuging at 25,000 g and 4 °C for 30 min using a Beckman ultracentrifuge and resuspended in a minimum amount of 20 mM Tris-HCl buffer, pH 8.0. After almost fermentation of production for 4 days the crude thermolabile enzyme was subjected to centrifugation for clear supernatant at 4°C and stored in -80°C for further analysis. (NH4)2SO4 was added to supernatant at 30% and was further treated with up to 90% concentration of ammonium salt. Precipitated thermolabile protein after centrifugation at 4°Cwas resuspend in buffer of tris-HCl and was taken in dialyzing bag and placed in buffer of phosphate salts at low temperature of 4°C for almost 1 day against three changes of the phosphate buffer. The dissolved proteins were applied to an ion exchange chromatography (IEX: SOURCEQ 10/10) column equilibrated and the proteins on the basis of charges was eluted by using buffer of tris-HCl with 8 pH. Fractions collected was analyzed for proteolytic activity and those with the high catalytic activity was selected and subject for further purification on the bases of molecules sizes gel chromatography to get the homogenous mixture of thermolabile protein molecules (Bae *et al.*, 2014).

Pseudomonas peli sp. a psychrotolerant microorganism when cultivated in the fermentation medium it was witnessed that in the bacterial stationary growth curve the highest thermolabile protease molecules was experimental subsequently in 96 hours. The thermolabile alkaline protease molecules was purified with distinctive purification practice is concise in Table 4.7. Precipitated $(NH_4)_2SO_4$ partially purified molecules was obtained were subjected to dialysis for further removal of excess of salts and impurities. The dialyzed extract enzyme was first subjected onto an Ion exchange chromatography technique (IEX SOURCEQ 10/10) and the two peaks of thermolabile purified proteins was eluted showed in (Figure 4.61). Hydrolytic activity of both the points was assayed and the fraction with high catalytic activity was subjected to Gel Filtration Chromatography (GFC: Superdex 200 PG 16/60) (Figure 4.62) and the purified thermolabile fractions with high enzymatic catalysis from both the peaks was eluted and subjected on SDS-PAGE analysis (Sharma *et al.*, 2017) (Figure 4.63). Under silver staining protease protein showed purity with an expected 32 KDa molecular mass (Arpigny and Jaeger, 1999 and Lin *et al.*, 1996 and Sharma

et al., 2017), respectively. Purified monomer proteins were observed from the protein analysis. Specific activity of 1381.22 U/mg with purification fold of 68.42 and yield recovery of 20.52 % was observed from the overall purification procedure. Lipases with thermolability properties characterized high hydrolytic activity but at low temperature declined thermostability. In most of the cases, advantageous for some industrial applications such as in low temperature production of unsteady compounds that need inactivation of enzymes by modest proliferation of temperature (Joseph *et al.*, 2008, Feller *et al.*, 1996)

Thermolabile alkaline hydrolytic activity and lipase stability was investigated toward distinctive temperature such as 4, 10, 20, 30, 40, 50 and 60 °C. The maximum catalysis of proteins in the reaction mixture of enzyme was observed for 30 °C and by increasing the temperature lipase activity was gradually decrease 57, 32 and 11% of relative hydrolytic activity was observed for the thermolabile lipase enzyme (Figure 4.64). The activity of cold active lipase was inhibited at 4 °C which was observed as 6% hydrolytic activity against casein. To investigate the thermostability properties of the purified hydrolytic thermolabile enzyme, purified molecules of enzyme was allowable to stand at 8 pH for 240 minutes with distinctive temperatures such as 25, 35, 45, 55, 65, 75 °C and also for 240 minutes at low temperature 4 °C. Subsequently the catalytic activity after pre-incubation such as residual catalytic activity toward substrate was analyzed (Figure 4.65). Catalytic activity of purified molecules of lipase was observed active at temperatures beneath at 35 °C with approximately no decline in hydrolytic activity, and the activity was observed retained nearly 50% hydrolysis against 45 °C and 55 °C. thermolabile molecules denatured and showed with no activity at 65 °C and 75 °C after incubation at 210 and 150 minutes. The results of the present finding are in accordance to the early observations of (Gokbulut and Arslanoglu, 2013) who experimented that thermolabile lipase showed activity at temperature range from 15 to 65 °C and highest catalysis observed at 45 °C, at pH 8.0.

Lipase observed retained 100 and 70% of stability following pre-incubation of 45 min and 1 hour and 45 min at temperature of 45 °C and tris-Hcl buffer of pH 8. Present are in plausible with previous finding of (Dey *et al.*, 2014) they observed catalytic activity was maximum at 22 °C. Lipase molecules was highly stable against 20 °C, residual hydrolysis was diminished slightly toward 30°C temperature after

preincubation of enzyme. Half of the residual activity was retained after approx. 2 hours and 1 hour and 30 min pre-incubation against 50 °C and 60 °C correspondingly. completely inhibition was resulted at 70 °C after incubation for 120 mins. After purification using chromatographic techniques and maximum lipolytic activity for purified lipase was at 15 °C and the activity was highly dependent on pH (Bae *et al.*, 2014). Present research was in accordance of previous observation of (Joseph and Ramteke, 2013) experimented highest catalytic activity against temperature for thermolabile purified lipase observed at 15 °C and thermolabile lipase was observed stable in the temperature stretched from 20 to 30 °C. Most of the cold-active lipases was observed to have their highest catalytic activity against temperatures amongst 25 and 45 °C and around 30% of the highest catalytic hydrolysis at 5 to 20 °C (Gokbulut and Arslanoglu, 2013, Kavitha and Shanthi, 2013). Thermolabile lipase from *Pseudoalteromonas sp and Pseudomonas sp.* was observed stated stable up to 40 °C, and *Bacillus sphaericus* purified lipase was stable up to 30°C (Joseph and Ramteke, 2013, Wang *et al.*, 2012)

The catalysis of peptide bonds was assessed in the wide-ranging pH 6 to 12, and the highest catalytic activity was observed at pH 9 Tris-HCl buffer (Figure 4.66). Residual activity was retained against 56%, 30% and 8% with Tris- HCl buffer of pH 10, 11 and 12 correspondingly. The catalysis in the acidic assortment, substantial decrease in catalytic activity of purified molecules was experimented. For illustration, pH 6 the residual activity of purified lipase having only 16 % of its highest hydrolytic activity. In imperative to research the pH stability, the distinctive pH was observed to allowed alkaline lipase to stable for 1 hour at wide ranging pH 6–12 at 30 °C, after preincubation of enzyme molecules retained residual activity in the reaction mixture was analyzed (Figure 4.67). Purified thermolabile lipase exhibited highest stability at pH 9 Tris-HCl buffer retained 82 % residual activity. 50% residual catalytic activity was observed after preincubation against 7 pH and ongoing to declined activity under pH 6. Furthermore, protein hurriedly vanished some of its hydrolysis lasting catalysis of 59 % and 26 % at Tris-Hcl buffer of pH 10 and 11. The outcomes of present study are in complete agreement with the previous finding of (Joseph and Ramteke, 2013) who experimented pH for lipase activity was optimum at 8.0, respectively. Lipase was found to have observed stable in the pH wide range from 6–9. Thermolabile llipolytic activity was observed in wide pH range from 6-8 for maximum hydrolytic activity.

Optimal pHs observed was different such as at 15 °C pH was 8.0 and at 30 °CpH was 7.5. It was examined that pH 7.5, the hydrolysis was high up to 25 °C and diminished over 30 °C, while the hydrolysis with pH 8.0 exhibited greater activities at 15 °C compared to pH 7.5 decreased more considerably subsequent resulting into reduced hydrolysis as compared to pH 7 over 20 °C. 80 % of the original activity for thermolabile lipase was stable with the pH ranges between 5.0 and 8.0 at 15 and 30 °C and the enzyme was denatured significantly below 50% over pH 9 after 24hour incubation 15 and 30 °C. (Bae *et al.*, 2014). Two optimum pH for hydrolytic activity was resulted at 3.5 and alkaline pH 8.5 (Dey *et al.*, 2014). Observations of present study are in complete agreement to previous finding of (Gokbulut and Arslanoglu, 2013) who observed the optimum hydrolysis at potassium phosphate buffer of pH 8. 65 and 33 % of its highest activity at 9 pH and 10 pH was observed.

In the acidic conditions, substantial diminution in hydrolysis was resulted and citric acid buffer at pH 6 the thermolabile lipase retained 6 % catalytic activity was resulted. To investigate the diverse metal ions, stimulate or disrupt the catalysis of purified enzyme, the influence of $CaCl_2$, $MgCl_2$, KCl, NaCl, $MnSO_4$, $ZnSO_4$, $FeCl_3$, $MnCl_2$ and EDTA as chelator against 1 mM concentration on hydrolytic activity was researched with results summarized in (Figure 4.68). From the Results, $CaCl_2$ stimulated the enzyme activity by 2.0 %. Though, decent stimulation effect on catalytic activity was experimented in the existence of of $MgCl_2$, KCl, NaCl, $MnSO_4$, $ZnSO_4$, $MnCl_2$ and having more than 72 % residual activity after pre-incubation to the above-mentioned ions. $MnSO_4$, and $FeCl_3$ slightly inhibited the activity by 37 % and 31%. EDTA was observed negative effect on hydrolysis, enzyme deteriorated around 58% of its highest catalytic activity (58%) toward substrate, recommending that the thermolabile alkaline enzyme was a metalloenzyme. From the present research thermolabile organic solvent tolerant lipase from *Pseudomonas peli PAK03* is extremely active in the existence of diverse metal ions. Similarly, the hydrolysis of substrate by lipase in the existence of $NiCl_2$ observed differentiating quality associated to lipase from other diverse species of *Pseudomonas*, and the hydrolytic catalysis was inhibited by 24% and activity was retained by 76%, since they were usually galvanized only by $CaCl_2$, but not by the $NiCL_2$ investigated by the previous finding of (Joseph *et al.*, 2008).

Lipase hydrolysis observed was at 1 mM of metal ion. Cu and Co observed marginally augmented catalytic activity with 31 and 38%. In distinction, slightly inhibition was observed by 14, 18, 19, 24 and 31% hydrolytic activity for Zn, K, Na, Al, and Fe. Lipase was special metal-stimulated because not requisite ions for hydrolytic activity, certain salts heightened hydrolysis (Bae *et al.*, 2014). $MgCl_2$ with different concentrations was observed radical influence on catalysis. Hydrolytic activity was somewhat declined and having 10 to 20 % of the residual catalysis against $ZnSO_4$ and $FeSO_4$. distinctive ionic strengths Cu exhibited resilient negative influence on hydrolysis. Chelating elements, EDTA disturbed catalytic activity and having 50 % of catalytic activity was retained by preincubation with EDTA (Joseph and Ramteke, 2013).

Stability toward solvents is a desired characteristic such as transesterification or esterification (Gupta *et al.*, 2004). To investigate organic solvents, influence on the hydrolysis of enzyme, acetonitrile, n-hexane, DMSO, methanol, acetone and 2-propanol and ethanol against concentration of 30% were investigated. DMSO have stimulation effect on catalytic activity and have 83 % retained preincubation with DMSO (Figure 4.69). 34%, 69%, and 50% activity retained in the company of different solvents e.g. ethanol, acetone, and methanol. Acetonitrile, n-hexane and 2-propanol was observed inhibited the catalytic activity with 26, 17 and 23% residual activity. From the present observation there are some conceivable justifications for the stimulatory effects of different solvents on hydrolysis of lipase that solvent might augment catalysis of protein by lipase enzyme deprived of instigating denaturation by transforming interface of water and oil. In second place, solvents may construct transformations structure of 3-dimensional configurations or might preclude disaggregation of lipase amino acid (Ahmed *et al.*, 2010). In contrast different solvents displaying undesirable influence on hydrolysis might alter functional catalytic site of enzyme, consequently blocking substrate compounds from efficiently retrieving catalytic domain of enzyme for hydrolysis (Peng *et al.*, 2010).

Lipases correlated with catalytic enzymes from other diverse psychrotrophic strains such as *Pseudomonas* sp. B11-1 solvent such as DMSO was a discriminating feature as preference of the enzyme (Joseph *et al.*, 2008). Organic solvent resistant lipase enzyme was observed to have the catalytic activity of 83% in the presence of DMSO. The lipase from *Pseudomonas peli* was examined to have conceivable

utilization of biochemical synthesis reactions and chiral resolution effects engage usage of DMSO solvents. In organic solvents stability and hydrolytic activity are significant features of lipases in organic synthesis. (Joseph and Ramteke, 2013) studied that lipase slightly inhibition in hydrolysis with contact to different solvents. Most of the lipases secreted from *Bacillus species* was observed exhibited stable catalytic activity against different solvents (Schmidt-Dannert *et al.*, 1994; Nawani *et al.*, 1998 and Hun *et al.*, 2003). Further thermolabile protein from *Acinetobacter sp.* observed was more than 90 % hydrolysis in water-misciblility of diverse kinds of solvents (Snellman *et al.*, 2002). Triacylglycerol for the hydrolysis of lipase are not soluble in solution, and organic solvents surges the substrate solubility. Activity was inhibited in existence of acetonitrile that might because of solvents destructiveness (Sikkema *et al.*, 1994). Nevertheless, activity was resistant to solvents could be studied in different industrial applications, fatty acid esters making, oligosaccharide derivatives, and peptides synthesis. Influence of diverse organic solvents on hydrolytic activity at strength of 30%, stimulation of catalytic activity of lipase was observed 191 and 113% pre-incubation against DMSO and dimethylformamide. Residual catalysis with values of 25%, 75%, and 59% activity inhibited in the existence of ethanol, acetone, and methanol solvents. Acetonitrile and 2-propanol sturdily hindered catalysis and n-hexane was observed absolutely constrained hydrolysis (Gokbulut and Arslanoglu, 2013)

5.13. Kinetic constants and Catalytic Efficiencies

Km assessment was observed with 0.68 mg/ml for different concentration of substrate such as p-nitrophenyl palmitate correspondingly. Maximum velocity Vmax of enzyme was explained as the reciprocal intercept of Lineweaver-Burk graph. Vmax 294 U/ml/min was observed for the triacylglycerol substrate. inferior deceptive Km designates that the purified thermolabile lipase has higher affinity for triacylglycerol substrate of p- NPP. Turnover number, k_{cat} and kinetic efficiency were calculated as 47.07 min^{-1} and 69.18 $min^{-1}mg^{-1}ml^{-1}$, respectively (Figure 4.70). The values of thermodynamic parameters for alkaline lipase was calculated at changed temperatures from 30 to 80 °C. $E_{a(P)}$ calculated for lipase enzyme form *pseudomonas peli* was 40.17 KJ/mol (Figure 4.71). The lower $E_{a(P)}$ suggested that lesser energy was required by alkaline hydrolytic protease enzyme to reach the transition state of p nitrophenol formation. kinetic investigation of the purified thermolabile lipase was

observed by (Bae *et al.*, 2014), catalytic activity was observed using numerous p-NPB substrate observed was 0.5 to 1.2 mM concentration. K_m and V_{max} values of the purified lipase resulted was 1.7 mM and 8 μmol/min/mg. Associating hydrolytic enzyme extracted from *Yarrowia lipolytica* was stated 0.022 mM of K_m and 0.1 μmol/min/mg V_{max}. K_m high value designated that enzyme substrate affinity extracted from *P. lynferdii* was comparatively weak, however it includes in K_m range of most industrial lipases (Florczak *et al.*, 2013).

5.14. Alkaline Lipase from Psychrotrophic *Pseudomonas peli* PAK03 for Detergent Additive Capability

The alkaline lipase enzyme from the experiment showed decent catalytic activity in existence of Tween 80 and SDS and the hydrolysis retained was 86 and 90% activity, the same findings were also resulted for surfactant tolerant lipases from *Rhizopus* sp. and *Aspergillus* sp. (Derewenda *et al.*, 1994 and Saisubramanian *et al.*, 2006). Effective use against in tough commercial detergent conditions, alkaline nature lipolytic activity required be effective and stable with different constituents of detergent formulation such as surfactants (Kamini *et al.*, 2000). Amongst innumerable detergents was tested, better enhanced hydrolytic activity of lipase was observed from the present experiment with Gain with retained activity was 87% and alkaline hydrolytic lipase revealed 86% residual activity after 1 hour of incubation with SDS (Figure 4.72). Though, catalytic activity of enzyme from *Ralstonia pickettii* (Hemachander and Puvanakrishnan, 2000) and SDS has an inhibitery effect on hydrolytic activity of lipase from the *Aspergillus carneus* was observed by the finding of (Saxena *et al.*, 2003) although hydrolytic activity was amplified just in case of *H. lanuginosa* (Omar *et al.*, 1987).

The hydrolysis of ester bonds of oil substrate in the existance of All, Purex, Xtra, Sun, Arm and Hammer was investigated and approximately half of its activity was retained was 58, 57, 53, 59, 62%. Stability of alkaline lipase with oxidizing agents was experimented in occurrence of sodium perborate, sodium peroxide, sodium hypochlorite and hydrogen peroxide. Results exhibited that thermolabile lipase was greatly active regarding oxidizing elements at 1.5% strength for 1h at 25 °C and 83% of residual activity was preserved at 2.0% H_2O_2 concentration, by rising in concentration of sodium perborate, sodium hypochlorite and sodium peroxide from 1.0 to 2.0% the hydrolytic activity was observed to inhibited up to 43, 48 and 43%

though results showed that the activity was progressively decreased (Figure 4.73). Astonishingly, the results observed that the hydrolytic activity of lipase molecules exhibited suitable stability regarding concrete oxidizing agents specifically hypochlorite (70.5% hydrolysis at concentration of 1.0%). Relative hydrolytic catalysis demonstrated 43% relative activity after 60 minutes incubation from the observations presented by Rathi *et al.*, (2001). As it was concluded from the observations, that in presence of oxidizing agents' higher stability for the alkaline lipase make this hydrolytic alkaline lipase enzyme integrated as a commercial detergent additive improved activity. Higher oil exclusion 58% was investigated with Gain and selected for further analysis. Lipase was observed more efficient against nonionics than with anionics surfactants and observed greater catalytic activity inhibition toward anionic surfactants (Sajna *et al.*, 2013, Flipsen *et al.*, 1998).

Detergent and Lipase solution mixture catalysis of oily pigments was observed better than with only Buffer with Detergent, indicated advantage of lipase molecules presence in the detergent as an additive. The usage of psychrotrophic *pseudomonas peli* excreting cold active alkaline lipase with 0.6% Gain and tide recovers the oil removal from stained tested cotton cloth by 50% and 48% (Buffer + Detergent) to 58% and 56% (Buffer + Detergent + Lipase) utilizing 100 U of purified lipase at 25 °C after incubation at 30 minutes projecting the catalytic ability of lipase in detergent additive (Figure 4.75). Distinctive lipase concentration and oil removal association was demonstrated in (Figure 4.76). It was observed from the results that in both cases, with lipase concentration oil elimination rises till accomplishing the stability at a strength of more than 60 units. Attainment of equilibrium after detergent stable Unit/ml concentration of lipase rely on the initial catalytic frequency of hydrolysis of ester bonds of triglycerides-based lipase on the boundary region amongst unsolvable triglyceride ester bonds and lipase aqueous mixture. Surface area of a particular quantity of olive oil was examined to be persistent afterward an evident concentration of hydrolytic lipase with which saturation was observed for interface. As shown in (Figure 4.77), an improvement with the addition of the lipase was observed 26% to 63% without the detergent and with existence of the detergent. results observed for the lipase was Enhanced in combination with marketable detergent which was also in harmony with the previous finding of (Sajna *et al.*, 2013) who studied the identical case of *Pseudozyma* sp. and *Pseudomonas aeruginosa* lipases (Grbavcic *et al.*, 2011).

The washing time consequence on oil removal was represented in Figure 4.78. It was detected from the results that on washing prolonged time barely Buffer and Lipase solution functioned properly for removal of oil from muddied fabric. The solution of Buffer with Detergent used for oil removal from the results was observed to be persistent subsequently after 30 min. From the outcomes it was observed that exploitation of the solution comprising Buffer with Detergent along with Lipase in encouraged oil removal of 71% of the outcomes using 40 min washing cycle. It was also observed that the noteworthy contribution of lipase towards hydrolysis of ester bonds of triglycerides in washing analysis with prolonged incubation period with laundry detergent. Thermolabile lipase from the *Pseudozyma* sp. revealed that washing investigation of biosurfactant from pigment removal experimented was more with increased in the washing time (Sajna *et al.*, 2013). The same findings were also observed by the Grbavcic *et al.*, (2011) who observed by heightening with increasing clean period in case of *Pseudomonas aeruginosa* lipase enzyme biodetergent analysis.

5.15. CONCLUSION

- High catalytic activity of enzymes at cold and temperate temperatures proposals potential economic benefits. Capability of bacteria to growing over wide-ranging temperature makes them remarkable for biotechnological and industrial purposes.
- The existing microbial consideration concluded that the alkaline cold tolerant protease and lipase isolated from *Stenotrophomonas sp.* PAK01 MG662181 and *Pseudomonas peli* PAK03 MG687270 are thermolabile enzymes and was observed stable at high alkaline conditions. These characteristics designate the potentials of these protease and lipase as an additive in detergent formulation for cold laundry to improve the cleansing of the proteinaceous and greasy stains, respectively.
- We had purified an alkaline thermolabile protease and lipase from the culture supernatant of a psychrotrophic *Stenotrophomonas sp* PAK01 *and Pseudomonas peli* PAK03. The highest catalytic efficiency of protease enzyme was achieved at cold environment in comparison with other commercial proteases and half of its activity was inhibited at 60 °C after incubation of the reaction mixture at 90 min and the thermolabile enzymes was supposed to have lability toward high temperature. Its concluded from the temperature results that enzymes from PAK01 exhibited thermolabile characteristics. Maximum proteolytic activity for protease enzyme was observed at 9 pH and showed stability against pH distending from 6 to 10.
- $CaCl_2$ at concentration of 15 mM stimulated the catalytic activity significantly up to 76% and was completely inhibited by $FeSO_4$. Protease enzyme was stable in the presence of nonionic surfactant and was observed stimulated by Tween-80 at 6% concentration after preincubation at 36 hours. Residual activity for protease enzyme was observed to be 87% and 79% after preincubation with 0.5% SDS and 1% H_2O_2. Moderate stability was observed against inhibitors whereas PMSF was observed completely inhibited the activity.
- Reasonable stability was observed toward organic solvents at 5% and 10% concentration whereas 28% and 15% residual activity was observed with ethyl acetate. Highest catalytic efficiency K_{cat}/K_m was observed for 25°C as

compared to 4 and 15°C which are considered as cold enough to hindered catalytic efficiency of purified protease enzyme.

- Maximum lipolytic activity was observed at 9 pH and showed stability against wide-ranging pH distending from 6 to 10. Highest activity was observed at 30°C and thermostability properties was shown by lipase enzyme from 4 to 55°C
- Reasonable stability was observed against different organic solvents at concentration of 30% and 42% of the residual activity was observed in buffer system provided by EDTA where DMSO inhibited only 17% activity and 17% and 23% residual activity was observed toward hexane and propanol
- The protease and lipase were active as well as stable at a low temperature below 30°C, which might be useful properties for a potential application in the fields of the high salt wastewater treatment, bioremediation in fat and proteinaceous contaminated cold environment and would also be important to test alkaline protease and lipase in complete enzyme-detergent systems.
- The use of thermolabile alkaline protease and lipase from *Stenotrophomonas sp* and *pseudomonas peli* as an additive in industrial applications such as the detergent industry is desirable.
- The outcomes of this analysis exhibited that thermolabile alkaline protease from *Stenotrophomonas sp.* progresses the blood and egg yolk pigment removal from dirtied cotton fiber as an additive to laundry detergent formulation, specifically, 1% 1% of 15 mg/ml purex under optimized conditions of 100 U/ml of protease in 50 mM Tris-HCl, pH 9 as laundry temperature and time seems to be equivalent to 25 °C and 20 and 15 minutes for complete exclusion of blood and yolk pigments
- Thermolabile alkaline lipase from *Pseudomonas peli* progresses the triglycerides exclusion from stained cotton cloth by 24% as an ingredient to laundry additive detergent, with reaction mixture (0.6% Gain under optimized situations of 60 U of lipase in Tris-HCl buffer (pH 8.5) as washing temperature and washing time seems to be equal to 25 °C and 40 mins) respectively.
- Thermolabile enzymes from *Stenotrophomonas sp. strain* MG662181 and *Pseudomonas peli* MG687270 was an ideal contender for practice in laundry

detergent formulations, subsequently its stability in hypochlorite and detergents and washing performance, seems to be a potential enzyme for washing additive in detergent reaction formulations.

- Additive consequence was detected more toward nonionic as compared against anionic surfactant creating it a novel alkaline protease and lipase for further utilization toward commercial purposes as a potential ingredient in detergent additive formulations.

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