ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Production and characterization of alkaline chitinase and protease from an alkaliphilic actinomycete

By
Firew Kassa

A Thesis submitted to the School of graduate studies of Addis Ababa University in partial fulfillment of the requirements of the Degree of Master of Science in Biology (Applied Microbiology)

July 2007

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Declaration

I, the undersigned, declare that this thesis is my original work. It never been submitted in any institution and that all sources of materials have been acknowledged

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Date_____

This thesis has been submitted for examination with our approval as
Advisor Amare Gessesse, PhD
Signature _____
Date_____

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Abbreviations

ATCHIT4	*Arabidopsis thaliana* chitinase 4
CCA	Colloidal Chitin Agar
ChiA	Chitinase A
ChiB	Chitinase B
EARI	Ethiopian Agricultural Research Institute
ES	excretory–secretory
GlcNac	acetyl glucosamine
PCD	programmed cell death
PMSF	Phenylmethylsulfonyl fluoride
rpm	Revolutions per min
SmF	Submerged fermentation
SSF	Solid state fermentation
TCA	trichloroaceticacid
U/ml	Units per millilitre
UDA	*Urtica dioecia*
YE	Yeast Extract

Abstract

Chitinase and protease are enzymes produced by a number of organisms and have wide industrial applications. This study was aimed at production and characterization of alkaline chitinase and protease from alkaliphilic microorganisms and evaluation of their possible applications. Alkaliphilic actinomycetes isolated from alkaline soda lakes were screened for the production of chitinase and protease. Out of 122 isolates tested 54 % produced appreciable chitinase activity indicating the presence of diverse chitinolytic microorganisms in these habitats. Based on the level of chitinase and protease production, isolate designated as A1 was selected for further study. When grown in colloidal chitin, the isolate A1 showed peak production of chitinase after 72 h while it was 120 h for protease production. The maximum amount of chitinase obtained at the peak production time was 22.6 U/ml for and 110.4 U/ml for protease. Both of the enzymes showed wide temperature and pH optima where the peak pH for chitinase activity was 8.5 while it was 10.5 for protease. The optimum temperatures for chitinase and protease activity were 45 ℃ and 55 ℃ respectively. Chitinase was stable over the wide range of pH and maintained more than 80% of its original activity after incubation in different buffers of pH 6.5-11 for one hour at room temperature. Protease A1 was stable in a wide range of pH and temperature. It has kept over 75% of its original activity in the pH range of 9-11 after one hour at 50℃. Protease does not require $CaCl_2$ for its stability. It showed good stability in the presence and absence of $CaCl_2$ where its stability was maintained after 60 min of incubation at 45℃. Both protease and chitinase can be used in wide applications of biotechnology and various industries.

Key words: Alkaline, Alkaliphilic, Protease, Chitinase, enzyme

1. Introduction

1.1. Chitin

Chitin is a polymer of N-acetylglucosamine which is one of the most abundant biopolymers in nature. It is suggested to be the second most abundant polysaccharide only next to cellulose (Tsujibo *et al.*, 2003; Agullo *et al.*, 2003; Mejia-Saules *et al.*, 2006). Chitin is a linear, fibrous, unbranched, and aggregated polymer of β-1,4-linked (Fig. 1) amino sugars (Nawani *et al.*, 2002). It exists in the exoskeleton of insects, molluscs, coelenterates, nematodes, and protozoans. Moreover, it is the main structural component of most fungi and plays a vital role, such as cell wall development, in these organisms (Horn *et al.*, 2006). Chitin is also one of the principal structural components of insect exoskeleton and crustaceans like shrimp and crab (Matsumiya *et al.*, 2002). Some reports estimated that the annual production of chitin accounts for more than one hundred trillion metric tons in the aquatic biosphere alone (Tsujibo *et al.*, 1997; Bhushan, 2000; Horn *et al.*, 2006; Mejia-Saules *et al.*, 2006). Despite its strong resistance against chemicals, insolubility, and abundant production, chitin does not accumulate in most ecosystems suggesting that chitin is degraded in natural processes (Tsujibo *et al.*, 1997; Kawada *et al.*, 2007).

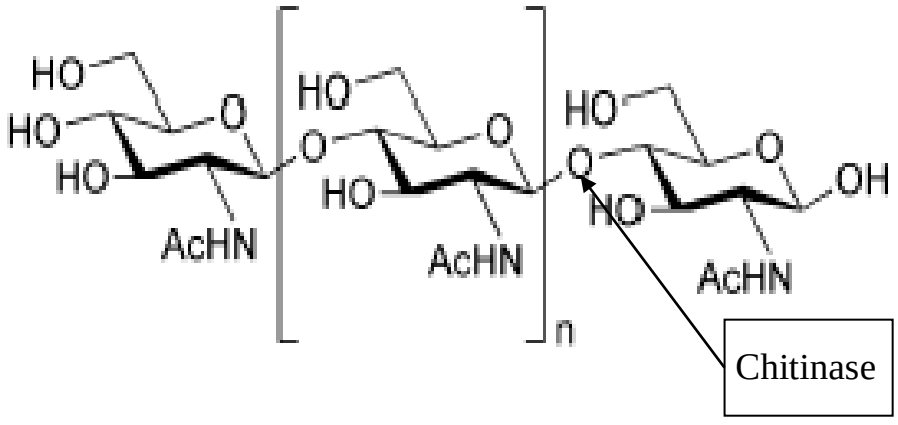

Figure 1. The structure of chitin catalysis site of chitinases (Andersen *et al.*, 2005)

1.2. Chitinases

Chitinases are enzymes that hydrolyse chitin and its oligomers into the monomers of amino sugars, N-acetylglucosamines. These enzymes cleave the bond between the C1 and C4 of the two consecutive N-acetylglucosamines of the chitin molecule. Complete hydrolysis of chitin is, therefore, performed by an enzymatic system consisting of groups of chitinases such as endo chitinases, exochitinases, and chitobiases (Nawani *et al.*, 2002; Driss *et al.*, 2005).

Endo-chitinases are enzymes that split the substrate randomly at internal points over the entire length of the chitin micro-fibril. They cleave β-1,4-glycosidic bonds within the oligosaccharide chain leading to the products of varying length. The final products formed by endo-chitinases are soluble and low-molecular mass multimers of N-acetylglucosamine such as chitotetraose and chitotriose with the dimer di-acetylchitobiose being predominant (Andersen *et al.*, 2005).

Exo-chitinases are those enzymes releasing chitobiose from the non-reducing end of the chitin. These groups catalyze the progressive release of di-acetylchitobiose in a stepwise fashion and no monosaccharides or oligosaccharides are formed as their reaction products. They cleave the chitin micro fibril only at the non-reducing end and this group of enzymes is referred to as chitobiosidase. Chitobiase, also called the β-1,4-N-acetylglucosaminidase splits di-acetylchitobiose as well as chitotriose and chitotetraose into N-acetylglucosamine monomers in an exo type of action (Sahai and Manocha, 1993).

1.2.1. Distribution and classification

Chitinases are widely distributed in nature where they are produced by a broad range of organisms including bacteria, fungi, insects, plants, and animals. These enzymes differ from each other in amino acid sequence, structural arrangement, and mechanism of action (Andersen *et al.*, 2005). Based on the amino acid sequence similarities of their catalytic domains, chitinases are divided in to two families such as family 18 and family 19 of the glycosyl hydrolases classification system.

Family 18 chitinases contain enzymes from mammals, insects, plants, nematodes,

fungi, and bacteria. Therefore, the enzymes of this family are evolutionarily diverse. The family 19 chitinases, on the other hand, contain the enzymes that are only present in plants. Recently, however, a family 19 chitinases have been discovered in *Streptomyces griseus* HUT6037 (Itoh *et al.*, 2003).

The plant-type family 18 chitinases are endo-chitinases which randomly cleave the β-1,4-glycosidic bonds within the oligosaccharide chain and lead to the formation of varying lengths of oligosaccharides. The bacterial-type chitinases are usually grouped under the family 18 and they are exo-chitinases. Therefore following their catalytic action, the cleavage of the specific β-1,4-glycosidic bonds along the oligosaccharide chain progressively releases acetylchitobiose ($GlcNAc_2$) or acetylchitotriose ($GlcNAc_3$) from the non-reducing end of chitin (El-Tarabily *et al.*, 2000; Andersen *et al.*, 2005).

Familial differences in chitinase catalytic domain sequences also reflect the mechanisms of chitin hydrolysis that result in either retention or inversion of the anomeric configuration of the product (Colussi, 2005). When the β-1,4-glucosidic linkages are hydrolyzed by the catalytic action of the family 18 chitinases, the oligosaccharide fragments are produced by retaining mechanism, while it is by inverting mechanism in the case of family 19 chitinases. Moreover, the family 18 chitinases are known to be sensitive to allosamidin, the common inhibitor of chitinase activity, while family 19 chitinases are not sensitive for this inhibitor (Ueda *et al.*, 2003; Colussi, 2005).

1.2.2. Sources and characters of Chitinases

1.2.2.1. Bacterial chitinase

Most chitin degrading bacteria are assumed to produce varying amount of chitinase and hydrolyze chitin into its monomeric units in order to grow readily on chitin substrate. However, the industrial enzyme production is based on quantity and quality of products in order to get maximum benefits. In this regard, *Serratia marcescens* is one of the most intensively studied chitinolytic bacteria (Horn *et al.*, 2006). When grown on chitin, *S. marcescens* produce four chitinases, ChiA, ChiB, ChiC1 and

ChiC2 which are used for the efficient degradation of chitin (Suzuki *et al.,* 1999).

The bacterial chitinases have wide optima of activity depending on their species and types of enzymes. For instance, the optimum pH ranges from 4.5 to 6.5 for chitinases produced by *Clostridium thermocellum* while it ranges from 5.0 to 8.0 in the case of chitinases from *Aeromonas hydrophilia*. Moreover, chitinases produced by actinomycetes are in acidic and neutral pH range though few alkaline chitinases were reported (Tsujibo *et al.,* 2003). The optimum temperature of chitinases also ranges from 35ºC to 60ºC for various chitinase-producing bacteria (Konagaya *et al.,* 2006). Bacterial chitinases are inhibited by common metal ions and allosamidin – the common chitinase inhibitor (Gooday *et al.,* 1992; Tsujibo *et al.,* 1997).

1.2.2.2. Fungal chitinase

It is supposed that almost all fungi can produce chitinase. *Aphanocladium album, Aspergillus nidulans, Candida albicans,* and *Mucor rouxii* are among the known fungal chititinase producers whose enymes are well characterised. Fungal chitinases are characterized by their activity at slightly acidic pH levels and they have high apparent temperature optima. However, exponentially growing mycelia of *Mucor rouxii* have produced endochitinases, which unlike other fungal chitinases are basic or neutral (Sahai and Manocha, 1993).

Fungal chitinases have high degree of stability and show both endochitinase and some exochitinase activity during catalysis. Their molecular masses differ considerably with higher molecular masses present in *Saccaromyces cerevisiae* (130 kDa), *Candida albicans* (70 kDa), and *Verticillium alboatrum* (64 kDa). These enzymes are inhibited by copper and mercury salts, lack ionic cofactors and are competitively inhibited by allosamidin (Gooday *et al.,* 1992).

1.2.2.3. Plant Chitinase

Most plants produce chitinase as a part of their general disease resistance mechanism (Sahai and Manocha, 1993) and their chitinases are studied extensively in order to maximize industrial application in biocontrol. The chitinase from yam is used against

fungal pathogens in straw berry farms and showed significant improvement against powdery mildew (Karasuda *et al.,* 2003). Moreover chitinases from tomato (Wu *et al.,* 2001), barley (Troedsson *et al.,* 2005), tobacco (Dani *et al.,* 2005), pumpkin (Arie *et al.,* 2000), and other plants are studied and characterized for their application in biocontrol against phytopathogens.

Plant chitinases are generally endochitinase (Andersen *et al.,* 2005) and have molecular masses of approximately 30 kDa. They are both acidic and basic in their nature where, the acidic forms are secreted into the apoplast or extracellular environment and the basic forms accumulate intracellularly in the vacuole. The chitinases from plants show properties including possible involvement as an active defense mechanism against chitin bearing plant pathogens such as fungi or insects. Disease resistance by hydrolases can occur by the hydrolysis of the pathogen cell wall, by the liberation of substances which elicit defense reactions, or by digestion of vital structures of the pathogen (Arie *et al.,* 2000).

1.3. Roles of chitinases

1.3.1. Roles in medicine and public health

Until recently, it has been generally stated that mammals lack chitinases. However, the identification of chitotriosidase in the serum has revealed that man can synthesize a chitin-degrading enzyme, which tightly binds to chitin particles. Moreover, human saliva forms a well-known system of defense against oral invasion of pathogens such as bacteria and fungi for it contain lysozymes and chitinases (Van Steijn *et al.,* 1999). The salivary chitinase activity was shown to be largely derived from salivary glands, not from serum or oral bacteria. The presence of chitinase in saliva explains its possible role for destruction of chitin containing pathogens, such as *Candida albicans, Aspergillus fumigatus, and Cryptococcus neoformans* as well as other pathogenic fungi (Van Steijn *et al.,* 2002).

The systemic infection of laboratory animals with chitin containing fungus, *Aspergillus fumigatus,* resulted in an increase of chitinase activity in serum. This increase was reduced by injecting the animals with antifungal drugs such as Amphotericin B or Itraconazole. The activities of chitinases were also decreased up

on the treatment of the periodontal patients suggesting that chitinases play due roles in defense against systemic infection (Van Steijn *et al.,* 2002). Moreover the normal guineapig spleen was found to have high activity of chitinase of all the tissues reflecting that this enzyme functions for defense against systemic infections.

1.3.2. Bioconversion

Chitinases are largely used in the bioconversion of chitin wastes from seafood industries into single cell protein, ethanol, and fertilizer (Bhushan, 2000; Nawani *et al.,* 2002). Moreover, chitinases are useful components in the industrial applications like production of N-acetylchitooligosaccharides $(GlcNAc)_n$ as biological active substances (Matsumiya *et al.,* 2002; Agullo *et al.,* 2003).

1.3.3. Morphogenesis

Chitinase is a morphogenetic enzyme in fungi where it plays an integral part in cell wall metabolism. The growth of cell wall including growth at the apex in filamentous fungi has been suggested to be the result of a harmonious balance between the processes of synthesis and lysis by the enzymes such as chitin synthase and chitinase respectively (Sahai and Manocha, 1993). Chitinase and chitin synthase counterbalance activities with the synthase replenishing chitin lost through chitinase action during cell separation.

Chitinase is also important for budding in yeast cell (Colussi *et al.,* 2005), separation of daughter yeast cell (Gooday *et al.,* 1992; Colussi *et al.,* 2005), the extension of hyphae (Sahai and Manocha, 1993), control of chitin metabolism at the site of hyphal branch initiation (Rast *et al.,* 1991).

Moreover chitinase plays a role in the moulting of crustaceans in marine ecosystem (Matsumiya *et al.,* 2002). It also has the role of degradation of chitin during the moulting process in insects (Zhang *et al.,* 2002).

1.3.4. Penetration of hosts

The chitinolytic activity in parasitic and pathogenic microorganisms is potentially needed for penetration of their respective chitin containing host. *Nomuraea rileyi*, a parasitic fungus on larvae of the cabbage looper, showed increased levels of endochitinase and β-1,4-N-acetylglucosaminidase as compared to an avirulent mutant strain grown over a period of 30 days (Sahai and Manocha, 1993). In the virulent isolates, the chitinase activity to total protein ratio during germination (2 days) was as much as 35 times greater than that found in conidia at day zero. Zhang *et al.*, (2002) have also observed that the chitinase activity was present at the onset of the blastospore stage (3 days), which is a stage critical to penetration of the chitin-ladened host insect cuticle.

1.3.5. Defense against phytopathogens

Plant chitinases are antifungal in nature and involved in the defence of plants against chitin containing pathogens. Heterologous expression of chitinase genes in transgenic plants has increased their resistance to certain plant pathogens (Karasuda *et al.*, 2003). They play roles in the lysis of the host cell wall of the plant pathogenic fungi and this is an important step in the mycoparasitic effect towards applications of bio-control (Seidal *et al.*, 2005).

Chitinases have, therefore, attracted increased attention especially for their use in the role they play as a biocontrol agents in agriculture and considered to be an attractive alternative to the strong dependence of modern agriculture on fungicides, which may cause environmental pollution, and selection of resistant strains (Seidal *et al.*, 2005).

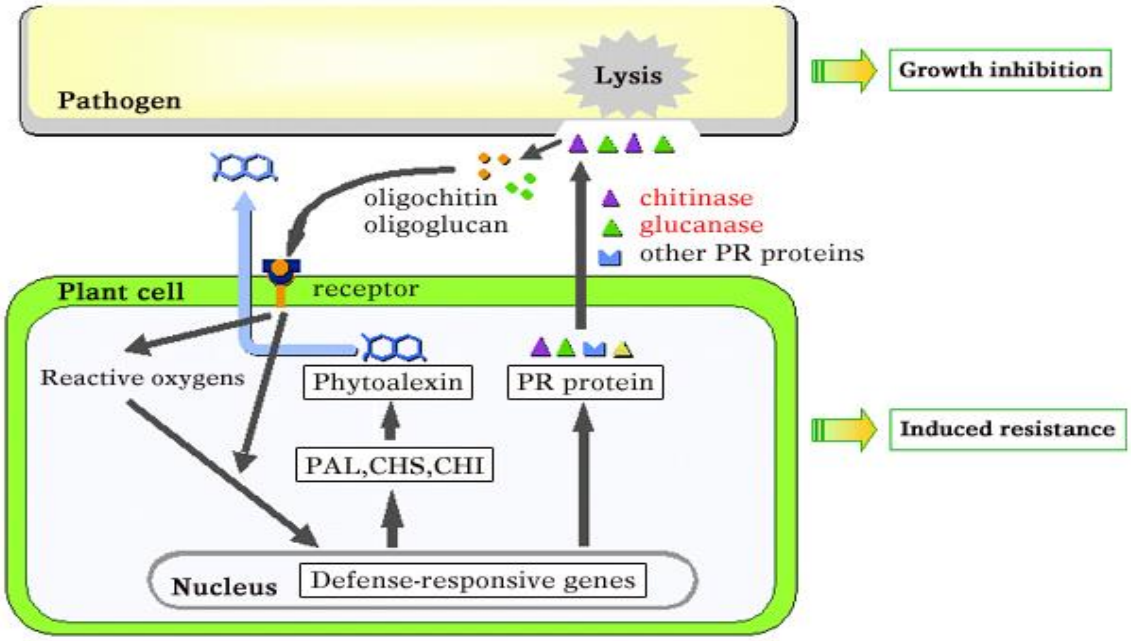

Figure 2. Model of plant-fungus interaction **(www.glycoforum.gr.jp).**

1.3.6. Bio-control of phytopathogenic organisms

1.3.6.1. Bio-control of phytopathogenic protozoa

Phytomonas francai is a protozoan which causes empty root disease in cassava and *Phytomonas staheli* is an other protozoan seriously affect coconut and palm crops. Since the chitin is an important structural component of many invertebrates including protozoa, chitinase is used in the control of diseases caused by such organisms. Two plant chitinases from *Arabidopsis thaliana – chi4* (ATCHIT4) and *Urtica dioecia – chia5* (UDA) were purified and found to bind to the cell surface and trim chitin present in phytopathogenic protozoans, suggesting that plant chitinases could represent an attractive alternative biocontrol over chemical control against protozoan attack (Godoi *et al.*, 2002; Gomes *et al.*, 2003).

1.3.6.2. Biocontrol of insect pests

The gram-positive bacterium *Bacillus thuringiensis* is used worldwide as a bio-control of pests for it acts on the susceptible insects by producing parasporal crystals containing delta-endotoxins during sporulation. Pathogens have to cross chitin rich barrier of fungal cell wall or insect exoskeleton to exert their virulence. Since *B. thuringiensis* is a known chitin-degrading microorganism and chitinase producer (Driss *et al.,* 2005), it is a potential organism for application in bio-control of insect pests.

1.3.6.3. Bio-control of phytopathogenic fungi

Sclerotia minor and *S. sclerotium* are fungal phytopathogens, which cause the most common and serious basal drop disease of lettuce (*Lactuca sativa*) and other wide range of hosts. Various bacteria: *Seratia marcescens*, *Streptomyces viridodiasticus*, and *Micromonospora carbonacea* produce effective chitinases and β-1,3-glucanase which cause extensive plasmolysis and cell wall lysis of *S. minor* hyphae in-vitro. All the three bacteria were antagonistic to *S. minor* when applied individually or in combination and significantly reduced disease incidence under controlled glass house condition (El-Tarabily *et al.*, 2000).

Gomes *et al.,* (2001) reported five strains of *Streptomyces spp*, which are effective against phytopathogenic fungi. In antagonistic test, the growth of phytopathogenic fungi (*Collelotrichum gloesporiodes*) was totally inhibited by the crude extracts from strains 68, 70, and 80 of *Streptomyces spp*. When the antifungal activity test was performed using the growing actinomycetes, all the strains were active against the fungi tested and strain 68 of the *Streptomyces spp* was very efficient in their endochitinolytic activity. *Streptomyces spp* are therefore considered to play a major role in the degradation of chitin from fungal mycelia in acidic soil and litter. *Streptomyces* RC1071 has also showed antifungal activity when tested against seven phytopathogenic fungi from acidic soils of Brazilian forest (Gomes *et al.,* 2001).

Trichoderma spp are other known groups of fungi which attach to the host hyphae by

coiling and penetrate the host cell wall by secreting basic proteins of hydrolytic enzymes such as, β-1,3-glucanases, and chitinases. The chitinase produced by *Trichoderma spp* was found to inhibit the phytopathogenic fungi, *Sclerotium rolfsii*. Moreover, the synergistic effect of β-1,3-glucanase with chitinase was reported (El-Katatny *et al.,* 2000) for efficient inhibition of fungal phytopathogens.

For the control of Grape vine dieback fungus *Eutypa lata,* Schimidt *et al.,* (2001) reported the activity of fungal hydrolases (chitinases, proteases, and cellulases) produced by *Erwinia herbicola, Bacillus subtilis, and Seratia plymuthica*. The activities of fungal hydrolases produced by these organisms were highly correlated with reduced growth of mycelia on the wood of grape vine.

The *Stenotrophomonas maltophilia* produces chitinases and showed antifungal activity against leaf spot disease (*Bipolaris sorokinians*). There were close association between chitinolytic enzymes produced by the *Stenotrophomonas maltophilia* strain C-3 and inhibition of spore germination. Chitinolysis is therefore believed to be the sole mechanism by which this strain can affect the growth of *B. sorokiniana* and inhibit leaf spot development *(*Zhang and Yuen, 2000).

Karasuda *et al.,* (2003) reported the powdery mildew infecting the straw berries was degraded mainly by spraying commercial chitinase extracted from yam (*Discorea opposite)* and the disease did not appear again for two weeks. Their result demonstrated that plant chitinase is a safe and biodegradable biocontrol agent for use instead of conventional fungicides.

Moreover at the assay condition for antifungal activity, transgenic chitinase (ChiC) from *Streptomyces griseus* to rice plant has clearly inhibited hyphal extension of *Trichoderma reesii.* This suggested that ChiC is a promising candidate as a tool of fungal disease resistance in plants (Itoh *et al.,* 2003).

The deuteromycete *Aphanocladium album* is a hyperparasite of *Pucinia graminis* var. *tritici*. This filamentous fungus produces large amount of chitinases when grown on chitin substrates. The crude extracellular protein preparations from this fungus

efficiently inhibited the phytopathogenic fungi. However, its purified chitinases were only partially hydrolyzed the cell wall of *P. graminis.* This indicates that chitinases are not solely responsible for the observed antifungal activity (Studer *et al.,* 1992). Proteases and glucanases are suggested to act against such pathogens with chitinases.

1.4. Protease

Proteases are enzymes that catalyse the hydrolysis of proteins in to peptides and amino acids. They are found everywhere in nature from the smallest virus and bacteria to the higher organisms such as plants and animals. Proteases have diverse physiological roles which range from food digestion to controlling signal cascades, blood clotting, and apoptosis (Gossas, 2007). Hence, proteases consist of one of the most useful enzyme groups in both biochemical investigations and industrial applications. In this regard, the alkaline proteases produced mostly by alkaliphilic micro-organisms are becoming attractive (Kumar and Takagi, 1999; Miyaji *et al.*, 2005).

Proteases have well-defined substrate specificity for their catalytic activity. The substrate specificity is determined by the substrate binding site, which is lined with pockets in which the residues of the substrate peptide can bind. These are named as P1, P2, etc. from the scissile bond toward the N-terminus (Fig. 3). From the scissile bond toward the C-terminus the residues are named P1', P2' etc. The corresponding binding-pockets in the protease are called sub sites and they are named with an S instead of the P as in S1 or S1' (Sajid and McKerrow, 2002; Gossas, 2007).

1.4.1. Classification and mechanisms of action

Proteases are classified according to the natural substrates upon which they act and their known physiological functions. Based on this distinct classes of proteases are named after their active catalytic centre residues as serine, cysteine**,** aspartic, glutamic, and therionine proteases. Other proteases also are named after their dependence on co-factors and grouped as metalloproteases for use of metal ion in their catalytic mechanism (Sajid and McKerrow, 2002; Antao and Malcata, 2005).

There are several mechanisms of protease activities which have evolved to handle the

peptide bond hydrolysis workload of the cell. Among these, serine, threonine, and cysteine proteases rely upon the nucleophilic character of the serine, threonine, and cysteine side chains respectively to directly attack the peptide bond and form a transient covalent enzyme-substrate intermediate. Aspartic and metalloproteases on the other hands employ a water molecule for nucleophilic attack and do not form a covalent bonded intermediate (Klemba *et al.*, 2002; Antao and Malcata, 2005).

Proteases can further be categorized based on their mode of action and ability to cleave at the N or C end of the substrate (exoproteases) or internally over the entire length (endoproteases). Proteases, particularly endoproteases usually bind their substrates in an extended conformation, with the side chains of the substrates fitting into sub sites, or binding pockets, along the protease active site (Klemba *et al.*, 2002).

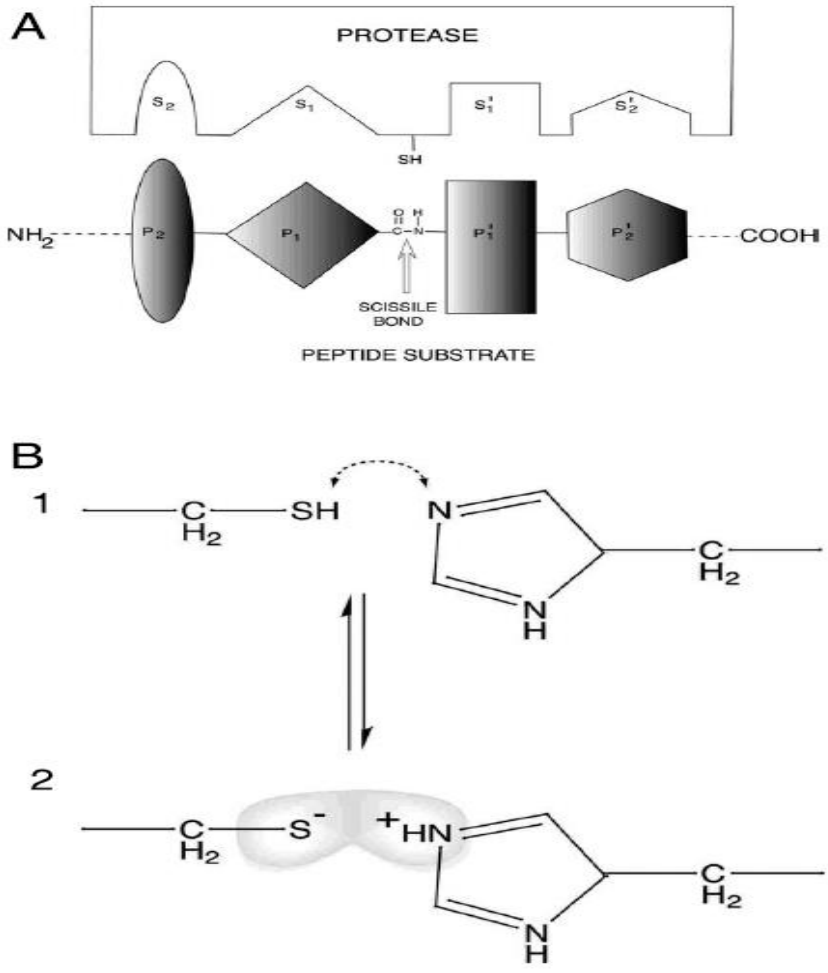

Figure 3. Peptide substrate interaction with protease (Sajid and McKerrow, 2002)

Amino acid residues from the peptide are denoted by 'P' and the sub-sites that the peptide interact with is given the letter 'S'. The active site cysteine sulfhydryl nucleophile is represented as -SH, and the corresponding peptide bond that is attacked prior to hydrolysis (scissile bond) is labelled.

1.4.2. Biological Roles of Proteases

Proteases participate in a number of biological processes such as the degradation of abnormal proteins, control of transcription factors, precursor processing, development and differentiation of cells, programmed cell death (PCD) in higher organisms, regulation of the cell cycle and apoptosis in animals among others (Huesgen *et al.*, 2005; De Castro *et al.,* 2006). They also encompass a broad class of hydrolytic enzymes that play essential roles in cellular, developmental and digestive processes, blood coagulation, inflammation, wound healing and hormone processing (De Castro *et al.*, 2006).

The serine proteases of insects play the roles in their immune system, (Gorman *et al.*, 2000; Gorman and Susan, 2001). The proteases of parasites function to facilitate the invasion of host tissues, aid in the digestion of host proteins, help parasites to evade the host immune response and mediate molting in parasitic nematodes. As a result, parasite proteases are considered as a potential targets for the development of immunotherapeutic, chemotherapeutic and serodiagnostic agents for the antiparasite interventions (Klemba and Goldberg, 2002).

Moreover, plant-parasitic nematodes cause severe damages to agriculture all over the world pushing to the use of chemical pesticides which can cause a series of environmental problems. The natural enemies of nematodes, nematode-trapping fungi, which can immobilize and digest nematodes, have been suggested as a potential biocontrol agent to control the harmful nematodes. Infection of nematodes by nematode-trapping fungi proceeds by adherence and trapping of the nematode followed by penetration and immobilization and finally digestion of the nematode (Yang *et al.,* 2005 cited in Wang *et al.,* 2006). The combination of mechanical activity and hydrolytic enzymes including proteases, chitinases and collagenases have been assumed to take part in the penetration of nematode egg shell and cuticle (Wang *et al.*, 2006; Khana *et al.*, 2004).

1.4.3. Industrial applications of alkaline proteases

Alkaline proteases are vast groups of enzymes which have considerable industrial potentials and applications in detergents, leather processing, silver recovery, medicine, food processing, feeds, and chemical industries, pharmaceutical industries, as well as waste treatment. These enzymes contribute to the development of high value-added applications or products by using enzyme-aided digestion (Kumar and Takagi, 1999; Denizci *et al.*, 2004).

1.4.3.1. Detergent additives

Microbial extracellular alkaline proteases are important enzymes which are used in detergents mainly to facilitate the release of proteinaceous stains such as blood, milk, egg and meat. Therefore these proteases account for approximately 40 % of the total worldwide enzyme sale (Sharma *et al.*, 2006). The increased usage of these proteases as detergent additives is mainly because of the cleaning capabilities of these enzymes, their environmental friendliness, and for they are non-phosphate detergents. In addition to improved washing efficiency, the use of the enzymes allows lower wash temperatures and shorter periods of agitation, often after a preliminary period of soaking (Kumar and Takagi, 1999).

Moreover, alkaline proteases used in detergent formulations have high activity and stability over a broad range of pH and temperature. These enzymes should be effective at low levels (0.4–0.8%) and compatible with various detergent components along with oxidizing and sequestering agents in addition to a long shelf life (Kumar and Takagi, 1999).

1.4.3.2. Tannery industry

The leather industry consumes a significant proportion of the world's enzyme production. Alkaline proteases are used to remove hair from hides. Relatively large amounts of enzyme are required (0.1-1.0 % (w/w)) and the process needs close control to avoid reducing the quality of the leather. After dehairing, hides are bated, a

process often involving enzymes that increases their flexibility and improves the softness of their appearance (www.lsbu.ac.uk).

Possessing elastolytic and keratinolytic activity, alkaline proteases offer an effective biotreatment of leather especially the dehairing and bating of skins and hides. The alkaline conditions enable the swelling of hair roots and subsequent attack of proteases on the hair follicle protein to allow easy removal of the hair. Despite the strong alkaline conditions, this process is pleasant and safer than traditional methods using sodium sulfide treatment, which contributes to 100% of sulfide and over 80% of the suspended solids in tannery effluents. The bating following the dehairing process involves the degradation of elastin and keratin, removal of hair residues, and the deswelling of collagen. This finally produces good and soft leather mainly used for making leather clothes and goods where microbial alkaline proteases are known to take large and efficient share in the process (Kumar and Takagi, 1999).

1.4.3.3. Food industry

Proteases are among the most important groups of industrial enzymes, with considerable application in the food industry (Merheb *et al.,* 2007). They could be used at various pH values, and may be highly specific in their choice of cleavable peptide links or quite non-specific. The action of proteases generally increases the solubility of proteins at their isoelectric points of food items. Alkaline proteases can hydrolyze proteins from plants, fish, or animals to produce hydrolysates of well-defined peptide profile.

The commercial alkaline protease, *Alcalase*, has a broad specificity with some preference for terminal hydrophobic amino acids. Kumar and Takagi (1999) have reviewed the uses of *alkalase* such as tenderise meat mainly beef, production of methionine rich hydrolysate from chickpea protein, hypoallergenic infant food formulation, and manufacturing of protein-rich therapeutic diets among others.

1.4.3.4. Waste treatment

Alkaline proteases provide potential application for the management of wastes from various food processing industries and household activities. They can solubilize proteins in wastes through a multi-step process to recover liquid concentrates or dry solids of nutritional value for fish or livestock (Kumar and Takagi, 1999).

Feathers constitute approximately 5-7% of the body weight of poultry (Kumar and Takagi, 1999; Amare Gessesse *et al.,* 2003) and can be considered as high protein source for animal feed provided their rigid keratin structure is completely destroyed. Pretreatment with NaOH, mechanical disintegration, and enzymatic hydrolysis resulted in total solubilization of the feathers. The end product is therefore, a heavy, greyish powder with a very high protein content which could be used as a feed additive (Kumar and Takagi, 1999).

In general the use of enzymes, particularly from alkaliphilic micro-organisms, has gained considerable attention during the last few years in different applications (Mehta *et al.,* 2006). These enzymes are now a days replacing chemical catalyst in manufacturing of chemicals, textiles, pharmaceuticals, paper, food and agricultural chemicals.

The Ethiopian Rift Valley soda lakes could represent a unique environment with huge potentials of extremophilic microorganisms. Now a days, there is a promising development of industries in the country and would demand some biotechnological measures to ensure environmental as well as public welfare sustaining their contributions to the development of the country. Although there are research efforts to combat possible problems the emerging industries could pose, much work should be done continuously through exploring biotechnological options.

2. Objectives:

- To screen alkaliphilic actinomycetes for chitinase and protease production
- To characterise chitinase and protease of the best isolate
- To evaluate potential application of these enzymes

3. Materials and methods

3.1. Sampling sites and sample collection

The samples of lake shore mud and sediments were collected from Lake Abijata, Shalla, and Chitu which are located in the Rift Valley of Ethiopia. Lake Shalla is located at an altitude of 1558 m and covers an area of 329 km^2. It has a maximum depth of 266 m while the salinity of the lake is 18.1 g/l and pH 9.7. Lake Abijata is also one of the Rift Valley lakes located at an altitude of 1578 m. The area coverage of the lake is 176 km^2 with the maximum depth of 14 m, salinity of 26.4 g/l, and pH 9.9. Lake Chitu is found at an altitude of 1600 m covering the area of 0.8 km^2 where its maximum depth is 21 m. The salinity of Lake Chitu is 44.9 g/l and it has pH10.2 (Fig. 4) (Elizabeth Kebede, 1996).

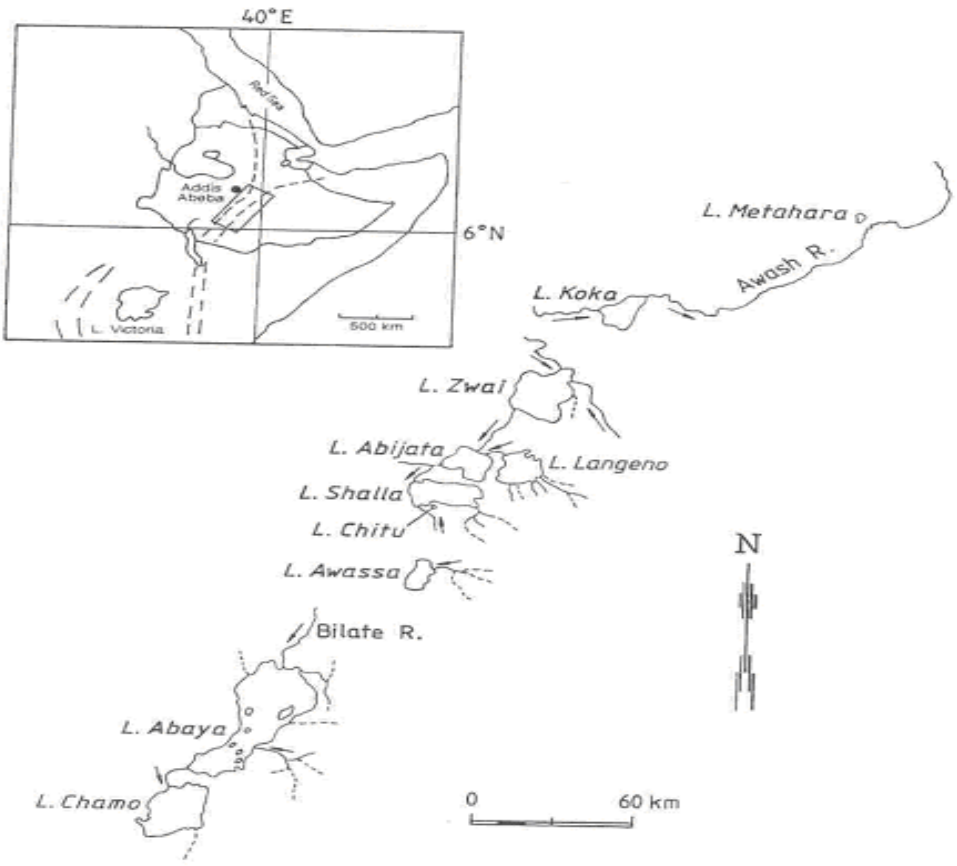

Figure 4. Map of sampling sites (Elizabeth Kebede, 1996) representing the geographical sites of Ethiopian rift valley lakes.

3.2. Media and Growth Condition of the organisms

Mud and sediment samples were collected using sterile plastic cans and transported to applied microbiology laboratory of Addis Ababa University where they kept in refrigerator until use. The samples were inoculated by spreading on separate actinomycete selective colloidal chitin agar medium (Hsu and Lockwood, 1975) containing (g/l): colloidal chitin 4.0, K_2HPO_4, 0.7; KH_2PO_4, 0.3; $MgSO_4$-$7H_2O$, 0.56; $FeSO_4.7H_20$, 0.01; $ZnSO_4.4H_2O$, 0.01; $MnCl_2$, 0.01; and agar, 20. The Na_2CO_3 was separately prepared (Amare Gessesse *et al.,* 2003); autoclaved and added in the media to final concentration of 1% in order to keep the medium alkaline (pH 10.3). Plates were incubated at 30°C for 96 h so as to get fully grown colonies. A total of 62 isolates capable of growing on colloidal chitin agar medium were obtained and taken for further analysis. Another 60 isolates which were screened for different research in the Department of Biology from the fore mentioned lakes also were tested and found to grow on colloidal chitin agar medium and selected for further evaluation.

3.3. Colloidal Chitin preparation

The stock solution of colloidal chitin was prepared from coarse chitin powder according to the method of Li *et al.,* (2002). Ten grams of chitin powder were added slowly into 180 ml of HCl (37 %, *w/v*) at 25 ºC under vigorous stirring for 2 h. The suspension was poured into one liter of ice-cold 95 % alcohol under vigorous stirring for 30 min, and stored at -20 °C until use. To prepare colloidal chitin substrate, 10 ml of the suspension was centrifuged (MSE Mistral 1000) at 4500 rpm for 5 min and the precipitate was washed 3 times with 50 ml of 0.1 mol/L potassium phosphate buffer (pH 7.0) and used consequently.

3.4. Chitin hydrolysis test

A total of 122 isolates of actinomycetes that were selected for their capacity to grow on colloidal chitin agar were purified by repeated streaking on starch casein agar containing g/l: Soluble starch 10.0, Casein 0.3, KNO_3 2.0, K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01, and agar 15. Each of the representative colonies was transferred to colloidal chitin agar media to evaluate their capacity of clear zone

formation. The colonies with clear zones around due to colloidal chitin hydrolysis were evaluated as chitinase producers.

3.4.1. Chitinase induction in Liquid State Fermentation

Based upon best clear zone formation, three isolates (A1, A29, and A47) were evaluated for chitinase production on liquid state (submerged) fermentation. A loop full of these isolates were transferred into nutrient broth in a 250 ml Erlenmeyer flask and incubated at ambient temperature on a rotary shaker (120 rpm) for 48 h. A 2 ml of the 48 h culture was aseptically transferred in to colloidal chitin broth and incubated at ambient temperature for 4 days (84 h). The samples were taken every 12 h to test for chitinase production (U) and activity (%). The cell free culture filtrate was obtained after centrifugation (Wagtech, Centurion K_2 Series) at 10000 rpm for 5 min. Following standard assay procedures, the amount of chitinase produced by each isolate was recorded by measuring absorbance at 540 nm against blank.

An alkaline protease was also produced from alkaliphilic actinomycete (A1) isolated for chitinase production under chitin media. The culture was incubated at ambient temperature for 144 h on rotary shaker (120 rpm). The culture filtrate was separated by centrifugation (Wagtech, Centurion K_2 Series) at 10000 rpm for 5 min and cell-free supernatant was obtained for the enzyme assay.

3.4.2. Chitinase induction in Solid State Fermentation

The three isolates (A1, A29, and A47) were also evaluated for chitinase production using solid state fermentation in colloidal chitin medium (Hsu Lockwood, 1975), with a little modification, containing g/l: colloidal chitin 4.0, K_2HPO_4, 0.7; KH_2PO_4, 0.3; $MgSO_4$-$7H_2O$, 0.56; $FeSO_4.7H_20$, 0.01; $ZnSO_4.4H_2O$, 0.01; $MnCl_2$, 0.01; and supplemented with 10g of wheat bran. Two ml of a 48 hour culture was inoculated in solid state fermentation medium and the moisture content of the medium was kept to be (1:1.5 w/v) in order to maintain the solid state condition. The cell free filtrate was obtained by adding 100 ml distilled water at 4^{th} day of incubation and subjected to 10000 rpm centrifugation (Wagtech, Centurion K_2 Series) for 5 min. The isolate which produced more chitinase than the others was selected for further study.

3.4.3. Protease induction in Solid State Fermentation

Isolate A1 was selected from previous experiments and evaluated for its protease production capacity on colloidal chitin media for solid state fermentation. Two ml of the 48 hour culture was inoculated in the solid state fermentation medium with similar proportion mentioned earlier for chitinase production. The moisture content of the medium was kept at 1:1.5 (w/v) so as to maintain solid state of the media. The cell free culture filtrate was obtained by adding 100 ml distilled water at 6^{th} day and subjecting to 10000 rpm centrifuge (Wagtech, Centurion K_2 Series) for 5 min.

3.5. Enzyme production using mushroom as carbon and nitrogen source

Oyster mushroom (*Pleurotus ostereatus*) was dried to constant weight, ground, and used to replace colloidal chitin as a carbon and nitrogen source for protease and chitinase production of selected microorganism.

3.6. Chitinase assay

The activity of chitinase in the culture filtrate was measured by the dinitro-salicylic acid (DNS) method (Miller, 1959) using colloidal chitin as the substrate. The culture mixture containing 500 µl of enzyme solution was added to an equal amount of colloidal chitin solution (0.4 % v/v colloidal chitin solution prepared in 100 mM Tris-HCl buffer pH 8.5). The mixture was incubated at 50 °C for 20 min to allow reaction of enzyme with chitin substrate. The reaction was terminated by the addition of 2 ml of DNS mixture (containing g/l: sodium sulfite 0.5, phenol 2, sodium hydroxide 10, potassium-sodium tartarate 200, and the 3-5, dinitro-salicylic-acid 10). The reaction mixture was then incubated in boiling water to investigate the colour change of the mixture by reaction with simple sugars. It was then kept to cool before the absorbance was measured at 540 nm against blank and the result was related to the chitinase activity. One unit of chitinase activity (U) was defined as the amount of enzyme liberating 1µg of reducing sugar equivalent to glucose per min under the assay conditions.

3.7. Characterization of Chitinase

3.7.1. Effect of pH on activity and stability of chitinase

The activity of chitinase was measured at different pH values by the standard assay method using colloidal chitin as the substrate (Miller, 1959). The crude culture filtrate was used as the enzyme source and the pH of the reaction mixtures was varied using the following buffers (100 mM): citrate phosphate (pH 5-7), Phosphate (pH 7-7.5), Tris-HCl (pH 7.5-9), and glycine-NaOH (pH 9-11). The pH stability of the enzyme was evaluated by incubating the enzyme solution for 1 h at various pH values indicated above at room temperature. The residual enzyme activity was determined under the standard assay conditions as before.

3.7.2. Effect of temperature on activity and stability of chitinase

The effect of temperature on activity of chitinase was determined by incubating the reaction mixtures of 500µl colloidal chitin with equal volume of Tris-HCl buffer pH 8.5 at different temperatures ranging from 35 to 75 °C in 5 °C intervals for 20 min. For the determination of the effect of temperature on the stability of chitinase, the solution of culture filtrate was exposed to different temperatures (35-75 °C) for 30 min. The residual enzyme activity was then measured under standard assay conditions using colloidal chitin as the substrate. The four best values of this assay condition were further evaluated for their stability for one hour in 10 min interval keeping the temperature constant.

3.8. Protease assay

The activity of protease was determined by using casein as a substrate. To 450 µl of 1% casein prepared in100 mM glycine-NaOH buffer pH 10.5, 50 µl of enzyme source was mixed in test tubes and incubated for 30 min at 50^{o}C. The reaction was terminated by adding 500 µl of 10 % trichloroaceticacid (TCA). It was then incubated at room temperature for 20 min and centrifuged (Wagtech, Centurion K_2 Series) at 10000 rpm for 5 min. Then, 0.5ml of the clear supernatant was mixed with 2.5ml of

0.5M $NaCO_3$ and 1N Folin- Ciocalteau's phenol reagent. After 30 min of incubation at room temperature, absorbance was measured at 660 nm against a reagent blank. One unit of protease activity was defined as the amount of enzyme that liberates 1μg of amino acid equivalent to tyrosine per min under the standard assay conditions (Amare Gessesse, *et al.* 2003).

3.9. Time course of protease production

The effect of culture conditions for maximum protease production by the isolate was evaluated. A 50 ml of colloidal chitin medium was prepared in an Erlenmeyer flask of 250 ml capacity. The pH of the media was raised to 10.3 by adding 1 % of Na_2CO_3 sterilized separately. Isolate A1 was inoculated and incubated at ambient temperature on rotary shaker (120 rpm). Samples were collected between 12 h intervals for 144 h and enzyme activity was recorded after measuring absorbance against blank at 660 nm.

3.9.1. Effect of chitin concentration on protease production

The effect of chitin concentration on protease production was investigated by varying the level of chitin in the culture. Four levels of chitin were used from 0.2 % to 0.5 % the amount of protease produced in each level was measured following the standard assay procedures as before. After incubation at ambient temperature on rotary shaker for 144 h, the cell free culture filtrate was obtained by centrifuging at 10000 rpm for 5 min. The activity of enzyme was assayed following the standard procedures and its amount was determined by measuring the absorbance against blank at 660 nm.

3.9.2. Effect of nitrogen source on protease production

The effect of nitrogen source on protease production was studied using 0.1 g/l of peptone, yeast extract, peptone + yeast extract, and casein against the non supplemented control. The culture was incubated at room temperature on rotary shaker at 120 rpm for 144 h. The samples were taken every 12 h in order to investigate the protease production across the time. After centrifugation (Wagtech, Centurion K_2 Series) at 10000 rpm for 5 min, the cell free culture filtrate was obtained for enzyme assay. The enzyme activity was assayed following the standard

procedures and the amount of the enzyme was determined by measuring the absorbance at 660 nm against blank.

3.9.3. Effect of carbon source on protease production

The colloidal chitin culture medium was supplemented with 1 g/l of glucose, sucrose, maltose, and lactose against the non supplemented control. The culture was grown on shake-flask condition for 144 h and samples were collected every 12 h to test the amount of protease production. The cell free filtrate for enzyme assay was obtained by centrifuging (Wagtech, Centurion K_2 Series) the culture at 10000 rpm for 5 min. The standard enzyme assay procedures (Amare Gessesse *et al.,* 2003) were followed and the relative activity was determined by measuring the absorbance against blank at 660 nm.

3.10. Characterization of Protease

3.10.1. Effects of pH on protease activity and stability

The effect of pH on the activities of protease was studied using casein as a substrate. The test tubes of reaction mixtures containing 450 µl of 1 % casein as substrate in 100 mM buffers with varying pH: phosphate pH 7-8, Tris-HCl pH 7.5-9, and glycine-NaOH pH 9-11 were incubated with 50 µl of culture filtrate at 50 °C for 30 min. The standard enzyme assay procedures (Amare Gessesse *et al.,* 2003) were followed and the relative activity was determined by measuring the absorbance against blank at 660 nm.

The pH stability of the protease was investigated by exposing the culture filtrate to equal volume of different buffers with varying pH: citrate phosphate pH 4-7, phosphate pH 6-8, tris-HCl pH 7.5-9, and glycine-NaOH pH 9-11. The mixtures were incubated at ambient temperature for one hour and each mixture was assayed using casein as a substrate following the standard procedures (Amare Gessesse *et al.,* 2003). The residual activity of the enzymes at each exposure to different pH values was determined by measuring the absorbance against blank at 660 nm.

3.10.2. Effects of temperature on the activity and stability of protease A1

The optimum temperature was determined by exposing the reaction mixture containing 450 µl of 1 % casein prepared by using 100 mM Glycine-NaOH buffer, pH 10.5 and 50 µl of culture filtrate for the temperatures ranging from 35°C to 75°C in 5 °C intervals. After 30 min of incubation the enzymatic activity in each temperature was assayed following standard procedures (Amare Gessesse *et al.,* 2003) and relative activity was determined by measuring the absorbance against blank at 660 nm.

The stability of the enzyme in different temperature was determined by incubating it at varying temperature values (35-65 °C in 5 °C intervals) for 30 min keeping the time constant in the presence and absence of $CaCl_2$. The thermal stability was also determined by incubating the culture filtrate for 60 min at constant temperatures (45 and 50 °C) where samples were taken in 10 min intervals. The effect of $CaCl_2$ on thermal stability was also investigated by measuring residual activity under standard conditions in the presence and absence of 5mM $CaCl_2$. The residual enzymatic activity was assayed following standard procedures (Amare Gessesse *et al.,* 2003) before measuring the absorbance against blank at 660 nm.

3.11. Standard curves

The standard curves for both chitinase and protease assay was prepared using the standard proportions of glucose and tyrosine respectively (Miler, 1959; Amare Gessesse *et al.,* 2003). The standard proportions for glucose was prepared by adjusting the final concentration to 10 µg/ml as 0, 1, 2.5, 5, 7.5, 10, and following the DNS method (Miller, 1959) where the actual proportions of glucose to water was 0:100, 10:90, 25:75, 50:50, 75:25, and 100:0 where the X represents glucose while Y stands for water. The final concentration for tyrosine was adjusted to be 100 µg/ml, as 0, 5, 10, 25, 50, 75, and 100. Its proportion of tyrosine to water was 0:500, 25:474, 50:450, 125:375, 250:250, 375:125, and 500:0. The assay procedures of Amare Gessesse *et al,* (2003) were followed before measuring the absorbance.

The standard formula, Y= aX + b, was developed for both enzymes based on the result of respective standard curves (Fig. 3) where:

Y= the amount of glucose equivalent and
X= absorbance value.

The final value that was equivalent to glucose or tyrosine equivalent was obtained by multiplying Y with the formula values of DF X P/IT where:

DF= Dilution factor for culture filtrate
P= Actual proportions of culture filtrate in the reaction mixture
IT= Incubation time (min) during enzyme assay

3.12. Identification of the organism

The isolate was characterized on the basis of its morphological (shape, gram reaction), cultural (color, texture) and some biochemical (oxidase, catalase, oxygen tension) properties. All these properties were then compared with the standard characteristics described in Bergey's Manual of Systematic Bacteriology (Bergey, 1984).

4. Results

4.1. Isolation and screening of the organisms

A total of 122 alkaliphilic isolates capable of growing on colloidal chitin agar as sole source of carbon and nitrogen were screened where 62 were isolated and 60 of them were added from previous culture collection. Among these, 66 isolates (54%) produced chitinase on colloidal chitin agar plates (Table 1). Based on the relative size of clear zone formation, three of them were selected for further study.

Table 1. Number of isolates screened for chitinase production.

Qualitative clear zone	Number of isolates
4X	3
3X	10
2X	16
1X	37
0X	56
Total	122

"X" indicates relative clear zone forming areas. The 1-4X shows varying relative clear zones formed. "0X" indicates colonies forming no clear zones but showed growth on colloidal chitin medium.

4.2. Enzyme production using submerged and solid state fermentation

The three best clear zone forming isolates were compared for their enzymatic production in submerged fermentation. Isolate A1 produced chitinolytic enzyme of 9.7 U followed by 2.8 U and 2.7 U of isolates A29 and A47 respectively (Table 2). Isolate A1 was, therefore, found to be the best producer of all the three isolates. This isolate is used in the whole course of the study. Two of the rest isolates produced lower amount of chitinase than their counter part.

Table 2. Chitinase and protease production by using the three isolates under SmF and SSF.

Isolates No.	Enzyme production (SmF)		Enzyme production (SSF)	
	Chitinase (U/ml)	**Protease (U/ml)**	**Chitinase (U/mg)**	**Protease (U/mg)**
A1	9.7	15.6	37.3	59.6
A29	2.8	ND	ND	ND
A47	2.7	ND	ND	ND

ND=Not determined, SmF=Submerged fermentation, SSF=Solid state fermentation, U/ml= Units of enzyme per ml, U/mg=Units of enzyme per mg

4.3. Organism identification

The colony appearance of isolate A1 was creamy yellow with mucosy appearance. The vegetative cells had rod shape with mycelial and branched nature and found to be gram positive. It requires oxygen to grow and positive for both catalase and oxidase tests (Table 3). In addition to the features mentioned here, this isolate grew in the selective media for actinomycetes (Hsu Lockwood, 1975). Based on these features isolate A1 was grouped as *Actinomycete spp*.

Table 3. Some morphological and biochemical features of the isolate A1

Characteristics	Isolate A1
Colony colour	Creamy Yellow
Colony texture	Mucosy appearance
Vegetative cells	Mycelial and branched
Cell shape	Rod
Gram staining	Gram positive
Oxygen relationship	Aerobic
Oxidase test	+
Catalase test	+

(+) = indicates positive result and (-) =indicates negative result for the tests conducted

4.4. Evaluation of mushroom as carbon and nitrogen source for chitinase and protease production

Isolate A1 produced chitinase and protease using oyster mushroom (*Pleurotus ostereatus*) as a carbon and nitrogen source under submerged fermentation (SmF) which were revealed by relative enzymatic activity (Table 4).

Table 4. Effect of mushroom as chitin substitute for enzyme production.

Substrates	**Protease (U/ml)**	**Chitinase (U/ml)**
Colloidal chitin	15.6	9.7
Mushroom	22.8	10.4

(U/ml)= unit per ml protease and chitinase.

4.5. Standard curve for chitinase estimation

The standard curve was used to estimate the amount of glucose equivalent liberated per min by the action of chitinase. The standard formula obtained was Y=28.8X + 0.049 based on the result of standard curve (Fig. 3) where:

Y= the amount of glucose equivalent and
X= absorbance value.

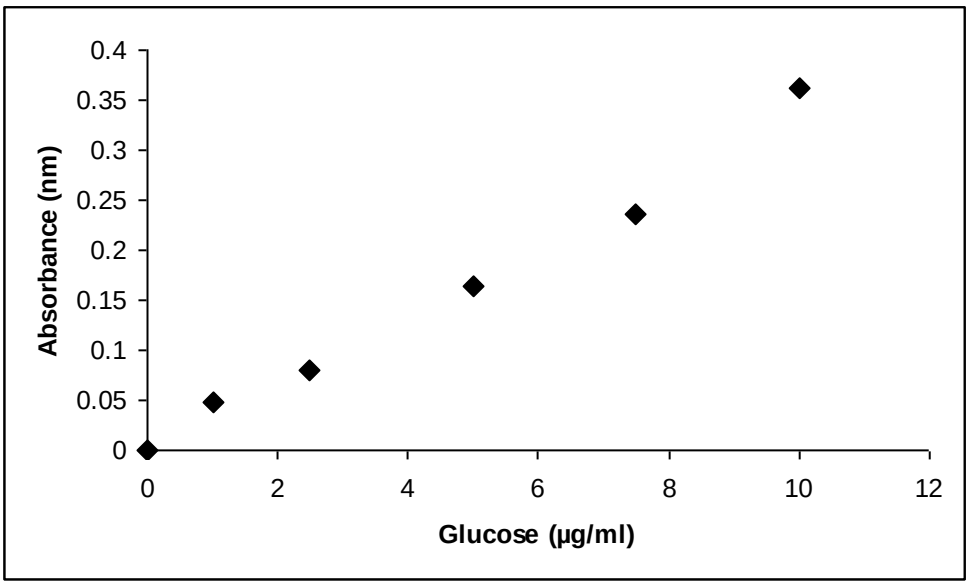

Figure 5. The standard curve of used to estimate chitinase activity. It was developed

by varying the proportion of glucose to the final concentration of 0, 1, 2.5, 5, 7.5, 10, and following the DNS method (Miller, 1959) where the respective average values of spectrophotometer readings were 0, 0.048, 0.081, 0.165, 0.236, and 0.362.

4.6. Time course of chitinase production

Chitinase production of isolate A1 started after 48 h of incubation and reached a maximum at 72 h. After 72 h there was rapid decline in enzyme production (Fig. 5).

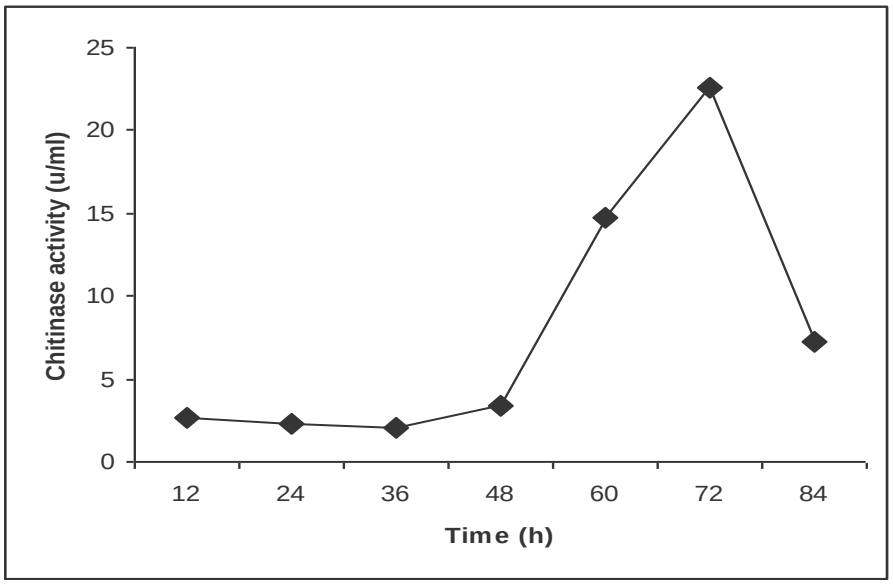

Figure 6. Time course for chitinase production. The enzyme was obtained by subsequent sampling in 12 h intervals assayed for activity accordingly. The time course of chitinase production was determined by incubating the enzyme at 50 °C for 20 min using colloidal chitin (prepared by mixing with Tris-HCl buffer pH 8.5) as a substrate.

4.6.1. pH profile and stability of chitinase of A1

Chitinase from actinomycete A1 was active in a broad pH range. More than 80% of the peak activity was displayed in the pH range of 8-11. The optimum pH for chitinase activity was 8.5 and its activity in the pH ranges between 6 and 11 was over 75% of its maximum value. Tris-HCl was by far the buffer that maintained the highest relative activity followed by glycine-NaOH.

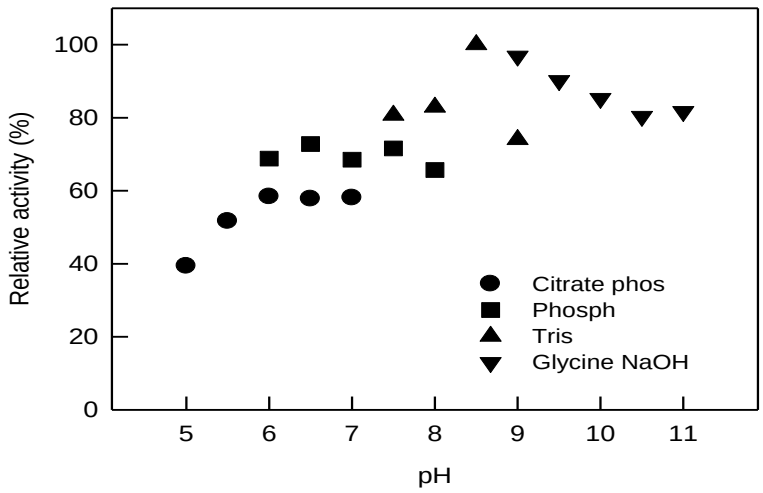

Figure 7. pH profile of chitinase activity. Effect of pH on enzyme activity was determined by incubating the enzyme in different buffers (citrate phosphate pH 5-7, phosphate pH 6-8, Tris-HCl pH 7.5-9, and Glycine-NaOH pH 9-11) for 20 min at 50 °C and following standard assay procedures.

Chitinase A1 was also stabile in alkaline pH ranges until pH 11 making its maximum residual activity (97.9) at pH 9. It has retained ≥ 80% residual activity between pH of 7.5-11. At pH values below neutrality, chitinase A1 was less stable and stability continuously dropped as pH goes acidic until tested values (pH 5) (Fig. 7).

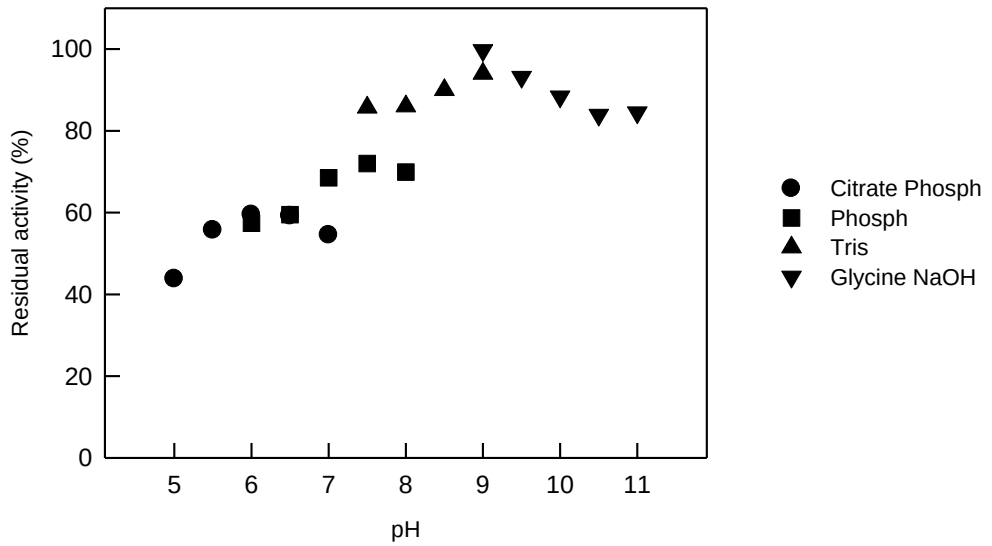

Figure 8. pH Stability of chitinase. The stability of chitinase A1 at pH was studied by incubating enzyme with different buffers (citrate phosphate pH 5-7, phosphate pH 6-8, Tris-HCl pH 7.5-9, and Glycine-NaOH pH 9-11) for 1 h at ambient T °C followed with standard assay procedures using each as a source of enzyme.

4.6.2. Temperature profile and thermal stability of chitinase

Chitinase A1 was active in the temperature range of 35-55 °C with maximum activity at 45 °C. Above 55 °C the activity was dropped gradually until it was about 20% at 75 °C (Fig. 8).

The stability of chitinase A1 was also lowered against temperature increase which was indicated by its low residual activity. However, this enzyme was stable at 35 °C and 40 °C after 30 min of incubation retaining about 90% of its original activity (Fig. 9).

Under constant temperature conditions, the stability of chitinase A1 was dropped suddenly to 50 % and 70 % of its original activity after 10 min incubation at 50 °C and 40 °C respectively. It was however relatively stable at 40 °C until 30 min incubation retaining about 90 % of its original activity (Fig. 10).

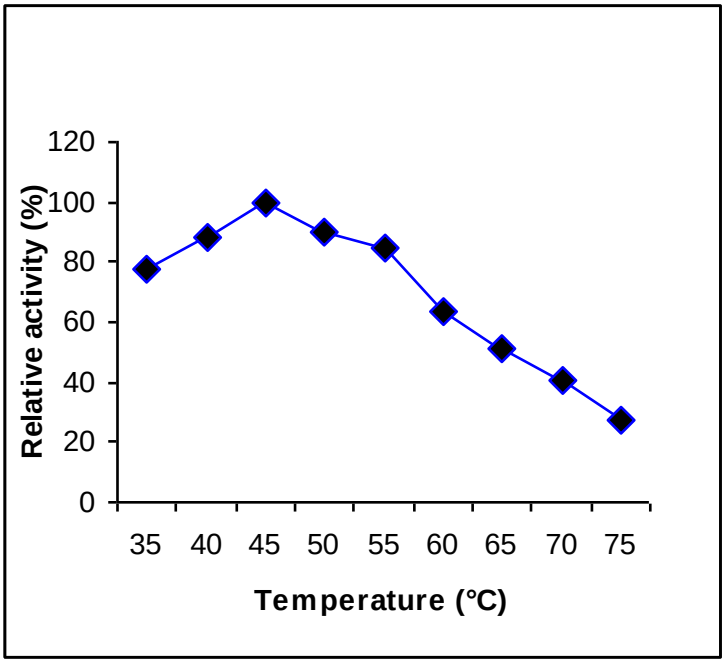

Figure 9. Effect of temperature on activity of chitinase. The temperature profile of chitinase A1 was determined by assaying enzyme activity at different temperature values using 100 mM tris-HCl buffer pH 8.5.

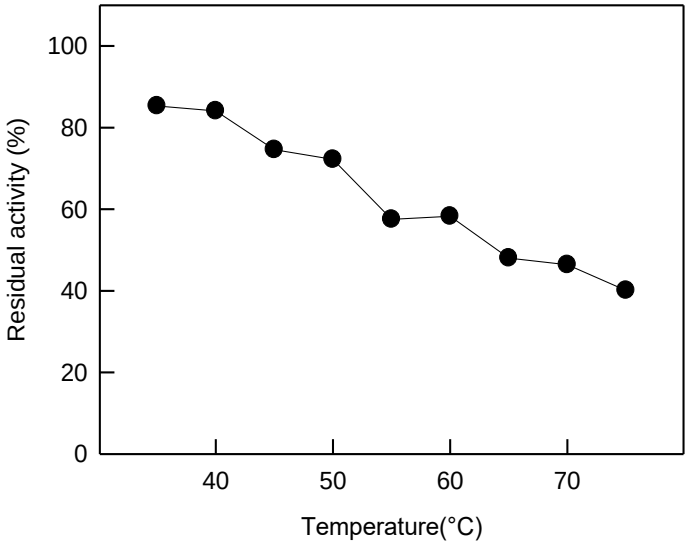

Figure 10. Thermal stability of chitinase after 30 min incubation. Effect of temperature on the stability of chitinase was evaluated by incubating the enzyme at different temperature values for 30 min and residual activity was assayed following standard procedure (Fig. 9).

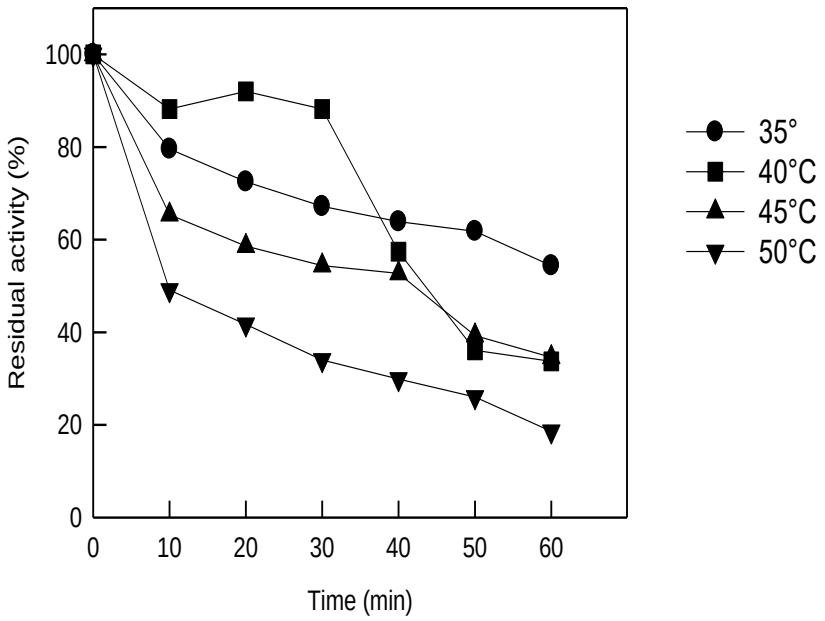

Figure 11. Thermal stability of chitinase across 60 min of incubation. The enzyme was incubated 35, 40, 45, and 50 °C for an hour and residual activity was measured following standard assay procedure.

4.7. Standard curve of protease estimation

The standard curve was used to estimate the amount of tyrosine equivalent liberated per min by the action of protease. The standard formulae Y=108X - 0.825 was obtained based on the result of standard curve (Fig. 3) where:

Y= the amount of glucose equivalent

X= absorbance value.

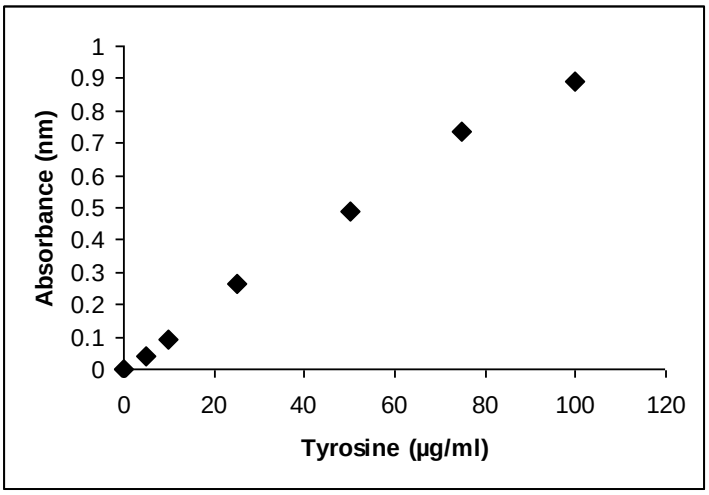

Figure 12. The standard curve of used to estimate protease activity. It was developed by varying the proportion of glucose to the final concentration of 0, 5, 10, 25, 50, and 100, before following the method of Amare Gessesse *et al*, (2003) for respective values of spectrophotometer readings 0, 0.039, 0.091, 0.263, 0.487, 0.738, and 0.891.

4.8. Time course of protease production

Protease production by isolate A1 reached a maximum at bout 120 h of incubation. Enzyme production declined after the incubation for 120 h which was evident from a decline of its activity. A sharp drop of protease activity was observed beyond 132 h of incubation (Fig. 11).

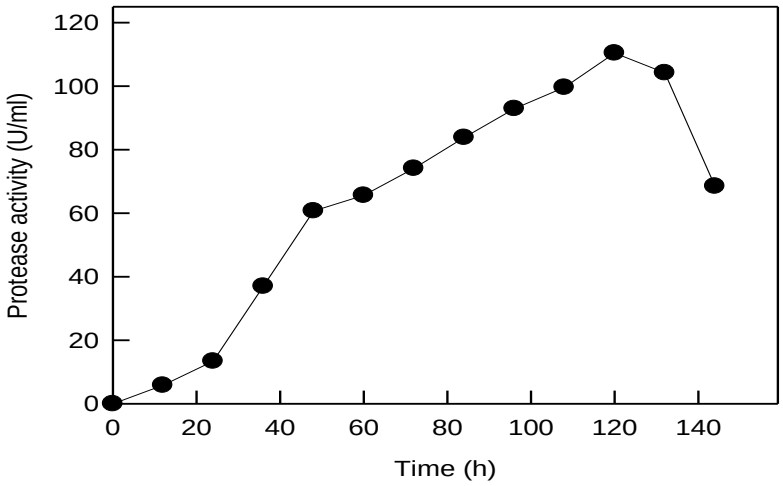

Figure 13. Time course of protease production. The time course of protease production was determined by incubating the enzyme at 50 °C for 30 min using colloidal chitin (prepared by mixing with glycine-NaOH buffer pH 10.5) as a substrate. The enzyme was obtained by subsequent sampling in 12 h intervals for 144 h and assayed for activity accordingly.

4.8.1. Effect of nitrogen source on protease production

Addition of 0.01% yeast extract to the basal medium (colloidal chitin) showed protease yield increment of strain A1 by 22.4 %. The culture supplemented with peptone showed lower protease production and casein supplemented culture showed minimum protease production than the others. The non supplemented control produced considerable amount of protease though lower than that of the culture supplemented with YE (Fig. 12).

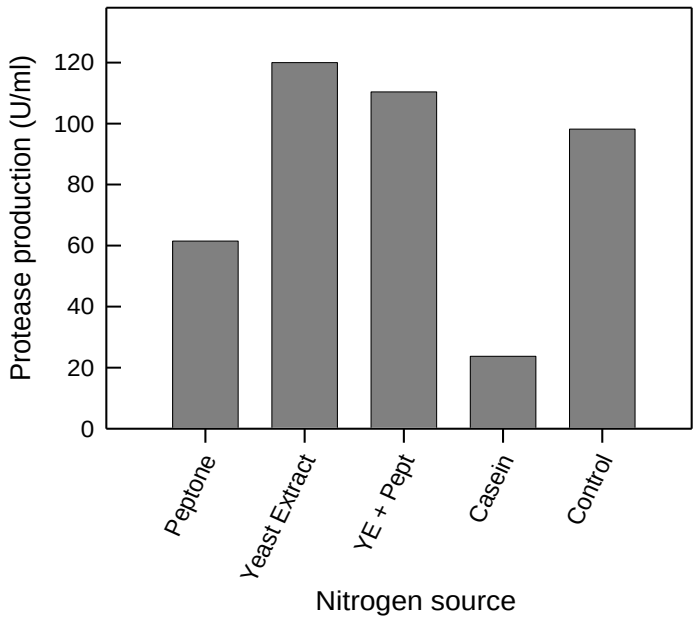

Figure 14. Effect of nitrogen source on protease production. Protease production of isolate A1 tested on basal medium containing (g/l): colloidal chitin 4.0, K_2HPO_4, 0.7; KH_2PO, 0.3; $MgSO_4$-$7H_2O$, 0.56; $FeSO_4.7H_20$, 0.01; $ZnSO_4.4H_2O$, 0.01; $MnCl_2$, 0.01.) Peptone, yeast extract, casein, and peptone + yeast extract was supplemented as nitrogen source and compared against the control (no supplement) for protease production after 144 h incubation.

4.8.2. Effect of carbon source on protease production

Supplementing glucose, sucrose, maltose and lactose showed no yield increment on protease production over the non supplemented control. The maltose supplemented culture showed peak production after 60 h of incubation while peak production of glucose supplemented culture was after 96 h. Sucrose supplemented culture showed lower protease amount during incubation time and the culture with no supplement of carbon source gave the highest protease production (Fig. 13).

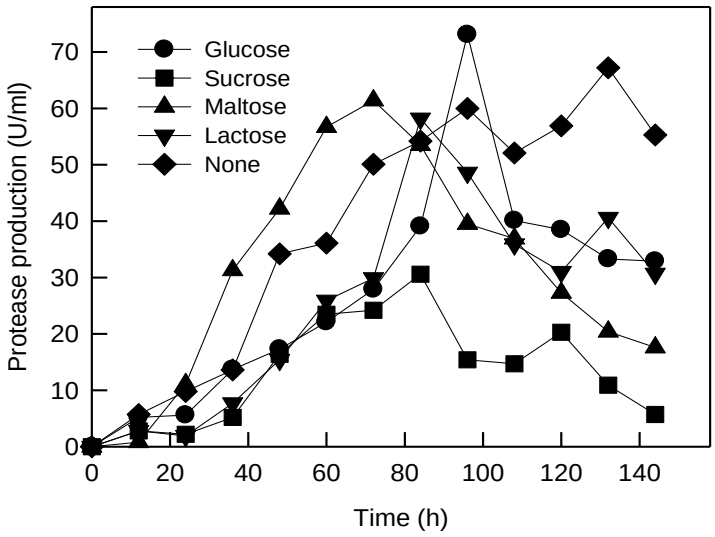

Figure 15. Effect of carbon source on protease production where the basal medium was supplemented with glucose, sucrose, maltose, and lactose and incubated for 144 h.

4.8.3. Effect of chitin concentration of protease production

The overall protease production in the level of 0.2 % was low while no significant difference was observed among others (0.3 %, 0.4 %, and 0.5) (Fig. 14).

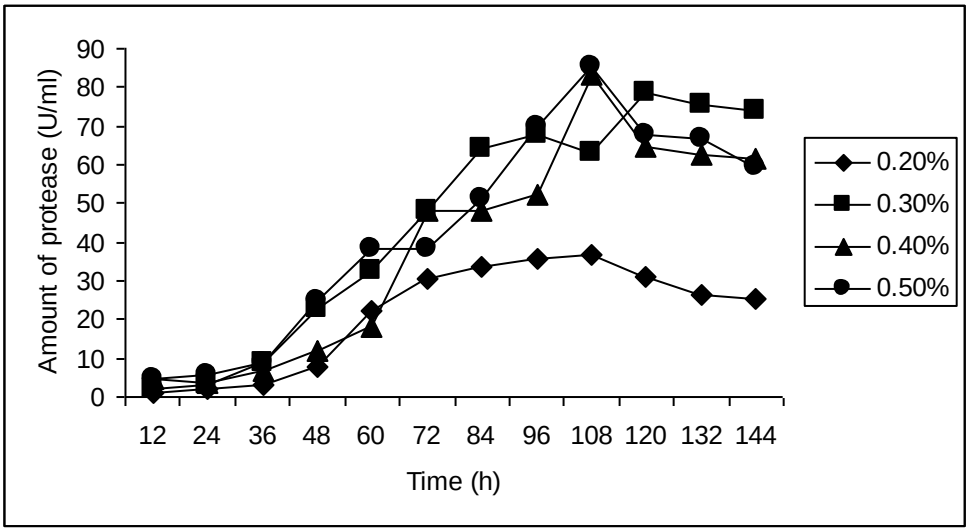

Figure 16. Effect of chitin concentration on protease production where 0.2, 0.3, 0.4, and 0.5 % of colloidal chitin was included in the media for 144 h.

4.8.4. Properties of protease A1

4.8.4.1. Effect of pH on activity and stability

Protease A1 was optimally active in the alkaline pH range (9.5-11) where it showed more than 90% of its peak production with maximum activity at pH 10.5. At pH 9, the activity was close to 80% and became lower below this pH (Fig. 15). Glycine-NaOH buffer showed better buffering capacity for the enzyme maintaining most of the activity of protease A1.

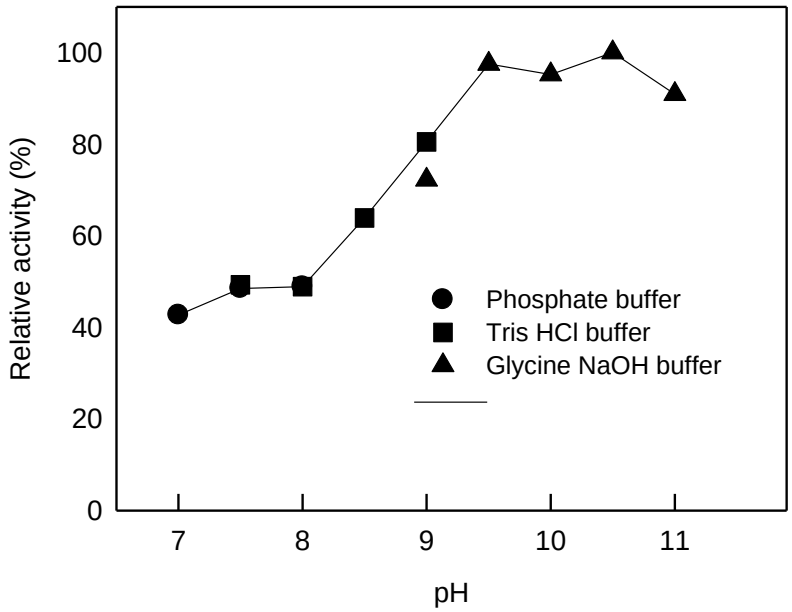

Figure 17. pH profile of protease activity. Effect of pH on enzyme activity was determined by incubating the culture filtrate in different buffers (phosphate pH 7-8, Tris-HCl pH 7.5-9, and Glycine-NaOH pH 9-11) for 30 min at 50 °C and following standard assay procedures.

The protease A1 also showed good stability in alkaline pH range where the maximum residual activity was 87% at pH 10.5. Below pH 8, the protease A1 was less stable and as pH goes acidic, the residual activity of the enzyme was also lowered (Fig. 16). The enzyme (culture filtrate) incubated in citrate phosphate buffer showed the lowest residual activity.

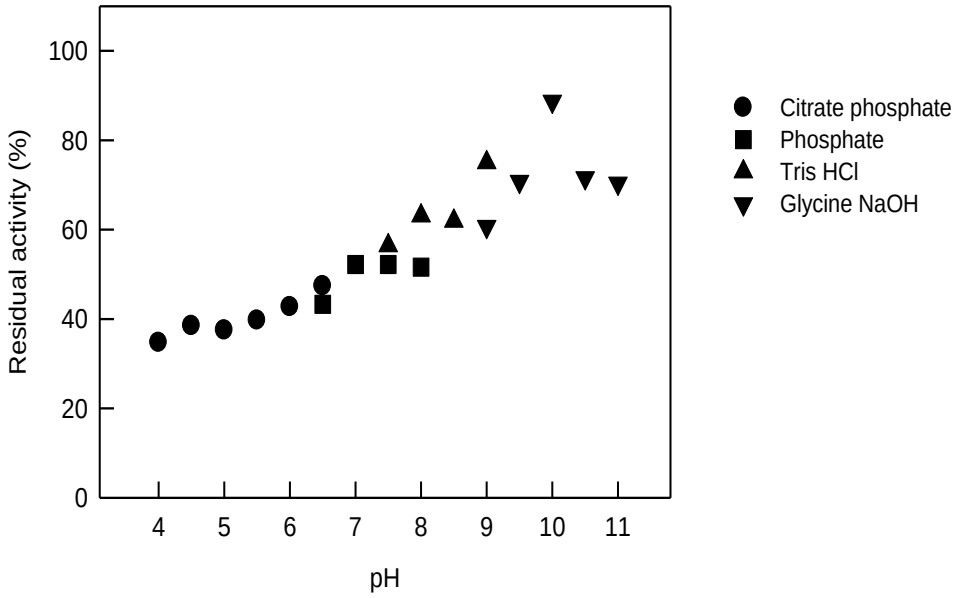

Figure 18. pH stability of protease A1 was determined by incubating the enzyme with the following buffers: citrate phosphate: pH 4-7, phosphate: pH 6-8, Tris-HCl: pH 7.5-9, and glycine-NaOH: pH 9-11 for 1 h at 50 °C against the control.

4.8.4.2. Effect of temperature on activity and stability of protease A1

Protease A1 was optimally active at 55°C and beyond this its activity declined rapidly (Fig. 17). More than 70 % of the original activity was observed between 50 and 65 °C. The lowest enzymatic activity was observed below 45 °C and above 70°C. This enzyme was stable within temperature ranges of 35-45 °C where it showed the residual activities more than 75 % after incubation for 30 min with 5mM of 10 µl $CaCl_2$. Protease A1 was also stabile in the absence of $CaCl_2$ in 35-45 °C where more than 80 % of the residual activities were maintained after 30 minutes of incubation. Therefore, addition of $CaCl_2$ did not bring about significant difference on protease stability after 30 min incubation in the temperatures of 35-65 °C (Fig. 18).

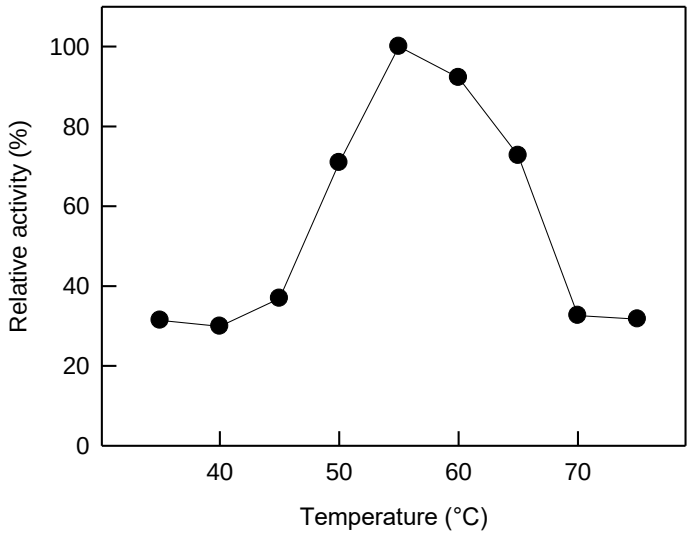

Figure 19. Temperature profile of protease activity where casein (pH 10.5) used as substrate and incubated with protease A1. The temperature profile of protease A1 was determined by assaying enzyme activity at different temperature values using 100 mM glycine-NaOH buffer pH 10.5.

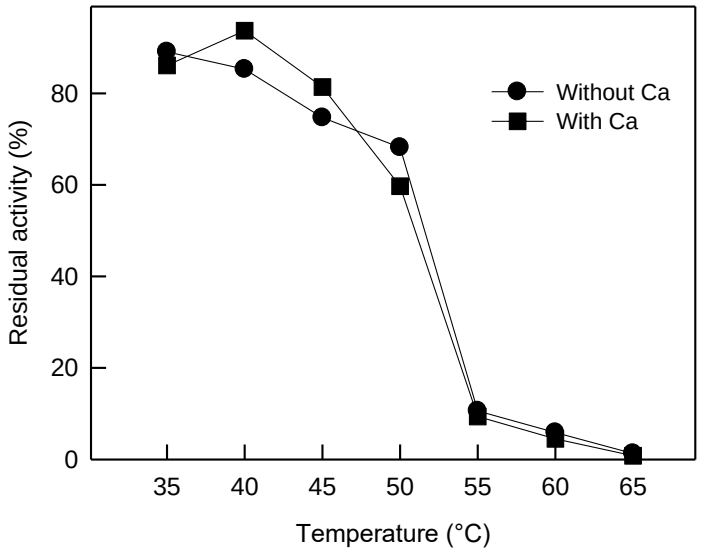

Figure 20. Temperature stability after 30 min of incubation in the presence and/or absence of $CaCl_2$. The temperature stability of protease A1 was determined by incubating the enzyme for 30 min under different temperatures and following standard assay procedure to evaluate residual activity.

Protease A1 also exhibited high stability at 45 ºC both in the presence and/or absence of $CaCl_2$ when incubated for 60 min keeping the T°C constant (Fig. 19). Residual activity was dropped to below 50 % after 20 min incubation at 50 °C.

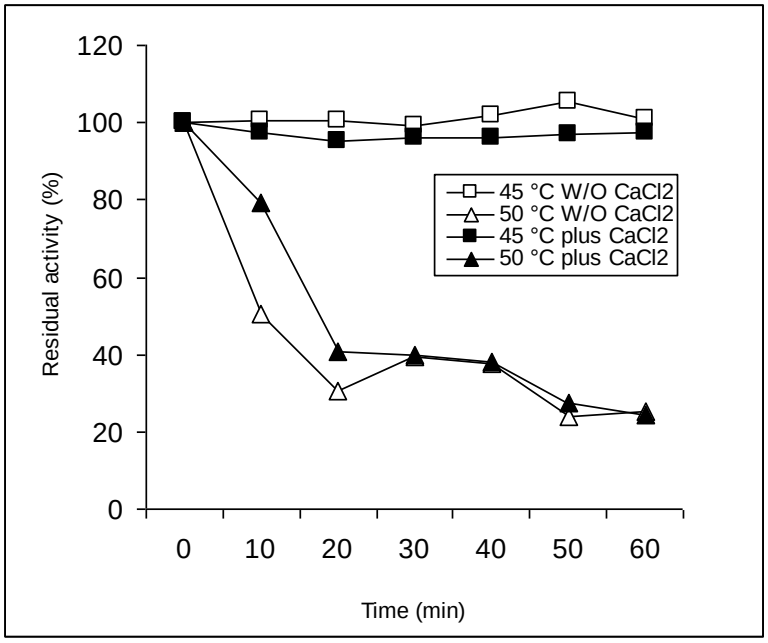

Figure 21. Thermal stability of protease across 60 min incubation in the presence and absence of $CaCl_2$.

5. Discussion

Chitinases are enzymes produced by various organisms in order to hydrolyse chitin for nutritional and morphogenetic purposes and have various biotechnological and industrial applications. This study was initiated to isolate chitinolytic and proteolytic alkaliphilic actinomycetes from alkaline soda lakes.

Out of a total of 122 alkaliphilic actinomycete isolates grown on the enrichment media and screened, 54 % produced detectable chitinase activity based on clear zone formation around colonies. This indicates that Ethiopian alkaline soda lakes have a diverse group of alkaliphilic chitinase producing actinomycetes. The alkaline soda lakes of Ethiopia are highly productive which could support a high diversity of heterotrophic microorganisms that are involved in the decomposition of various biopolymers, thus playing a vital role in nutrient recycling (Amare Gessesse and Birhanu Gashe 1997).

Out of chitinolytic microorganisms, three isolates were found to display large clear zones and selected for further work. The most effective isolate A1 was further characterised by some morphological, cultural and biochemical characteristics. Consequently, it was found to be gram positive and rod shaped bacteria. Isolate A1 was oxidase and catalase positive with creamy yellow and mucosy texture. It was also aerobic and the characters are comparable with the genus Actinomycete spp (Bergey, 1984).

In addition to chitinase, this isolate produced high amount of protease when grown on colloidal chitin. The organism also produced high amount of protease when colloidal chitin was replaced by mushroom. This indicates that mushroom can serve as an alternative carbon and nitrogen source for isolation of chitinolytic microorganisms and production of chitinolytic and proteolytic enzymes.

The maximum chitinase production was attained after 72 h of incubation and it was after 120 h for protease production. Comparing the slow growing property of actinomycetes, this might indicate that isolate A1 can be seen as good enzyme yielder with in short period of time. This could therefore suggest that using isolate A1 might

offer significant economic advantage reducing the cost of production for these enzymes.

The chitinase from isolate A1 was active in a broad range of pH with maximum activity at 8.5 and displayed more than 80 % of its peak activity between pH 8 and 11. The enzyme also showed good stability in the alkaline pH range maintaining 97 % of its original activity at pH 9. Most chitinases known so far are optimally active at slightly acidic to neutral pH (Wang and Chang, 1997; Kim *et al.,* 2003). To date few chitinases are reported to be active and stable in the alkaline range (Tsujibo *et al.*, 1997). Since chitin wastes from sea food industries are alkaline in their nature (Agullo, *et al.* 2003), chitinase A1 has tremendous potential for chitin hydrolysis. The hydrolysate can be used for the production of single cell protein, ethanol, fertilizer and other useful bioactive molecules (Bhushan, 2000).

Most chitinases are active and stable in the temperature range of 35-50 °C (Konagaya *et al.,* 2006; Tsujibo *et al.,* 2003). Only few chitinases from thermophillic bacteria were reported to show optimum activity between 70-90 °C (Sakai *et al.,* 1998; Bhushan, 2000). Chitinase A1 in this study showed maximum activity at 45 °C and above 55 °C its activity dropped gradually.

Chitinase A1 did not show much thermal stability, especially, at higher temperatures of over 50 °C where more than half of relative activity was lost. Low thermal stability could be due to the presence of proteases in the culture. The protease present in this culture might be mixture of enzymes which need inhibition using multiple inhibitors. One option to improve thermal stability and other qualities could be to clone the gene and express in appropriate hosts which could also help to avoid degradation by protease.

There was no increase in protease production by A1 was observed up on addition of different nitrogen sources such as peptone and casein. Although addition of casein was expected to increase protease production, nevertheless it showed lower yield and the reason is unclear. However, protease production by isolate A1 was increased by 22.4 % up on addition of yeast extract. This might be for the fact that other than serving as a nitrogen source, yeast extract provides some growth factors, such as

vitamins, required for growth. Other organisms are also known to require yeast extract for improved enzyme production (Mehta *et al.,* 2006).

Isolate A1 can, therefore, be considered as an efficient consumer of colloidal chitin as a nitrogen source for protease production with no need of supplementation. Since chitin is cheap and abundant natural biopolymer, isolate A1 could offer remarkable opportunity for profitable industrial protease production. Moreover, using enzymes of this isolate could provide vast possibility for bioconversion of industrial chitin wastes to valuable industrial products and for environmental wellbeing.

Supplementation of glucose (0.5 %) as a carbon source has been reported to improve protease yield of alkaliphilic actinomycete (Mehta *et al.,* 2006) where beyond this concentration it showed repressing effect. However, incorporation of different carbon sources did not bring about an increase in protease production in this study. The overall protease production by an un-supplemented control was similar to and/or greater than the supplemented cultures. This might show that isolate A1 can produce significant amount of alkaline protease without the need of supplementation of carbon or energy source. What is interesting here is that protease production by isolate A1 was induced by chitin, and mushroom and chitin have supported production of both chitinase and protease. This indicates that the genes for production of protease and chitinase might be controlled by the same promoter.

The chitin level of 0.4 % is reported to be optimum for growth of actinomycetes from soil and water (Hsu Lockwood, 1975). In this study, varying the level of colloidal chitin from 0.3 % to 0.5 % did not show any difference on protease production by isolate A1 except for reduced amount in the level 0.2 %. Therefore this might indicate that the chitin level of 0.3% can sufficiently support protease production.

The optimum pH range of alkaline proteases is generally between pH 9 and 11 with few exceptions of higher optima at pH 11.5-13. They are also commonly stable between pH 6 and 12 (Kumar and Takagi, 1999; Miyaji *et al.,* 2005). Detergent and tanning industries use alkaline enzymes with good activity and stability in the alkaline pH range since high activity offer effective cleaning capacity of proteinaceous matters. Protease A1 was optimally active at pH 10.5 and has broad range of pH

activity and stability. Hence, this enzyme can effectively be applied in detergent industry. Moreover, alkaline conditions enable the swelling of hair roots and subsequent attack of proteases on the hair follicle protein allowing easy removal of the hair. Therefore its activity and stability over broad range of pH makes protease A1 a promising candidate in dehairing and bating processes of tanning industries.

Alkaline proteases are characterised by their optimum temperatures between 50 °C and 70 °C (Kumar and Takagi, 1999). Protease A1 was active and stable over a wide range of temperature with the maximum activity at 55 °C. Since temperature stability is one of the limiting factors of enzymatic activity under application conditions (Amare Gessesse *et al.,* 2003), protease A1 can be a promising alternative for detergent industries.

Protease A1 did not require Ca^{2+} for activity and stability. Previous works showed that alkaline proteases commonly require divalent cations like Ca^{2+} for activity and stability. It is believed that these cations protect the enzyme against thermal denaturation and play a very important role in maintaining active conformation of the enzyme at high temperatures (Kumar and Takagi, 1999). Consequently, since it was stable with out the need of $CaCl_2$, the enzyme from this isolate can be taken as a good alternative for industrial application where higher temperature can affect enzymatic activity.

In the process of detergent formulation, where alkaline proteases are commonly added, chelating agents are included to overcome the problem of water hardness. In the presence of such chelating agents, however, the Ca^{2+} from the weak binding site can easily be stripped off thus greatly affecting the thermal stability of the detergent enzyme under application conditions. Enzymes that do not require Ca^{2+} for stability could therefore offer tremendous potential for detergent application (Amare Gessesse, *et al.* 2003) and protease A1 could be an attractive alternate in this regard.

6. Conclusion

- This work showed that Ethiopian soda lakes may be rich in alkaliphilic chitinolytic actinomycetes with vast potential in industrial applications.

- Isolate A1 grew in high pH value which might have advantages to reduce the level of contamination during production process since only few organisms are capable of growing under alkaline conditions.

- Isolate A1 produced alkaline chitinase and protease optimally active and stable in alkaline pH ranges which can have wide industrial applications.

- Isolate A1 produced alkaline chitinase and protease using chitin as sole source of carbon and nitrogen which could have economic advantage for enzyme production since chitin is abundant and cheap industrial waste.

- Protease A1 is optimally active and stable in wide ranges of temperature with out need of Ca^{2+} and might have significant advantages for industrial application.

- Since temperature stability is one of the limiting factors of enzymatic activity under application conditions, protease A1 can be a promising alternative for detergent industries.

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