Addis Ababa University
School of Graduate Studies
Institute of Biotechnology

Optimization, Characterization, and Potential Industrial Applications of Alkaline Protease isolated from Alkaliphilic *Actinobacteria* BACC-15

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Abstract

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Microbial alkaline proteases are stimulating tremendous interests in the research and enzyme market since there is a great demand for developing biotechnological alternatives for many industrial processes. Hence, this study was conducted with the aim of screening for the best alkaline protease producing alkaliphilic Actinobacteria*, optimizing enzyme production in SSF; characterizing and investigation of the different industrial applications. One best producer isolate (*Actinobacteria *BACC-15) was selected based on different parameters. Alkaline protease production by BACC-15 in SSF was maximum at 37℃ (591.67±12.99 U/g) and 1:1.8 initial moisture content (595.75±9.60 U/g). The enzyme was active and stable in the pH ranges of 6.0-11.5, with optimum activity at pH 10.0 and stability at pH 7.5-9.5. It was active in temperature range of 35-75 ℃, with optimum activity at 60 ℃ and 65 ℃ in presence of 5mM Ca^{2+} and highly stable at 30-50℃. The enzyme was active and stable in presence of NaCl, and 8.62% enhancement was observed at 3M. Presence of high concentrations of H_2O_2, $NaBO_3.H_2O$, SDS, Tween-80, Triton X-100, sodium citrate and saponins had no effect on enzyme stability. The protease (84.21U/g) alone or 25% protease + 75% endod preparation in combination completely removed proteinaceous stains. Using 1% w/v E/S ratio, BACC-15 alkaline protease hydrolyzed SBPI (DH, 77.09%) and MSPI (DH, 66.19%). SBPH and MSPH were suitably used for growing pathogenic bacteria with PR of ≥1 compared to commercial peptone. Therefore, BACC-15 alkaline protease is potential candidate in many industrial applications as alternative to environmentally unfriendly chemicals and production of valuable products.*

Key words: Alkaline protease, biodetergent formulation, isolates BACC-15, protein hydrolysates, SSF.

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List of Abbreviations and Acronyms

ANOVA	Analysis of variance
DH	Degree of hydrolysis
EDTA	Ethylene-diamine tetra acetic acid
g	Gram
KDa	Kilo dalton
L	Liter
M	Molar
Mb	Mega base pair
µg	Micro gram
µl	Micro liter
ml	Milliliter
mM	Milli mololar
MSPH	Moringa seed protein hydrolysates
MSPI	Moringa seed protein isolate
nm	Nanometer
O.D	Optical density
PMSF	Phenylmethylsulphonyl fluoride
PR	Productivity ratio
rpm	Revolutions per minute
SBPI	Soy bean protein isolate
SBSPH	Soy bean seed protein hydrolysates
SDS	Sodium dodecyl sulphate
SmF	Submerged state fermentation
SSF	Solid state fermentation
TCA	Tri -Chloro Acetic acid
U	Protease activity unit
U/g	Unit per gram
U/ml	Unit per milliliter

1. Introduction

Many chemical transformation processes using inorganic catalysts in various industries have inherent drawbacks from an economic, energy and environmental point of view (Jegannathan and Nielsen, 2012; Li *et al.,* 2012). However, all of these drawbacks can be virtually eliminated by using biocatalysts and or industrial enzymes (Buchholz *et al.*, 2012). Biocatalysis offer green and clean solutions to chemical process and are emerging as a challenging and revered alternative to chemical technology (Prakash *et al*., 2013). As a result, enzymatic conversion has recently received attention and is becoming a favorable alternative to inorganic catalysts.

In the 21st century, extensive application of bioprocesses has created conducive environment for many scientists and engineers to expand knowledge and interest in biotechnology. One of the useful applications of biotechnology is the use of microorganisms to produce enzymes which are used in the laboratory, diagnostic and many industrial processes (Buchholz *et al.,* 2012).

Biotechnology industry is a dynamic and rapidly changing industry. Enzyme industry, at the heart of biotech industries, is growing dynamically and providing wide varieties of job opportunities and income globally (Binod *et al.,* 2013). The global market for industrial enzymes was valued at $3.1 billion in 2009 and $3.6 billion in 2010. The market for 2011 totaled $3.9 billion. This market is expected to reach $6 billion by 2016 with a compounded annual growth rate (CAGR) of 9.1% in 2016 (BCC, 2012). Of industrial enzymes, 75% are hydrolytic enzymes (Li *et al.*, 2012). Proteases constitute the largest product segment in industrial enzymes market with 60-65% and alkaline protease accounts at least 25% of the total worldwide sales of enzymes, of which two –thirds of proteases produced commercially being of microbial origin (Sarrouh *et al.,* 2012).

Although proteolytic enzymes are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth and the limited space required for their cultivation (Ray, 2012). Further, microbial proteases can easily be produced in large quantities, and genetic manipulation to increase their activity is relatively simple to

achieve (Gupta *et al.,* 2002). Alkaliphilic *Actinobacteria*, inhabiting in the soda lakes, are one of these microorganisms which grow at high pH and salt levels.

The *Actinobacteria* are aerobic or facultative anaerobic filamentous bacteria that form branching mycelia superficially similar to those of the fungi. They have high GC content, and are gram-positive bacteria with small mycelia (Hogg, 2005). They are well known for the production of antibiotics (Nanjwade *et al.*, 2010). Several strains of *Actinobacteria* were shown to have extraordinary capacity for the production of different industrial enzymes. Xylanase (Porsuk *et al.,* 2013) and alkaline protease (Hameş-Kocabaş and Uzel, 2007; Gohel and Singh, 2013; Manivasagan *et al.*, 2013) with high activity are some of these enzymes to mention.

Amongst the industrially important enzymes, microbial alkaline proteases are stimulating tremendous interests in the enzyme market owing to the fact that there is a great demand for developing biotechnological alternatives for many industrial processes (Singhal *et al.,* 2012). Kumar and Takagi (1999) have indicated that alkaline protease mediated process provides a viable alternative to the use of hazardous, expensive, and environmentally unsafe chemicals used in many industries. Today alkaline proteases are the largest groups of industrial enzymes and find application in detergent (Haddar *et al.,* 2010), leather tanning (Verma *et al.,* 2011), textile (Chen *et al.,* 2007), and food and feed processing industries (Li and Youravong, 2010). Other applications include silver recovery from used X-ray films and gelatin decomposition (Nakiboğlu *et al.,* 2009), cosmetics, medicine and pharmaceuticals (Simkhada *et al.,* 2010; Mukherjee and Rai, 2011), waste management (Amare Gessesse *et al.,* 2003a), and bioremediation processes (Jensen *et al.,* 2010; Kornilłowicz-Kowalska and Bohacz, 2011).

There is a need for production of enzymes particularly alkaline proteases in line with the flourishing number of industries in Ethiopia like leather industries, to minimize the use of toxic chemicals. To produce enzymes at industrial level, conducting continuous stream of new research on screening of hyper producer extremophilic organisms from rich diversity in Ethiopia is needed.

Currently extensive investigations were carried out on enzymatic and physicochemical properties of alkaline protease from genus *Bacillus, Vibrio* and other alkaliphilic bacteria. However, there is lack of research on the production, scale - up and applications of alkaline proteases from the alkaliphilic *Actinobacteria* in soda lakes of Ethiopia. Therefore, the present study was conducted with the aim of producing, characterizing and investigating different applications of alkaline protease from alkaliphilic *Actinobacteria* isolated from Chitu, Ethiopian Soda Lake.

2. Objectives of the study

2.1. General objective of the study

- The main objective of this study was to identify alkaline protease producing alkaliphilic strains, optimize enzyme production through solid state fermentation, and evaluate its industrial application.

2.2. Specific objectives of the study

- To select alkaline protease producing alkaliphilic strains and optimize enzyme production through solid state fermentation ;
- To characterize the enzyme and assess its potential biotechnological applications.

3. Literature Review

3.1. *Actinobacteria*

The *Actinobacteria* are filamentous bacteria that form branching mycelia superficially similar to those of the fungi (Hogg, 2005) and have elevated G+C content in their genome. Their name was derived from the fact that many members of the group have the tendency to form filaments or hyphae (*actinis*, Greek for ray or beam (Okafor, 2007). *Actinomycetes* defined on morphological basis as microorganisms with thin, elongated cells or filaments that show some degree of true branching or with the ability to form branching hyphae at some stage of their development (Goodfellow and Williams, 1983).

Actinobacteria were originally classified as fungi because of possession of branching filamentous hyphae which somewhat resemble the mycelia of the fungi. In fact they are unrelated to fungi, but are regarded as bacteria for the following reasons. First they have peptidoglycan in their cell walls, and second they are about 1.0 μm in diameter (never more than 1.5 μm), whereas fungi are at least twice of that size in diameter (Okafor, 2007). Thirdly, the hyphal filaments of *Actinomycetes* are finer than are those for most fungi, wing from 0.5 to 1.0 pm in diameter (Hogg, 2005).

Currently it is evident that possession of branching filaments does not automatically place an organism in the *Actinobacteria*, nor does the absence of branching filaments necessarily exclude from this group of bacteria (Goodfellow and Williams, 1983). Therefore, the use of morphological, physiological and phylogenetic characteristics gives broader and wider concepts and modifies traditional view. As a result, the *Actinobacteria* broadly defined as a group of gram- positive bacteria; mostly aerobic and some facultative anaerobes as in case of *Actinomyces israelii (*Hogan*,* 2013*)* with thin elongated and branching filaments or no filamentous and rich in G+C content (57-75%) in their genome. *Actinobacteria* genome sizes range from the 2.5-Mb skin commensal strain *Micrococcus luteus* to the 9.7 Mb environmental strain *Rhodococcus jostii* (Miao and Davies, 2010).

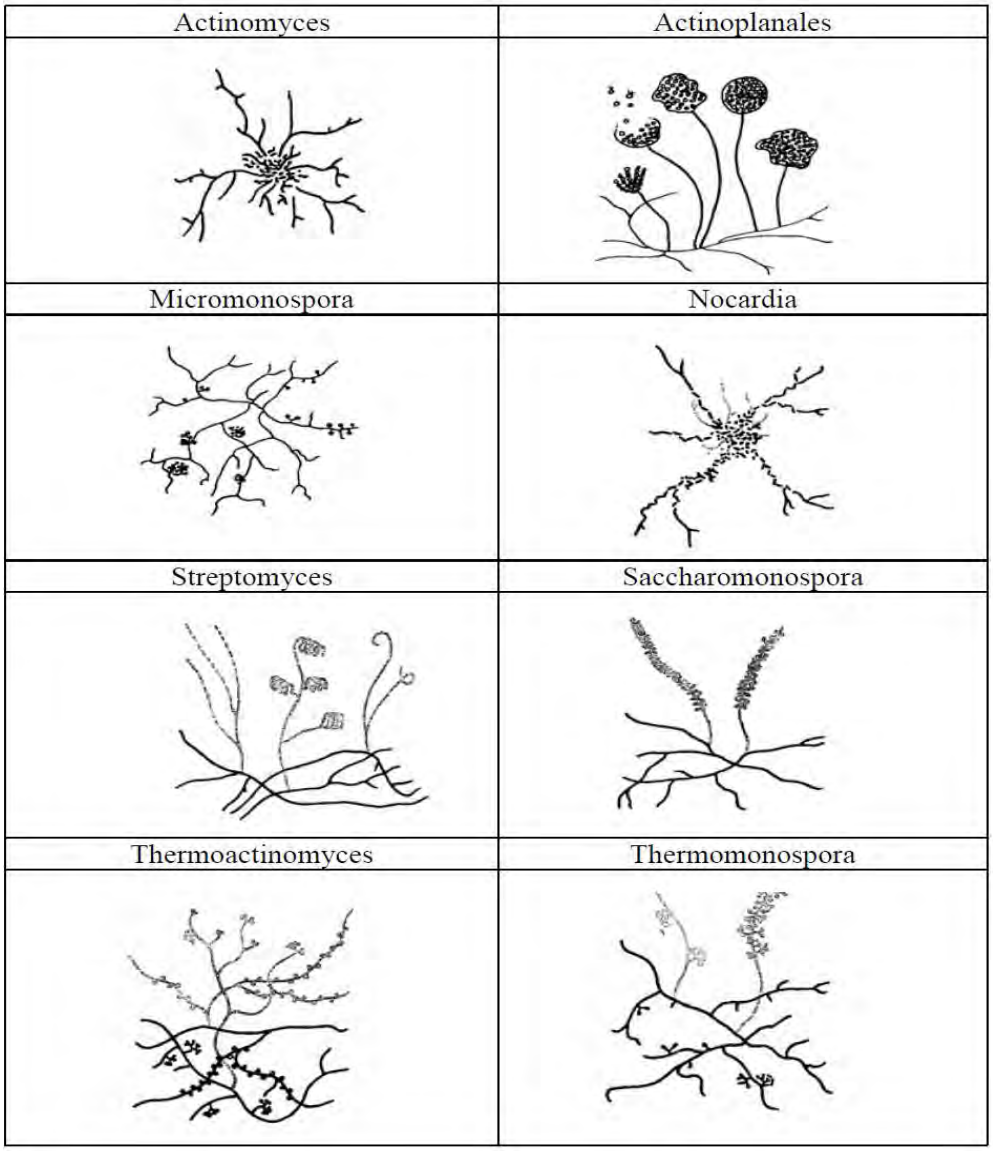


Fig 1. The morphology and structure of different Actinobacteria, Source: (Okafor, 2007).

3.1.1. Morphological and physiological diversity of *Actinobacteria*

The *Actinobacteria* exhibit a wide range of morphology from coccoid or spherical cells (*Micrococcus*) or rod-coccoid, slender and long (genera *Arthrobacter* and *Cellulomonas)*, fragmenting hyphal forms (*Nocardia*) to those with permanent and highly differentiated branched mycelium (genera *Streptomyces* and relatives*)*. Many *Actinobacteria* form spores, which range from motile zoospores to specialized propagules (Goodfellow and Williams, 1983; Gao *et al.,* 2006; Glazer and Nikaido, 2007; Okafor, 2007).

Actinobacteria are also physiologically very diverse as evidenced by their production of numerous extracellular enzymes for degradation of many polymers, and by the thousands of metabolic products (including antibiotics) they synthesize and excrete (Gao *et al.,* 2006). They are aerobes or facultative anaerobes (Goodfellow, 2012).

3.1.2. Ecology of *Actinobacteria*

The *Actinobacteria* has widespread occurrence in soils, freshwater and marine ecosystems but they are primarily soil dwellers (Okafor, 2007). The *Actinobacteria* are a successful group of bacteria that live in extreme environments (Goodfellow and Williams, 1983; Miao and Davies, 2010; Singh *et al.,* 2013). Alkaline soda lakes are one of these extreme environments with respect to pH and salinity. They are rich sources of many *Actinobacteria.* In a research conducted at different soda lakes in Kenya (Mwirichia *et al.,* 2010), in Russia in gradient of soda lakes of the Kulunda Steppe (Foti *et al.,* 2008), Hungary (Borsodi *et al.,* 2005), Turkey (López-García *et al.,* 2005) and in Egypt in hyper saline lake of Wadi An Natrun (Mesbah *et al.,* 2007) hundreds of novel *Actinobacteria* species were reported.

3.2. Extracellular enzymes production potentials of *Actinobacteria*

Apart from the ability of production of diverse range of antibiotics and secondary metabolites, *Actinobacteria* also produce different extracellular enzymes. *Actinobacteria* extracellular enzymes are more attractive than enzymes from other sources because of their high stability and unusual substrate specificity (Prakash *et al.,* 2013). The potential of thermophilic *Actinobacteria* in producing extracellular hydrolytic enzymes ranging from amylase, protease, lipase, and cellulase to xylanase was reported by Singh *et al.* (2013). Table 1 shows extracellular enzymes from *Actinobacteria*.

Table 1. List of some of industrial enzymes from *Actinobacteria* and their characteristics

Enzyme	Producing strain	pH stability	Thermo-stability	Substrate specificity	References
Amylase	*Nocardiopsis sp.*	5.0–10.0	35–45 ℃	Starch	(Stamford *et al.,* 2001)
Protease	*Streptomyces albidoflavus*	6.0-11.0	40 ℃	Casein	(El-Shafei *et al.,* 2010)
	Streptomyces microflavus	7.0	40 ℃	Casein	(Rifaat *et al.,* 2005)
	Nocardiopsis prasina	6.0-12.0	40-55 ℃	Casein	(Ningthoujam *et al.,* 2009)
	Actinobacterium Actinoalloteichus	8.0-12.0	50-60 ℃	Feather powder	(Manivasagan *et al.,* 2013)
Collagenase	*Streptomyces exfoliatus*	7.0-9.0	30-80 ℃	Azocollagenase	(Jain and Jain, 2010)
Xylanase	*Streptomyces* species	5.0-8.0	50℃	Spent xylan	(Porsuk *et al.,* 2013)
Chitosanase	*Streptomyces* species	5.5	45 ℃	Chitosan	(Sinha *et al.,* 2012)
Pectinase	*Streptomyces* species	6.0-9.0	50-60℃	Citrus pectin	(Kuhad *et al.,* 2004)
Lipase	*Streptomyces* species	7.5	50-60℃	Tributyrin	(Meena *et al.,* 2013)
Cellulase	*Streptomyces* species	7.0	-	CMC	(Meena *et al.,* 2013)

3.3. Cultivation conditions for alkaline protease production

Actinobacteria are well known to produce alkaline protease in two different states of fermentation, *Viz*. solid state fermentation (SSF) (Siddique *et al.,* 2001; Lazim *et al.,* 2009; Vijayaraghavan *et al.,* 2012) and submerged state fermentation (SmF) (Ferracini-Santos and Sato, 2009; Lazim *et al.,* 2009; El-Gammal *et al.,* 2012).

3.3.1. Solid state fermentation (SSF)

Solid state fermentation is defined as the growth of microorganisms on moist solid supports in the absence or near-absence of free water. However, the substrate must possess enough moisture to support the growth and metabolic activity of the microorganism. The solid matrix could be both the source of carbon (and other nutrients), or it could be an inert material to support the growth of the microorganisms on it (Hölker *et al.,* 2004; Pandey *et al.,* 2008; Thomas *et al.,* 2013). Solid-state fermentation involves the utilization of water insoluble material for microbial growth and metabolism and is usually carried out in solid or semi solid systems in the shortage of available water (Pandey, 2003; Hölker *et al.,* 2004). Recently, SSF has generated much interest, because of lower manufacturing costs by utilizing unprocessed or moderately processed raw materials.

Solid state fermentation has a number of advantages over submerged fermentation. Its advantages include superior volumetric productivity, higher concentration of enzyme to facilitate recovery, simplicity of process operation, and easier contamination control. In addition, lower energy requirements; use of inexpensive agro-industrial by-products as substrates, low waste water output, and less solvent requirement for recovery of the enzyme significantly reduce enzyme production costs (Hölker *et al.,* 2004; Sandhya *et al.,* 2005; Pandey *et al.,* 2008; Nigam and Pandey, 2009; Paranthaman *et al.,* 2009; Chen, 2013; Thomas *et al.,* 2013).

3.3.2. Submerged Fermentation (SmF)

Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. Industrial enzymes can be produced using this process. This involves growing carefully selected micro organisms in closed vessels containing a rich broth of nutrients (the fermentation medium) and a high concentration of oxygen. As the microorganisms break down the nutrients, they release the desired enzymes into solution (Renge *et al.,* 2012). Approximately 90% of all industrial enzymes are produced in SmF, frequently using specifically optimized, genetically manipulated microorganisms. In this respect SmF

processing offers an insurmountable advantage over SSF (Hölker *et al.,* 2004; Chen, 2013).

Generally, the identification of the physiology of the microorganisms and the physico-chemical factors where it grows leads to the development of process parameters which are required for its optimal growth. These factors include temperature, pH, aeration, salinity, water activity and moisture, nature of solid substrate employed, including the particle size, etc. These must be optimized using different biotechnological tools (Nigam and Pandey, 2009; Chen, 2013).

3.4. Chemistry of proteases

3.4.1. General introduction to proteases

Proteases are hydrolytic enzymes that catalyze the hydrolysis of peptide bonds in proteins. They are necessary for the survival of all living things and are encoded by about 2% of genes in all kinds of organisms (Rawlings *et al.,* 2007). There are about 500 human genes that encode peptidases and their homolog (Polgar, 2005). They are ubiquitous found in a wide diversity of sources such as plants, animals and microorganisms (Gupta *et al.,* 2002). Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields (McAuliffe, 2012).

Proteases are important for many cellular and biological processes including digestion of food proteins, recycling of intracellular proteins, blood coagulation and lysis of fibrin clots, and antigen presentation (Rawlings *et al.,* 2007; McAuliffe, 2012). They also conduct highly specific and selective modifications of proteins such as activation of zymogenic forms of enzymes by limited proteolysis (Rawlings *et al.,* 2007). Furthermore, they perform processing and transport of secretory proteins across the membranes and activation of a variety of proteins, including enzymes, peptide hormones, and neurotransmitters (Rao *et al.,* 1998; Rawlings *et al.,* 2007). In addition, pathogenic viruses and bacteria use proteases in their life cycle for infection of host cells. Therefore,

proteases are important targets for drug design against a diverse set of diseases (Vandeputte-Rutten and Gros, 2002).

Proteases represent one of the three largest groups of industrial enzymes and account for about 60-65% of the total worldwide sale of enzymes (Sarrouh *et al.,* 2012). There are many industrial uses for proteases, though often mixtures (crude proteases) rather than purified enzymes are used (Rawlings *et al.,* 2007). They are used in cheese making, meat tenderization, clarifying beers, production of protein hydrolasates, leather industry in bating and soaking of the leather, cosmetics, silver recovery, waste treatment, peptide synthesis, detergents as cleaning agents, drugs and medicine to remove gastrointestinal parasites (anthelminthics), removal of dead skin from burn patients (debridement), determination of blood groups, and for relief of back pain by digesting the cartilage content of herniated inter vertebral discs (chemonucleolysis) (Rao *et al.,* 1998; Kumar and Takagi, 1999; Gupta *et al.,* 2002; Rawlings *et al.,* 2007; McAuliffe, 2012). In the laboratory they are used for proteolysis of proteins, processing of recombinant fusion proteins, and protein sequencing (Rawlings *et al.,* 2007; McAuliffe, 2012). Therefore, proteases are an exceptionally important group of enzymes in biology, medical research and biotechnology.

3.4.2. Classification of proteases

Since there is huge diversity in action and structure among proteases, it is not easy to classify them. According to Rao *et al.* (1998) proteases are classified based on three major criteria: *viz.* type of reaction they catalyze, chemical nature of the catalytic site, and their composition and structure.

Proteases can be divided into two overall categories based on catalytic site on substrates, *viz.* exoproteases (i.e., peptidases) that hydrolyze terminal peptide bonds releasing free amino acids, and endopeptidases that hydrolyze internal peptide bonds and convert proteins into smaller peptides (McAuliffe, 2012). Based on site of action at the N or C terminus, exoproteases are classified as amino- and carboxypeptidases. Aminopeptidases act at a free N- terminus of the polypeptide chain and liberate a single amino acid residue,

a dipeptide, or a tripeptide. Aminopeptidases are mostly intracellular enzymes. Conversely, carboxypeptidases act at C -terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases again subdivided into three major groups; serine carboxypeptidases, metallocarboxypeptidases, and cysteine carboxypeptidases based on the nature of the amino acid residues at the active site of the enzymes (Rao *et al.,* 1998; Rawlings *et al.,* 2007; McAuliffe, 2012).

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini. Based on catalytic mechanism or functional group present at active site, endopeptidases are classified into Serine proteases (EC 3.4.21), Cysteine proteases (EC 3.4.22), Aspartic acid proteases (EC 3.4.23) and Metalloproteases (EC 3.4.24). This classification is determined by amino acid sequence and through reactivity toward inhibitors that act on particular amino acid residues in the active site region of the enzyme (Rao *et al.,* 1998; Rawlings *et al.,* 2007).

Based on pH optima, proteases are classified in to (a) acidic proteases are active in the pH range of 2.0 – 6.0. They are mainly obtained from fungi. They include aspartic proteases and also some of the cysteine proteases and metalloproteases; (b) neutral proteases are active in the pH range of 5.0-8.0. They are of mainly plant and few of microbial and fungal origins. They include more of cysteine proteases and some metallo-proteases (Rao *et al.,* 1998); (C) Alkaline proteases are with the pH optima of 8.0-13.0. They are mainly of microbial origin especially from *Bacillus* and *Streptomyces* species. They mostly belong to serine proteases and some metallo-proteases (Rao *et al.,* 1998; Kumar and Takagi, 1999). Based on sources proteases are also classified as animal, plant and microbial proteases. Microbial proteases have highest commercial value and comparative advantage over plant and animal proteases (Rao *et al.,* 1998).

3.4.3. Mechanism of catalysis

Proteases catalyze amide (peptide) bond hydrolysis in protein or peptide substrates (Fig 2). The catalytic type of a protease is related to the chemical groups responsible for its catalysis of peptide bond hydrolysis (Rawlings *et al.,* 2007). Therefore, based on catalytic residue on active sites the mechanism of catalysis differs from one type to another.

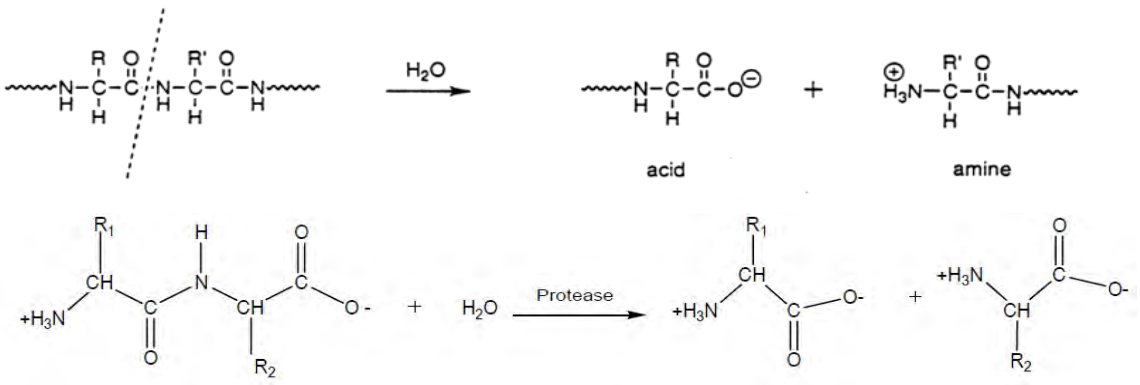
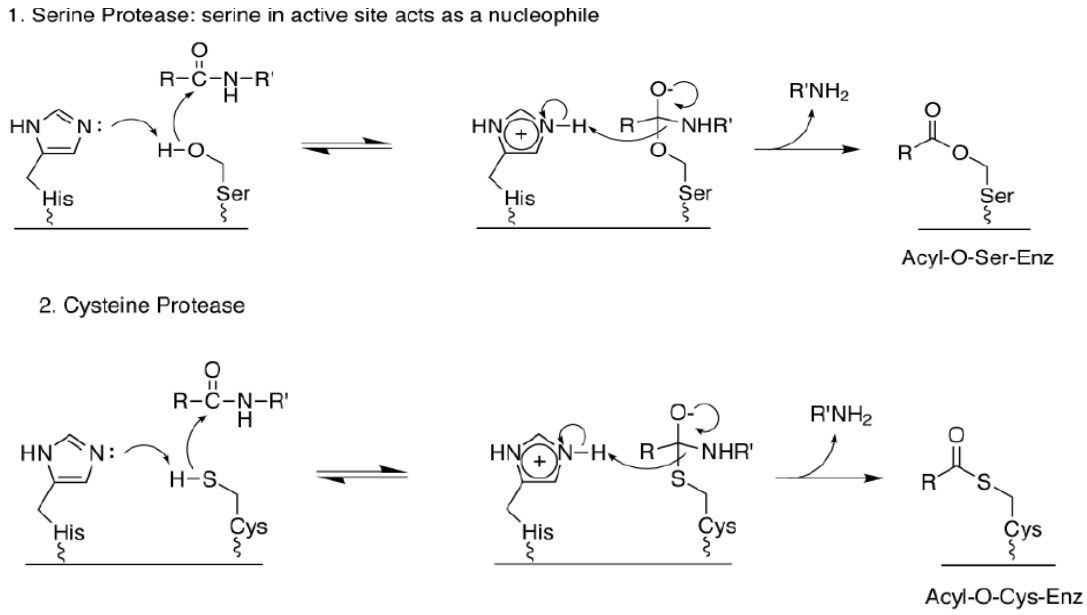


Fig 2. Schematic representation of peptide bond hydrolysis by proteases
Source: Frey and Hegeman (2007).

There are four specific catalytic types that are recognized, *viz.* serine, cysteine, aspartic, and metallo- proteases (Rao *et al.,* 1998; Rawlings *et al.,* 2007). In proteases of serine and cysteine type, the catalytic nucleophile is the reactive group of an amino acid side chain, either a hydroxyl group (serine peptidases) or a sulfhydryl group (cysteine peptidases) (Polgar, 2005; Rawlings *et al.,* 2007). In aspartic and metallo- peptidases, the nucleophile is commonly an activated water molecule. In aspartic proteases, the water molecule is directly bound by the side chains of aspartic residues. In metallopeptidases, one or two divalent metal ions hold the water molecule in place, and charged amino acid side chains are ligands for the metal ions (Rawlings and Barrett, 2004; Rawlings and Barrett, 2013). The metal is most commonly zinc, but may also be cobalt, manganese or copper. A single metal ion is usually bound by three amino acid ligands. The activated water molecule is a fourth metal ligand, and the metal is described as "tetrahedrally co-ordinated". Where two metal ions are present, each is tetrahedrally co-ordinated, so that two activated water molecules are bound, and one amino acid residue ligates both metals (Rao *et al.,* 1998; Rawlings and Barrett, 2004; Rawlings *et al.,* 2007).

Besides the nucleophile, other residues are important for catalysis and maintaining the structure of the active site. The active site residues are very well conserved between all the active peptidases within a family (Rawlings *et al.,* 2007). In general terms, cleavage of a peptide bond has been described as an example of an acid/base reaction, in which the charged nucleophile is the proton donor and a residue known as the general base is the proton acceptor. In serine and cysteine peptidases the general base is often a histidine, but can be a lysine. When the general base is a histidine, usually a third residue orientates the imidazolium ring of the histidine and helps charge one of the nitrogen atoms in the ring (Hedstrom, 2002; Polgar, 2005; Rawlings *et al.,* 2007). In serine and cysteine peptidases, a fourth residue is often important because it helps stabilize the transitional acyl-intermediate that forms between the peptidase and the substrate as a first stage of catalysis. Residue forms a hydrogen bond with the negatively charged oxygen atom, and this catalytic sub site is known as the oxyanion hole (Hedstrom, 2002; Rawlings *et al.,* 2007). Fig 3 below shows catalytic mechanisms of four different types of proteases.



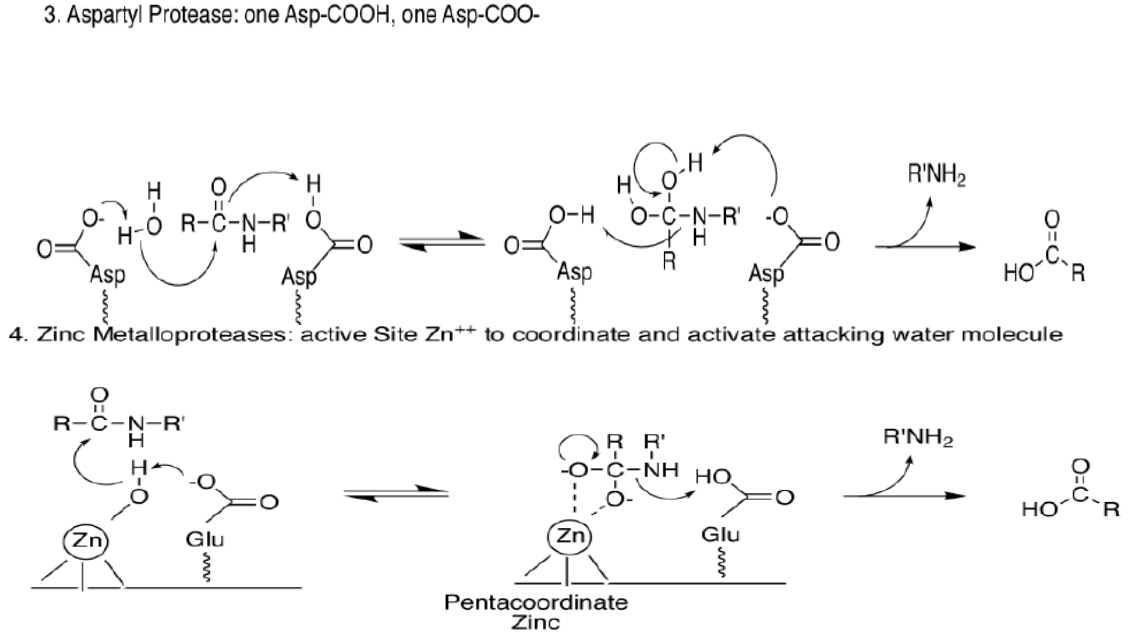


Fig 3. Schematic representation of four mechanistic variations of proteases to activate a peptide bond for hydrolysis.
Source: Frey and Hegeman (2007).

3.5. Characteristics of alkaline proteases

Proteases of commercial importance especially from microorganisms have enormous applications in a range of processes which take the advantage of the unique physical and catalytic properties of the individual proteolytic enzyme types (Rao *et al.,* 1998).

Bacterial alkaline proteases are characterized by their broad substrate specificity and activity against a number of synthetic substrates and natural proteins. They are active against casein than against azocasein, hemoglobin or bovine serum albumin (BSA) (Jisha *et al.,* 2013). Alkaline proteases are also specific against aromatic or hydrophobic amino acid residues such as tyrosine, phenylalanine or leucine at the carboxylic side of the cleavage site (Rao *et al.,* 1998; Gupta *et al.,* 2002).

The optimal temperature of alkaline proteases for activity is around 60 ℃ (Rao *et al.,* 1998), but majority have temperature ranges between 50 and 70 ℃ with a few exceptions of extreme temperatures between 80–90 ℃ (Gupta *et al.,* 2002). The pH range of alkaline proteases is generally between 9.0 -11.0 with a few exceptions of higher pH optima of

11.5-13.0. They are generally stable between pH 6.0 and 12.0. Alkaline proteases from *Bacillus* species, *Streptomyces* species and *Thermus* species are quite stable at high temperatures, and the addition of Ca^{2+} further enhanced enzyme thermostability (Rao *et al.,* 1998; Kumar and Takagi, 1999).

Most alkaline proteases have a high iso-electric point which is near to their pH optimum in the range of 8.0–11.0 (Gupta *et al.,* 2002). Kumar and Takagi (1999) indicated that the molecular mass of alkaline proteases ranges from 15 to 30 kDa with a few exceptions like with lowest molecular weight of 8 kDa to as high as 45- 90 kDa in SDS-PAGE analysis.

Alkaline proteases require a divalent cation like $Ca^{2+,}$ $Mg^{2+,}$ and Mn^{2+} or a combination of these cations, for maximum activity and thermal stability. These cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures (Kumar and Takagi, 1999). On the other hand, various studies have shown in the inhibitory effects of heavy metals like Cd^{2+}, Hg^{2+}, and Cu^{2+}.

Inhibition studies are important to know the nature of enzyme, its cofactor requirements and the nature of active site (Rawlings *et al.,* 2007; McAuliffe, 2012). Most alkaline proteases from bacteria belong to serine proteases and rarely metallo-proteases. As a result they are completely inhibited by serine inhibitors phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP) (Rao *et al.,* 1998). Alkaline proteases are also inhibited by metallo protease inhibitors like ethylene-diamine tetra acetic acid (EDTA) and 1, 10 - phenantroline (Kumar and Takagi, 1999; Gupta *et al.,* 2002; McAuliffe, 2012).

3.6. Industrial applications of alkaline proteases

Alkaline proteases (E.C. 3.4.21-24, 99) are physiologically and commercially most important group of enzymes and find multiple applications in various industries. They find a potential industrial applications in detergent formulations, leather processing, textile processing, peptide synthesis, silver recovery, medical purposes, food and feeds processing, waste treatment, and chemical industries (Rao *et al.,* 1998; Kumar and Takagi, 1999; Gupta *et al.,* 2002; Donlon, 2007; Rawlings *et al.,* 2007).

3.6.1. Applications of alkaline protease in biodetergent formulations

The largest industrial application of alkaline proteases is in detergents detergent industry (Donlon, 2007). Proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures (Rao *et al.,* 1998). Alkaline proteases have long been of interest to the detergent industry for their ability to aid in the removal of proteinaceous stains and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies (Gupta *et al.,* 2002). The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes (Rao *et al.,* 1998).

The ideal detergent protease should possess broad substrate specificity to facilitate the removal of a large variety of stains due to food, egg, blood, milk, and other body secretions (Saeki *et al.,* 2007). They must be active and stable at high alkaline pH and temperatures and in the presence of sequestering agents, oxidizing agents, chelating agents, and surfactants added to the detergents (Rao *et al.,* 1998; Donlon, 2007). Among various classes of proteases, alkaline serine proteases are most appropriate and potentially suited to inclusion as detergents additives since they fulfill the above mentioned criteria (Donlon, 2007; Saeki *et al.,* 2007).

3.6.2. Applications of alkaline protease in leather processing

Leather industry is currently facing tremendous pressure from various pollution control bodies because of huge amount of pollution associated with processing (Kanagaraj, 2009). However, it has been contributing significantly to economic development, employment, and export potentials in many developing countries (Sundararajan *et al.,* 2011). In this industry, pre-tanning and tanning processes contribute 80–90% of the total pollution and generate noxious gases, such as hydrogen sulfide, as well as solid wastes, such as lime and chrome sludge (Thanikaivelan *et al.,* 2004).

Soaking, liming, dehairing, deliming, bating, degreasing and pickling are the steps carried out to obtain processed leather. All these steps are carried out by using quite toxic chemicals *viz.* lime, sodium sulphide, salt, solvents, etc. Thus, this contributes to environmental pollution (Kumar and Takagi, 1999). As a result, the use of enzyme-based technologies for leather processing has potential advantages over conventional methods for the reduction of pollution problem (Thanikaivelan *et al.,* 2004; Kanagaraj, 2009; Østergaard and Olsen, 2011). In addition, this biotreament method also enhances the speed, provides an easy control over the process, and finally produces clean and quality leather (Ward *et al.,* 2009).

Soaking is the process to restore moisture in the raw hide, making the hide flexible, removing salts and globular proteins and improving the penetration of chemicals later used (Østergaard and Olsen, 2011). It has been found that time for soaking is greatly reduced by applications of microbial alkaline proteases because they improve the water absorption by degrading noncollagenous protein (Ward *et al.,* 2009). Liming is a process to degrade the epidermal structure of the hide and skin, including the hair. The primary objective of this operation is to remove hair (deharing) and flesh and open up the fiber structure suitably to get the desired properties in the final leather. Conventional liming process involves the use of lime and sodium sulphide which results in the destruction of the hair and other protein materials (Thanikaivelan *et al.,* 2004; Kanagaraj, 2009; Sundararajan *et al.,* 2011). Currently, alkaline proteases have been used in the dehairing process, where it is carried out at pH values between 8 and 10 (Ward *et al.,* 2009).

Hence, they are used in this process as auxiliaries to speed-up the reactions (Østergaard and Olsen, 2011).

Bating is a treatment with proteolytic enzymes to make the leather pliable and takes place before tanning. The treatment softens hide and skin by dissolving and digesting the interfibrillar matrix proteins (fibrous proteins) and globular proteins partially or completely. It washes out residues of non-collagen protein (Rao *et al.,* 1998; Østergaard and Olsen, 2011). The extent of removal of these constituents decides the characteristics of the final leather such as durability and softness (Kumar and Takagi, 1999). Specificity of an enzyme towards the target matrix proteins like keratin and elastin and the type of leather (soft or hard) are the deciding factors in selection of the type and amount of enzyme (Kumar and Takagi, 1999; Ward *et al.,* 2009).

3.6.3. Applications of alkaline protease in protein hydrolysates production

Protein hydrolysates are the products obtained after hydrolysis of proteins with the aid of partial digestion by enzymes, acid or alkali which results in an organic nitrogen in readily available form. They are commonly known as peptones or peptides, and used in a wide variety of products in food, pharmaceutical, fermentation, and biotechnology industries (Pasupuleti *et al.,* 2010).

The protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations, specific therapeutic dietary products, and the fortification of fruit juices and soft drinks (Rao *et al.,* 1998; Ward *et al.,* 2009). Moreover, they find applications in an animal cell culture to enhance biosynthesis of therapeutic proteins and monoclonal antibodies (Siemensma *et al.,* 2010). They are used in animal nutrition as regular diets as well as prescription diets as hypoallergenic, flavors and palatants for companion animals (McCalla *et al.,* 2010). In large-scale industrial fermentations they are used as sources of nitrogen and growth factors for starter culture (Ummadi and Curic-Bawden, 2010). It is also used in the diagnostic media to grow microorganisms in Petri plates and detect pathogens (Ranganathan *et al.,* 2010).

Now a day, protein hydrolysates are prepared from many sources using alkaline proteases isolated from different microbial sources. Kong *et al.* (2007) have studied on alcalase assisted production of wheat gluten hydrolysates with high protein recovery (81.3 ± 0.1%) and degree of hydrolysis (DH 5.0- 15.0%). Yust *et al.* (2010) also reported the improvement of functional properties of chick pea proteins by hydrolysis of immobilized alcalase enzyme with DH of 10%. Hrckova *et al.* (2002) reported production of soy protein hydrolysates (SPH) with (DH, 35.1%) with aid of alcalase digestion. In another study conducted by Kong *et al.* (2008) soy protein hydrolysates with lower molecular weight were prepared by several commercial alkaline proteases.

3.6.4. Applications of alkaline proteases in textile processing

In recent years, textile processing based on biotechnology has gained importance in view of stringent environmental and safety conditions (Parvinzadeh and Ashrafi, 2002). Alkaline proteases are used in many processes in textile industry (Kumar and Takagi, 1999). The use of these enzymes to improve some physical and mechanical properties such as wool anti-shrinkage, anti-felting, drapeability, dyeing affinity and water absorbency is particularly interesting (Silva *et al.,* 2005; Parvinzadeh, 2007). They are also used in removal of scale cuticles or smoothing the edges, better whiteness, improved handling, and dyeing of wool and silk fibers (Rao *et al.,* 1998; Freddi *et al.,* 2003; Smith and Shen, 2011). Likewise, they are used in scouring process of cotton fibers to remove proteinaceous impurities (Hsieh and Cram, 1999; Karapinar and Sariisik, 2004). Parvinzadeh and Ashrafi (2002) reported the application of proteases in surface degradation of wool fiber to improve dyeability. In this study they found decreased tensile strength and lightness, increased dyeability, and no change in wash and light fastness properties of treated wool yarns with proteolytic enzymes. In this experiment protease catalyzes degradation of epicuticle that produces the fiber with more amine terminal groups. As a consequence, improved susceptibility of dyes attraction was observed.

In textile industry alkaline proteases are used to remove the stiff and dull gum layer of sericine from the raw silk fiber to achieve improved luster and softness (Freddi *et al.,* 2003). More recently, Sumana *et al.* (2013) also reported degumming of raw silk fabric with help of marine extracellular protease in a dosage of (0.2-1.0 U/cm^2 fabric). Within 4 h at room temperature with enzyme concentration of 0.8unit/cm^2, they found maximum results in degumming loss, anti shrinkage, tensile strength and yarn count and color fastness to light/water compared to conventional treatment. Therefore, protease treatments can modify the surface of wool and silk fibers to provide new and unique finishes.

3.6.5. Applications of alkaline proteases in photographic industry

Photographic films and plates essentially consist of an emulsion on a firm support of cellulose, acetate, or polyester, or glass. The emulsion is composed of a suspension of minute silver halide crystals in gelatin (Grzonka *et al.,* 2007). Alkaline proteases play a crucial role in the bioprocessing of spent X-ray or photographic films for silver recovery. These waste films contain 1.5–2.0% silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes (Kumar and Takagi, 1999). In this process alkaline proteases hydrolyze gelatin of the scraped film and allowing to recovery of silver present. Additionally, enzymatic hydrolysis of gelatin not only helps in extracting silver, but also the polyester film base can be recycled (Gupta *et al.,* 2002). Nakiboğlu *et al.* (2009) reported recovery of silver (0.4013 g) with purity of 99.16 ± 0.11 % at pH =8.0 and 50 ^{0}C from waste photographic films using alkaline protease produced by *Bacillus subtilis* ATCC 6633.

3.6.6. Applications of alkaline protease in waste management

There are different kinds of proteinaceous wastes that accumulate in nature from various sources and human activities. Wastes that are generated by fibrous proteins such as feathers, hairs, horns, nails, etc are some of the abundant proteinaceous wastes. Alkaline proteases are useful in degradation of these waste materials into useful products, thus help to lower the biological oxygen demand of aquatic systems (Gupta *et al.,* 2002). Amare Gessesse *et al.* (2003a) reported production of alkaline protease which degrade

poultry feather i.e. keratinicious fibrous protein which in turn used as nutritional supplements as amino acids for domestic animals. Again, Korniłłowicz-Kowalska and Bohacz (2011) carried out an excellent review on various alkaline proteases from different sources which are used in complete or partial degradation of keratin waste. Moreover, Khardenavis *et al.* (2009) reported the application of alkaline keratinase from *Serratia sp. HPC 1383* in processing of poultry feathers.

The use of alkaline protease in the management of wastes from various food-processing industries and household activities opened up a new era in the use of proteases in waste management (Gupta *et al.,* 2002). Lasekan *et al.* (2013) have studied on the applications of alkaline proteases in combination with other enzymes in production of protein hydrolysates from fish, livestock, and chicken by-products obtained from livestock product processing plants. Similarly, Jensen *et al.* (2010) have indicated the application of protease with other hydrolytic enzymes in degradation of municipal solid waste.

3.6.7. Applications of alkaline protease in medicine and pharmacy

The wide diversity and specificity of proteases are used to a great advantage in developing effective therapeutic agents. Oral administration of proteases from *Aspergillus oryzae* (Luizym and Nortase) has been used as a digestive aid to correct certain lytic enzyme deficiency syndromes (Rao *et al.,* 1998). As indicated by Kumar and Takagi (1999); Gupta *et al.* (2002) alkaline proteases with elastolytic activity are used for the preparation of elastoterase, which in turn applied for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses. In addition, peptidases are used in medicine as targets to many medicine; to remove gastrointestinal parasites (anthelminthics), removal of dead skin from burn patients (debridement), determination of blood groups, and for relief of back pain by digesting the cartilage content of herniated intervertebral discs (chemonucleolysis) (Rawlings *et al.,* 2007).

Neutral to slightly alkaline proteases (bromelain and papain) are used in pharmaceutical industry and medicine to kill the lymphatic leukemia cells, bacteria, parasite, and bacillus tuberculars. In doing so they help to diminish inflammation, normalize the functioning of

the gallbladder, alleviating pain and promote digestion (Grzonka *et al.,* 2007). In study conducted on novel and highly potent fibrinolytic protease from *Streptomyces species* by Simkhada *et al.* (2010) and Uesugi *et al.* (2011) indicated potential application in therapy of thrombosis was reported.

Another interesting application of alkaline protease in medicine and / veterinary medicine is associated with degradation of prion proteins (PrP) (Booth *et al.,* 2013). Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are a group of invariably fatal neurodegenerative disorders. It is associated with infectious agent of misfolding and aggregation of the normal cellular prion protein (PrPC) which is changed to infectious isoform prion protein (PrP^{Sc}) (Morales *et al.,* 2012). It can affect sheep and goats (scrapie), cattle (bovine spongiform encephalopathy (BSE) or 'mad cow' disease) and humans (Creutzfeldt–Jakob disease (CJD) and koru) (Booth *et al.,* 2013). Prions are remarkably resistant to inactivation by different treatments and can persist and remain infectious in the environment for years (Morales *et al.,* 2012). However, in studies conducted by Hui *et al.* (2004); Pilon *et al.* (2009) reported complete degradation, decontamination, and inactivation of infectious PrP^{Sc} protein to normal prion protein (PrPC) forms by alkaline proteases and consortia of alkaline protease producing alkaliphilic microorganisms.

3.6.8. Other miscellaneous applications of alkaline proteases

Besides to the above mentioned applications, alkaline proteases are also used to lesser extent in many areas of technical and industrial processes. Largely proteases are known to catalyze the cleavage of peptide bonds. However, catalysis of the reverse of hydrolysis reaction resulting in synthesis of peptides was also reported by many researchers. Using alkaline proteases to catalyze peptide synthesis in organic solvents is reported by Kumar and Bhalla (2005); Narai-Kanayama and Aso (2009); Ruiz *et al.* (2010).

Amino acids are important as dietary supplements for both humans and domestic animals. Only the L-amino acids can be assimilated by living organisms, since the chemical synthesis of amino acids produces a racemic mixture, it is necessary to separate

the isomers before commercial use. Resolution is one of the best ways to produce optically pure amino acids. Alkaline proteases have the ability to cause resolution of the racemic mixture of D-L amino acids (Ordóñez and Cativiela, 2007; Komeda and Asano, 2008).

Alkaline protease from non - toxigenic *Vibrio* species (V26) is reported to substitute trypsin used in the applications of alkaline protease in cell culture i.e. in detaching of growing cells from plasma clots, dissociation of tissues for primary cell cultures, maintenance of cell lines (Najafi *et al.,* 2005; Manjusha, 2011). Moreover, Moran *et al.* (2001) reported the potential application of heat liable protease in DNA isolation for digestion of cellular proteins and purifying products between enzymatic steps.

Alkaline protease with gelatinase and collagenase activity find application in cosmetics and skin care products for skin rejuvenation, wrinkle smoothing and dandruff removal, prophylactic on senile skin changes and also removal of the atrophied epithelium from the skin surface (Najafi *et al.,* 2005).

4. Materials and Methods

Most of the studies were carried out in the Microbial and Molecular Biotechnology Laboratories of the Institute of Biotechnology, AAU (IOB-AAU). Nitrogen and / protein content determination using Kjeldhal process was conducted using facilities at food science and nutrition laboratory at Ethiopian Institute of Public Health (EIPH), Addis Ababa.

4.1. Materials

The fruit berries of Endod (*Phytolacca dodecandra*) were collected from Aklilu Lemma Institute of Pathobiology- AAU. Animal blood samples were taken from Addis Ababa Abattoirs Enterprise, while Ariel detergent, eggs and soy bean (*Glycine max)* were purchased from super markets around AAU - Arat kilo campus. The seed and/ flour of different indigenous cultivars of Moringa plant (*Moringa stenopetala*) collected from different regions of Ethiopia were kindly provided by Yihune Ayele (a Ph.D. fellow at Center for Food Science and Nutrition, AAU). White cotton cloth was purchased from Merkato market center. All the remaining reagents and chemicals are laboratory grade and produced by internationally known chemical industries.

4.2. Experimental organisms

The experimental organisms for this study were taken from the collection of bacterial (*Actinobacteria*) isolates maintained in the Microbial Biotechnology Laboratory of the Institute of Biotechnology, Addis Ababa University (IOB – AAU). The organisms were previously isolated from mud and water samples of Lake Chitu, Ethiopian Rift Valley soda lake, and studied for their potentials in antibiotic production (Biniam Wondale, 2008). They were kindly given by Dr. Amare Gessesse. A total of 90 isolates were randomly selected from deep freeze (-80℃), refreshed and subjected to screening for proteolytic activity.

4.2.1. Refreshing and screening for extracellular protease production

The bacterial isolates stored in the deep freeze (-80 ℃) in the straw were refreshed as previously described by Amare Gessesse *et al.*(2003a) in a peptone yeast extract agar medium containing(w/v) of peptone(0.5%), NaCl(0.5%), yeast extract(0.5%), $MgSO_4$. $7H_2O$(0.02%), $CaCl_2$. $2H_2O$ (0.02%), and K_2HPO_4(0.1%). The media components were mixed and boiled in a hot plate until the crystals of the components are dissolved. Then pH was adjusted to 7.5-8.0 by using 1M NaOH and agar (2%) was added and allowed to boil. Next to this all the media components were autoclaved at 121 ℃ for 15 min. Twenty five percent (25%) Na_2CO_3 (1%) and 25% glucose (0.5%) were prepared, autoclaved separately and added to the medium components after cooling approximately to 60℃. Finally, the media was poured to the plate aseptically. Then isolates maintained in the straw were streaked and the plates were incubated in culture box (Electrical heat Thermostatic Culture Box DH5000B) at 30 ℃ for 24-36 h. Then the cultures were preserved and maintained in refrigerator (4 ℃) until further use.

Screening of best bacterial isolate for extracellular protease production on a plate was carried out following the method described previously by Amare Gessesse *et al.* (2003a) on a casein agar medium containing (w/v): $CaCl_2.H_2O$ (0.02%), $MgSO_4.7H_2O$ (0.02%), K_2HPO_4 (0.1%), NaCl (0.5%), and casein (1%). These components were dissolved by stirring and heating, and after cooling pH was adjusted to 7.5-8.0 by using 1M NaOH. Then agar (1.5%) was added and allowed to boil. Next the medium was autoclaved at 121℃ for 15 min. Twenty five (25%) of Na_2CO_3 stock solution was prepared and autoclaved separately. After cooling approximately to 60℃, 1% (w/v) Na_2CO_3 was added to the mixture. Each refreshed pure culture was inoculated on to casein- agar media in a uniform manner by making short streaks. Then the plates were incubated in culture box at 30 ℃ for 24-36 h. Formation of clear halo zone around the colonies, resulting from casein hydrolysis was taken as positive for proteolytic activity. The ratio of the diameter of clear halo zone to colony was calculated and used to evaluate the performance of extracellular protease production. Isolates with large clear halo- zone were selected and used for further study in SSF and SmF studies.

4.2.2. Screening for extracellular protease production in submerged fermentation

To select an isolate which produces extracellular alkaline protease with highest activity in submerged fermentation, a small amount of refreshed culture from casein - agar plate was taken and inoculated in to 50 ml of same medium mentioned above but without agar in 250 ml Erlenmeyer flask and this was used as starter culture. Then from a 24 h old starter culture 1ml was transferred to 50ml of fresh media and the flasks were incubated in the rotary shaker (121 rpm) at 30 ± 1 ℃ for 3-12 days. Then 2.5 ml of the fermented broth was taken within each 24 h difference aseptically and centrifuged at 10,000 rpm for 5 min and the cell free supernatant was used as crude enzyme.

4.2.3. Screening for extracellular protease production in solid state fermentation

To select an isolate which produces extracellular alkaline protease with highest activity in solid state fermentation, 1ml of 48 h old starter culture was taken and transferred in to SSF medium containing (g/g): wheat bran (10), K_2HPO_4 (0.2), NaCl (0.25), $MgSO_4.7H_2O$ (0.02), $CaCl_2.2H_2O$ (0.02),and casein (1). The SSF medium was prepared in a 250 ml Erlenmeyer flasks and the solid substrate to moistening agent ratio was adjusted to 1:1.5 using distilled water. Then the components were autoclaved at 121℃ for 15 min. Following, 1% (v/v) of sterile Na_2CO_3 autoclaved separately was added. Finally, after mixing and cooling at room temperature the medium was inoculated and incubated at 30 ± 1 ℃ for seven days.

After seventh days of solid state fermentation, alkaline protease was extracted by soaking the fermented solid substrate with 10ml of distilled water per gram of wheat bran, which was a total of (100ml) in shaking 121 rpm for 30 min at room temperature (Paul, 2005). The solids again mixed thoroughly by using glass rod. Then the suspension was hand squeezed through a double layered muslin cloth and the particulate materials clarified by centrifugation at 3700 rpm for 32 min at 4℃. Finally, the harvested supernatant was used as crude enzyme and preserved at -20℃ until further use.

From totally refreshed 90 isolates, three isolates with best extracellular protease production efficiency in both SSF and SmF were selected. From these three isolates, one best isolate was further studied depending on efficiency in enzyme production in SSF and the characteristics of the enzyme suitability to different industrial applications needed. The characteristics of enzymes used during screening were pH, temperature, and calcium ion dependency for activity and thermal stability.

4.3. Characterization of the *Actinobacteria*

Morphological identifications and biochemical characterizations like Gram test, Catalase test, and Oxidase tests were conducted according to Manjusha (2011). The form/configuration, transparency, elevation and margin of colonies, pigment formation was identified by direct observation and using dissecting microscope. Cellular morphology, filaments structure and color of an isolate were determined by using light microscope.

Gram test was conducted by taking 48 h old colony from casein agar plate. A small amount of the culture was picked with loop and placed on a clean slide and smeared with toothpick for 2-3 min and mixed with 3% of KOH added drop by drop. Then the viscous cell parts dragged up ward using metal loop. Absence of stretching/extending viscous material shows positive result while presence indicates gram negative reaction.

Catalase test was performed by pouring 2-3 drops of 3% of hydrogen peroxide solution on to the loop full of 48 h old colonies placed on clean microscopic slide. When rubbing with a metal loop, formation of air bubbles indicates the presence of Catalase enzyme. Catalase negative reaction is indicated by the absence of formation of air bubbles.

Oxidase (Cytochrome Oxidase activity) of the BACC-15 *Actinobacteria* was conducted by taking 48 h old colony. A smear of cultures were made on whatman filter paper and placed on a Petri dish. Then the compact smear was moistened with 2-3 drops of 1% solution of *N, N, N, N′*-tetramethyl-*p*-phenylenediamine (TMPD). A positive reaction is indicated by the formation of intense deep blue/ purple color within 30 seconds. Catalase

negative reaction is indicated by the absence of the characteristic colors within ten seconds which shows absence of phenyle-amine derivatives to be oxidized by cytochrome C to produce a bluish color.

Anaerobic test was conducted by incubating BACC -15 *Actinobacteria* in the anaerobic jar for 72 h. Casein and gelatin hydrolysis was performed by growing the organism in 1% of casein or gelatin broth medium as described in section 4.2.1.

4.4. Optimization of culture conditions under SSF

The optimum conditions for maximum alkaline protease production under solid state fermentation were carried out according to the method described by Paul (2005). These includes optimization of initial moisture content (1: 0.5 - 1: 4.0), incubation period (4-12 days), temperature (25, 30, 37 and 40 ℃), inoculums preparations (1-6%), and fermentation conditions (SSF and SmF).

During optimization studies of fermentation process parameters in SSF, one factor at a time strategy was used. Each parameter was optimized independent of the others and subsequently optimal conditions were employed in all experiments.

4.5. Enzyme extraction

After the required period of incubation, the enzyme was extracted according to the method mentioned in section 4.2.3.

4.5.1. Effect of extractant

The effect of extractant on activity and yield of alkaline protease was evaluated by using distilled water, physiological saline water, carbonate- bicarbonate buffer (pH= 9.0), Tris-HCl buffer (pH= 8.0), Glycine -NaOH buffer (pH=10.0), methanol, ethanol and acetone.

4.5.2. Effect of volume of extractant

In this study, distilled water was used as extractant for protease recovery. The optimal volume of extractant that has to be used for maximum extraction of BACC-15 alkaline protease was studied by using different volumes 5, 10, 15, 20, 30 and 40ml of extractant per gram of Wheat bran.

4.5.3. Effect of repeated extraction

Once, twice and three times of repeated extractions were carried out as confirmatory test whether most of protease could be recovered in one extraction. Then percent recovery was calculated from the crude supernatant protease activity by dividing total activity of at each squeezing stage to the overall protease activity at three stages (Paul, 2005).

4.6. Concentrating the enzyme

The solid state fermented medium was extracted as described in section 4.2.3. Then the harvested broth filtered through double layered muslin cloth and centrifuged at 3700rpm for 30 min. The pellet was discarded and clear supernatant was obtained. To the supernatant, Acetone, Ethanol and Methanol (30%) were added in a ratio of (1:3 v/v). Then the mixture was thoroughly hand shaken and stored at 4 ℃ until the enzyme precipitates. The protein precipitate was separated by centrifugation at 10,000 rpm for 5 min and dissolved in 50mM Tris-HCl buffer, pH 8.0. Finally, the dissolved enzyme stored in clean glass bottles at 4 ℃ until further use.

4.7. Physico-chemical characterization of the alkaline protease

4.7.1. Enzyme assay

The activity of protease was measured using casein as substrate according to Amare Gessesse (1997) and Amare Gessesse *et al.* (2003a). First 2% of casein and 100mM of Glycine-NaOH buffer pH 10.0 were prepared and mixed together by vortex to give final concentration of 1% casein and 50mM Glycine-NaOH (pH 10.0) buffer. Then the reaction mixture containing 450 µl of the above prepared substrate was added to three

clean labeled eppendorf tubes; two duplicates of enzyme and one enzyme blank. Similarly, one reagent blank was prepared except 50 µl of distilled water, which was used instead of crude enzyme in others before incubation in the water bath. To the reaction mixture eppendorf tubes labeled as enzyme, 50 µl of crude enzyme was added and incubated at 50 ℃ for 30 min in the water bath. After 30 min 500 µl of 10% TCA (Tri Chloro Acetic acid) was added to stop the reaction. Following this, 50 µl of crude enzyme was added to the enzyme blank. Then the reaction mixture left at room temperature for 10-15 min to allow undigested casein to precipitate. After this, the reaction mixtures were centrifuged at 10,000 rpm for 5 min. Then 500 µl of clear supernatant from each reaction mixture was transferred to the respective labeled clean test tubes and 2.5 ml of 0.5 M sodium carbonate solution was added to it. Next 500 µl of 1:10 diluted folin- Ciocalteau's - phenol reagent was added to the solution and mixed thoroughly by vortex and incubated at dark room for 25 min. Finally, optical density (OD) was measured using spectrophotometer (JENWAY 6300 UV/Vis) at 660 nm. Enzyme activity was measured against enzyme blank and reagent blank. One unit of alkaline protease is defined as the amount of enzyme that releases 1µg of amino acid equivalent to tyrosine per 1min under standard assay conditions (Amare Gessesse *et al.,* 2003a).

Relative activity was calculated as the percentage enzyme activity of the sample with respect to the sample for which maximum activity is obtained.

$$\text{Relative Activity} = \frac{\text{Activity of sample (U)} \times 100}{\text{Maximum enzyme activity (U)}}$$

Residual activity was calculated as the percentage enzyme activity of the sample with respect to activity of control (untreated enzyme).

$$\text{Residual Activity} = \frac{\text{Activity of sample (U)} \times 100}{\text{Activity of control (U)}}$$

4.7.2. Preparation of Tyrosine standard curve

In order to compute the activity of protease in unit basis, the standard curve of tyrosine was prepared using the standard proportions of tyrosine (Amare Gessesse *et al.,* 2003a). The stock concentration of tyrosine used in the solution was 200 µg/ml (Table 2).

Table 2. Standard concentrations of tyrosine for determination of unit of alkaline protease

S. No.	200 µg/ml of Tyrosine	Buffer(µl)	[Tyrosine] (µg/ml)
1	0	500	0
2	62.5	437.5	25
3	125	375	50
4	187.5	312.5	75
5	250	250	100
6	312.5	187.5	125
7	375	125	150

Then to generate the standard curve; 0.5M of Na_2CO_3, Glycine-NaOH buffer (50mM , pH 10.0), 1:10 diluted 2N Folin Ciocalteau's phenol reagent and the tyrosine solution mixtures given in the table 2 were used. First 200 µg/ml of stock solution of tyrosine was prepared and appropriately diluted to attain a given concentrations of tyrosine as given in the above table. Then 2.5 ml of 0.5M Na_2CO_3 was added in each test tube including blank. Five hundred (500 µl) of the above mixture was added on it. Following the mixtures vortexed and kept at room temperature for 10 min. After this 500µl of 2N Folin Ciocalteau's phenol reagent was added in each test tube. Then solution was vortexed immediately and kept at room temperature for 30 min. Finally, the absorbance (OD 660_{nm}) was measured with spectrometer and standard curve was plotted by using Origin 8.0 software as shown in Figure 4.

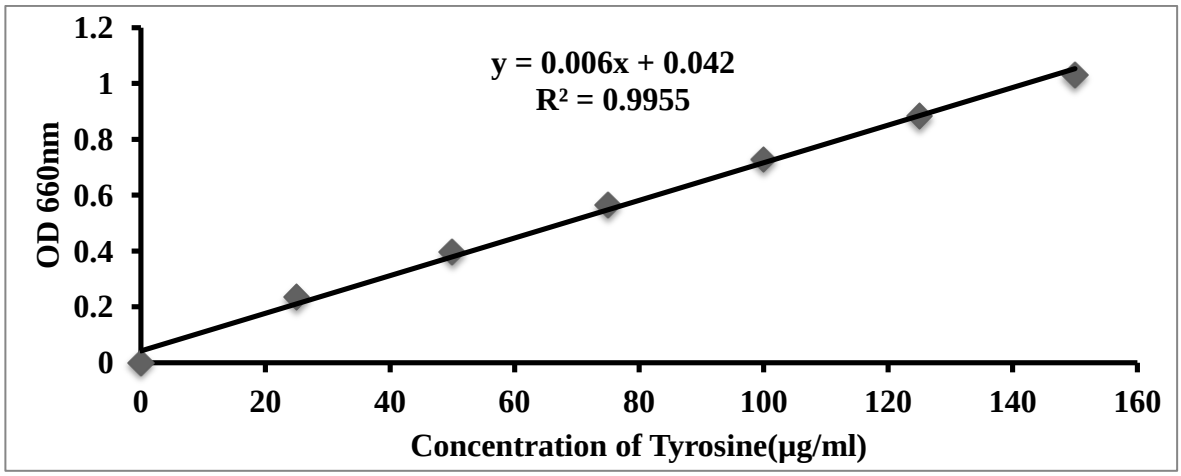


Fig 4. Tyrosine standard curve

The OD $_{660nm}$ values from the above concentrations results in a regression equation of **Y= 0.006x + 0.042**, with the regression coefficient (**R^2= 0.9955),** which is strongly positively correlated. Then by rearranging, solving and substituting the absorbance values (not shown here) in to the above equation, we got

$$\text{Enzyme Units (U/ml)} = \frac{(166.67 \times OD_{660nm} \times \text{Dilution factor}) - 7}{\text{Time of incubation}}$$

The enzyme unit of BACC-15 alkaline protease extracted from SSF was explained in U/g basis. All the procedures are similar except the mass of fermented bran was dried until constant mass was obtained by oven. Then the unit of enzyme obtained per gram of wheat bran was calculated.

4.7.3. Effect of enzyme concentration

The effect of dilution of alkaline protease on the proteolytic activity was determined. The original stock enzyme preparation was diluted by 100mM of Glycine - NaOH buffer (pH=10.0) to the ratio of 1:1,1:2,1:5,1:7,1:10,1:15,1:20,1:25 and 1:50. Then casein hydrolysis was determined by standard protease assay procedure following the procedures described in section 4.7.1 for its activity.

4.7.4. The effect of pH on activity and stability of the enzyme

The effect of pH on activity of alkaline protease was examined by assaying the enzyme at different pH values. The buffers used are: KH_2PO_4 and K_2HPO_4 (pH=6.0- 8.0), Tris-HCl (pH=7.5-9.0), Glycine –NaOH (pH=8.5-10.5) and Na_2PO_4 and NaOH (pH=10.5-12.0). All the buffers were used at concentration of 50mM and within 0.5 differences at 50 ℃ using casein as substrate. The enzyme assay procedure was similar to the procedure described at section 4.7.1 and relative activity was calculated.

The pH stability of BACC-15 alkaline protease was studied both at pH 10, its optimum pH for activity and pH 8.0 the requirement of many industrial applications at which protease is tested and applied. The effect of pH on stability of the protease was conducted according to the method described by Amare Gessesse *et al.* (2003a). The crude enzyme sample was mixed with different buffers and incubated at 50 ℃ for 1 h and residual activity was measured using casein in Glycine - NaOH(pH=10.0) and Tris - HCl (pH=8.0) as substrate using standard protease assay procedure. Buffers used and their concentrations were similar to the above mentioned in the enzyme activity part. Finally, residual activity was measured by taking untreated enzyme as 100%.

4.7.5. The effect of temperature on activity and stability of the enzyme

Temperature profile of the protease was determined by incubating the enzyme at different temperatures from 30-85 ℃ with in 5 ℃ interval in a water bath by using 1% casein in 50mM Tris-HCl buffer as substrate (pH =8.0). Then activity was determined following standard protease assay procedure. Relative active activity was calculated to determine the optimum temperature for proteolysis.

Thermal stability of the enzyme was determined according to Amare Gessesse *et al.* (2003a). The enzyme appropriately diluted with Glycine-NaOH buffer (pH=10.0, 0.1M) and Tris-HCl (pH=8.0, 0.1M) and then the aliquot was incubated at temperatures from 30-65 ℃ for 1h in a water bath. Following the enzyme samples withdrawn every 10 min

and stored at 4 ℃ . Then the residual activity was determined by using casein as substrate following standard protease assay procedure. Untreated enzyme was taken as 100%.

4.7.6. Temperature profile in the presence of 5mM $CaCl_2$

In order to examine the effect of Ca^{2+} ion on thermal activity of enzyme 5mM of $CaCl_2$ was added to the reaction mixture and compared with the one without $CaCl_2$ (0mM Ca^{2+}). All the assay procedures were similar to the thermal activity experiment except addition of 5mM $CaCl_2$ to check Calcium ion dependency for thermal activity.

4.7.7. The effect of Sodium Chloride on activity and stability of protease

The ionic strength of the alkaline protease was determined by adding 0-3M (with in 0.5M interval) of NaCl to the reaction mixture and then by carrying out standard protease assay at pH 8.0 and 10.0. Finally, relative activity was calculated by taking the one with largest activity as 100%.

The stability of alkaline protease in the different concentrations of sodium chloride was examined by preparing 0-3M NaCl (with in 0.5 intervals) according to modified method of Jadhav *et al.* (2013). First a required concentrations of NaCl prepared by dissolving it in 100mM Tris-HCl buffer (pH=8.0) and Glycine-NaOH buffer (pH=10.0). Then equal volumes of different [NaCl] in buffers and enzyme mixed together in clean eppendorf tubes and incubated at 30℃ for 1 h. After 1 h the mixture withdrawn and standard protease assay was carried out. The enzyme not treated with NaCl but incubated in the similar condition was taken as 100%.

4.7.8. The effect of protease inhibitors on activity of alkaline protease

The effect of inhibitors on protease activity was studied based on the method described by Amare Gessesse (1997). First equal volumes of the enzyme diluted by 50mM Tris-HCl (pH = 8.0) and Glycine-NaOH (pH = 10.0) buffer and 0, 1, 5 and 10mM inhibitors were mixed. The inhibitors used were: PMSF (serine protease inhibitor), EDTA (metallo-protease inhibitor), and 1, 10- Phenanthroline (zinc metallo protease inhibitor). Then

enzyme inhibitor mixture was pre - incubated at 30 ℃ for 1 h. Following 50 µl of the mixture was taken and used as enzyme source. The residual activity was determined by standard protease assay and untreated enzyme was taken as 100%.

4.7.9. The effect of oxidizing and bleaching agent on stability of the protease

The stability of the alkaline protease in the presence of oxidizing agent was examined according to Haddar *et al.* (2010) by using 0-30% concentrated H_2O_2. Equal amount of enzyme preparation and 0-30% hydrogen peroxide (with 5% difference) was pre-incubated at 30 ℃ for 1 h. Then residual activity was determined by taking 50 µl of the mixture and following standard protease assay procedure. Control or the enzyme without H_2O_2 was taken as 100%.

Similarly the stability of protease in the presence of bleaching agent was examined according to Haddar *et al.* (2010) by using 0 - 50mM of sodium per borate ($NaBO_3.H_2O$). All the procedures were done similarly to the above mentioned in H_2O_2 experiment.

4.7.10. The effect of detergents and sequestering agents on stability of the protease

The effect of different surfactants on stability of the proteases was investigated according to Wang *et al.* (2007) and Oliveira *et al.* (2010). The protease preparation was pre-incubated with different concentrations of surfactants at 40 ℃ for 1 h and the remaining enzymatic activity was determined under normal assay conditions. The protease activities of the samples without any surfactants (control) were taken as 100%. The surfactants used were: SDS (0-30% w/v) anionic detergent, Triton x- 100 and Tween-80 (non-ionic surfactants) (0-25% v/v).

The effect of sequestering agent on different washing soaps and detergents on stability of protease was examined according to Amare Gessesse *et al.* (2003b). The protease preparation was pre-incubated with 0-2M of sodium citrate at 40℃ for 1 h and the remaining enzymatic activity was determined under normal assay conditions.

4.7.11. The effect of Endod (*Phytolacca dodecandra*) on stability of protease

The effect of Endod (Ethiopian traditional soap) on stability of protease was examined. First ripe endod berries were collected from Aklilu Lemma Institute of Pathobiology campus, Addis Ababa University. Then the berries were allowed to dry in the sun and grinded to fine size by using pistil and mortar. Then the grinded flour was sieved with 450 µm sieve. The resulting powder was dissolved in distilled water (10% w/v). Following different concentrations of enzyme preparation and endod extract was mixed. The enzyme endod preparations pre-incubated at 40 ℃ for 1 h, at room temperature for 1-2 days. The remaining enzymatic activity was determined by taking 50 µl of pre - incubated aliquot as enzyme source under standard assay conditions.

4.8. Potential biotech applications of the alkaline Protease

4.8.1. Detergent applications and stain removal

Washing performance of the alkaline protease towards proteinacious stain and its compatibility with commercial detergent(Ariel) and Ethiopian traditional soap, Endod, was examined according to the method mentioned by Rai and Mukherjee (2009). During this experiment cleaning of white cotton cloth was tested using commercial detergent (Ariel), buffer, and enzyme alone, and varied composition enzyme and Endod mixtures.

Ariel was diluted in tap water (7mg/ml) according to manufacturer's specification to simulate washing conditions. The endogenous enzymes contained in the Ariel were inactivated by heating the diluted solution for 1 h at 80 ℃ prior to the addition of the enzyme preparation. BACC-15 crude alkaline protease was incubated in Ariel detergent solution for 1 h at 40 ℃, and then the remaining activity was determined following the standard protease assay conditions. Similarly, the stability of enzyme in Endod preparations was also tested. The enzyme activity of a control, incubated under the similar conditions without detergent, was taken as 100%.

The effect of Endod on the washing performance and efficiency of the enzyme towards proteinaceous stains was evaluated. The washing performance of enzyme alone (84.21U/g, OD =0.8), enzyme and Endod mixtures, and Endod alone were examined. First different types of stains *viz* blood and egg stains painted to white cotton cloth (14 cm x14 cm) and allowed to dry at room temperature for 72-120 h. Then the stains were washed with distilled water and buffer alone(control), 100% Ariel detergent, 100% Endod within buffer, 100% enzyme within buffer, 25% enzyme and 75% endod , 50% Endod and 50% enzyme, 75% enzyme and 25% endod preparations. Then pieces of clothes were rinsed thoroughly by tap water and allowed to sun dry. Finally, the dried pieces of clothes were observed for cleanness visually.

4.8.2. Application of alkaline protease in protein hydrolysates production

4.8.2.1. Preparation of Protein isolates (PIs)

Alkaline protease was evaluated for its application in preparations of protein hydrolysates from Soy bean seed (*Glycine max)* (L.) Merr. (SBPH), and Moringa seed (*Moringa stenopetala*) (Bak. f.) Cufod. (MSPH). Preparation of protein isolates from the defatted flour of the above samples were conducted according to slightly modified method of Li *et al.* (2005); Kudre *et al.* (2013). The whole procedure of protein isolates preparation (PIs) is given in Figure 5 (Page 41).

4.8.2.2. Enzymatic hydrolysis

Enzymatic hydrolysis of the protein isolates (PIs) were conducted according the modified method of Hrckova *et al.* (2002); Li *et al.* (2005). Protein isolates were suspended in carbonate bicarbonate (Na_2CO_3 / $NaHCO_3$, 10mM, pH =9.0) buffer to obtain 10 % w/v protein solution of SBPI and MSPI. The solutions were preheated at 50 ℃ for 30 min and then equilibrated at BACC-15 alkaline protease optimum temperature (45℃) and pH (9.0 by using 1M NaOH) for activity and stability before addition of the enzyme. Then hydrolysis was carried out using 1% (v/v) acetone concentrated BACC-15 alkaline protease. Hydrolysis was performed for 3.5 h at 45 ℃ in the water bath with continuous shaking. The samples were withdrawn and heated at 85 ℃ for 15 min to inactivate the

enzyme. Then the samples were centrifuged at 3700 rpm and pellet was discarded. The clear supernatant obtained was used for analysis of degree of hydrolysis.

4.8.2.3. Degree of Hydrolysis (DH)

At the end of hydrolysis time (3.5 h), an aliquot of 5 ml was withdrawn and mixed with 5 ml of 10% Tri- chloro acetic acid (TCA) and then centrifuged at 3700 rpm for 30 min at 4℃. The supernatant was decanted and analyzed for nitrogen content by the micro Kjeldhal method following Tecator Kjeldhal manual AOAC (2000). The degree of hydrolysis (%) was calculated as:

$$\text{DH (\%)} = \frac{\text{10\% TCA soluble } N_2 \text{ in the sample}}{\text{Total } N_2 \text{ in sample}} \times 100$$

Degree of hydrolysis (DH) is defined as the percentage ratio between the number of TCA soluble free nitrogen after enzymatic hydrolysis and the total number of nitrogen in the reaction mixture (protein isolates) studied. The total protein content was determined by micro Kjeldahl method (the conversion factor is 6.25 x % N_2).

4.8.2.4. Assessment of bacterial growth on SBPH and MSPH in comparison with commercial peptone

The clear supernatant obtained after BACC-15 alkaline protease hydrolysis which was prepared as described in section 4.8.2.2 was adjusted to the pH of 7.10 by using 1M NaOH. Then the supernatant directly subjected to freeze drying. Finally, freeze dried protein hydrolysates were grinded to fine powder and used as *homemade* peptone for growing pathogenic microorganism as alternative to *commercial* bacteriological peptone.

The microorganisms used for comparison of the SBPH and MSPH with commercial bacteriological peptone were *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (29212), and *Salmonella* (NCTC 8385). All these five microorganisms were taken from microbial culture collection of IOB-AAU.

Each of the above bacterium was separately cultured on nutrient agar for 24 h. Pure colonies were taken and inoculated to nutrient broth and incubated for 16 h and then 1 ml was serially diluted in physiological saline water (0.85% w/v NaCl in distilled water). From the 10^{-6} dilution, 100µl was used to inoculate on agar medium prepared from MSPH, SBPH and commercial peptone. All tested microorganisms were grown at 37 ℃. Bacterial colony counts were determined by the pour plate technique. For each the above pathogen (n=3) plates were prepared.

The above pathogenic bacteria were grown on 1% w/v of SBPH and MSPH supplemented with 0.5% w/v glucose dissolved in distilled water. After dissolving by stirring and heating, the medium was allowed to cool at room temperature. Following pH was adjusted to 7.10 and 1.5% w/v of agar was added as solidifying agent. Commercial peptone agar medium was used as control and prepared based on the slightly modified method described by Berhanu Andualem and Amare Gessesse (2013). The commercial peptone agar (CPA) medium containing (w/v) of glucose (0.5 %), peptone (0.5%), yeast extract (0.5%), and agar (1.5%) dissolved in distilled water was prepared. pH was adjusted to 7.10 by using 1M NaOH. Comparison of bacterial growth was carried out by colony counts following pour plate technique as described above.

Productivity ratio of the media at present study were measured by dividing total colony count obtained on the test culture medium to total colony count obtained on the control medium (commercial peptone).

$$\text{Productivity ratio} = \frac{\text{No. of colonies on test medium x dilution factor}}{\text{No. of colonies on control medium x dilution factor}}$$

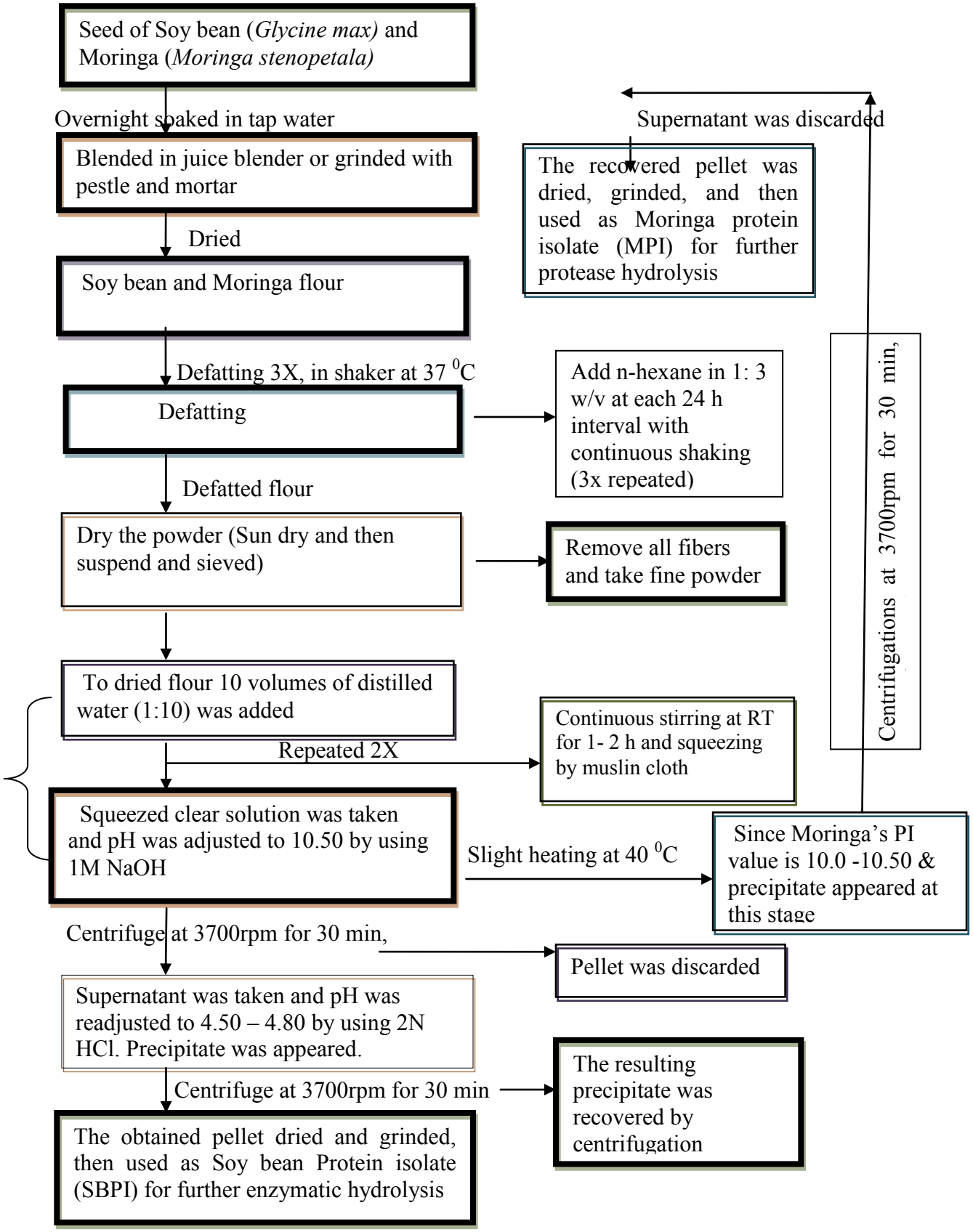


Fig 5. Schematic representation of protein isolates (PIs) preparation

4.9. Data analysis

Data generated from the optimization and application studies of alkaline protease were analyzed by using one way analysis of variance (ANOVA). All the experiments were carried out in triplicates in order to confirm the validity of the results obtained. Mean of the results were compared using post- hoc multiple comparison analysis performed using Duncan homogenous test using SPSS 20.0 version for windows at a significance level of $p< 0.05$. The results of optimization of process parameters under SSF and /some of characterization studies were given as mean ± standard deviation.

The results of the characterization studies of alkaline protease both on activity and stability were given by mean of two independent measurements and explained by graphs and columns using Origin pro 8 data analysis and workspace, version 8.0 for windows software; and Microsoft office Excel worksheet 2007.

5. Results

5.1. Screening of extracellular alkaline protease producers

Out of the tested 90 isolates, 32 isolates (32.55%) showed clear zone on casein - agar plate. Based on the ratio of the diameter of clear halo - zone (cm) to the size of colony (cm) four isolates designated as BACC-5, BACC-15, BACC-101 and 10R-7 relatively with large quotient were selected for further study (Table 3).

Table 3. The ratio of the diameter of clear halo zone to colony of the four isolates in casein agar plate.

Isolate	Diameter of colony (cm)	Diameter of clear zone(cm)	Ratio of clear zone to colony
BACC-5	1.0	4.4	4.40
BACC-15	0.9	4.8	5.33
BACC-101	0.6	4.7	7.83
10R-7	1.0	4.0	4.00

Three isolates namely BACC-5, BACC-15, and BACC -101 were found to grow and secrete large amount of alkaline protease at 96 h of incubation under SmF. All these 3 isolates were again found to produce appreciable amount of extracellular alkaline protease in SSF medium after 96 h of incubation. Based on results of (Table 4), isolate BACC-15 excels others in majority of the studied parameters and selected for further study.

Table 4. Summary of the results of screening parameters of three best isolates grown under SSF

Isolate	AP production U/g	AP production in SmF (U/ml)	Optimum temperature (^{o}C) for activity	Optimum pH for activity	Time course for maximum AP production	Ca^{2+} dependency for activity and stability
BACC-5	56.42	5.24	60	10.5	$\geq$ 120 h	Shifted 5 ^{o}C
BACC-15	62.78	5.63	60	10.00	$\geq$ 120 h	Shifted 5 ^{o}C
BACC-101	50.98	4.70	65	10.5	$\leq$ 96 h	No influence

All the measurements were mean of the three independent measurements where AP is alkaline protease production.

Actinobacteria BACC-15 produces appreciable amount of alkaline protease both under SmF (Fig 6 left) and effectively colonizes surface of wheat bran under SSF (Fig 6 right) after five day of incubation at 30℃.

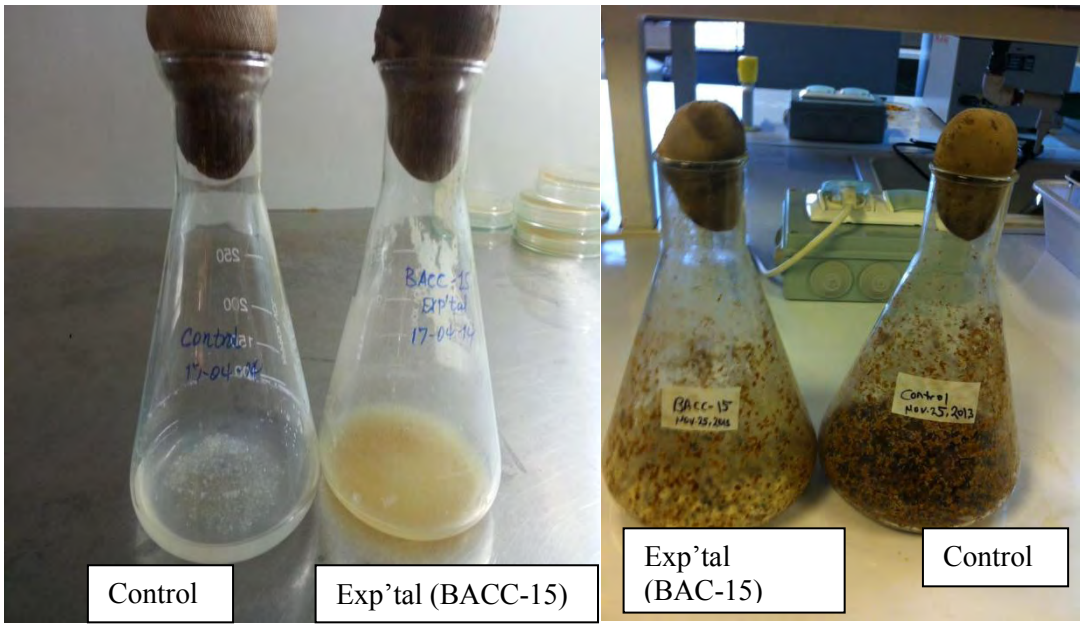


Fig 6. Growth of selected isolate (BACC-15) in SmF and SSF for alkaline protease production

5.2. Characterization of the *Actinobacteria*

5.2.1. Morphological and biochemical characterization

Actinobacteria BACC-15 had long filamentous, septate and branched white colored mycelia. The colonies were characterized as opaque, circular configuration, entire margin, and white- creamy pigmented (Table 5). A long non-motile and straight rod shaped cells were observed under light microscope with 100X magnification power in an oil immersion within 48-72 h. The strain was also found to be facultative in nature and has the ability to hydrolyze casein and gelatin. Some of the morphological, physiological and biochemical characteristics of the colony of *Actinobacteria* BACC-15 have indicated in Table 5 and 6 respectively.

Table 5. Morphological characteristics of isolate BACC-15

Test	Colony morphology
Configuration/form	Circular
Margin	Entire
Texture	Rough
Elevation	Umbonate
Mycelium	Branched, White
Filaments	Long , white colored, septate
Colony transparency	Opaque
Pigment of colony	Creamy white
Odor	Moist earthy smell
Cell shape and arrangement	Rod shaped and single arranged
Gram test	Positive

Table 6. Biochemical and physiological characteristics of isolate BACC-15

Biochemical test/ physiological test	Result
Motility	Negative
Oxygen requirement	Facultative
Catalase	Positive
Oxidase	Positive
Gelatin hydrolysis	Positive
Casein hydrolysis	Positive

5.3. Optimization studies of alkaline protease production under SSF

The results of the optimization studies of the fermentation process parameters under SSF were taken from three different flasks studied (n=3) and the unit (U/g) was reported. Results were reported as mean ± standard deviation in the table.

5.3.1. The effect of temperature of incubation

All temperatures selected for the study supported growth and alkaline protease production by BACC-15 *Actinobacteria* under SSF. The optimal temperatures for maximum alkaline protease production 575.32 ± 13.77 and 591.67± 12.99 U were 30 and 37 ℃ respectively. Beyond this range resulted in declined alkaline protease production. Interestingly, BACC-15 *Actinobacteria* produced 431.44 ± 22.32 U at room temperature (25℃) at seventh day of incubation (Table 7).

Table 7. The effect of temperature of incubation on the alkaline protease yield in SSF

Temperature of incubation	Alkaline protease yield (U) (M± SD)
25 ℃	431.44 ± 22.32^{b}
30 ℃	575.33 ± 13.77^{a}
37 ℃	591.67 ± 12.99^{a}
40 ℃	425.22 ± 8.32^{b}

Values followed by different superscripts are significantly different at (P<0.05)

Alkaline protease production (U) was not significantly varied at 25 and 40 ℃, and also between 30 and 37 ℃ incubations.

5.3.2. The effect of initial moisture content on alkaline protease production

Maximum alkaline protease production was attained when the initial moisture content was 1: 1.0 to 1: 1.8 solid to liquid ratio. Further increase in initial moisture content resulted in sharp reduction in enzyme yield and large accumulation of biomass. Moisture content of less than 1: 1.0 resulted in least protease yield. Initial moisture contents above 1:2.5 also resulted in significant reduction of alkaline protease production where as moisture content level of 1:4.0 totally blocked enzyme production (Table 8).

Table 8. The influence of moisture content on the BACC-15 alkaline protease production capacity in SSF

Moisture content(ratio)	BACC-15 alkaline protease yield (U/g) (M± SD)
1:0.5	027.96 ± 11.67^{f}
1:1.0	557.06 ± 24.89^{a}
1:1.5	582.18 ± 12.12^{a}
1:1.8	595.75 ± 09.60^{a}
1:2.0	418.22 ± 16.83^{b}
1:2.5	274.72 ± 5.41^{c}
1:3.0	168.55 ± 39.67^{d}
1:3.5	159.60 ± 5.60^{d}
1:4.0	116.24 ± 6.72^{e}

Values followed by different superscripts are significantly different at ($P<0.05$)

Significant difference in enzyme yield was obtained at all initial moisture content levels studied except 1:1.0 to 1:1.8.

5.3.3. Effect of incubation time on alkaline protease production in SSF and SmF

Extracellular alkaline protease secretion was detected after 72 h of incubation at SmF and 96 h of incubation at SSF. The alkaline protease production reached maximum level at 7th day both in SSF and SmF with 633.78 ± 9.32 U/g and 91.21 ± 1.66 U/ml respectively (Table 9).

Table 9. The effect of time course of incubation of BACC- 15 on alkaline protease yield

Time of incubation (Days)	Alkaline protease yield in SSF (U/10g) (M± SD)	Alkaline protease yield in SmF(U/50ml) M±SD
4	119.88 ± 7.32^{d}	72.21 ± 3.10^{e}
5	336.99 ± 60.40^{c}	81.10 ± 0.12^{d}
6	489.66 ± 69.10^{b}	NA
7	633.78 ± 9.32^{a}	91.21 ± 1.66^{a}
8	629.22 ± 19.32^{a}	87.10 ± 1.43^{b}
9	606.89 ± 8.66^{a}	85.44 ± 0.21^{bc}
10	583.22 ± 50.10^{ab}	$84.32 \pm 5.21^{b-d}$
11	579.78 ± 59.43^{ab}	$84.21 \pm 0.77^{b-d}$
12	563.89 ± 9.32^{ab}	82.44 ± 5.32^{cd}

Where NA is not available, the data is not collected at this day.

Values within the same column followed by different superscripts are significantly different at ($P<0.05$).

Further incubation up to 12th day showed no significant difference in alkaline protease yield in SSF. Analysis of variance (ANOVA) also showed that there was no significant difference in enzyme production of BACC-15 *Actinobacteria* from 10 - 12 days of incubation and optimum level production was attained between 7th and 9th days in SSF (Table 9). On the other hand, under SmF alkaline protease yield was decreased from its maximum level 91.21 ± 1.66 U/ml at seventh day to 82.44 ± 5.32 U/ml at twelfth day.

5.3.4. Effect of concentration of inoculums preparations on protease production

Maximum alkaline protease production was attained by *Actinobacteria* BACC-15 isolate at 7th day in 3% w/v inoculums preparation with total yield of 611.89 ± 31.66 U/g under SSF. Minimum alkaline protease production was recorded at 1% of inoculums preparation with enzyme yield of 549.67 ± 49.55U/g (Table 10).

Table 10. The effect of concentration of inoculums preparation on alkaline protease yield under SSF

[Inoculums preparation] (%w/v)	BACC-15 alkaline protease yield (U/g) (M± SD)
1	549.67 ± 49.55^{b}
2	587.00 ± 15.32^{ab}
3	611.89 ± 31.66^{a}
4	594.00 ± 11.43^{ab}
5	592.45 ± 23.10^{ab}
6	591.67 ± 37.88^{ab}

Values followed by different superscripts are significantly different at ($P<0.05$)

As shown in Table 10 above, alkaline protease production increased with increasing concentration of inoculums up to 3% w/v. Further increasing resulted in slight reduction in enzyme yield and remains constant up 6% w/v.

5.4. Optimization of extraction parameters for alkaline protease recovery

5.4.1. Effect of Extractants

Different extractants were used for alkaline protease recovery. Carbonate/bicarbonate buffer (pH= 9.0, 50mM) was found to be the most suitable followed by distilled water and methanol (Table 11).

Table 11. Effect of extractants

S.No	Extractants	Alkaline protease recovery (U/g)	Relative activity (%)
1	Acetone (30%v/v)	623.56 ± 2.62^{b}	86.23
2	Ethanol (30%v/v)	699.00 ± 1.04^{a}	96.66
3	Methanol(30% v/v)	713.00 ± 4.64^{a}	98.60
4	Glycine - NaOH buffer(pH 10.0)	708.34 ± 9.38^{a}	97.96
5	Tris-HCl buffer(pH=8.0)	709.11 ± 8.84^{a}	98.06
6	Carbonate-bicarbonate buffer(pH 9.0)	723.11 ± 9.15^{a}	100
7	Distilled water	722.34 ± 9.31^{a}	99.89
8	Physiological saline water	692.78 ± 8.99^{a}	95.80

Values followed by different superscripts are significantly different at ($P<0.05$)

As shown in Table 11 there was no significant difference in enzyme recovery among the extracts used except acetone. The ANOVA also confirmed the same result at $p < 0.05$. During extraction of BACC-15 alkaline protease in SSF better enzymatic activity was obtained by using extractants Carbonate/bicarbonate buffer > distilled water > Methanol > Tris-HCl buffer > Glycine-NaOH buffer > Ethanol > physiological saline water > Acetone respectively (Table 11).

5.4.2. Effect of volume of extractant

Alkaline protease recovery increased when the volume of extractant increased from 5 ml to 15 ml distilled water per gram of wheat bran (Table 12). Maximum recovery of BACC-15 alkaline protease (596.33U/g) was obtained when 15 ml of extractant per gram

wheat bran was used. Least protease recovery (29.32 U/g) was achieved when the volume of extractant was 40ml/g of wheat bran (Table 12).

Table 12. The effect of volume of extractant on alkaline protease recovery

ml of Extractant/g of wheat bran	Alkaline protease yield(U/g)	Relative activity (%)
5	485.89	81.48
10	553.56	92.83
15	596.33	100
20	429.89	72.09
30	395.66	66.35
40	29.32	4.91

5.4.3. Effect of repeated extraction

It was seen that out of three cycles of repeated extraction, 100, 82.34 and 66.36% of activity was retained in 1st, 2nd and 3rd cycles of extraction. Beyond third cycle, the enzyme activity sharply drops. The first three cycles were found to be effective for recovery of BACC-15 alkaline protease (Table 13).

Table 13. Effect of repeated extraction on alkaline protease yield

No. of cycles of extraction	Enzyme recovery(U/g)	Relative enzyme recovery (%)
1	596.3	100
2	491.33	82.34
3	395.66	66.36
4	103.99	17.47

5.5. Concentrating of the enzyme

Clarification of the alkaline protease from other solid state fermented broth products was achieved by using chilled acetone, ethanol and methanol precipitation (Table 14). The total alkaline protease activity was increased from 409.27 U/g in cell free crude extract to

578.44, 660.89, and 729.73 U/g after Ethanol, methanol and acetone, precipitation respectively. Acetone precipitated fraction showed maximum relative activity (100%) compared to cell free crude extract and other organic solvents (Table 14).

Table 14. Concentration of BACC-15 alkaline protease using organic solvents

Organic solvents used	Alkaline protease recovery (U/g)	Relative activity (%)
Crude extract	409.27	56.08
30% Acetone	729.73	100
30% Ethanol	578.44	79.23
30% Methanol	660.89	90.60

5.6. Physico-chemical characterization of alkaline protease

5.6.1. The effect of enzyme concentration

The activity of enzyme continuously increases with the increase of dilution up to 15X dilution and then decreases (Table 15). The minimum and maximum activity for BACC-15 alkaline protease (80.991 ± 12.32 and 843.780 ± 9.32 U/g respectively) was obtained at a dilution factor of 1X and 15X from original stock preparations.

Table 15. The effect of dilution (enzyme concentration) on BACC-15 alkaline protease activity

Dilution factor	BACC-15 alkaline protease activity (U) (M ± SD)
1X	80.99 ± 12.32^{h}
2X	161.33 ± 18.32^{g}
5X	421.44 ± 39.32^{e}
7X	568.00 ± 53.99^{c}
10X	669.56 ± 9.32^{b}
15X	843.78 ± 9.32^{a}
20X	637.11 ± 4.77^{b}
25X	509.22 ± 5.87^{d}
50X	281.22 ± 6.21^{f}

Values followed by different superscripts are significantly different at ($P<0.05$)

Where, X shows the number of times of dilution.

5.6.2. Effect of pH on activity and stability

BACC-15 alkaline protease was active in a broad pH range of 6.0-11.50. It showed ≥ 68% of its maximum activity at pH range of 7.0 -11.5, with an optimum activity at pH 10.0 as indicated by the peak in Figure 7. The activity of BACC-15 alkaline protease was found to increase progressively with the increase in pH range from 6.0 to 10.0 and slightly drop in activity beyond pH 10.0. The protease showed 76.98%, 95.88%, 92.33% and 93.57% of its maximal activity at pH 8.0, 9.5, 10.5 and 11.0 respectively. The activity of BACC-15 alkaline protease sharply declines above pH 11. Minimum relative activity was recorded at pH 6.0(37.68%) and pH 12.0(26.95%) (Fig 7).

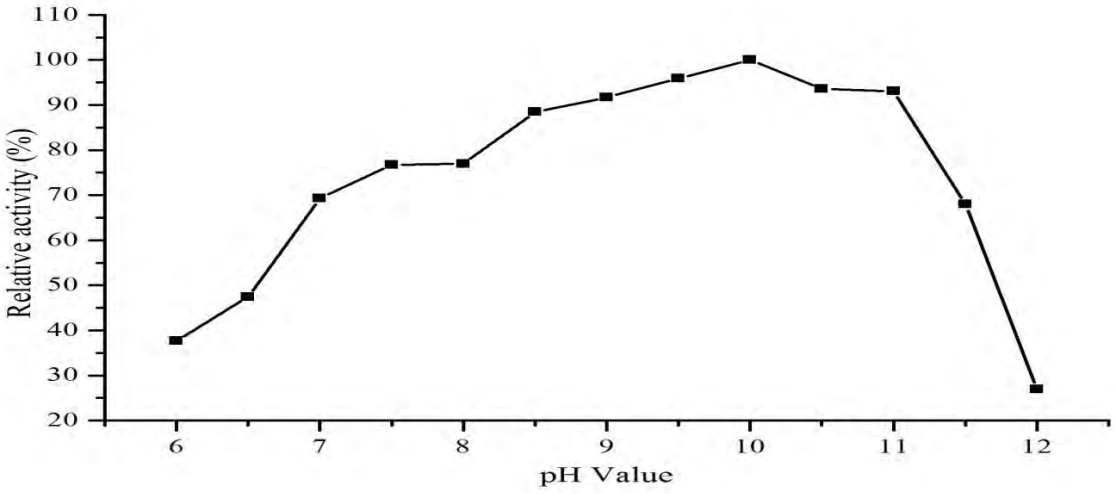


Fig 7. p*H profile of BACC-15 alkaline protease*

The protease exhibited > 60% of residual activity at pH range 6.0-10.5 with maximum stability at pH 8.0 when assay reaction mixture was at pH10.0. Likewise, >65% of original activity was retained at pH 6.0-10.5 and 100% of its original activity was retained at pH 8.0-9.0 when the assay reaction mixture was at pH 8.0 (Fig 8).

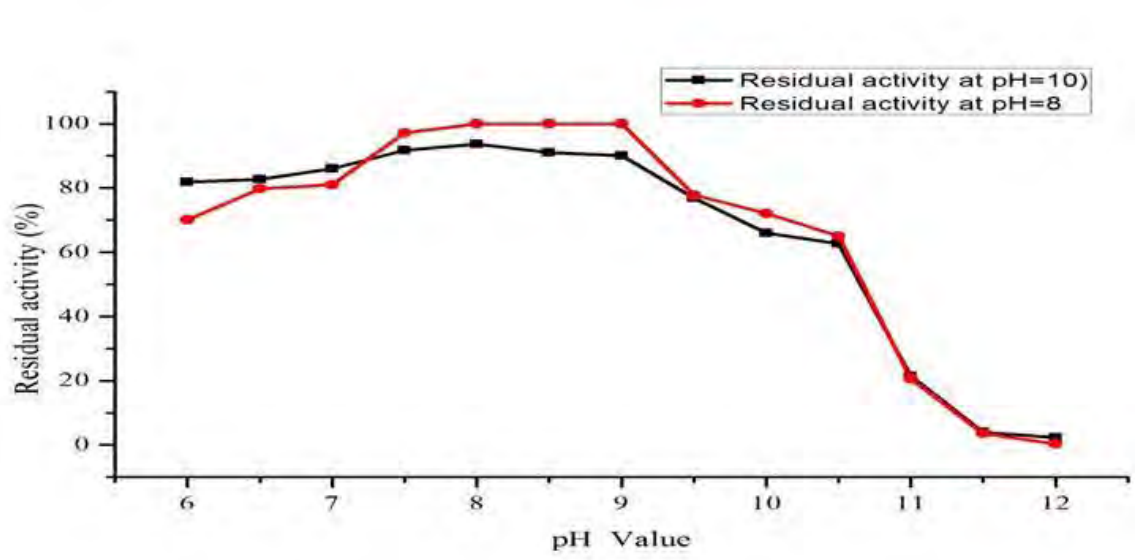


Fig 8. pH stability profile of BACC-15 alkaline protease

As shown in Figure 8 above, the maximum stability of BACC-15 alkaline protease achieved at pH of 6-10.5 and then sharply decreases beyond pH 10.5. The enzyme exhibited minimum stability in pH 11.0 with 21.42 % and 20.59% residual activity when the assay reaction mixture was pH 10 and 8.0 respectively. Beyond pH 11.0 the enzyme becomes totally inactivated.

5.6.3. Effect of temperature on activity and stability of the protease

The BACC-15 alkaline protease was found to be active at all temperatures tested between 35 and 75 ^{0}C with maximum activity at 60 ℃ in absence of 5mM $CaCl_2$ as shown as peak in Figure 9. The activity of protease progressively increased as temperature increased from 30 ℃ to 60 ℃ and then gradually decline beyond its optimum temperature. With in temperature range of 40-70℃, BACC-15 alkaline protease retained more than 54.2% of its maximum activity. At temperature of 30 and 80℃, the protease exhibited 32.46 and 31.15% of its optimum activity respectively. The minimum relative activity of protease was recorded at 85 ℃ (21.53%) (Fig 9).

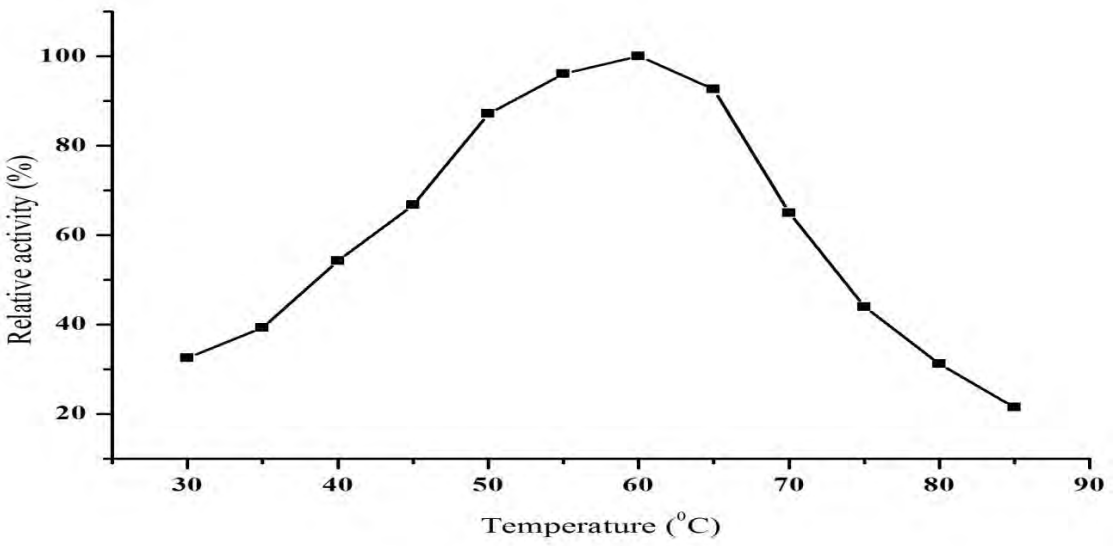
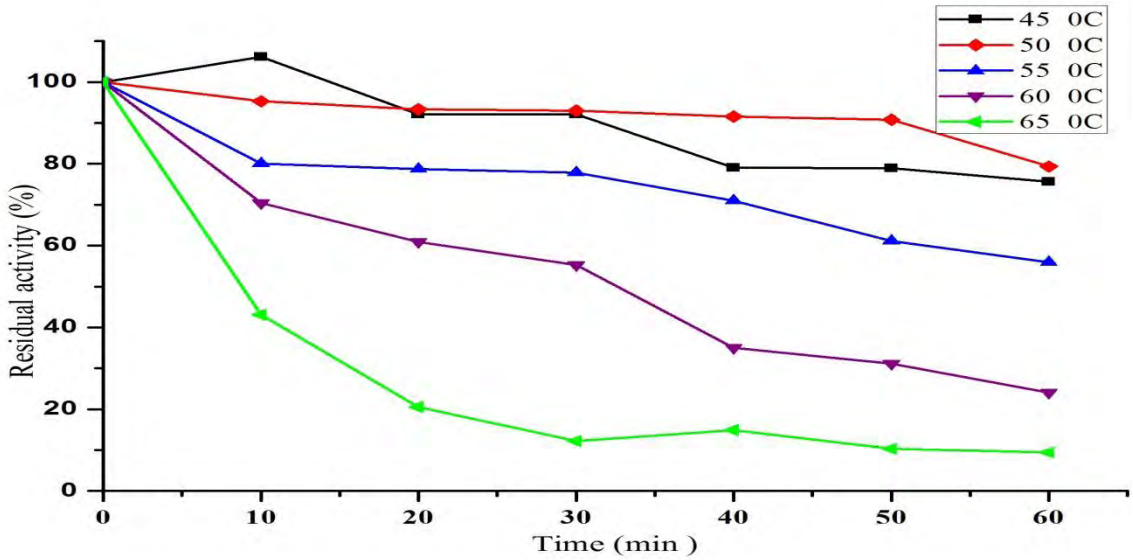
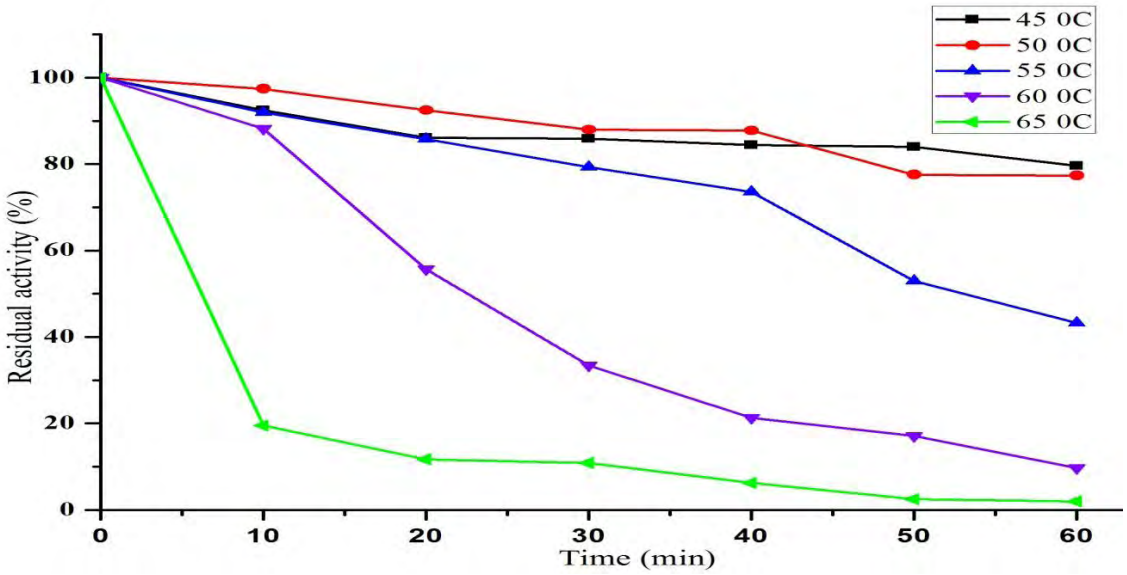


Fig 9. Temperature profile of BACC-15 alkaline protease

Thermal stability of BACC-15 alkaline protease was tested both at pH 8.0 and 10.0 as shown in Figure 10, a and b, respectively. The protease showed maximum thermal stability at 30-50 ℃ (>80% residual activity) at both pH 8.0 and 10.0 at 1h of incubation. After 30 min incubation beyond temperatures of 60 ℃ both at pH 8.0 and 10.0, the residual thermal stability sharply drops (<35%) and becomes inactivated at 1h of incubation at 65 ℃. Relatively higher thermal stability of BACC-15 alkaline protease was obtained at temperatures higher than 55 ℃ at pH 8.00 than 10.0 (Fig 10 a and b).



(a)Thermal stability at pH 8.0



(b) Thermal stability at pH 10.0

Fig 10. Thermal stability profile of BACC-15 alkaline protease (a) pH *8.0, (b)* pH *10.0*

5.6.4. Temperature profile in the presence of $CaCl_2$

Addition of 5mM $CaCl_2$ had not brought any increment on protease activity up to 65 ℃ and even relatively lower activity was exhibited in the presence of 5mM $CaCl_2$. However, addition of 5mM $CaCl_2$ changed the temperature maxima from 60 ℃ to 65 ℃. Addition of 5mM $CaCl_2$ enhanced activity up to 75℃. No significant differences were exhibited between 0 and 5mM $CaCl_2$ beyond 75 ℃ and even to some extent 0mM showed better activity (Figure 11).

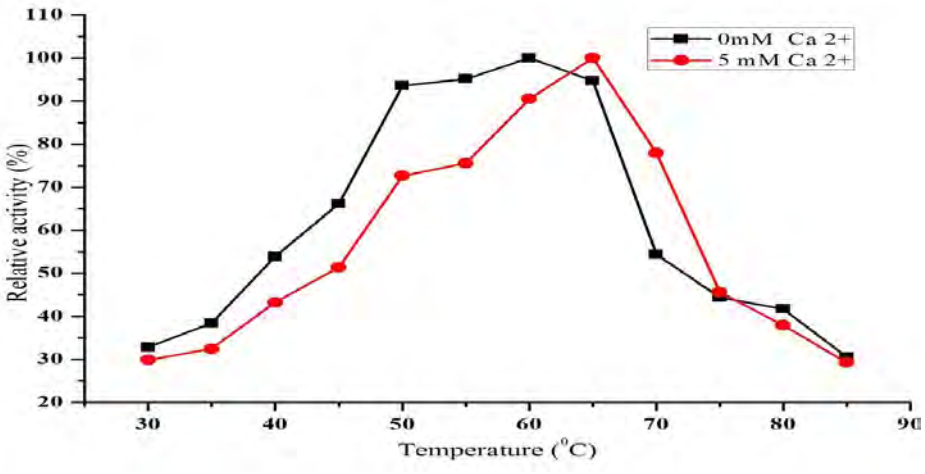


Fig 11. Calcium ion dependency profile of BACC-15 alkaline protease

5.6.5. Effect of Sodium Chloride on activity and stability

Maximum alkaline protease activity was obtained at 0.5M NaCl at both pH 8.0 (91.23%) and10.0 (96.13%). At 1.5M of NaCl, > 62% of optimum activity was recorded at pH 8 and 10. Even at highest concentration of NaCl (3M), BACC-15 alkaline protease showed 28.29% and 37.07% of the relative optimum activity at pH 8 and 10. Yet at both tested pH range, further increasing in NaCl concentration above 0.5M led to reduction in activity. In addition, at all studied concentrations of NaCl, comparatively higher relative activities were recorded at pH 10 than pH 8.0 (Fig 12a).

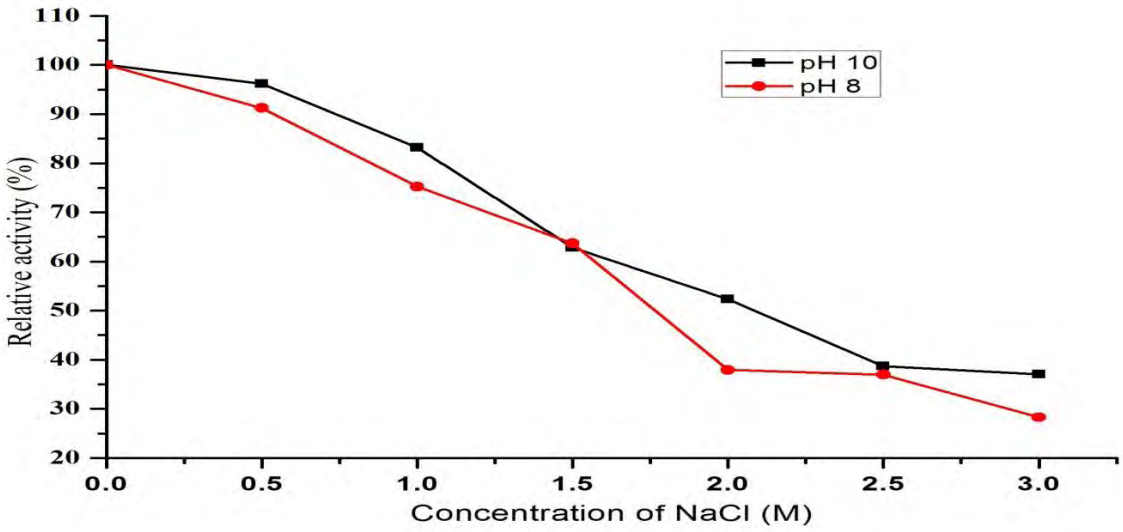


Fig 12. (a) The effect of [NaCl] on activity of BACC-15 alkaline protease

Unlike to the effect on activity at all tested concentrations of NaCl, enhancement on the stability of BACC-15 alkaline protease was revealed. After 1h of incubation at pH 8 and 10, more than 100% of original activity was retained between 0.5-3.0 M NaCl. At 11.68% w/v (2M) NaCl, 106.12 % (at pH 8) and 106.96% (at pH 10.0) of residual activity was recorded. As shown clearly in Figure 12(b), with increasing concentration of NaCl, the stability of BACC-15 alkaline protease increased and attained 108.62% and 108.39% of residual activity at pH 8.0 and 10 respectively in 17.53% w/v or 3M NaCl. Relatively higher residual activity or halo-stability was obtained at pH 10.0 than 8.0.

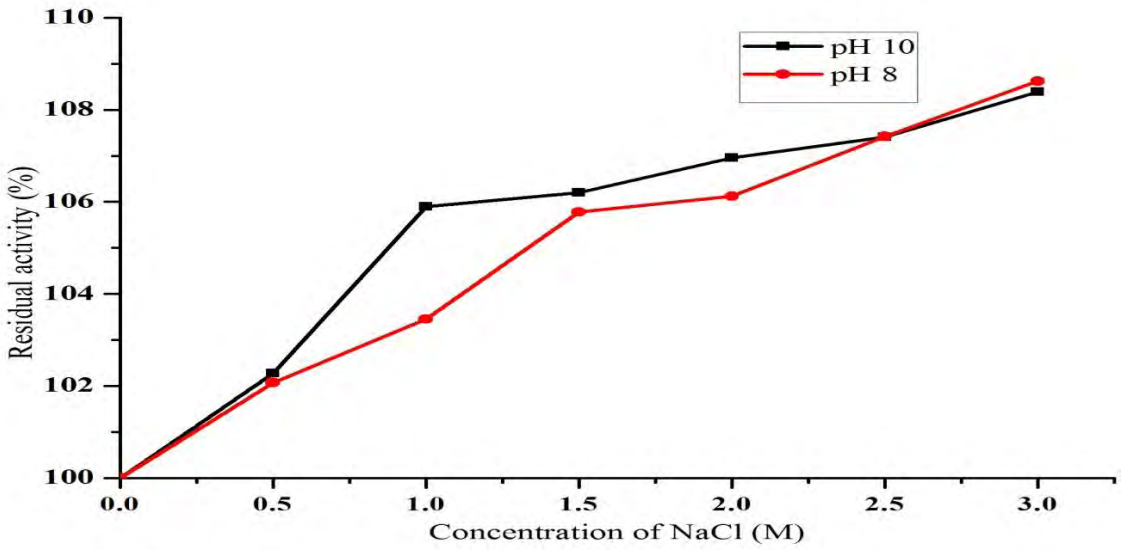


Fig 12. (b) The effect of [NaCl] on stability of BACC-15 alkaline protease

5.6.6. Effect of inhibitors on enzyme stability

BACC-15 alkaline protease was inhibited 39.06, 96.8 and 43.79% by 5mM of EDTA, PMSF and 1, 10- Phenanthroline respectively. Maximum inhibition (97.53%) was exhibited at 10mM of PMSF while EDTA and 1, 10-phenanthroline did not produce drastic inhibition. Comparatively maximum level of inhibition was exerted on BACC-15 alkaline protease by PMSF regardless of the concentration of inhibitors used while EDTA and 1, 10-phenanthroline inhibition capacity were concentration dependent (Table 16).

Table 16. The effect of inhibitors on BACC-15 alkaline protease stability

	Residual activity (%)			Inhibition (%)		
[Inhibitors mM	EDTA	PMSF	1,10-phenanthroline	EDTA	PMSF	1,10-phenanthroline
0 (Control)	100	100	100	0	0	0
1	70.80	5.88	59.96	29.20	94.12	40.04
5	60.94	3.20	56.21	39.06	96.80	43.79
10	59.61	2.47	53.35	40.39	97.53	46.65

5.6.7. Effect of oxidizing and bleaching agents on the stability of the protease

The stability of BACC-15 alkaline protease with oxidants and bleaching agents commonly added in present day detergent formulations were tested using strong oxidizing agent H_2O_2 (Figure 13) and bleaching agent $NaBO_3.H_2O$ (Figure 14). Maximum stability towards oxidizing agent was obtained at 1% v/v of H_2O_2, which in fact slightly enhanced the stability by 3.73% compared to the control. The BACC-15 alkaline protease was highly stable retaining 89.52, 89.37, and 87.07% of residual activity when incubated with 10, 15 and 20% v/v of H_2O_2 respectively. Even at the presence of a full strength (30%) v/v H_2O_2, the protease retained 83.2% of original activity (Figure 13).

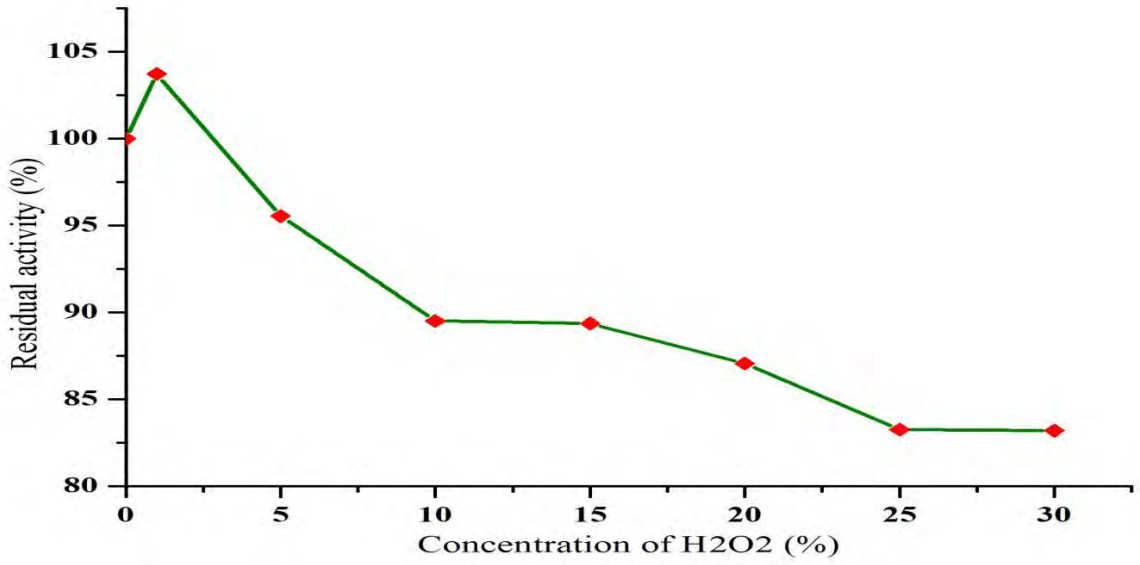


Fig 13. The effect of oxidizing agent (H_2O_2) on stability of BACC- 15 alkaline protease

The effect of bleach on BACC -15 alkaline protease stability was studied using sodium per borate. The enzyme was highly active and stable towards all the tested concentrations of sodium perborate and moderate enhancement on activity was recorded as shown in Figure 14. Interestingly, the protease retained greater than 100% of its original activity in presence of higher concentration v*iz*. 50 mM $NaBO_3.H_2O$ after 1h pre - incubation at 30^0C. At 15mM $NaBO_3.H_2O$, BACC-15 alkaline protease achieved 110.23% of original activity followed by 108.39, 105.99 and 105.21% of the original activity after 1h pre –

incubation at 20, 25 and 50mM. As clearly shown in Figure 14, beyond 15mM $NaBO_3.H_2O$ the residual activity remain $\geq$ 105% and shows no significant reduction in activity.

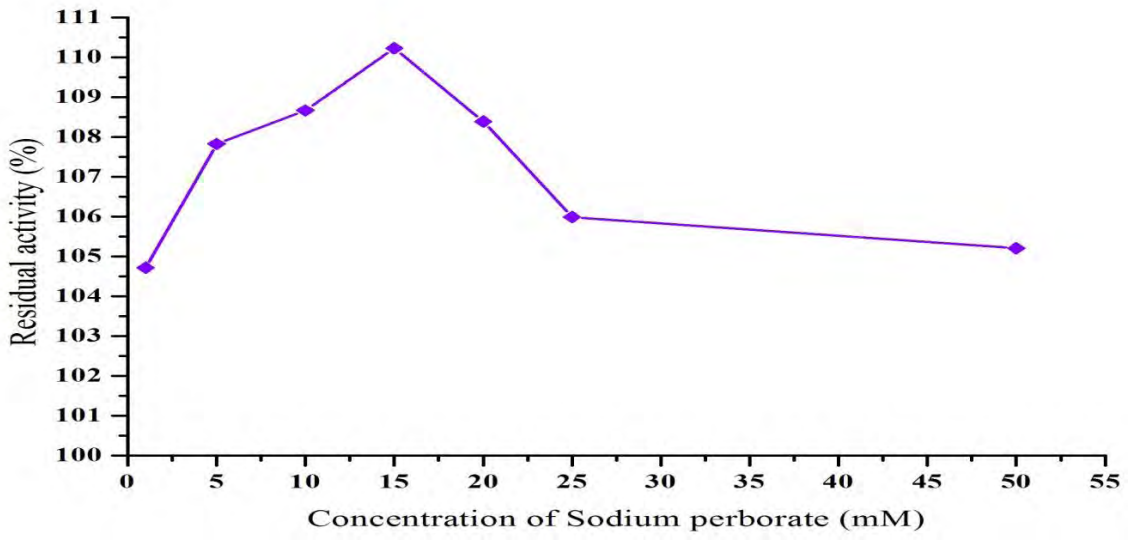


Fig 14. The effect of sodium per borate on stability of BACC-15alkaline protease

5.6.8. Effect of detergents and sequestering agent on stability of the protease

The protease was found to be quite stable at lower concentrations of SDS (anionic detergent), Tween-80 (non-ionic detergent), and Triton X-100 (non-ionic surfactant). With the increase in concentrations of detergents and surfactant studied, the inhibitory effect was also found to increase (Figure 15). The maximum residual activity towards SDS (anionic detergent) was obtained at 1% (w/v) (86.01%) and 85.95, 70.81 and 66.01% of original activity retained at 5, 15 and 25% (w/v) respectively. Even at presence of 30% w/v SDS, 43.18% of residual activity was retained. Likewise, highest residual activity towards Tween-80 was obtained at 1% v/v (88.93%) and 87.29, 66.5, 39.57% of original activity was retained at 5, 15 and 25% (v/v) respectively. Unlike to the SDS and Tween-80, minimum inhibitory effect was exerted on BACC-15 alkaline protease by Triton-X 100. The protease was highly stable in Triton-X 100, and it retained more than 95% of original activity at 20% v/v. Nevertheless, at presence of 25% v/v of Triton-X 100, BACC-15 alkaline protease retained 93.33% of its original activity.

Generally, maximum activity and stability was recorded at presence of Triton X-100 than SDS and Tween-80 after 1 h pre - incubation at 30 ℃ (Figure 15).

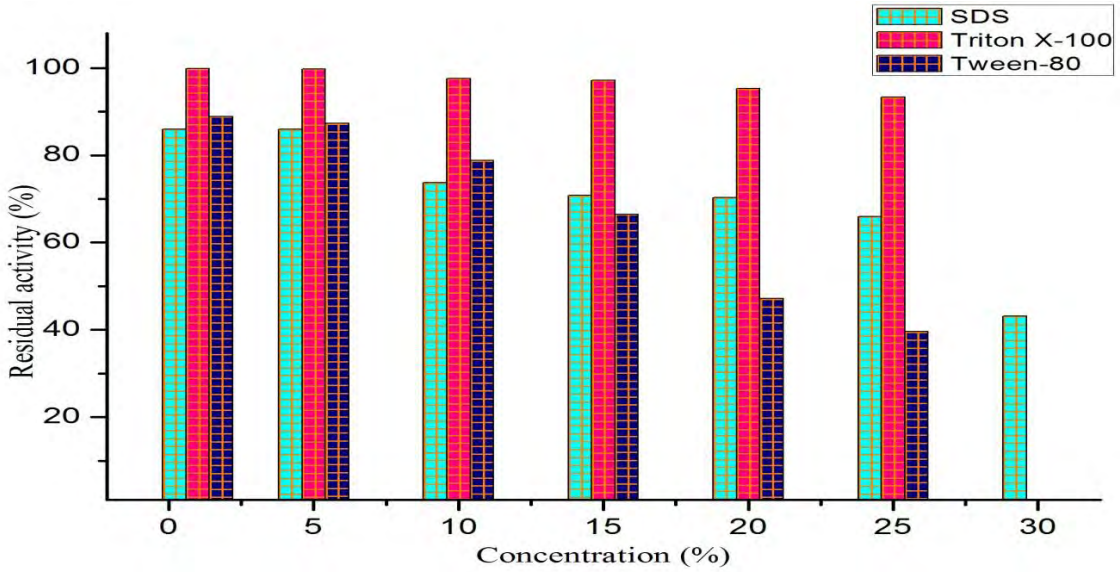


Fig 15. The effect of ionic and non ionic detergents on stability BACC-15 alkaline protease

Sodium citrate is one of the commonly used sequestering agents (builders) in most of washing detergents. Interestingly, the stability of protease was slightly enhanced in the presence of up to 2M sodium citrate (Figure 16). Maximum residual activity was obtained at 1.5 M sodium citrate with 106.04% of original activity and further increase in concentration brought slight reduction on stability.

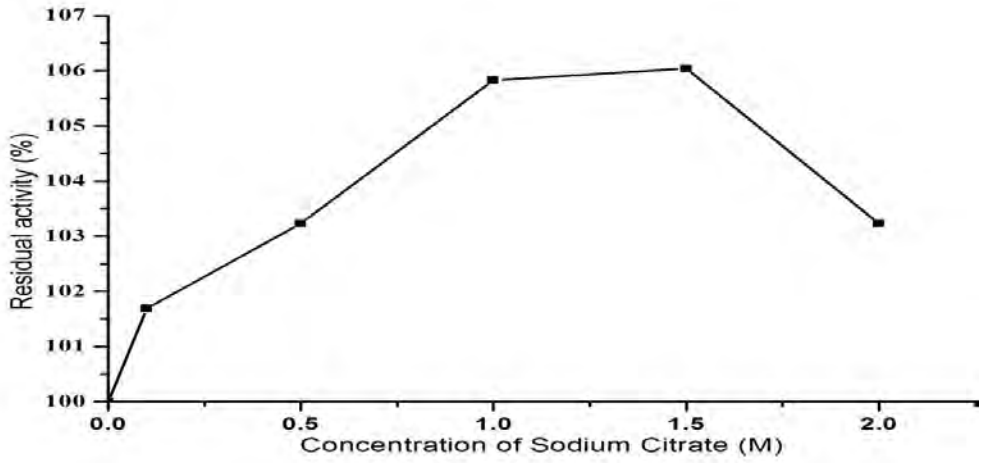


Fig 16. The effect of Sodium citrate on stability of BACC-15 alkaline protease

5.6.9. Effect of Endod (*Phytolacca dodecandra*) on stability of protease

It was reported by many researchers that Endod berries (*Phytolacca dodecandra*) contain saponins (ionic bioactive compounds) (Zimudzi, 2007). They are the source of foam/soap forming substances in these plants. It was seen that at 2 h of pre incubation at 40 ℃, BACC-15 alkaline protease retained 62.20, 97.7 and 114.20% of original activity in the presence of 75, 50 and 25% (v/v) of Endod preparations respectively (Table 17). This experiment clearly shows that the enzyme is compatible with Endod.

Table 17. The effect of *Phytolacca dodecandra* on stability BACC-15 alkaline protease

Protease to Endod ratio	Residual activity (%)
control	100
25% protease + 75% Endod	62.2
50% protease + 50% Endod	97.7
75 % protease + 25% Endod	114.2

5.7. Potential Biotech applications of BACC-15 alkaline protease

5.7.1. Detergent applications and stain removal

Before diving into stain removal efficiency of alkaline protease in removal of blood and egg stains, the stability and compatibility of BACC-15 alkaline protease with Ariel, solid commercial detergent and different concentrations of Endod were tested. The study showed that the protease was quite stable (>95% residual activity) in presence of both of them on 2 h of pre incubation at 30 ℃. In addition, the presence of protease in endod preparations alone and Ariel detergent was conducted using casein as substrate following standard protease assay procedures. Then the relative activity was calculated. The study had shown that neither Ariel detergent nor Endod preparations were found to contain active protease when the assay reaction mixture was at pH 7.0, 8.0 and 10.0 and also at room temperature and 40 ℃.

The set of pieces of clothes treated (washed) with Ariel detergent, distilled water and Glycine-NaOH buffer remained dirty after 30 min - 1h incubation at 40 ℃ by simulating washing conditions for both of them. Furthermore, with prolonged in incubation up to 2h, the stain was cooked on cotton cloths and become stubborn. Relatively only slight removal of blood and egg stain was seen on clothes washed with Ariel detergent than distilled water and buffer (Figure 17 a and b). Interesting result was obtained when the pieces of cotton clothes were washed with the mixture of BACC-15 alkaline protease and Endod preparations. The BACC-15 alkaline protease (84.21U/g) totally hydrolyzed both egg and blood stains at 1h incubation at 40 ℃. Keeping the concentration of enzyme and temperature similar, reducing time to 30 min and stimulating washing conditions resulted in relatively lower washing efficiency. Gradual removal of blood and egg stain from apiece of white cotton cloth was observed by increasing the contact time of enzyme broth and stained cloth. Similarly, using 10% (w/v) Endod preparations solution (100%) resulted in only slight removal of egg and blood stains (Table 18).

Another interesting finding was combination of enzyme and Endod preparations resulted in better stain removal performance than either enzyme or Endod alone (100%). After 30 min incubation, complete removal of egg and blood stains were observed by using 25% BACC-15 alkaline protease and 75% Endod preparations (Fig 17 a and b).

It was seen that at the BACC-15 alkaline protease ratio $\leq$ 50% in the combination to Endod preparations results in excellent washing performance and /degradations of blood and egg stains and improves the washing efficiency of Ethiopian traditional soap (Endod). Likewise, better stain removal performance was also achieved at Endod preparations concentrations $\geq$ 50% in the combination.

Table 18. Visual evaluation of washing performance of BACC-15 alkaline protease, Endod and different combinations of protease and Endod preparations.

Proportion of Enzyme to Endod preparations(treatments)	Inferences on performance of washing efficiency of stained white cotton clothes	
	Blood stain	Egg stain
Distilled water (Control)	-^{e}	-^{d}
Glycine-NaOH buffer (Control)	-^{e}	-^{d}
Endod + Buffer (100%)	+^{d}	+^{c}
Crude alkaline protease (100%)	++^{c}	+++^{b}
25% Enzyme + 75% Endod	++++ ^{a}	++++^{a}
50% Endod + 50% Enzyme	+++^{b}	+++^{b}
75% Enzyme + 25% Endod	++^{c}	++^{c}
Ariel detergent (100%)	+^{d}	-^{d}

Where '+' sign shows performance of washing efficiency (cleanness) or stain removal level, ++++ shows the stain was completely removed; whereas '–' sign shows the level of dirtiness (the stain was not removed). Treatments with letter of the same superscripts do not vary significantly visually.

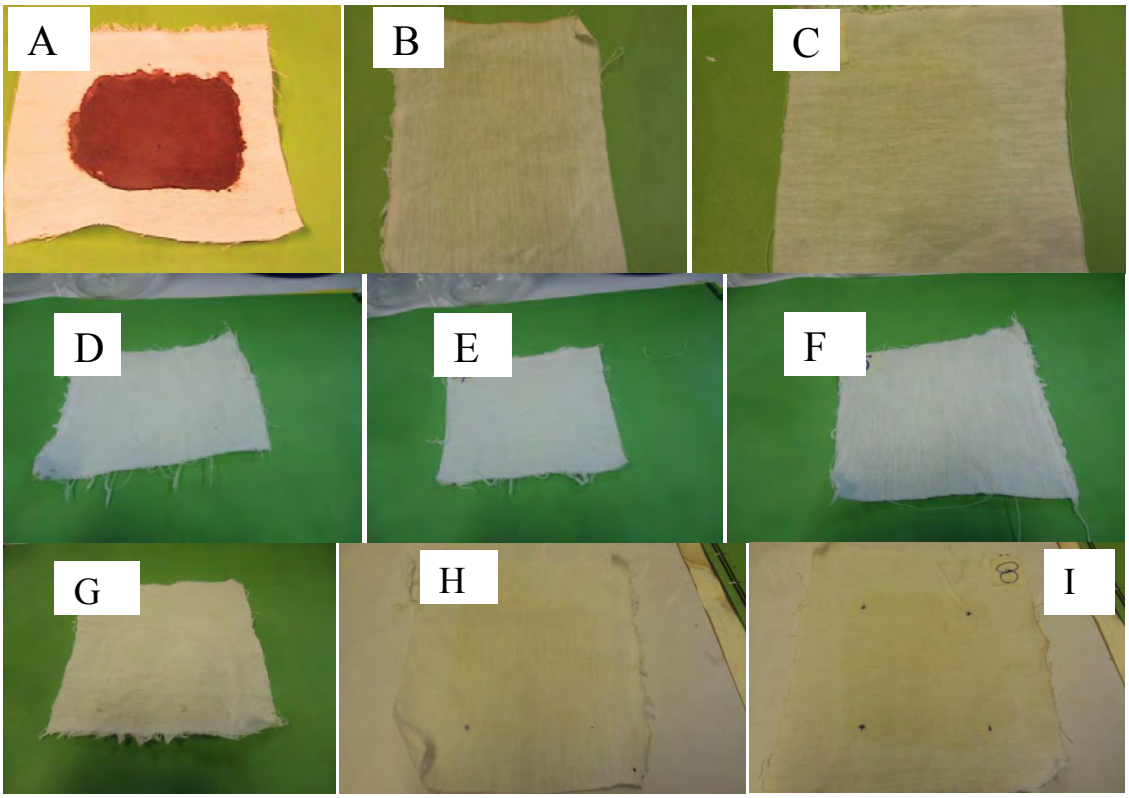


Fig 17. (a) Blood stain removal efficiency of treatments viz. different combinations of enzyme and Endod preparations, commercial detergent alone and control treatments. (A) stained piece of cotton cloth before any treatment was applied ; and then treated with (B) Control(distilled water only) (C) Endod preparations only (10%w/v) (D) BACC-15 alkaline protease only(84.21U/g), (E) 25% Enzyme + 75% Endod (F) 50% enzyme + 50% Endod (G) 75% Enzyme + 25% Endod (H) Ariel detergent (7mg/ml) and (I) Glycine- NaOH buffer 100mM and pH 10.0.

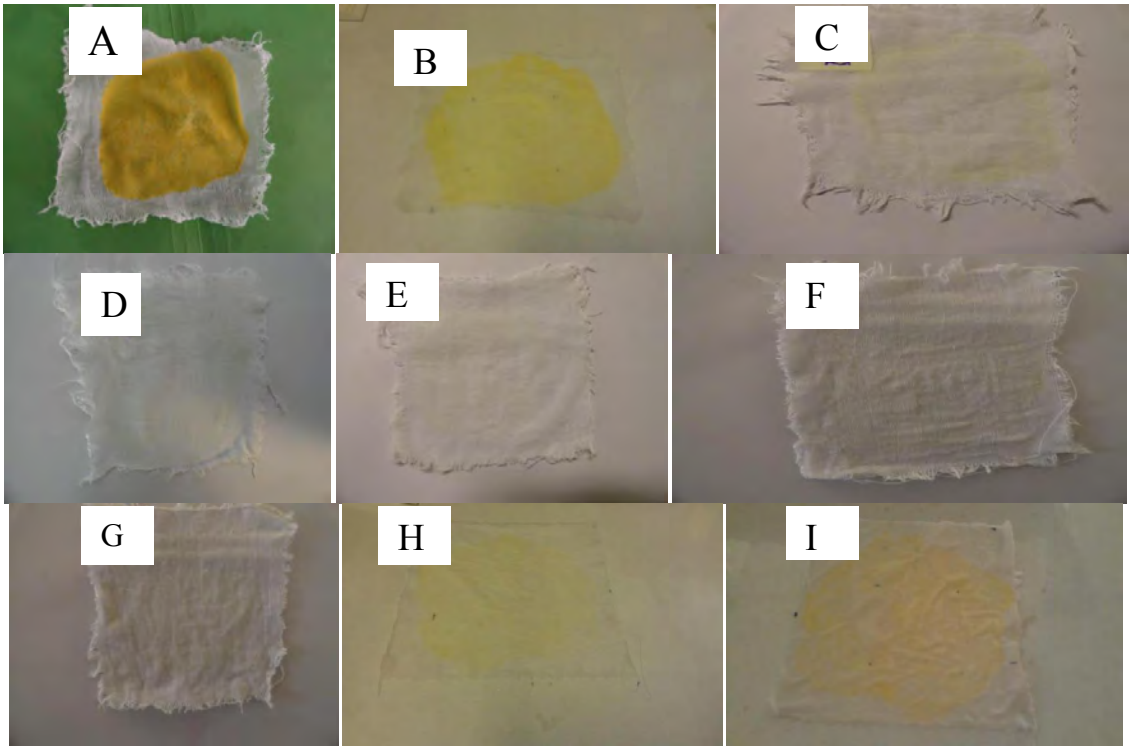


Fig 17. (b) Egg yolk stain removal efficiency of treatments viz. different combinations of enzyme and Endod preparation, commercial detergent and control treatments. (A) stained piece of cotton cloth before any treatment was applied ; and then treated with (B) Control(distilled water only) (C) Endod preparations only (10%w/v) (D) BACC-15 alkaline protease only(84.21U/g), (E) 25% Enzyme + 75% Endod (F) 50% enzyme + 50% Endod (G) 75% Enzyme + 25% Endod (H) Ariel detergent (7mg/ml) and (I) Glycine- NaOH buffer 100mM and pH 10.0.

5.7.2. Protein hydrolysates production

Protein digestibility of defatted Soy bean protein isolates (SBPI) and Moringa seed protein isolates (MSPI) treated by concentrated BACC-15 alkaline protease is shown in (Table 19). Using acetone concentrated BACC-15 alkaline protease the degree of hydrolysis of 77.09% for Soy bean protein hydrolysate (SBPH) and 66.19% for Moringa seed protein hydrolysate (MSPH) was obtained.

Table 19. The efficiency of BACC-15 alkaline protease in hydrolysis of SBPI and MSPI

Sample	Total Nitrogen content (%)	Total protein (%)	DH (%)
Soy bean Protein Isolate	14.93	93.31	77.09
Soy bean Protein Hydrolysate	11.51	71.94	
Moringa Seed Protein Isolate	9.91	61.94	66.19
Moringa Seed Protein Hydrolysate	6.56	41.00	

The protein content of BACC-15 alkaline protease digested hydrolysate from Soy bean protein isolate (71.94%) was higher than that of Moringa seed protein isolate hydrolysate (41.0%). After drying both Moringa seed protein hydrolysates and Soy bean protein hydrolysates were light brown in color.

5.7.2.1. Evaluation of bacterial growth on SBPH and MSPH in comparison with commercial peptone

The colony counts of pathogenic bacteria on SBPHA and MSPHA was compared with commercial peptone agar (Table 20). Statistically no significant differences were observed among SBPH, MSPH and commercial peptone (Oxoid, UK) across all the tested pathogenic bacteria except *Entrococcus faecalis* (ATCC 29212). Maximum CFU/ml of *Entrococcus faecalis* ($8.83 \times 10^7 \pm 6.80$) was recorded by using SBPH followed by CPA ($3.83 \times 10^7 \pm 3.25$) whereas MSPH strongly inhibited the growth of this pathogen. Growth of some pathogens in SBPHA, MSPHA and CPA was shown in Figure 18.

Table 20. Comparison of growth of some fastidious pathogenic bacteria on SBPHA and MSPHA in reference to CPA (CFU/ml)

Test bacteria	CPA (CFU/ml)	SBPHA (CFU/ml)	MSPHA (CFU/ml)
Escherichia coli	$6.33 \times 10^7 \pm 1.54^a$	$6.33 \times 10^7 \pm 3.51^a$	$7.66 \times 10^7 \pm 4.61^a$
Staphylococcus aureus	$6.66 \times 10^7 \pm 3.21^a$	$2.00 \times 10^7 \pm 1.00^a$	$3.00 \times 10^7 \pm 2.64^a$
Pseudomonas aeruginosa	$1.13 \times 10^8 \pm 1.09^a$	$1.10 \times 10^8 \pm 3.00^a$	$1.70 \times 10^8 \pm 3.00^a$
Salmonella(NCTC 8385)	$1.03 \times 10^7 \pm 1.52^a$	$7.33 \times 10^7 \pm 1.15^a$	$5.33 \times 10^7 \pm 4.93^a$
Enterococcus faecalis	$3.83 \times 10^7 \pm 3.25^b$	$8.83 \times 10^7 \pm 6.80^a$	0.00 ± 0.00^c

Where SBPHA = Soy bean protein hydrolysate agar, MSPHA= Moringa seed protein hydrolysate agar, CPA = commercial peptone agar, CFU/ml = colony forming unit per milliliter. Values are means of triplicate determinations; Values within the same row followed by different superscripts are significantly different at ($P<0.05$).

Growth productivity of microorganisms in the test medium was compared using productivity ratio method. This method is important to compare and contrast the productivity of culture media in the study with that of reference or standard commercial medium available in the market (Berhanu Andualem and Amare Gessesse, 2013). In this study, productivity ratio of greater than 1.0 was recorded in all test pathogenic bacteria except that of *Staphylococcus aureus* (0.3 at SBPHA), (0.45 at MSPH). Least productivity ratio was observed in *Enterococcus faecalis (*0.00 at MSPHA) (Table 21).

Table 21. Productivity ratio of SBPHA and MSPHA versus CPA

Test pathogen	Productivity ratio	
	SBPHA	MSPHA
Escherichia coli	1.00	1.21
Staphylococcus aureus	0.300	0.45
Pseudomonas aeruginosa	0.973	1.50
Salmonella(NCTC 8385)	7.11	5.17
Enterococcus faecalis	2.30	0.00

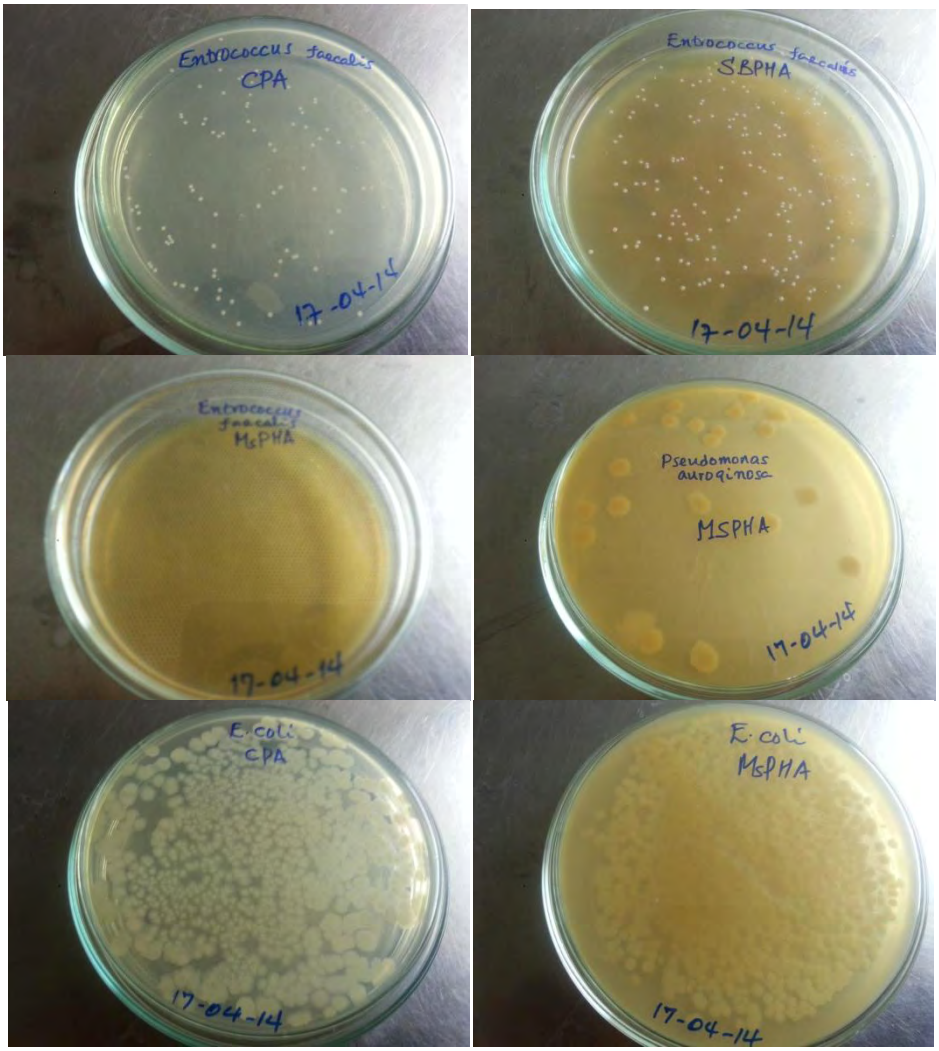


Fig 18. Colony of some selected pathogens grown on CPA, SPHA and MSPHA

6. Discussions

Isolation, screening, and identification of potentially useful microbial strains capable of producing the desired product, are the first critical step in the development of any industrial fermentation process (Glazer and Nikaido, 2007; Okafor, 2007). In this study an alkaliphilic *Actinobacteria* strain producing an alkaline protease with interesting potential application in different industrial processes was isolated from alkaline habitats in Ethiopia and characterized. Similarly Amare Gessesse (1997); Amare Gessesse and Gashe (1997); Amare Gessesse *et al.* (2003a) have also isolated different alkaliphiles producing industrially important alkaline enzymes. This shows that naturally occurring alkaline habitats of the East African Rift Valley (the soda lakes) harbor interesting microbial strains potentially useful for a variety of industrial applications.

Selection of a suitable microorganism is one of the most important criteria in SSF but also remains a tedious task, especially when commercially significant enzyme yields are to be achieved (Pandey *et al.,* 1999; Pandey *et al.,* 2000; Pandey, 2003; Nigam and Pandey, 2009). The vast majority of wild type microorganisms are incapable of producing commercially acceptable yields of the desired products in SSF (Pandey *et al.,* 2008; Nigam and Pandey, 2009*).* As a result, currently industrial demand of most of the enzymes is met by production using SmF, generally employing genetically modified strains (Pandey *et al.,* 2000; Hölker *et al.,* 2004). The cost of production in SmF is high and it is uneconomical to use many enzymes in several processes. However, in this study *Actinobacteria* BACC-15 isolated from natural environment produced high titers of alkaline protease in SSF using wheat bran as a low cost SSF substrate.

Optimization of fermentation process parameters is done to establish favorable conditions to the growing microorganism and thereby minimize unutilized components at the end of fermentation (Pandey *et al.,* 2008). No defined medium has been established for the optimum production of alkaline proteases from different microbial sources. Each organism or strain has its own special conditions and requirements for maximum enzyme

production (Paul, 2005). Therefore, *Actinobacteria* BACC-15 may also have its own special requirements for optimum alkaline protease production.

Solid state fermentation process is governed by a large number of physical, chemical and biological factors (Pandey *et al.,* 2000; Thomas *et al.,* 2013). Similarly, alkaline protease production is also governed by several factors in SSF processes. Among the various factors, some process parameters highly affect alkaline protease production by SSF such as selection of suitable strain and substrate, fermentation time, fermentation temperature, inoculum size and initial moisture content (Pandey *et al.,* 1999; Pandey, 2003; Paul, 2005; Özkan and Ertan, 2012; Thomas *et al.,* 2013). In this study these SSF process parameters were optimized for maximum alkaline protease production by *Actinobacteria* BACC-15.

In SSF system, water is used in limited quantity. When water is available in lower or higher amounts than the desired optimum, it affects the process productivity significantly (Tunga *et al.,* 1998). An increase in moisture level is believed to reduce the porosity of the substrate, alters particle structure of the substrate, and poses diffusional limitations and thus leading to poor oxygen transfer. It also promotes the growth of unwanted bacteria. Low moisture content causes reduction in the solubility of nutrients of the substrate, lower degree of swelling and higher water retention (Tunga *et al.,* 1998; Divakar *et al.,* 2006; Nigam and Pandey, 2009). Hence, optimal level of initial moisture content is required for maximum alkaline protease biosynthesis.

Isolate BACC-15 produced maximum alkaline protease (557.06± 24.89 to 595.75± 9.60U/g) when the initial moisture level was 1:1.0 to 1:1.8 wheat bran to moistening agents. Comparable levels of moisture contents were reported in studies of Bajaj and Singh (2010) who reported moisture content of 1:2.5 w/v for optimum xylanase production using *Streptomyces* sp 7b. Similarly, *Actinobacterium* PVJL produced maximum alkaline keranolytic protease when initial moisture level of 1:1.20 w/v was used (Vijayaraghavan *et al.,* 2012). *Rhizopus oryzae* RO IIT RB-13 maximum protease production was attained at moisture level of 1:1.4 w/v (Tunga *et al.,* 1998). Higher

moisture level requirement was reported by Saxena and Singh (2011) who reported initial moisture level as high as 1:3 w/v (300%) for optimum metallo - protease production by *Bacillus* strain. However, Mitra and Chakrabartty (2005); Sandhya *et al.* (2005); Lazim *et al.* (2009) have studied moisture levels as low as 1:0.4, 1:0.43, and 1: 0.6 w/v optimum for alkaline protease production by *Streptomyces nogalator, Aspergillus oryzae* and *Streptomyces* sp. CN902 respectively. Thus, the optimum moisture level in SSF depends on the requirement of the microorganism and type of end products (Nigam and Pandey, 2009).

The incubation time both in (SSF and SmF) is governed by characteristics of the culture / its metabolic profiles and is based on growth rate and enzyme production (Thomas *et al.,* 2013). The time employed may vary from 2 to 9 days depending upon the organism involved (Aikat and Bhattacharyya, 2001). BACC-15 alkaline protease production detected after 72 h in SmF and 96 h in SSF reached its optimum level at 7^{th} day. Protease production by *Trichothecium roseum* and *Streptomyces rimosus* was reported to be 7 and 9 days respectively after inoculation (Yang and Wang, 1999; Özkan and Ertan, 2012). However, shorter period of incubation of 3 to 4 days for maximum alkaline protease production was also reported for *Streptomyces* sp. 594 (Azeredo *et al.,* 2006); *Streptomyces* sp. CN902 (Lazim *et al.,* 2009) ; *Penicillium godlewskii* SBSS25 (Sindhu *et al.,* 2009) and *Streptomyces albidoflavus* (Narayana and Vijayalakshmi, 2008; El-Shafei *et al.,* 2010).

Importance of inoculum size on microbial fermentation processes is widely accepted and it has some optimum value depending on the microbial species and fermentation system (Tunga *et al.,* 1998; Sandhya *et al.,* 2005). A smaller inoculum extends the lag phase where as larger inoculum quantity favors more substrate degradation and biomass formation, perhaps because of the larger quantity of culture. Production of high alkaline protease titer by *Actinobacteria* BACC-15 was attained at 3% w/v inoculum level. Higher amounts of inoculum 10% and 20% w/v were reported for alkaline protease production using *Thermoactinomyces thalpophilus* PE 14 and *Actinobacterium* PVJL in SSF (Divakar *et al.,* 2006; Vijayaraghavan *et al.,* 2012).

The extraction efficiency is critical to the recovery of the enzyme from the fermented biomass; hence selection of a suitable solvent is necessary (Pandey, 2003). In this study carbobonate / bicarbonate buffer was found as best for BACC-15 alkaline protease recovery. Similar result was reported by Paul (2005) for recovery of alkaline protease from *Vibrio* species. However, 10% v/v ethanol to water mixture was found to be best for recovery of protease from *Rhizopus oryzae* (Tunga *et al.,* 1999).

The protease from *Actinobacteria* BACC-15 showed maximum activity at pH 10.0 and stability at pH 8.0-9.50 which entitles it to be classified under the category of alkaline protease. Proteases from most species of *Actinobacteria* in alkaline environments were reported to have their optimum pH at 9.0-11.0. Protease from salt-tolerant alkaliphilic *Actinomycete Nocardiopsis alba* OK-5 showed optimum activity at pH 10.0-11.0 (Gohel and Singh, 2013). Proteases from *Actinomycete* MA1-1 (Hameş-Kocabaş and Uzel, 2007); *Streptomyces gulbargensis* (Vishalakshi *et al.,* 2009); *Streptomyces albidoflabus* (El-Shafei *et al.,* 2010) showed optimum activity at pH 9.0 and with broad range of activity pH 8.0-13.0. Proteases active and stable at alkaline pH range especially (pH 8.0-12.0) are known for their applications in detergent formulations. Moreover, they find application in leather processing, peptide synthesis, silk industry, silver recovery from waste photographic and X-ray films, protein hydrolysates production, waste treatment (Rao *et al.,* 1998; Kumar and Takagi, 1999; Gupta *et al.,* 2002; Amare Gessesse *et al.,* 2003a; Ray, 2012; Jisha *et al.,* 2013). This indicates that BACC-15 alkaline protease can be potential candidate in such industrial applications.

Bacterial alkaline proteases are characterized by their high activity at temperature between 50℃ and 70℃ (Jisha *et al.,* 2013). The crude protease from *Actinobacteria* BACC-15 showed optimum activity at 60℃ and maximum thermal stability at 30-55℃. Similar ranges of optimum temperature for protease activity and stability from *Actinobacteria* was reported from *Streptomyces rimosus* K-0K-02 (Yang and Wang, 1999), *Streptomyces thermoviolaceus* (Siddique *et al.,* 2001), *Streptomyces* sp. 594 (Azeredo *et al.,* 2006), *Nocardiopsis prasina* HA4 (Ningthoujam *et al.,* 2009). The high activity and stability of BACC-15 alkaline protease at wide range of temperatures could

be very important characteristics for its eventual use at detergent industry as well as other industrial applications practiced at 30-75℃.

BACC-15 alkaline protease was highly stable attaining more than 100% residual activity with increasing concentration of NaCl (0-3M). Studies of Gohel and Singh (2013) shown high stability of protease from salt tolerant alkaliphilic *actinomycete* OM-6 up to 4M NaCl. Similarly, Joo and Chang (2005); Singh *et al.* (2011) reported full stability of protease from *Bacillus cereus* at 3-10 % (w/v) NaCl. The result of this study suggests that NaCl have significant role in conformational stabilization of BACC-15 alkaline protease. At higher NaCl concentration, the halophilic enzyme becomes stable because NaCl decreases unfavorable electrostatic repulsion (Mevarech *et al.,* 2000; Gohel and Singh, 2013). In addition, halophilic enzymes have high negative surface charge due to the presence of large number of acidic amino acids (glutamic and aspartic acids) with hydrated carboxyl groups which are shielded by high salt concentration. This avoids unfolding and maintain the solubility of these proteins (Madern *et al.,* 2000; Mevarech *et al.,* 2000).

The activity of most proteases from extremely halophilic microorganisms falls off dramatically and irreversibly when the enzyme is exposed to lower salt concentrations (Jadhav *et al.,* 2013). However, BACC-15 alkaline protease showed considerable amount of activity both in presence and absence of NaCl. Moreover, BACC-15 alkaline protease is stable at high concentrations of NaCl. The high salt tolerance is characteristic feature of proteases which has number of application in any biotechnological process that depends on high salinity or osmotic pressures. Therefore, these characteristics make BACC-15 alkaline protease an interesting candidate for applications in biotechnological processes, like in food, detergent and antifouling coating, leather processing, peptide synthesis, and proteinaceous saline waste water treatment industries.

BACC-15 alkaline protease was almost completely inhibited by PMSF (serine protease inhibitor). EDTA (metallo-protease inhibitor) and 1, 10-phenanthroline (Zinc specific metallo- protease inhibitor) moderately inhibited BACC-15 alkaline protease activity.

Since the protease activity was nearly completely blocked by PMSF, this enzyme must be an alkaline protease with serine residue at its active site. Many studies reported slight sensitivity of serine proteases to EDTA and other metallo protease inhibitors in addition to PMSF (Mitra and Chakrabartty, 2005; Rifaat *et al.,* 2005; Azeredo *et al.,* 2006; Jain and Jain, 2010; Singh *et al.,* 2011). This again suggests BACC-15 alkaline protease could be serine proteases.

BACC-15 alkaline protease was found to be highly stable in the presence of strong oxidizing agent (H_2O_2) and oxygenated bleaching agent (sodium perborate). Comparable results were reported by Oberoi *et al.* (2001); Moreira *et al.* (2002); Amare Gessesse *et al.* (2003b); Joo and Chang (2005). However, lower stability towards oxidants and bleaches was reported by protease of *Bacillus mojavensis* (Haddar *et al.,* 2009; Haddar *et al.,* 2010).

Commercially available proteases (Subtilisin Carlsberg, Subtilisin BPN', Alcalase, Esparase and Savinase) though have great stability in the presence of detergents, most of them are unstable in the presence of oxidants and bleaches (Moreira *et al.,* 2002; Haddar *et al.,* 2009; Singh *et al.,* 2011). BACC-15 alkaline protease is superior to these commercial proteases in stability and thus indicates its possible commercial application in detergent formulations. A possible mechanism underlying the oxidative stability of BACC-15 alkaline protease might be the slow oxidation of Methionine (Met) residue in the vicinity of the catalytic Serine (Ser) or longer distance between Methionine (Met) and the oxyanion hole (Saeki *et al.,* 2007).

SDS is known to be a strong denaturant of proteins including alkaline proteases. The monomeric detergents above critical micelle concentration (CMC) act as denaturants (Joo and Chang, 2005). As a result, SDS could unfold most proteins above CMC (7 mM in water), through the interactions between the sulfate group /charged head group of SDS and the positively charged amino acid side chains of proteins. Interactions may also occurs between alkyl chains of SDS and the non polar parts on the surface as well as in the interior of proteins (Otzen, 2002). Triton X-100 and Tween -80 are polyoxyethylene-

derived nonionic surfactants with an alkylphenyl hydrophobic group. They differ from SDS in their interaction to proteins. They are relatively non-denaturing rather inhibits protein-protein and protein-lipid bonding and in such a way they solublize membrane proteins (Kumar *et al.,* 2008).

BACC-15 alkaline protease is very stable in presence of higher concentration of either strong anionic surfactant (SDS) or nonionic surfactants (Triton X- 100 and Tween-80). Previous studies of Oberoi *et al.,* (2001); Moreira *et al.* (2002); Joo and Chang, (2005); Haddar *et al.* (2009); Rai and Mukherjee (2009); Haddar *et al.* (2010); Singh *et al.* (2011) reported similar findings. This suggests that BAC-15 alkaline protease has well-packed structure and its native conformation is very rigid. These stability patterns of BACC-15 alkaline protease to denaturation in the presence of anionic and nonionic surfactants further indicate its possible applications in commercial detergent formulations.

BACC-15 alkaline protease (84.21U/g) alone completely removed blood and egg stains from pieces of cotton cloth (14cm x 14 cm) after 1h incubation at 40℃. Similarly, studies on alkaline proteases from *Bacillus* sp. (Oberoi *et al.,* 2001), *Pseudomonas aeruginosa* (Najafi *et al.,* 2005), *Streptomyces albidflavus* (Abdel-Aziz *et al.,* 2011), *Streptomyces gulbargensis* (Vishalakshi *et al.,* 2009), *Aspergillus versicolor* (Choudhary, 2012) showed complete removal of proteinaceous stains from cotton cloths. In enzyme and Endod mixture, better stain removal performance was achieved at Endod preparations $\geq$ 50% of the combination. This might be due to higher concentrations of saponins expected to exists in the endod may act as surfactant and lubricates and solubilizes stubborn stains. Following subsequent attack by BACC-15 alkaline protease degrades proteinaceous stains. Based on this study we can conclude that, BACC-15 alkaline protease improves washing performance of Ethiopian traditional detergent (Endod).

Hydrolysis of proteins involves the cleavage of peptide bonds to give peptides of varying sizes and amino acid composition. This was most commonly explained in terms of percentage of degree of hydrolysis *(*DH %) (Kong *et al.,* 2008). In this study DH (%) obtained from SBPI and MSPI using BACC-15 alkaline protease was reasonably higher

than DH (%) obtained from many commercial alkaline proteases. Degree of hydrolysis (DH %) of $\leq$ 39.5 % from defatted soy flour (Hrckova *et al.,* 2002); 22% from Mung-bean Protein isolates (Li *et al.,* 2005); and 15.8% from wheat gluten protein isolate (Kong *et al.,* 2007) were reported. Furthermore, DH(%) of 18.36% from soy protein isolates (Kong *et al.,* 2008); 23% from peanut protein isolate; and <10% from chickpea protein isolates (Yust *et al.,* 2010) were also reported. Using crude protease extract from *Aspergillus oryzae,* DH of 43.4% on peanut meal proteins was obtained (Su *et al.,* 2011).

Comparable bacterial population growth and colonial characteristics of the test pathogenic organisms on the CPA, SBPHA and MSPHA was obtained. Studies of Hsieh *et al.* (1999); Uzeh *et al.* (2006); Berhanu Andualem and Amare Gessesse (2013); Hongfei *et al.* (2013) have reported production of homemade peptone using proteolytic enzymes. The result of productivity ratio also implies that SBPHA and MSPHA have better or equal potential with CPA to be used for isolation & identification of different fastidious microorganisms in the laboratory though detail study of optimization is needed for such uses.

7. Conclusions

Based on the result of this study the following conclusions were drawn

- Alkaline soda lakes of Ethiopia can be good source of isolates with alkaline protease production.
- Alkaliphilic *Actinobacteria* BACC-15 has shown good potential of producing alkaline protease in SSF and SmF.
- More efficient enzyme production of *Actinobacteria* BACC-15 was detected under SSF than in SmF.
- BACC-15 alkaline protease has excellent potential in removing blood and egg yolk stains which were stubborn on white cotton clothes. Therefore, it can be used as an ingredient in commercial and traditional washing agents.
- BACC-15 alkaline protease has a huge potential in production of protein hydrolysates with high N_2 content from Moringa seed (*Moringa stenopetala*) and soy bean (*Glycine max)* proteins. These hydrolysates in turn have a capacity to replace expensive commercial peptone.

8. Recommendation

- Since the Ethiopian soda lakes are showing potential as good source of alkaliphiles, further studies in screening for more efficient novel enzymes and isolates is suggested and in parallel conservation strategies and activities of the environment is crucial to maintain the current genetic resource potential.

- Further studies on the purification of the BACC-15 alkaline protease up to homogeneity level will give more information on the specific properties of the enzyme.

- Functional properties, nutritional qualities and other applications of MSPH and SBPH should be studied.

- The enzyme should also be studied for its additional potential applications.

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