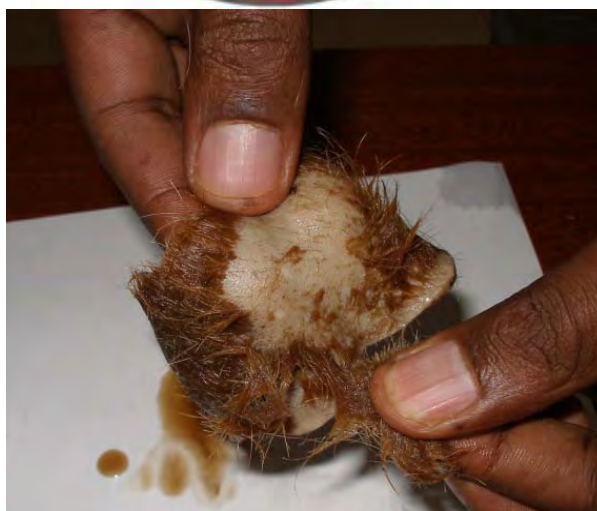


ALKALINE PROTEASE PRODUCTION BY NEW ALKALIPHILIC MICROBIAL ISOLATE UNDER SOLID STATE FERMENTATION



A thesis submitted to the school of graduate studies of Addis Ababa University in partial fulfilment of the requirement for the degree of Masters` of Science in Biotechnology.

**By Gizachew Haile Gidamo
August, 2009**

ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STADIES

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Abbreviations

SSF	Solid state fermentation
SmF	Submerged fermentation
U/g	Units per gram bran
TCA	Trichloroacetic acid
CYP	Casein-Yeast extract- Peptone

Abstract

A total of 240 alkaliphilic microorganisms isolated from samples collected from alkaline soda lakes of Ethiopia were screened for the production of alkaline proteases. Of these, 30% were protease positive indicating the abundance of protease producing microorganisms in these habitats. This again is a reflection of the abundance of protein substrates in the form of bird's feather and left over from dead cells of *spirulina* and other microorganisms. Out of the 80 protease positive isolates, 20 (25%) grew well and produce appreciable level of enzyme activity when grown in solid state culture. Of these, one isolate designated as C₄₅ was selected for further study. The protease produced by isolate C₄₅ was characterized to determine its potential industrial application. The enzyme was active in the pH range of 6.5-11.5, with optimum activity at pH 8-9; and stable at alkaline pH. The optimum temperature for activity was 40°C and 50°C in absence and presence of 5mM of Ca⁺², respectively. The enzyme displayed appreciable activity and stability at low temperature. These properties suggest that protease C₄₅ could find potential application for dehairing and detergent at moderate temperature. When protease C₄₅ was added to raw hide enabled dehairing, suggesting the potential usefulness of the enzyme in the leather industry. The commercial application of enzymes greatly depends on the cost of the enzyme which again is determined by the production cost of the enzyme. Currently most commercially available enzymes are produced through capital intensive submerged fermentation (SmF). An alternative method for the growth of microorganisms which is currently receiving significant attention is solid state fermentation (SSF). In this study, isolate C₄₅ was grown under solid state fermentation using wheat bran as the growth substrate. Maximum protease secretion was achieved at inoculum size of 20% (v/w), bran to moistening agent ratio of 1:2 when incubated at 30°C for 144 hr. Addition of inorganic nitrogen sources and organic carbon sources as a supplement of SSF medium repressed protease induction. These results indicate that the microbial isolate shows a good potential for production of low cost alkaline protease by using inexpensive substrate such as wheat bran alone and/or low cost complex nitrogen source such as *Millettia ferruginea* (Berbra) seed flour as supplement in SSF.

Key words: *Alkaline protease, isolate C₄₅, solid state fermentation (SSF).*

1. INTRODUCTION

Proteases are essential constituents of all forms of life on earth. Microbial proteases are among the most important, extensively studied groups since the development of enzymology. Alkaline proteases are so far exploited as industrial catalysts in various industrial sectors. Neutralophilic and alkaliphilic microbial alkaline proteases possess a considerable industrial potential due to their biochemical diversity and stability at extreme pH environments, respectively (Moon *et al.*, 1994). However, the demanding industrial conditions for technological applications and cost of alkaline proteases production resulted continuous exercise for search of new microbial resources.

Extreme environments such as soda lakes are important sources for isolation of micro organisms for novel industrial enzymes production (Kumar and Takagi, 1999). Ethiopia is endowed with a range of alkaline habitats in Rift valley region, that form rift from North East (forming Afar and Denakil depression) to South Lake Turkana, on the border to Kenya. The region is propitious for the development of unique alkaliphilic microorganisms. Such microbial diversity is important economic value for the country to use the microorganisms directly, or to develop products of microbial origin for industrial applications. However, only few reports have been found on isolation of alkaline protease producers from these soda lakes and their enzyme production using SmF (Amare Gessesse and Berhanu A.Gashe, 1997; Amare Gessesse *et al.*, 2003).

Enzyme cost is also the most critical factor limiting wide use of alkaline proteases for different applications. A large part of this cost is accounted for the production cost of the enzyme. Therefore, reduction in the production cost of enzymes could greatly reduce the cost of the enzyme. In submerged fermentation up to 40% of the total production cost of enzymes is due to the production the growth substrate (Enshasy *et al.*, 2008). In this regard, SSF which uses cheap agricultural residues have enormous potential in reducing enzyme production cost. So, studies on alkaline protease that are produced in SSF by alkaliphilic microorganisms are scarce in literature. As a result, it is of great importance to pursue such studies.

The objectives of this study were:

1. To isolate proteolytic alkaliphilic micro organism from Ethiopian soda lakes.
2. To evaluate the hydrolytic activities of the alkaline protease.
3. To confirm the use of the isolated alkaline protease as a hide dehairing agent.
4. To optimize cultivation conditions for protease production under SSF.

2 ALKALINE PROTEASES

2.1 *Introduction to proteases*

Proteases are hydrolytic enzymes found in every organism to undertake important physiological functions. These include: cell division, regulating protein turnover, activation of zymogenic preforms, blood clotting, lysis of blood clot, processing and transport of secretory proteins across membrane, nutrition, regulation of gene expression and in pathogenic factors. Proteases differ in their specific activity, substrate specificity, pH and temperature optima and stability, active site, and catalytic mechanisms. All these features contributed in diversifying their classification and practical applications in industries involving protein hydrolysis.

Proteases are classified based on chemical nature of the active site, the reaction they catalyze, and their structure and composition (Rao *et al.*, 1998). The major classes are again classified in to sub classes based on pH, catalytic site on polypeptide, occurrence, and so on.

Based on the catalytic site on the substrate, proteases are mainly classified in to endoproteases and exoproteases (Rao *et al.*, 1998). Endoproteases preferably act at the inner region of the polypeptide chain. By contrast, exoproteases preferentially act at the end of the polypeptide chain. Exoproteases are further classified in to amino peptidases (those proteases which act at the free N-terminus of the polypeptide substrate), and carboxypeptidases (those proteases which act at the free C-terminal of the polypeptide chain (Rao *et al.*, 1998).

Similarly, endoproteases are also classified based on the functional group present in active site and pH optimum. The different classes of proteases based on their catalytic active site include:

- 1) Serine proteases. Serine proteases are proteases having a serine group (-OH) in their active site.
- 2) Cystein proteases. Cystein proteases are proteases having a thiol (-SH) group in their active site.
- 3) Metalloproteases. Metalloproteases are proteases requiring divalent metal ion for their catalytic activity.
- 4) Aspartic protease. Aspartic proteases are proteases with aspartic residue at their catalytic active site.

5) Other rare proteases also contain other amino acid residues at their active site, such as threonine and glutamic acid.

Based on their optimal pH proteases are also classified as:

1) Acid proteases

Acid proteases are proteases which are active in the pH ranges of 2-6 (Rao *et al.*, 1998) and are mainly of fungal in origin (Aguilar *et al.*, 2008). Common examples in this subclass include aspartic proteases of the pepsin family. Some of the metalloprotease and cysteine proteases are also categorized in as acidic proteases.

2) Neutral proteases

Neutral proteases are proteases which are active at neutral, weakly alkaline or weakly acidic pH. Majority of the cysteine proteases, metalloproteases, and some of the serine proteases are classified under neutral proteases. They are mainly of plant in origin, except few fungal and bacterial neutral proteases (Aguilar *et al.*, 2008).

3) Alkaline proteases

Alkaline proteases are optimally active in the alkaline range (pH 8-13), though they maintain some activity in the neutral pH range as well (Horikoshi, 1999). They are obtained mainly from neutralophilic and alkaliphilic microorganisms such as *Bacillus* and *Streptomyces* species. In most cases the active site consists of a serine residue, though some alkaline proteases may have other amino acid residue in their active site (Rao *et al.*, 1998).

2.2. Industrial applications of alkaline proteases

Alkaline proteases have several industrial applications. These include: as processing aid in leather tanning industries, as detergent additive, in protein hydrolysis, in pharmaceuticals production, and in chemical synthesis (Gupta *et al.*, 2002). According to Rao *et al.*, (1998), alkaline proteases account more than 25% of the global enzyme market.

2.2.1. Application of alkaline proteases in leather industry

There are three series of unit operation that involve leather processing.

- 1) Pre-tanning operation stage is the stage that is used to clean hide or skin in leather industry. The non-collagen part of hide or skin proteins such as albumin, globulin, mucoids, and fibrous proteins such as elastin, keratin, and reticulin are removed during this stage (Sivasubramanian *et al.*, 2008).
- 2) The tanning operation stage is a step that is used to stabilise the skin or hide matrix and
- 3) Post-tanning or finishing operations is the stage that is used to add aesthetic quality of leather (Thanikaivelan *et al.*, 2005).

In pre-tanning stage, hide or skin undergoes series of treatment stages. These include: raw skin preservation, soaking, liming, dehairing and deliming, bating, degreasing, and pickling. The pre-tanning stage uses several chemicals such as sodium sulphide, sodium chloride, lime, chlorinated compounds and others that contribute for the generation of 80-90% of the total pollution load released by leather industry (Thanikaivelan *et al.*, 2004). Of these, dehairing accounts for one third of these total pollution generated by leather industry (Kamini, *et al.*, 1999). The use of alkaline protease in soaking, dehairing and tanning shown to greatly reduce the amount of pollution generated.

Soaking is a key step which prepares the hide for subsequent operation step by cleaning and softening of hides and skins with water. This process results in solubilisation and elimination of salts and globular proteins contained within the fibrous structure (Thanikaivelan *et al.*, 2004). It uses cured skin and is usually carried out under alkaline condition. Water, antiseptics such as sodium hypochlorite, sodium pentachlorophenate, formic acid, and so on are used in soaking. Addition of chemicals like sodium sulphide or sodium tetrasulphide aids the soaking process. It has been reported that the use of alkaline proteases aid the soaking stage by breaking soluble proteins of the in side matrix, and release salts and hyluaronic acid. As a result, water uptake facilitated at alkaline at an alkaline pH (Kamini *et al.*, 1999).

Dehairing is also an important operation in tanneries conventionally practiced using lime and sodium sulphide (Thanikaivelan *et al.*, 2004). In this process, the skin/hide is painted with sulphide which helps to reduce the disulphide bond that is responsible for attachment of hair keratin in epidermis. This brings about complete removal of hair, but the hair root remained with in skin (Sivasubramanian *et al.*, 2008). In this process, lime contributes to the dehairing process by opening up the collagen fiber structure. The use of alkaline protease has proven superior and efficient for selective removal of the non-collagen part of hide/skin (Kamini *et al.*, 1999).

Compared to conventional dehairing, the use of alkaline protease for dehairing has the following advantages (Thanikaivelan *et al.*, 2005; Mukhtar and Ul-Haq, 2008).

1. Significant reduction or even complete elimination of the use of sodium sulphide.
2. Their non-polluting effect and biodegradability.
3. Activity under mild conditions.
4. Reduction in dehairing time.
5. Their specificity.
6. Recovery of hair of good quality and strength with a good saleable feature that can be used to develop animal feed additives.
7. Creation of an ecologically conducive atmosphere for the workers. Thus, the skin/hide easily handled by work men.
8. Enzymatically dehaired leathers have shown better strength properties and greater surface area.
9. Simplification of pre-tanning processes by cutting down one step.

Therefore, introduction of alkaline proteases for leather processing have a potential to reduce environmental pollution, decrease processing time and improve leather quality.

2.2.2. Application of alkaline proteases as detergent additives

Removal of proteinaceous stains of blood, milk, egg, grass and sauces is very difficult using conventional surfactants. Removal of such a difficult- to- remove stain is now a day achieved by using alkaline proteases (Rao *et al.*, 1998; Maurer, 2004). Currently, detergent protease account for more than 30% of the world enzyme market (Horikoshi, 1998; Maurer, 2004). To be used as detergent additives proteases need to be active and stable at an alkaline pH, be stable in presence of chelating agents, and must possess broad substrate specificity. In addition, stability and residual activity in the presence of detergent additives such as surfactants, builders, bleaching agents, bleach activating agents, fillers, and fabric softeners are also required (Maurer, 2004).

2.2.3. Application of alkaline proteases for protein hydrolysis

Protein hydrolysates of high nutritional value that can be used in blood pressure regulation, protein-fortified soft drinks, and infant food formulations are produced by using alkaline proteases (Rao *et al.*, 1998; Agrawal *et al.*, 2004). In addition, alkaline proteases also contribute in improvement of fruit juice and in production of specific therapeutic dietary products from soya and other protein sources (Rao *et al.*, 1998).

2.2.4. Application of alkaline proteases in silver recovery from waste photographic and X-ray films

It is known that photographic and X-ray films are partly prepared using silver compounds. Black metallic silver spread in gelatin emulsion layer of waste X-ray films is about 1.5-2.0% silver by weight. Burning of X-ray films, and Striping methods are conventionally used to recover silver. But, the conventional burning and chemical alkali methods are not pollution free and result in the generation of foul smell. Use of alkaline proteases that degrade the gelatin in few minutes for silver recovery has been reported (Fujiwara *et al.*, 1991; Nakiboglu *et al.*, 2001). Using such enzyme treatment, about 99% pure silver recovery was achieved (Nakiboglu *et al.*, 2001). In addition to silver, recovery of base film made of polyester is also possible using alkaline proteases.

2.2.5. Synthetic and pharmaceutical application of alkaline proteases

Protease by themselves or their degradation products such as peptides can be used as therapeutic agents. For instance, subtilisin is used in the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses to speed up healing process by producing anti-inflammatory response in patients reported (Rao *et al.*, 1998). Peptides which can serve as chemotherapeutic agents, toxins, sweeteners, growth hormones, as inhibitors and antibiotics can be also synthesized using alkaline proteases (Crout *et al.*, 1992; Yust *et al.*, 2004; Guzman *et al.*, 2007).

2.2.6. Application of alkaline proteases in management of waste

Alkaline proteases have interesting potential applications in the management of wastes from households and food processing industries. These include: cleaning of hair clogged pipe lines containing hairs (Gupta, 2002), and in replacing alkali based feather processing from slaughterhouses to use as protein rich animal feed additive (Amare Gessesse *et al.*, 2003).

2.2.7. Miscellaneous applications

Silk Degumming

Alkaline proteases are important instruments in degumming silk before dyeing. Sercin is responsible component for silk compactness. Silk treatment with alkaline protease is known to reduce compactness and value smoothness to the silk (Freddi *et al.*, 2003).

Scale removal from wool fiber

Wool fiber exhibit felty and shrinking feature because of the presence of scales that are found toward fiber tips. Conventionally, chlorine-Hercosett is applied to remove the scales by oxidation, or coating of

fiber in polymers used. Wool treatment with hair and keratin degrading alkaline protease provide an option in the removal of scale tips from wool fibre (Rao *et al.*, 1998; Da Silva, 2005).

DNA isolation and in animal cell culture

Alkaline proteases of microbial origin are also reported as a substitute of other proteases for molecular biotechnology applications. For instance, alkaline protease from *Conidiobolus* species is reported to substitute trypsin that used in the preparation of animal cell cultures i.e. in dissociation of cells for primary cell cultures, maintenance of cell lines and in production of G-band in Meta phase chromosome (Chiplonkar *et al.*, 1985). Further more, Kwon *et al.*, (1994) reported the potential of alkaline proteases from *Vibrio metschnikovii* RH530 to be used as an alternative for Proteinase K in DNA isolation.

2.3. Sources of alkaline proteases

Alkaline proteases are widely distributed in animals, plants, fungi and bacteria. Several alkaline proteases from different sources are currently in the market, but almost all are products of microbial origin. Alkaline protease are produced by both neutralophilic and alkaliphilic microorganisms. These two groups represent almost all sources of commercial alkaline proteases currently available in the market (Moon *et al.*, 1994).

2.3.1 Alkaline proteases from neutralophiles

Neutralophiles are organisms that exhibit optimum growth at neutral pH range (Horikoshi, 1999). Their biochemical diversity significantly contributes in diversifying the protease applications, and their market value (Moon *et al.*, 1994). *Bacillus subtilis*, and *Bacillus licheniformis* are the major and highly exploited neutralophilic organisms for alkaline protease production. Members of the subtilisin super family of proteases that are used in almost all of the technical protease application areas today, are obtained from these groups (Maurer, 2004).

2.3.2 Alkaline proteases from alkaliphiles

Alkaliphiles are organisms isolated from extremely alkaline environments such as soda lake, having their optimum growth pH above 9 (Horikoshi, 1999). Examples of alkaliphilic microorganisms producing alkaline proteases include *Bacillus firmus*, *Bacillus lentus*, and alkaliphilic *Actinomycetes* (Moon *et al.*, 1994). Proteases from these sources are extremely

stable at high pH, as a result, they draw the attentions of many biotechnological companies and researchers in the world (Patel *et al.*, 2006).

2.4. Enzyme production

All commercially important alkaline proteases are produced by microorganisms. The enzymatic yield obtained from fermentation, cost of their production, and downstream processing cost determines the final cost of the enzyme. To develop a viable industrial process, lowering production cost, and increasing enzyme productivity are very important. Selection of best fermentation technique and optimization of culture conditions contribute much in achieving enzyme productivity (Barrios-Gonzalez *et al.*, 2005). Currently, enzyme production by microorganisms can be achieved using submerged fermentation or solid state fermentation.

2.4.1. Submerged fermentation (SmF)

Submerged fermentation is defined as the cultivation of microorganisms in liquid nutrient broth. The growth of the microorganism at a large scale involve use of closed large vessel (up to 100 cubic meters in volume) containing a rich nutrient broth and enough amount of oxygen (Enshasy *et al.*, 2008). Fermentation parameters such as medium composition, pH, and aeration are important variables that affect the production of enzyme in SmF (Maurer, 2004).

SmF has its own advantages and drawbacks. Serious pollution problems, low product concentration and high production cost are the major draw backs associated with the use of SmF. However, presence of high water content that is favorable for most bacterial growth, homogeneity of the fermentation system, ease in process parameter measurement and presence of well developed industrial equipment are some of the advantages of submerged fermentation (Holker & Lenz, 2005). At present, more than 90 % of the commercial microbial enzymes, including alkaline proteases, are produced using submerged fermentation (Underkofler *et al.*, 1958; Aguilar *et al.*, 2008).

2.4.2. Solid state Fermenttation

Solid state fermentation is defined as the growth of microorganisms on moist solid substrates in the absence of free flowing water. The microorganism obtain water, carbon, nitrogen, minerals, and other nutrients from the solid substrate. The substrate also provides anchorage for the micro organism, thus stimulate the growth condition occurring in nature (Pérez-Guerra *et al.*, 2003). Control of fermentation parameters such as pH, moisture content of the solid substrate, and

temperature during fermentation are important variables that affect enzyme yield in SSF (Pandey *et al.*, 1999).

Recently, production of industrial enzymes using solid state fermentation gaining more attention. This is because, compared to SmF, SSF has the following advantages (Holker & Lenz, 2005). First, cheap agricultural residues can be used as substrates, thus greatly reducing the cost of enzyme production.

Secondly, solid state fermentation requires easier operation. This includes requirement for a smaller space, simple aeration system, and less pollution. In addition, lower energy requirement for operation, possibility to use semi-sterilized condition, and reduced risk of contaminations are also important features of SSF that make the operation system simple (Pérez-Guerra *et al.*, 2003).

Thirdly, solid state fermentation significantly minimizes catabolic repression in production of hydrolytic enzymes in nutrient rich solid state medium. This is because of limited exposure of nutrients to microorganism, and decline in mass transfer that favour slower but constant microbial growth rate. In addition, the presence of active regulatory mechanisms in SSF is also believed to play important role in inducing high volumetric product biosynthesis (Barrios-Gonzalez *et al.*, 2005). More detail descriptions for some of the important biological, ecological and engineering aspects of SSF are shown in Table 1 below.

Table 1. Comparison of some important factors between submerged and solid state fermentation (adapted and modified from Raimbault, 1998; Pandey *et al.*, 1999; Pérez-Guerra *et al.*, 2003; Prabhakar *et al.*, 2005).

No.	Factor	Submerged fermentation	Solid state fermentation
1	Substrate	Soluble substrates (usually sugars)	Insoluble polymeric substrates (starch, cellulose, pectin, lignin)
2	Aseptic condition	Heat sterilization and aseptic control	Vapor treatment, even non-sterile conditions can be used
3	Water	High volumes of water consumed and effluents discarded	Limited consumption of water; low amount or, no effluent produced and discarded

4	Metabolic heating	Easy control of temperature	Low heat transfer capacity, difficulty in control of temperature
5	Aeration	Limitation by soluble oxygen, high level of air required	Easy aeration and high surface exchange air/substrate
6	pH control	Easy pH control	Buffered solid substrates
7	Mechanical agitation	Good homogenization	Static conditions preferred
8	Scale up	Industrial equipments available	Need for engineering and new design equipment
9	Inoculation	Easy inoculation	Spore inoculation(fungi), batch
10	Contamination	Risks of contamination for single strain bacteria	Risk of contamination for low rate growth fungi
11	Energetic considerations	High energy consuming	Low energy consuming
12	Volume of equipment	High volumes and high cost technology	Low volumes and low costs of Equipments
13	Effluent and pollution	High volumes of polluting effluents generated	No effluents, less pollution generated
14	Concentration of products	Low yield and diluted product	Highly concentrated product

3. MATERIALS AND METHODS

3.1. Isolation micro organisms

Mud sample was taken from Lake Arenguade, Lake Chitu and Lake Abijata and were kept in sterile tubes in refrigerator, at 4°C until used. Isolation of alkaline protease producing alkaliphilic microorganisms was carried after enrichment using solid state medium containing (g/g) wheat bran, 10; K_2HPO_4 , 0.1; $MgSO_4 \cdot 7H_2O$, 0.02; $CaCl_2$, 0.01; and casein, 1.0 in a 250ml Erlenmeyer flask. Moistening agents were added in such a way to give final bran to moisture ratio of 1:1.5, thoroughly mixed, and autoclaved at 121 °C for 15 minutes. After cooling to room temperature, sterile Na_2CO_3 was added to give a final concentration of 10 % (w/w). Then, each flask was inoculated with 10 % (v/w) aliquots of mud suspensions as inoculums and incubated at 37 °C for 5 days.

From thoroughly mixed enriched fermented solid substrate of each sample, 1g was taken and suspended in to 30 ml-glass tube containing 5 ml of sterilized distilled water. Then the glass tube was placed on a 121 rpm shaker for 30 minutes at room temperature. This suspension was serially diluted (10^{-1} to 10^{-7}) and spread on agar plates. Individual colonies were isolated and screened for alkaline protease production.

3.2. Screening for alkaline protease production

Screening of isolates for alkaline protease production was carried out using casein-yeast extract-peptone (CYP) agar medium containing (g/l): casein, 10; bacteriological peptone, 5; yeast extract, 1; K_2HPO_4 , 1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.1; Na_2CO_3 , 10; and agar, 15. Na_2CO_3 was autoclaved separately and added to the rest of the medium after cooling (Amare Gessesse *et al.*, 2003). After inoculation, the plates were incubated at 37 °C for 48 hours. Formation of halo zone around the colonies, resulting from casein hydrolysis was taken as positive for proteolytic activity. These colonies were isolated and streaked repeatedly in fresh plates until single uniform colonies were obtained.

To select an isolate which gives protease with high activity, a loop full of culture from agar plate was taken and inoculated in to 30 ml glass tube containing 5 ml of alkaline protease production medium and incubated overnight at 121 rpm at room temperature. Then, 5 % (v/v) of the 16 hr inoculum was inoculated in to 50 ml of same medium in 250 ml Erlenmeyer flask and incubated with rotary shaking

(121 rpm) at room temperature for 5 days. Five ml of the fermented broth was taken and centrifuged at 6000 rpm for 5 minutes and the cell free supernatant was used as enzyme source.

3.3. Solid state fermentation

SSF medium containing (g/g): wheat bran, 10; K_2HPO_4 , 0.1; $MgSO_4 \cdot 7H_2O$, 0.02; $CaCl_2$ 0.01; and casein, 1.0 was prepared in a 250 ml Erlenmeyer flask and the solid substrate to moistening agent ratio was adjusted to 1:1.5, unless stated otherwise. After autoclaving, sterile sodium carbonate was added to give a final concentration of 10% (w/w), inoculated and incubated at 37°C for 5 days.

From the fermented substrate, alkaline protease was harvested by soaking the fermented solid with ten volumes of distilled water per gram solid substrate (wheat bran), in shaking (121rpm) condition for 30 minutes at room temperature (Ikasari and Mitchel, 1996). At the end of the extraction, the suspension was hand squeezed through a double layered muslin cloth and the particulate materials clarified by centrifugation at 10,000 rpm for 5 minutes. Recovery efficiency 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 (Roussos *et al.*, 1992).

3.4. Optimization of culture conditions.

To select the optimum condition for maximum enzyme production, the level of parameters optimized, where the optimum value determined for one parameter was applied in the subsequent experiment. The initial values used for investigation of the parameters were: initial pH (8.0-10.2), moisture content (1: 0.5- 1: 4 (Amare Gessesse and Gashew Mamo, 1999), temperature (25°C, 30°C and 37°C), incubation time (24-244 hrs), nitrogen sources (10% w/w), and carbon sources (7% w/w (Niadu and Devi, 2005)).

3.5. Analytical Methods

3.5.1 Protease assay

Proteolytic activity in the culture supernatant was determined by using casein as a substrate. A mixture of 450 µl of 1% (w/v) of casein in 50mM Tris-HCl buffer, pH 8.5 and 50 µl crude enzyme extract were incubated in a water bath at 40°C for 20 minutes. After 20 minutes, the enzyme reaction was terminated by the addition of 500µl of 10% (w/v) Trichloroacetic acid (TCA) and was kept at room temperature for 15 minutes. Then, the reaction mixture was centrifuged to separate the unreacted casein at 10,000 rpm for 5 minutes. Samples containing 500 µl supernatant taken, and mixed with 2.5 ml of 0.5M Na_2CO_3 . After adding 0.5 ml of 1N Follin Ciocalteus phenol reagent, the mixture incubated in the dark for 30 minutes and absorbance measured at 660 nm against a reagent blank. One U corresponds to the amount

of enzyme required to release 1 micromole of amino acid equivalent to tyrosine per minute under the standard assay conditions. Protease production was expressed as U/g.

3.5.2 Characterization of alkaline proteases

The protease activity of C₄₅ at different pH value was tested at 40°C with the following buffers: Phosphate (6-8), Tris-HCl (7.5-9), and Glycine-NaOH (8.5-11.5) each at a concentration of 50mM were used. pH stability was determined by incubating the crude supernatant at buffers of varying pH(6-11.5) for 1 hr at 25°C following enzyme assay at 40°C. Temperature effect on protease activity was determined at different temperature (30°C -70°C) as procedures described in *assay* section (3.5.1). To determine thermal stability, the samples were pre-incubated with buffers at different temperatures (30°C -70°C) following determination of residual activity every 10 minutes. To determine the protease ionic strength, 2M NaCl used under mentioned assay conditions by pre-incubating enzyme for 1hr at 25°C. Relative protease activity (expressed in %) was defined as the percent protease activity compared with the maximum value.

3.6. Test for potential use in enzymatic dehairing.

Two set of cow hides were washed and cut into 10x15 cm pieces. One set from the pairs (controls) was put in to flask containing distilled water. The other halves were put in to flask containing enzyme solution by keeping liquid (ml) to gram hide proportion one to one (Najafi *et al.*, 2005; Macedo *et al.*, 2005; Malathi and Chakraborty, 1991) and incubated in shaker (121rpm) at room temperature for 24 hrs. To asses dehairing extent, one flak at a time taken for hair removal trail with fingers. The skin pieces after treatments were examined for dehairing time, dehairing extent, and scud.

3.7. Experimental design and statistical analysis

Optimization of culture conditions such as inoculums size, sodium carbonate concentration, temperature, fermentation time, moisture level, and carbon and nitrogen sources selected as main influencing factors in the protease production. Optimum conditions were determined from the examination of magnitude of enzyme activity. The enzymes activity was analyzed by taking duplicate values and their means. Microsoft office Excel work sheet 2007 was used in the analysis of generated data and in preparation of graphs.

4. RESULTS

4.1. Isolation and screening of proteolytic microorganisms

Out of a total of 240 microbial isolates tested, 80 isolates showed clear zone on casein agar plates. Because of the absence of correlation between zone of clearing around colonies on a casein agar plate and protease quality, all the positive isolates were cultivated in liquid medium and screened for the quality enzyme secreted. Twenty isolates selected based on their enzyme property and secretion level. To screen isolates capable of growing in SSF medium, all the 20 isolates were grown in casein enriched SSF medium. All the twenty found to grew well and produced appreciable protease, of which four isolates produce high enzyme level in SSF. In addition to secretion of sufficient enzyme activity in SSF, isolates were also compared on the property of the enzyme, such as residual activity in the presence of high salt concentrations, high protease activity at moderate temperature and so on. One isolate designated as C₄₅, which was isolated from Lake Chitu, was selected for further study.

Table 2. Summary of results of protease producers screening for the four best selected isolates under SSF medium containing casein, 10% (v/w) inoculum, 10% (w/w) Na₂CO₃ and final bran to moisture level of 1:1.5.

NO.	Isolate	Protease production (U/g)	High protease activity pH range	High protease activity temperature range	Residual activity in presence of 10mM EDTA, and 2M NaCl
1	R ₁₅	12	9-11	50°C-70°C	>80%
2	C ₃₉	10.3	8-10	40°C-60°C	>80%
3	C ₄₁	13.5	9-11	50°C-70°C	>70%
4	C ₄₅	15.2	8-10	30°C-50°C	> 90%

4.2. Effect of environmental conditions on alkaline protease production.

In this section, protease production using isolate C₄₅ was optimized with respect to inoculum size, temperature, initial pH of the medium, moisture level, incubation time, and nitrogen and carbon source supplements in SSF.

4.2.1. Effect of inoculum size on protease production

In order to determine the effect of inoculums size on production of protease C₄₅, the SSF culture was inoculated with 10% (v/w), 20% (v/w), 45% (v/w) and 90% (v/w) bacterial inoculums. The optimum inoculation ratio was 20 % (v/w) which corresponded to 1x10⁶ cells/ gram bran (Table 3). At 10% (v/w) and 90 % (v/w) inoculums sizes only about half of its maximum protease activity was detected.

Table 3. Effect of inoculation ratio on the protease production by C₄₅ isolate in SSF. The inoculated SSF medium containing 10% (w/w) Na₂CO₃, final moisture ratio of 1:1.5 incubated at 37°C for 5 days.

No.	Inoculation ratio(% v/w)	Log colony forming unit (CFU)	No. of cells/g bran	Protease production(U/g)	Relative protease production (%)
1	10	6.6	4x10 ⁵	15.2	49.2
2	20	7	1x10 ⁶	30.9	100.0
3	45	7.3	2x10 ⁶	25.5	82.5
4	90	7.6	4x10 ⁶	18.6	60.2

4.2.2. Effect of incubation temperature

In order to determine effect of incubation temperature on alkaline protease production, the SSF culture was subjected to 25°C, 30°C and 37°C incubator after addition of 20% (v/w) inoculum. Results in Fig 1 below indicated that the optimum temperature for the production of C₄₅ alkaline protease was 30°C (47.9 U/g) followed by 29.1 U/g and 26.1U/g at 25°C and 37°C,respectively.

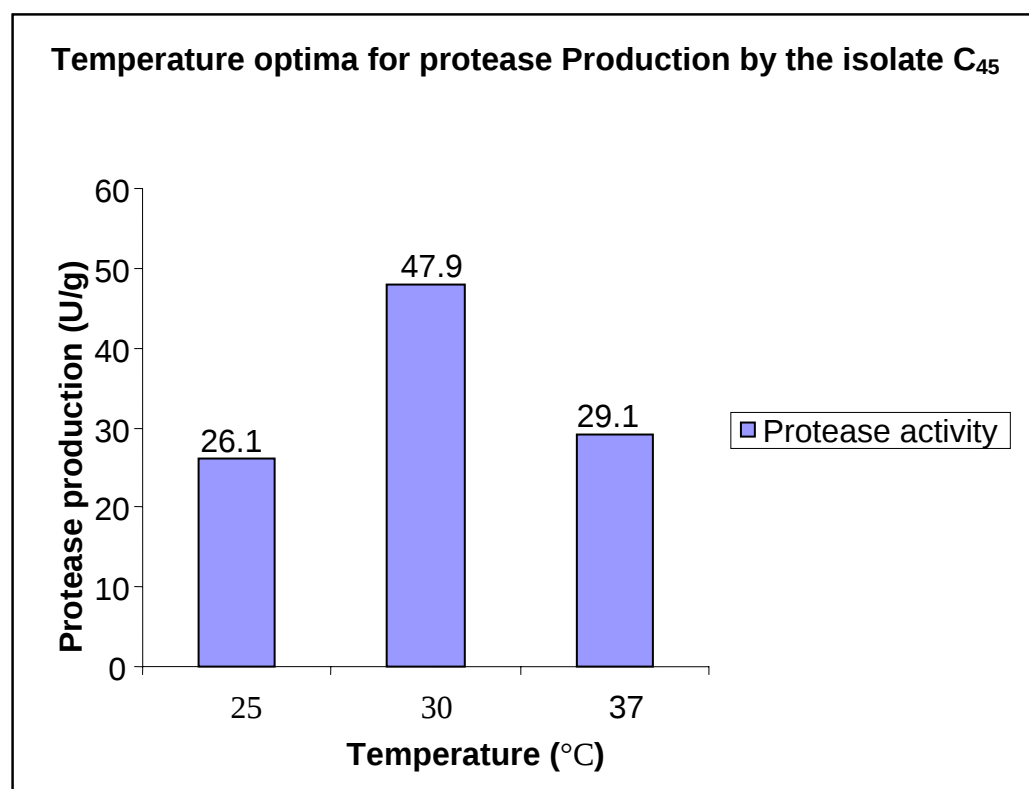


Fig 1. Effect of incubation temperature on the protease production by the isolate C₄₅ in SSF. The SSF medium containing 10% (w/w) Na₂CO₃, final bran to moisture ratio of 1:1.5, inoculated with 20% (v/w) inoculum incubated for 5 days.

4.2.3. Effect of sodium carbonate concentration

In order to adjust the pH of the medium to alkaline range, sodium carbonate was used. In SSF culture medium, sterile Na₂CO₃ was added to give a final concentration of 2.5% (w/w), 5% (w/w), 10% (w/w) and 15 % (w/w). As indicated in Table 4, 15% (w/w) sodium carbonate concentration was found to induce maximum protease production (57.5 U/g). At 2.5% (w/w) sodium carbonate concentration, only 40 % (24.8 U/g) of its maximum yield of protease production achieved. While, protease production at 5% (w/w) and 10% (w/w) sodium carbonate concentrations are relatively comparable.

pH values of the fermented extract (Table 4) also shows the extent of pH change during the course of fermentation by the organism. pH of the media starts to drop as the micro organism starts to grow. In almost all the tested sodium carbonate concentrations for high protease production, medium pH dropped in a range between 8-9.

Table 4. Effect of sodium carbonate concentration on the protease production by the isolate C₄₅ in SSF. The SSF medium containing final bran to moisture ratio of 1:1.5, and inoculated with 20% (v/w) inoculum incubated at 30°C for 5 days.

No.	Na ₂ CO ₃ concentration (%w/w)	Initial pH of the medium	Initial pH of extract (before fermentation)	pH of fermented extract	Protease production (U/g)	Relative protease production (%)
1	2.5	8.8	7.8	7.4	24.8	43.1
2	5	9.4	9.0	8.1	45.9	79.8
3	10	10	9.7	8.6	48.9	85.0
4	15	10.2	9.9	8.8	57.5	100.0

4.2.4. Effect of solid to moistening agents ratio

In order to study the effect of moisture level on alkaline protease production under SSF, moistening agents were added to give final wheat bran to moistening agent ratio of 1: 0.5, 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5 and 1:4. Maximum alkaline protease production (75.2 U/g) was obtained at bran to moistening agent ratio of 1:2. At bran to moistening agent ratio of 1:0.5, protease production was very low (Fig 2). While at bran to moistening agent ratio above the optimum, low protease production was observed.

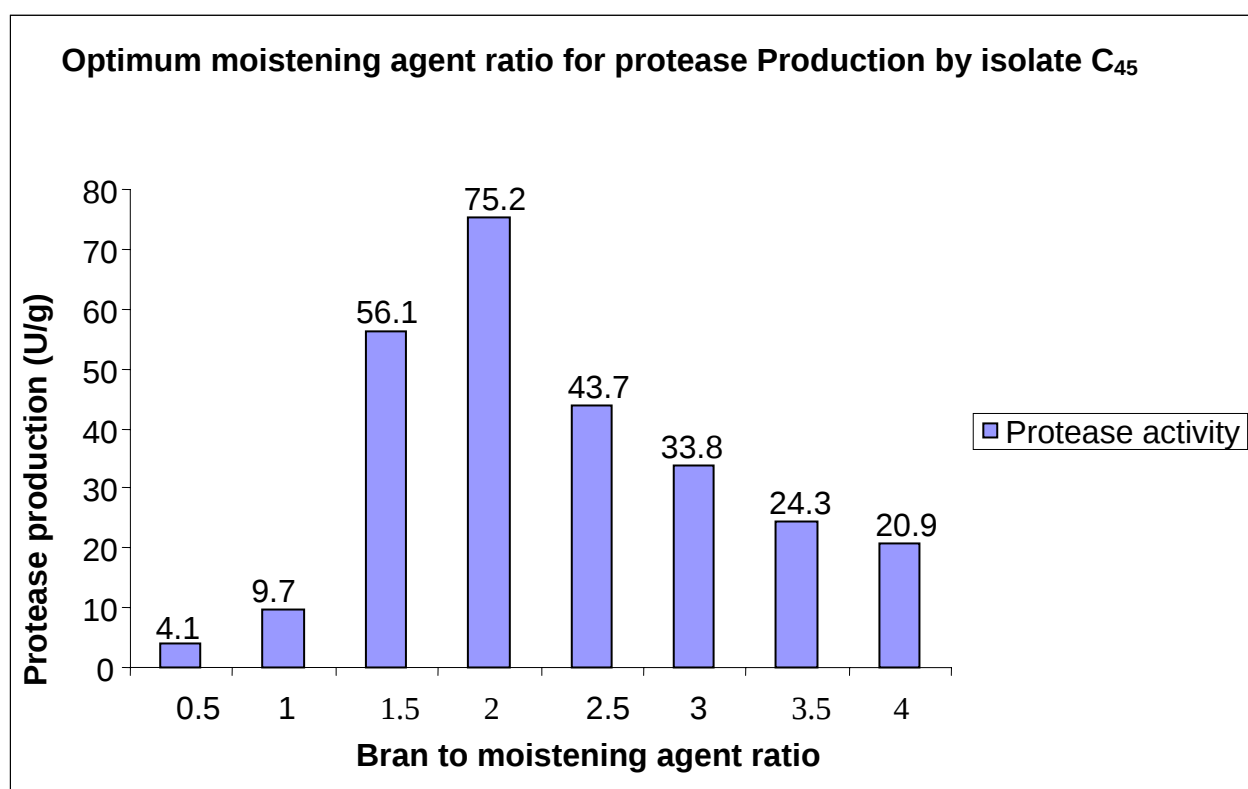


Fig 2. Effect of bran to moistening agent ratio on protease production by the isolate C₄₅ in SSF. The SSF medium containing 15% (w/w) Na₂CO₃, 20% (v/w) inoculum size incubated at 30°C for 5 days.

4.2.5. Time course of alkaline protease production

The effect of incubation time on protease production under SSF was determined by incubating 10 flasks in parallel at 30°C for different times, one flask taken every 24 hr for analysis of enzyme production. Enzyme induction was detected throughout the course of fermentation (Fig 3), but rapid increase in enzyme secretion was detected by the isolate up to 72 hr (Fig 3) and maximum production was achieved after 144 hrs incubation time (74.6 U/g). As fermentation time increases above the optimum incubation time, no further increase as well as no pronounced rapid drop in protease production observed (Fig 3).

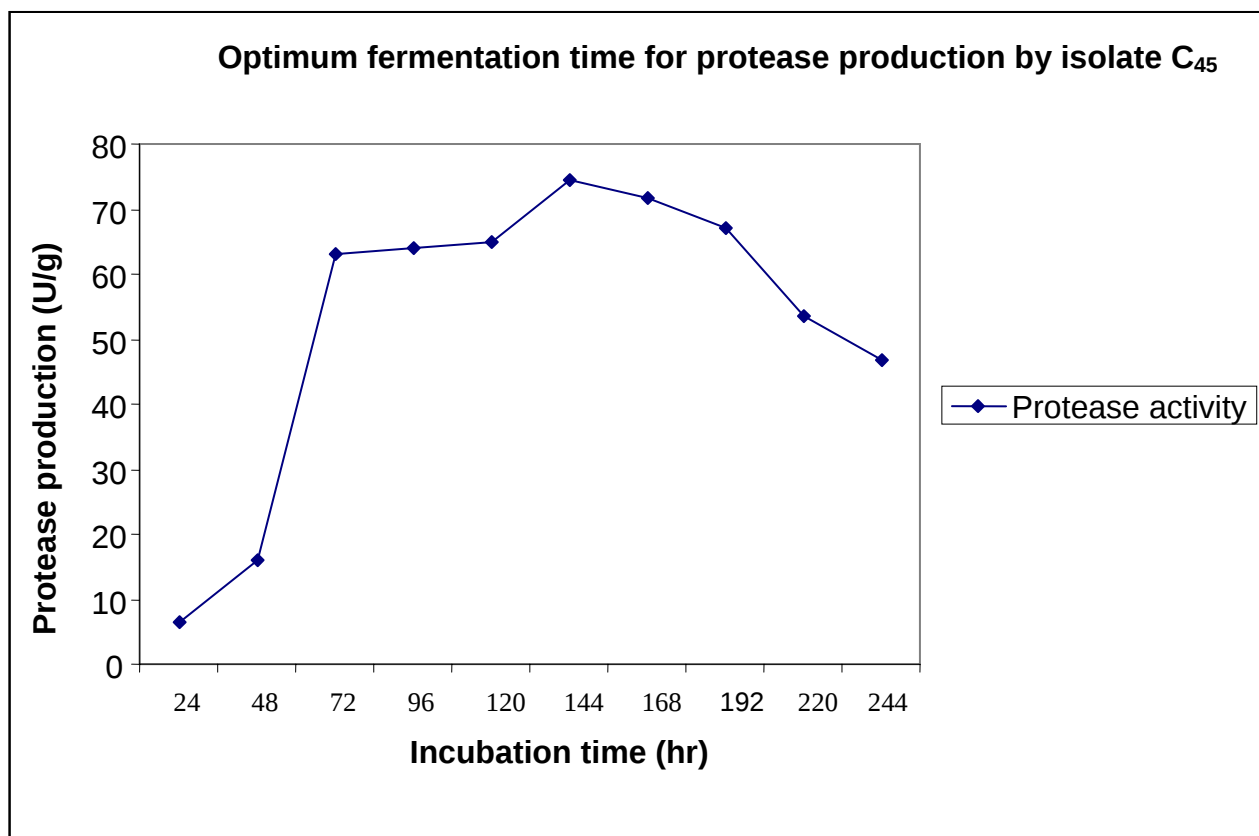


Fig 3. Effect of fermentation time on protease production by the isolate C₄₅ in SSF. The SSF medium containing 15% (w/w) Na₂CO₃, final bran to moistening agent ratio of 1: 2, and 20% (v/w) inoculum size incubated at 30°C.

4.2.6 Effect of nitrogen sources

In order to determine the effect of nitrogen sources on alkaline protease production, 10 % (w/w) nitrogen sources was incorporated in the growth medium. The tested organic and inorganic nitrogen sources include: NH₄Cl, NH₄NO₃, urea, peptone, yeast extract, casein and local protein source raw seed flour from *Millettia ferruginea* (Berbra). In general, the organic nitrogen sources neither induce nor repress protease production while the inorganic nitrogen sources used in this study repress protease production. The highest level of protease production was observed in solid state medium supplemented with casein and Birbra flour (Table 5). It was also observed that significant level of protease production was also achieved in the absence of any nitrogen supplement. Thus, the increase in the level of protease production up on addition of casein and Berbra flour was only 10% and 7%, respectively.

Table 5. Effect of different nitrogen supplements on the protease production by the isolate C₄₅ in solid state fermentation. The SSF medium containing 15% (w/w) Na₂CO₃, final bran to moistening agent ratio of 1: 2, and 20% (v/w) inoculum size incubated at 30°C for 6 days.

No.	Nitrogen source (10% w/w)	Protease production (U/g)	Relative protease production (%)
1	None	67.6	91.7
2	NH ₄ NO ₃	1.5	2.0
3	NH ₄ Cl,	1.6	2.2
4	Urea	1.6	2.2
5	Yeast extract	61.9	83.9
6	Peptone	51	69.2
7	Casein	73.7	100
7	<i>Millettia</i> (Berbra) raw seed flour	72.9	98.9

4.2.7. Effect of different carbon sources

Isolate C₄₅ was grown on the growth medium supplemented with different carbon source that make up 7% (w/w) to determine their effect on protease production. Among all the tested carbon sources, sodium acetate was found to increase protease production (85.7U/g). Other organic carbon sources except fructose, did not significantly affect protease production (Table 6). Protease induction by fructose was relatively high, approximately higher by a minimum of 10% compared with other organic carbon sources.

Table 6. Effect of different carbon source supplements on the protease production by the isolate C₄₅ in solid state fermentation. The SSF medium containing 15% (w/w) Na₂CO₃, 10% (w/w) casein, final bran to moistening agent ratio of 1: 2, and 20% (v/w) inoculum size incubated at 30°C for 6 days.

No.	Carbon source (7% w/w)	Protease production (U/g)	Relative protease production (%)
1	None	76.0	88.7

2	Sodium acetate	85.7	100
3	Fructose	78.3	91.4
4	Glucose,	63.7	74.3
5	Sucrose	65.5	76.4
6	Starch	71.3	83.2
7	Maltose	57.3	66.9

4.3. Recovery pattern of the alkaline protease

Alkaline protease from the fermented solid was harvested by adding distilled water under shaking condition for 30 minute. Then, the well mixed fermented solid substrate was squeezed and filtered using muslin cloth. The results for the first two leaching pattern by squeezing through a double layered muslin cloth was presented in Table 7. The 31.8 g moist fermented solid (10g bran with 100 ml distilled water) yielded 90 ml leachate in the first stage. Using 90ml of water and solid from the first squeezing yielded 85.5 ml of leachate in the second stage. About 10 ml in the first and 4.5 ml water in the second stage was held up in the solid. One (v/w) in the first and 0.45 (v/w) ratio of absorbed water to solid in the second stage extraction was obtained (Table 7). About 100% enzyme recovery was achieved using two stage squeezing.

Table 7. Recovery pattern of the protease produced by the isolate C₄₅ from fermented solid substrate in two squeezing stages using distilled water.

No.	Squeezing stage	Recovered Volume	Ratio of absorbed water to solid	Leachate to fermented solid ratio(v/w)	Protease activity		
					U/ml leachate	Total activity(U/g)	Recovered percent
1	First	90 ml	1	2.8	7.8	70.2	87.6
2	Second	85.5 ml	0.45	2.7	1.2	9.9	12.4
Over all		175.5 ml	1.45	5.5	9.0	80.1	100

Total enzyme activity was 80.1 U/g. The leaching recovery at first and second stages of extractions was 90% and 95%, respectively from the total water present.

4.4. Properties of the proteases

4.4.1 Effect of temperature on activity and stability of the protease C₄₅

The effect of temperature on activity of alkaline protease C₄₅ was determined by assaying enzyme activity in the temperature range of 30°C-70°C in absence and presence of 5mM of Ca⁺². The enzyme was optimally active at 40°C and 50°C in the absence and presence of 5mM of Ca⁺², respectively (Fig 4). At 65°C the enzyme displayed 50% and 10% of the maximum activity in the presence and absence of 5 mM of Ca⁺², respectively (Fig 4).

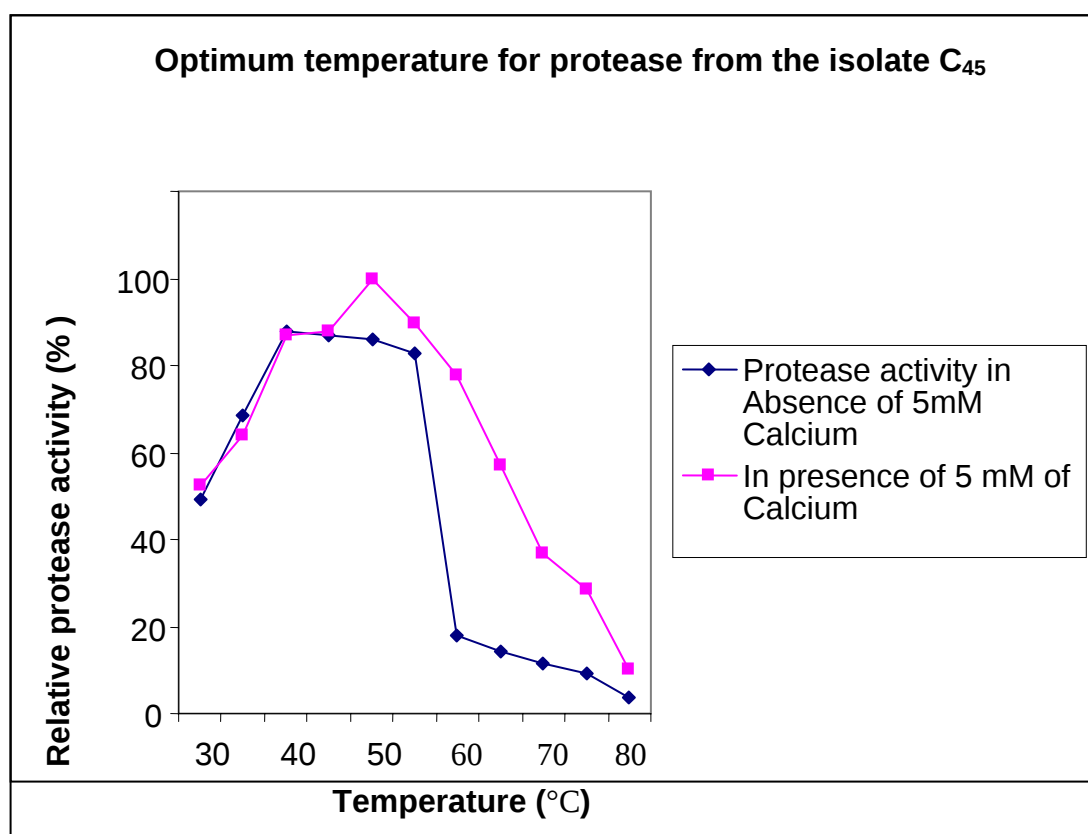


Fig 4. Temperature profile of the protease C₄₅ in presence and absence of 5mM Ca⁺².

The alkaline protease was stable at moderate temperatures (30°C to 50°C (Fig 5)). The enzyme was completely inactivated after 30 minute incubation at 60°C and 10 minutes incubation with buffer at 70°C.

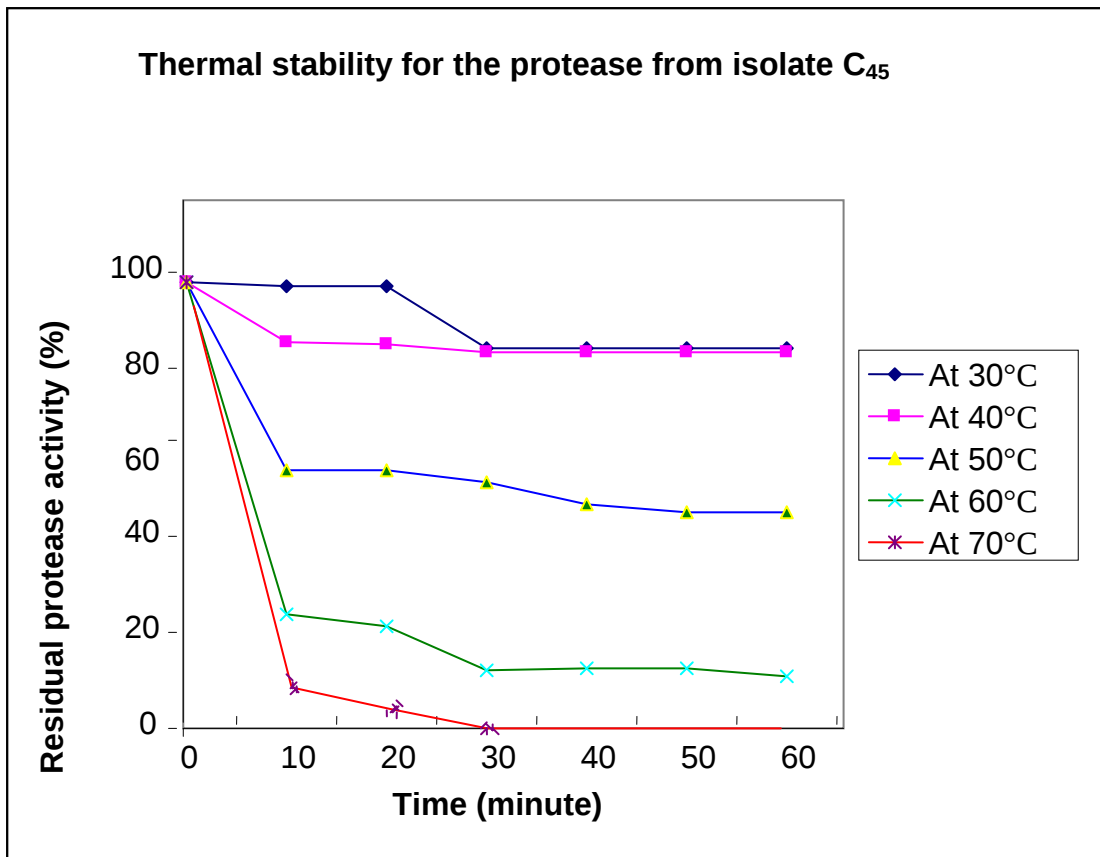


Fig 5. Thermal stability of the protease C₄₅ in presence of 5mM Ca⁺².

4.4.2 Effect of pH on activity and stability of the protease C₄₅

Protease C₄₅ was active in a broad pH range of 6.5-11.5, with optimum activity in the pH range of 8-9 (Fig 6).

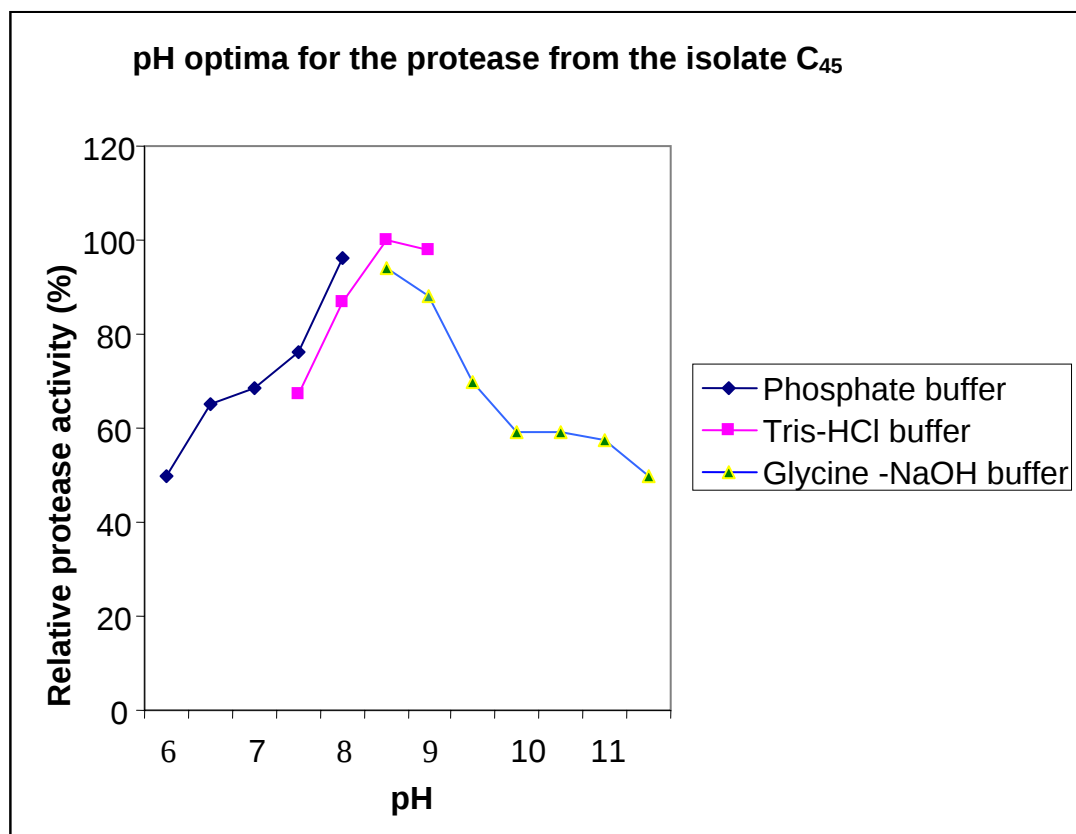


Fig 6. pH profile of the protease C₄₅. The protease activity was assayed at 40°C. Buffers: Phosphate (6-8), Tris-HCl (7.5-9), and Glycine-NaOH (8.5-11.5) each at a concentration of 50mM were used.

Protease C₄₅ was stable in a broad pH range (Fig 7). After 1 hr incubation at different pH values at room temperature, the enzyme maintain more than 80% of its original activity in the pH range of 7.5-11.5

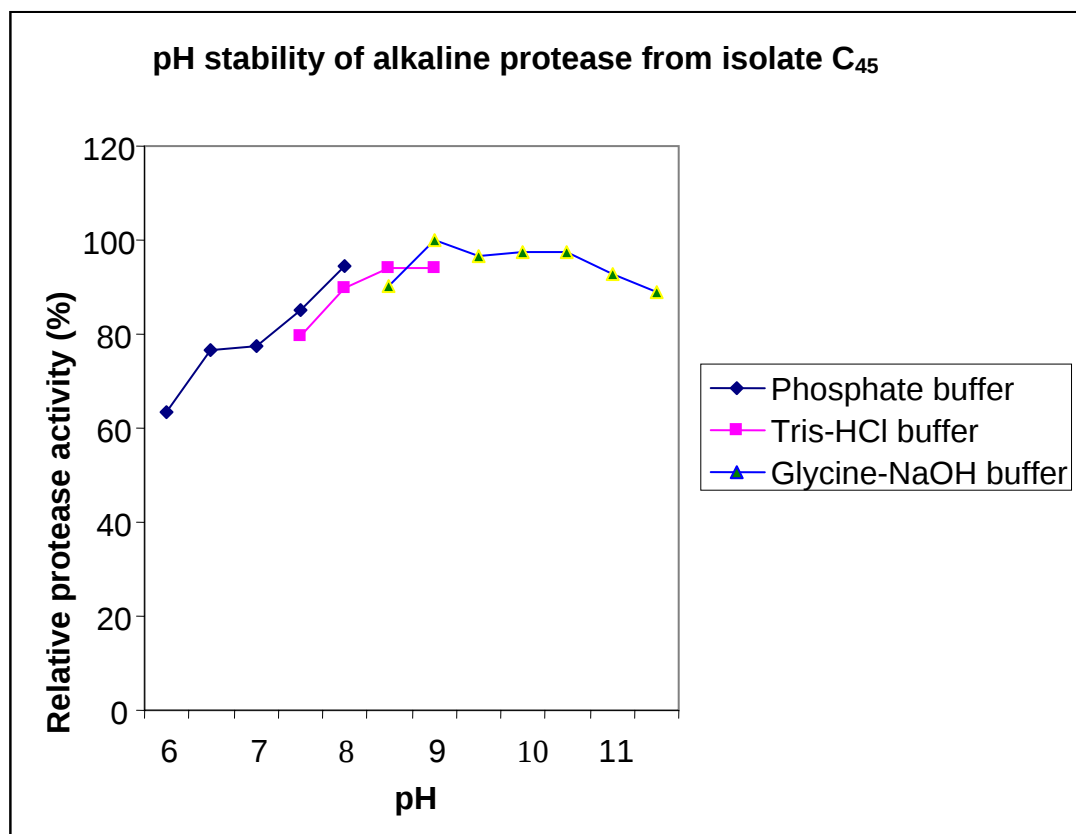


Fig 7. pH stability of the protease C₄₅. The protease activity was assayed at 40°C. Buffers: Phosphate (6-8), Tris-HCl (7.5-9), and Glycine-NaOH (8.5-11.5) each at a concentration of 50mM were used.

4.4.3. Effect of Sodium chloride on protease C₄₅ activity.

To study the effect of sodium chloride on enzyme activity, the enzyme was incubating with 2M NaCl at 25°C for 1 hour followed by protease assay. The protease was stable and maintained 100% of its original activity after an hour incubation with sodium chloride.

4.5. Enzymatic cow hide dehairing.

To evaluate the potential use of this enzyme as a hide depilating agent in leather industries, pair of cow hide was taken and added to 250 ml flask containing enzyme, its pH adjusted to 8.5 (in one to one proportion of ml of liquid to gram of hide) and placed on shaker (121 rpm) for 24 hr. One at a time from each pair taken and hair removal trial done. As shown in Fig 5 below, complete dehairing of the enzyme treated skin was achieved in 12 hr, at room temperature, with 7.8 U/g cow hide resulting pelt (hide) of natural pore (grain) on dehaired surface (Fig 8).



1)



2)



3)



4)

Fig 8. Results of cow hide dehairing experiment: (1) raw hide before dehairing, (2) control, (3) enzyme treated cow hide dehairing with fingers, (4) dehaired hide after few hours showing removal of scud and presence of natural hide pore on surface.

5. DISCUSSIONS

In this study, microorganisms isolated from Lakes Abijata, Chitu, and Arenguade screened for alkaline protease production. More than 30% of all the alkaliphilic microorganisms were protease positive. Isolation of alkaline protease from this region has been also reported in both *Bacillus* and *Nesterenkonia* sp. (Amare Gessesse and Berhanu A.Gashe, 1997; Amare Gessesse *et al.*, 2003). Out of a total of 80 proteolytic alkaliphilic microbial isolates, 20 isolates (25%) grew on SSF.

Enzyme production through SSF has enormous potential in reducing the cost of enzyme production. The selected isolate, Isolate C₄₅, grew well and produced three fold higher protease under SSF than SmF (data not shown). Since cheap agricultural residues as a media substrate can be used, production cost could be minimized that indirectly minimize enzyme cost. Thus, growth and production of appreciable amount of protease by isolate C₄₅ under SSF offer better option over SmF for its large scale production.

The amount of inoculum used to culture the microorganism in SSF affect protease production. The optimum inoculum size was 20% (v/w) that corresponded to 1×10^6 cells/g 16 hr inoculum age. At inoculum size higher or lower than the optimum a decrease in enzyme production was noted. Decrease in protease production at inoculum size below the optimum may be due to extended lag phase that result in insufficient number of microorganisms to ferment the solid substrate (Shafee *et al.*, 2005). As a result, higher fermentation time may be required to reach maximum protease production stage. At inoculum sizes above the optimum, the growing microbial cells may have created stressful conditions such as depletion of nutrients, pH fluctuation, change in availability of oxygen, and competition to access limited resources to result low protease production (Kumar *et al.*, 2008). This implies importance of controlling inoculum size in yielding high protease. A slightly different protease production behaviour and inoculum sizes compared with the observed result has been reported elsewhere. 3.6×10^6 spores/g reported by Aikat and Bha-itachryya, (2000) in *Rhizopus oryzae*, 50% (v/w) 24 hr inoculum age in *Bacillus subtilis* used to produce keratinase using solid bio-waste (horn meal) by Kumar *et al.*, (2008), and 10^8 spores/g in alkaliphilic *Streptomyces* sp.CN902 for thermostable alkaline protease production (Lazim *et al.*, 2009) in SSF. These authors suggested importance of such a high inoculum in manipulating generation of dense vegetative and protease productive morphology (hyphal tip in fungi and actinomycetes), that is a site for enzyme secretion.

Like most of the alkaliphilic bacteria in nature, the isolate C₄₅ grow and produce maximum alkaline protease at temperature of 30°C. In addition, its protease was active and stable at moderate temperature (30°C - 50°C) in the absence and presence of Ca⁺², respectively (Fig 4 and Fig 5) showing mesophilic nature of the isolate. This indicates potential applicability to reduce operating energy cost in protease assisted industrial process such as washing and dehairing (Rao *et al.*, 1998; Oliver *et al.*, 2006). Many reports showed bacterial and fungal alkaline protease production at lower ($\leq 25^{\circ}\text{C}$) and moderate (30°C-40°C) temperatures, to mention are in *Aspergillus* and *Bacillus* strains preference of such temperatures under SSF (Malathi & Chakraborty, 1991; Kumar and Takagi, 1999; Niadu and Davi, 2005; Soarese *et al.*, 2005). Thus, with out need of incubation instrument and reduced energy cost for production, high amount of protease production could be obtained.

Initial pH of medium is an other important factor that significantly influence production of alkaline protease under SSF (Da Silva *et al.*, 2007). Since soda lakes contain high concentrations of sodium carbonate, sodium carbonate containing media commonly used to adjust pH above 7.5 (Kumar and Takagi, 1999). Isolate C₄₅ requires 15% w/w Na₂CO₃ concentration for maximum alkaline protease production that corresponded to pH 10.2 (Table 4). Production of protease at high pH may be important to reduce contamination risk during fermentation that is commonly a problem in neutral, weak acidic and weak alkaline fermentation systems (Amare Gessesse and Gashew Mamo, 1999). High sodium carbonate concentration (10% w/w) is required for maximum xylanase production by alkaliphilic *Bacillus* sp.AR-009 reported by Amare Gessesse and Gashew Mamo, (1999). Likewise Martha Kebede, (2007) reported the maximum alkaline amylase production by *Actinomycetes* isolated from Lake Abijata at 10% (w/w). This indicates that, isolate C₄₅ requires relatively higher sodium carbonate concentration.

Moreover, slight drop in medium pH as well in fermented extract that falls in the range 8-9 during growth and protease induction was observed (data not shown). This change during fermentation might be associated with utilization of nitrogenous compounds and liberation of acidic compounds in the medium (Kumar and Takagi, 1999). Thus, it was most likely protease inducing factor and might be useful to know the starting and ending period for alkaline protease secretion. The medium pH change is a common phenomenon in fermentation processes and has been reported in *Bacillus subtilis* (Soarese *et al.*, 2005) which is in the range 5-10, for growth and alkaline protease production.

Moisture content of SSF is also a critical factor in production of alkaline protease. Maximum alkaline protease production by the isolate was obtained at bran to moistening agent ratio of 1:2. Marked

improvement in alkaline protease production (75.2 U/g) was achieved by the isolate C₄₅ (Fig 2) due to optimization of moisture content. At bran to moistening agent ratios below the optimum level, low protease production might be due to the inability of the micro organism to access nutrient because of reduced nutrient solubility, which in turn result poor microbial growth (Kumar *et al.*, 2008). The low protease production at moistening agent ratio above the optimum might be due to the decrease in oxygen availability as a result of reduced substrate porosity in solid substrate, and change in substrate particle structure. Different workers showed difference in optimization of moisture content of SSF: 100% (v/w) for *Bacillus subtilisin* using horn meal as a solid substrate incubated at 37°C and pH 7 for 48 hr (Kumar *et al.*, 2008), and 60% (v/w) for *Streptomyces* sp.CN902 using wheat bran that incubated at 45°C for 5 days (Lazim *et al.*, 2009), for maximum alkaline protease production. The present work demonstrated slightly higher bran to moistening agent ratio for maximum protease production compared with other workers. This may be due to the difference in the nature of the solid substrate used for fermentation.

The production of alkaline protease by isolate C₄₅ was directly related to fermentation time, just like other secondary metabolites. In all the fermentation period, protease production detected showing bimodal maximum protease production trend at 72 hr and 144 hr (Fig 3). After 144 hr of incubation time, no further increase in protease production; and no pronounced drop in protease production was observed. This might be due to the decrease in microbial growth associated with the depletion of available nutrient, loss of moisture content, production of toxic metabolites and autolysis of produced protease (Aikat and Bha-itachryya, 2000; Sumantha *et al.*, 2006). Seventy two hour fermentation time was important in terms of cost. Thus, knowing maximum protease induction period may be advantageous in managing production cost associated with fermentation time. Different fermentation time has been also reported by other workers: 216 hr in *Rhizopus oryzae* (Aikat and Bha-itachryya, 2000), 72 hr in *Rhisopus* strain RRL 2710 (Sumantha *et al.*, 2006), 96 hr in *Bacillus* sp. to produce subtilisin (Bhaskar *et al.*, 2008), and 120 hr in *Streptomyces* sp.CN902 to produce thermostable alkaline protease (Lazim *et al.*, 2009) for maximum alkaline protease production under SSF.

Another factor influencing protease production by the SSF culture under study is the nitrogen sources of the growing medium. Both organic and inorganic nitrogen sources as additives were tested on growth and alkaline protease production. The inorganic nitrogen sources were found to repress protease production. But, moderate and good protease production was observed for media supplemented with simple and complex organic nitrogen sources, respectively (Table 5). However, no significant difference between wheat bran alone and supplements with casein, yeast extract and *Millettia*

ferruginea (Berbra) raw seed flour observed on protease induction. This implies existence of close relationship in the type of nitrogen sources used as a supplement and amount of protease production. Similarly, Niadu and Devi, (2005) also reported repressing ability of inorganic nitrogen sources in the *Bacillus* isolate K-30. These authors suggested inability of the *Bacillus* isolate to utilize inorganic nitrogen sources.

Microbial growth medium for enzyme production at industrial scale takes about 30-40% production cost (Enshasy *et al.*, 2008). By using wheat bran alone, appreciable amount of protease production can be achieved, implying presence of enough nutrients in wheat bran that support the growth of the isolate and alkaline protease production. Furthermore, local complex protein sources like *Millettia ferruginea* (Berbra) raw flour found to induce almost comparable enzyme yield with casein. This indicates the potential of Berbra raw flour to use as a cost effective medium ingredient that supplement wheat bran for higher protease induction. Thus, by using wheat bran alone or/ and local complex organic protein sources in combination with wheat bran is very important in making the protease production system viable and cost effective.

Among the tested carbon sources, all the organic carbon sources did not significantly affect protease production (Table 6). Protease production analysis also showed no significant difference between the different organic carbon sources on protease production. By contrast, high protease production was observed in SSF medium supplemented with sodium acetate. This might be because of the alkaline pH stabilizing ability of the sodium acetates in the SSF medium that favor high protease induction (Kumar and Takagi, 1999). This finding was in accordance with the earlier well documented reports in literature for protease production (Kumar and Takagi, 1999).

Enzyme recovery is the main obstacle in production of high amount of alkaline protease under SSF. In first stage of extraction, 87.6 % recovery and in the second stage, 12.4% recovery of protease was achieved using distilled water as extractant. The 87.6% protease recovery in the first stage might be due to high solubility of the protease with distilled water. High enzyme recovery in single stage and using water as extractant may be important in reducing cost associated with enzyme harvesting and in reducing amount of liquid used for extraction. Use of more efficient extraction procedures and equipments may even increase the recovery percent in the first stage. Similarly, Malathi and chakraborty, (1991) used distilled water for alkaline proteases extraction from fermented solid. However, high protease recovery has been also reported using organic solvent such as ethanol-glycerol

mixture and sodium chloride (Tunga *et al.*, 1999), and buffers (Aikat and Bha-itachryya, 2000) as protease extractant.

The enzyme produced by the isolate C₄₅ under SSF was further characterized to evaluate its potential usefulness for different applications. The protease from this isolate was optimally active between pH 8-9 and stable at alkaline pH. Proteases active in the pH range of 8-12 and stable at alkaline pH are known as potential candidates for detergent application, dehairing of hides, and silver recovery from waste X-ray and photographic films (Rao *et al.*, 1998; Gupta *et al.*, 2002; Maurer, 2004). This indicates that protease C₄₅ has a good potential for such applications.

An other interesting property of protease C₄₅ was its ability to maintain 100% of its original activity in the presence of 2M NaCl. The observed stability at high ionic strength was due to the presence of high proportion of acidic amino acids such as glutamic acid on its surface (Madern *et al.*, 2000). This high salt tolerance of the enzyme implies its importance to use for bioorganic synthesis for protease assisted peptide synthesis (Martinek and Semenov, 1981). Halophilic protease production has been also reported in alkali-tolerant *Bacillus patagoniensis* (Oliver *et al.*, 2006), and by other halophilic and alkaliphilic bacterial isolates (Patel *et al.*, 2006; Dodia *et al.*, 2006) at 2M NaCl. Therefore, stabilising ability of active site conformation even in presence of high competing solutes for water and at dry state by halophilic enzymes show their usefulness in proteases assisted synthesis of unique chemicals.

Results of enzymatic cow hide dehairing showed successful use of this enzyme as a dehairing agent. Complete dehairing of hide was achieved at 12 hr. Because of specificity to hydrolyse non-collagen protein part at hair roots in hide, proteases are very important in shortening hide dehairing time and in production of high quality full grain leather having natural hair pores on the surface (Sivasubramanian *et al.*, 2008). Cow hide usually treated with dehairing chemicals in a drum for 24 hr (Thanikaivelan *et al.*, 2004). Shortening of dehairing time has been also reported, 20 hr for *Aspergillus flavus* protease by Malathi & Chakraborty, (1991), and 9 hr for keratinases of *Bacillus subtilis* S14 by Macedo *et al.*, (2005). Thus, protease C₄₅ has a potential to substitute environmentally objectionable dehairing chemicals for hide/skin dehairing in leather industries and for production of quality leather products.

6. CONCLUSIONS AND RECOMMENDATIONS

Based on the results of this work, the following conclusions can be drawn:

- The new alkaliphilic microbial isolate C₄₅ shows higher protease production at pH 10.2.
- The alkaline protease produced by this isolate was active and stable at high alkaline and salt concentration.
- The protease stability and high enzyme activity at moderate (30°C-50°C) and at room temperatures, respectively, was an attractive feature to develop enzyme based industrial processes at room temperatures.
- Hide dehairing experiment at laboratory also confirmed the usefulness of this enzyme for application in leather industries.
- In addition production of the enzyme under SSF offer an advantage in minimizing production cost, because maximum amount of protease can be obtained by using wheat bran alone.

Therefore, the following activities for future research recommended:

1. Testing the remaining three of the selected best isolates for their maximum production potential under SSF.
2. Searching for more potent alkaline protease producers from Ethiopian soda lakes.
3. Testing the alkaline protease from C₄₅ for other suggested potential industrial applications.
4. Scale up studies.
5. Test of the enzyme dehairing capability at tannery experimental level.

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Declaration

I the undersigned candidate declare that this thesis is my original work. It has not been presented for degree in this university or other s and all source materials used for this thesis have been duly acknowledged.

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