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SCHOOL OF GRADUATE STUDIES

ALKALINE PROTEASE OF ALKALIPHILES ISOLATED FROM ETHIOPIAN RIFT VALLEY SODA - LAKES

BY

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ALKALINE PROTEASE OF ALKALIPHILES ISOLATED FROM ETHIOPIAN RIFT VALLEY SODA LAKES

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ABSTRACT

A proteolytic alkaliphilic bacteria was selected out of the bacterial isolates obtained from Riftvalley soda lakes. The isolate was obtained from lake Chitu water sample. The organism was Gram variable aerobic, rod shaped spore forming, motile bacterium. It has been identified to belongs to the genus *Bacillus*.

The growth of the isolate coded as CH-W_1 was observed in the pH and temperature ranges between 7-11 and 25-40^{0}C respectively. Protease production was observed shortly after inoculation reaching to maximum after 48 h. The crude enzyme had a temperature optimum of 55 °C and a pH optimum of 9. The enzyme was stable in a broad pH range of 8.5-10.5 after 1 hr incubation at 50^{0}C . It has a half-life of 30 min. at 60^{0}C . The enzyme was slightly activated by $CuSO_4$. Where as Ba^{++} ,Zn^{++}, Co^{++}, Hg^{++}, Mn^{++}, Fe^{++}, Na^{+}, $K^{+}$and Ca^{++} had very little or no effect on the activity of the enzyme. The enzyme was strongly inhibited by 1 mM PMSF showing that it belongs to the class of serine protease. EDTA at a concentration of 10 mM partially inhibits the activity. This shows the requirement of Ca^{++} for stability.

The organism was efficient in degrading Nug meal (*Guizota Abyssinia*) and feather when used as a sole carbon source.

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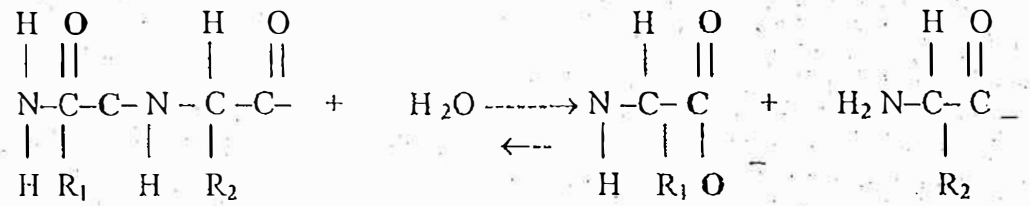
I. INTRODUCTION

The ability to utilize proteins as source of carbon is widely distributed among different microorganisms. Proteases hydrolyze proteins to amino acids and peptides. The actions on proteins occur by addition of water in the peptide linkage, resulting in protein hydrolysis.



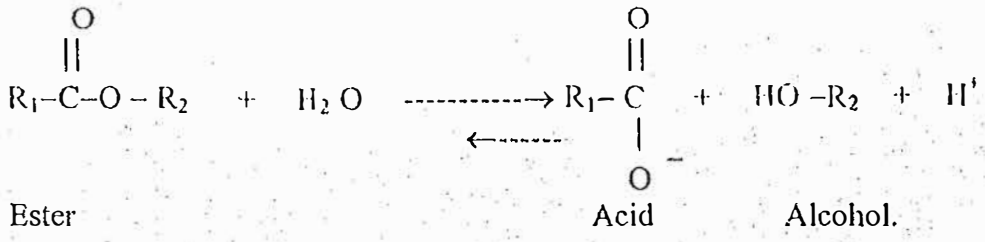
Peptide Carboxyl compound. Amino compound.

Protease often secreted as extracellular enzymes or may occur as intracellular enzymes (Leuschner *et al.* ,1995).

Proteases are the most important group of industrial enzymes and certainly form a major portion of world wide enzyme sales. As the result there are a wide variety of protease described in literature and available commercially. They are classified into groups depending on whether they are active under acidic, neutral or alkaline conditions and on the characteristics of the active site group of the enzyme (Ward, 1983; Morihara, 1992; Harley ,1960; North, 1982) .

Based upon the nature of their active center, proteases are categorized as:

1. <u>Serine Proteases;</u> These enzymes are characterized by the presence of a serine residue at their active site (Hartley, 1960) . Accordingly, they are inhibited by DFP (diisopropyl- fluorophosphate) and PMSF (phenyl methyl sulfonyl fluoride) but, possessing no metal ion requirement, they are resistant to EDTA (Priest, 1977) . They are however, stabilized at a high temperature by Ca^{++}. Serine proteases hydrolyze simple terminal esters (analogous to the specific peptide substrates).



All members of the group may be classified as endopeptidases since it is generally observed that the cleavage of terminal peptide bond is inhibited by the charge on the amino or carboxyl group of terminal residue.The alternative name alkaline protease, reflects, their high pH optimum of 9 to 11.

. The subtilisins are a group of alkaline protease originating from different strains of *Bacillus subtilis* or related bacteria. Three enzymes belonging to this were isolated in pure form from the Carlsberg, NOVO and BPN' strains of this organism (Barel and Glazer,1968). Analysis of complete amino acid sequence of these proteins has revealed that Carlsberg subtilisin differs from BPN' subtilisin in over 80 positions.

The enzymes obtained from NOVO and BPN' appear to be identical (Smith, *et al.* 1968).

2) **Metal Proteases** ; They are a group of metallo-endopeptidase that show maximal activity at or near neutrality. Metal proteases have distinct divalent cations and sensitive to metal chelating agents such as EDTA and do not possess esterase activity (Hartely, 1960). Metal ions are required for conformational stability of the enzyme that is the presence of calcium and other metallic ions greatly stabilize the enzyme from autolysis. EDTA is a potent inhibitor of the enzyme because it removes the catalytic metal ions from the enzyme. *Bacillus megazerium* (Millit and Aubert, 1966, Keay and Wildi, 1970) , *Bacillus thermoproteolytics*, *Bacillus polymyxa* (Keay *et al.*, 1970) secrete only the metal proteases.

Hybrid enzyme with characteristic of both the serine and metal protease (serine-metal protease) were reported to be produced by *Bacillus licheniformis* and *Bacillus pumilus* (Vitkoric and Sodoff, 1974).

3) Cysteine Proteases; They contain sulfhydryl (SH) groups which are inhibited by thiol reagents , heavy metal ions and alkylating and oxidizing reagents. They require a free sulfhydryl group for their catalytic activity. Activation is achieved by mild reducing agents such as cysteine, sulfide, sulfite as well as cyanide. They are inhibited by various reagents which show a pronounced reactivity toward the free sulfhydryl group (eg. PCMB Iodoacetate, H_2O_2, Heavy metals Hg^{2+}, Ag^{2+}, Cu^{+}).

Cysteine proteases appear to be restricted to the thermophilic fungi (Cowan *et al.* 1985).

4)Aspartic (Acid) - Proteases; They may be inactivated by alkylation of the aspartic residues of the center.Examples are pepsin and renin (Morihara, 1992). They are distinguished by their low optimum pH, which may imply a common mechanism (Hartely, 1960) . The typical inhibitors of aspartic protease are EPNP (1,2 -epoxy-3- (p-nitro phenoxy) propane and DAN (diazoacetyl -Dlnorleucine methyl ester).

The catalytic mechanism of a protease is an important consideration in commercial application Cysteine proteases are susceptible to inhibition by many reagents, particularly metal ions, and lose activity by oxidation. Optimal activity is often only obtained in the presence of reducing agents and such additions are often not industrially feasible. Neutral metallo proteases are rapidly inactivated by the removal of the catalytic metal ions and thus can not be used in application, where chelating agents are present. The serine proteases however are not readily inhibited, although many are stabilized by metal ions, and are used in most established biotechnological applications (Cowan *et al.* 1985).

Historically proteases have played a very important role in the processing of natural products. For many centuries protease containing cultures, extracts and waste products have been used in such diverse applications as tanning of hides and the production of condiment. Today, proteases are probably the most important of the

classes of industrial enzymes, with worldwide sales representing about 60 % of the total enzyme market (Cowan *et al.* 1985).

Proteases represent the largest proportion of enzymes sold in part due to the large use of alkaline protease in detergents to breakdown proteins, which bind the soil to fabrics. Therefore alkaline proteases are one of the most important group of industrial enzymes (Gonzalez, *et al.* 1992) . They are put to use in detergent, leather -tanning and food industries (Kelly and Fogarty, 1976; Godfrey and Reichelt, 1985; Bilinski *et al.* 1986 ; Hugenoholtz, *et al.*987; Boven, *et al.* 1988).

Today a large proportion of detergents in developed nations contain enzymes. The enzymes included in detergents are alkaline proteases, amylases lipases and cellulases. Earlier protease containing detergents were used for cleaning soiled laundry from slaughterhouses, fish processing industries, hospitals, etc. All contain a large amount of proteinaceous soil. Inclusions of protease in detergents help in degrading the protein and greatly improve the cleaning process (Sharp and Munster, 1986; Kalisz, 1988; Cowan, 1991).

The pH of detergents is in the range of 9 to 11 and mostly used washing temperature being in the range of 50^0C to 60^0C. Other components of detergents include oxidizing and sequestering agents, ionic or non ionic surfactants, perfumes and fragrances, builder and some other components (Barfoed, 1985; Kalisz,1988) .

Therefore an ideal detergent enzyme should be stable at high pH and temperature, withstand oxidizing and chelating agents, some of which are denaturing to proteins.

The alkaline protease subtilisin derived from a strain of *Bacillus licheniformis* and *Bacillus amyloliquifaciens* (Tilburg, 1984; Outtrup and Boyce, 1990) has been successfully used for a long time as a detergent additive (Kalisz, 1988) . This success has initiated the search for other more efficient enzymes. Hence extensive screening programs, have been launched for alkaline protease producing alkaliphiles (Horikoshi, 1990).

An alkaline protease from alkaliphilic *Bacillus* Sp.221 has been reported by Horikoshi (1971) as the first enzyme with an alkaline pH optimum produced by an alkaliphilic microorganism. The *Bacillus* strain 221 produces an alkaline protease that has high optimum pH (pH 11.5-12), thermostability at highly alkaline pH, and stability to detergents. Since this report was published, there have been extensive studies on the properties of alkaline proteases from other strains. Durham *et al.* (1987) purified and characterized two types of alkaline proteases from an alkaliphilic *Bacillus* sp. strain GX - 6638. Both enzymes showed good activity in broad pH range of 8 to 12 and have a temperature optimum of 65^0c. Takii *et al* (1990) reported the production of alkaline serine protease by *Bacillus alkalophilus*,subsp *halodurans Kp* 1239. The enzyme was most active at 60^0C and at pH 11.5.Shimogaki *et al* (1991) reported the production of a alkaline protease by *Bacillus* sp. strain y. The optimum pH for the enzyme was in the range of 10 to 12.5

with good stability between pH 6.5 and 13. The enzyme was stable in the presence of different detergent component and has temperature optima of 70°c.

Another alkaline protease isolated from alkaliphilic *Streptomyces* sp. Strain YSA-130 was reported to have pH and temperature optima of 11.5 and 60°C respectively (Yum, *et al.* 1994). Amare (1997) reported the production of alkaline protease by *Bacillus* sp. AR-009. The optimum pH for the enzyme activity was in the range of 9.5-11.5, with good stability in the pH range 5-12. It has temperature optima of 55°C.

All of these reports show that alkaline protease from alkaliphiles have better alkali resistance than those from neutrophilis, eg. *B.licheniformis.* Sequestering agents are usually added to complex with calcium and magnesium to avoid precipitation and binding to fabrics (Barfoed, 1985). The addition of sequestering agents in detergents poses one serious problem to detergent enzymes. Most alkaline proteases require calcium for stability. In the presence of these chelating agents the stability of the enzyme thus becomes a problem, since they precipitate the Ca^{++} ion. But alkaline soda lakes are known to have very low calcium concentration (Grant, 1992). Therefore protease from organisms isolated in these habitats are expected to require very little or no calcium.

The leather tanning industry is the second largest sector utilizing alkaline enzymes (Godfrey and Reichelt *etal.*1985; Sharp and Munsler, 1986; Grant *et al.*1990). Skin

(or hide) is composed of three distinct layers; the epidermis, the corium and the connective tissues of the under surface. The epidermis contains the hairs, which are composed of keratin and are imbedded to it by hair follicles. The corium represents the major portion of the skin being composed of collagen fibers. The corium is bound to the inner tissue of the animal by the connective layers, which contain all the blood vessels for nourishment.

Processing of animal skin (or hide)involves the following processes :

Soaking; Leather processing industries usually purchase the skin or hide in a dried form, through a process called curing. The first step in leather processing is therefore the rehydration and washing of the cured skin. The traditional process rely on the use of alkali for swelling. Addition of a small amount of alkaline protease to the soak liquor at this stage has been found to greatly improve water uptake, shorten the time required for soaking and help to produce skin suitable for subsequent operations (Kalisz, 1988; Grant, *etal.*1990; Horikoshi, 1990).

Dehairing and dewooling ; Hair removing is the next step in leather processing. The oldest method relies on the incubation of the skin in a steam chamber to make conditions favorable for the natural microflora to act on the hair. This method, in addition to being time consuming, has the danger of damaging the collagen fiber by the bacteria and result unnecessary loss. The dehairing process was improved by the use of very alkaline conditions using hydrated lime and addition of sodium sulfate. This method, although very cheap and simple, poses serious pollution problem due

to the formation of noxious gases like hydrogen sulfide in the effluent, thus necessitating subsequent expensive waste treatment operations. The use of alkaline proteases has been shown to greatly improve the dehairing process (Kalisz, 1988; Horikoshi, 1990). Enzymes help to remove the hair from the root rather than falling off from the surface. Dewooling is done by applying paint at the flesh side of the skin and incubating overnight at 20 - 30^0C. The dewooling paint is composed of hydrated lime , sodiumchloride , and proteolytic enzymes . The alkaline protases are the most preferred at this stage, thogh strict control on the proteolytic activity is needed depending on the type of dewooling (Godfery and Reichelt ,1985.)

Bating; the purpose of this step is to de-lime and de- swell the collagen of the skin, and to partially degrade the protein so as to make it soft, supple and able to accept an even dye and demonstrate the grain in an acceptable manner. In the old days bating was based on the use of bacterial protease from bird and dog feaces. Today animal derived protease and microbial alkaline protease from strains of *Bacillus* are used, often in combined preparation (Godfery and Reichet; 1985; Kalisz, 1988).Fibrous proteins, such as horn, feather, nail and hair are abundant and available as waste. All of these proteins have a great potential as source of food and / or feed after partial or complete proteolytic hydrolysis. Alkaline proteases have also been used for the production of protein hydrolysate from oil meals, fish waste, blood and several other proteins for the products that serve as food or feed (Cowan 1985; Dalev , 1990; Dalev and Simeanova, 1992).

Feather waste, generated in large quantities as a by-product of commercial poultry is nearly pure keratin protein. Because of a high degree of cross linking by cystine disulfide bonds, hydrogen bonding, and hydrophobic interactions, keratin is insoluble and not degradable by proteolytic enzymes, such as trypsin, pepsin and papain (Goddard and Michnelis, 1934; Harrap and Woods, 1964). Despite the unusual stability of keratin, feathers do not accumulate in nature. Keratinolytic activity has been reported for species of *Aspergillus*, *Ctenomyces*; and *Streptomyces* (Noval and Nickerson, 1959; Sen *et al.*, 1959).Currently, feather waste is utilized on a limited basis as a dietary protein supplement for animal foodstuffs. Prior to being used, the feather is steam pressure cooked or chemically treated to make it more digestible. This treatment processes require significant energy and also destroy certain amino acids (Papadopoulos, 1985).

Biodegradation by microorganisms possessing keratinolytic activity represents an alternative method to improve the nutritional value of feather waste. Alkaliphilic bacterial strains which grow on feather as the sole source of carbon and nitrogen have been reported (Xiang *et al.*, 1992; Williams, *et al.*, 1989; 1990). The enzyme from feather grown strains may find other applications such as in the detergent industry, thus making the over all enzyme production process cheaper.

The use of low cost growth substrate for the production of industrial enzymes is expected to greatly reduce the production cost. This is important for alkaline proteases, which account for over 25% of the total industrial enzyme marker. Oil

seed meals, which are byproducts of oil extraction are potentially useful low cost substrate for the production of different enzymes (Gattinger *et al.*, 1990; Bautista *et al.*, 1990).

New proteases are constantly being isolated. Although almost all types of protease have some potential application, one of the reasons that most are not even considered for commercial use is that any possible advantages are heavily out weighted by the necessary development costs. Even some which seem to have substantial advantages (eg. the alkaliphilic protease) have not yet found great commercial acceptance (Cowan *et al.*, 1985).

A large proportion of the commercial alkaline proteases is obtained from neutralophilic *Bacillus* species. Since the early 1970's many protease producing alkaliphiles have been reported from many laboratories (Horikoshi and Akiba, 1982; Outtrup and Boyee, 1990). A number of studies on microbial alkaline protease have been done in view of structure_ function relationships and industrial applications. Among them, alkaline proteases derived from alkalophilic Bacilli (Horikoshi, 1971; Durham *et al.* ,1987; Manachini *et al.*, 1988; Takali *et al.*, 1989), *Streptomyces* (Nakanishi and Yamato, 1974) and fungi (Sangita *et al.*, 1993) , are known to be active and stable in highly alkaline conditions . They have drawn the attention of many researchers to their industrial use, especially as laundry additives and to the fundamental questions why these protease are so active and stable under extreme conditions. However, most alkaliphiles known were isolated from neutral soil. But

naturally occurring alkaline environments, such as those found in the East African Rift valley, offer tremendous potential for the isolation of new microbial strains producing novel proteases of potential industrial importance. Such habitats are uniformly alkaline and organisms living there are adapted to live at alkaline conditions. To date few attempts have been made on the isolation of proteolytic alkaliphiles from these habitats in our country. Therefore the objectives of this study were

1) To isolate and characterize alkaline protease producing alkaliphilic bacteria from Rift Valley soda lakes.
2) To optimize cultivation conditions for optimum enzyme production; and
3) To characterize the crude enzyme(s) and evaluate their potential applications.

II. Materials and Methods

1.Isolation and screening

Samples of water and soil collected from lake Chitu, Shalla and Abjata were inoculated into 250 ml flasks containing 50 ml sterile nutrient broth supplemented with 0.5% casein and 4% Na_2CO_3. The flasks were incubated at 37^0C in an orbital incubator shaker for 24 h with the shaking rate of 180 rpm.

A loopful from each of these cultures was spread on casein and Na_2CO_3 containing nutrient agar plates and incubated at 37^0C until pure colonies were observed. After 24 h pure colonies were picked and transferred to fresh nutrient agar plates (supplemented with 0.5% casein), The purity of the isolate was ascertained through repeated streaking. Detection of protease producing bacteria on a plate were carried out by flooding the plates with saturated solution of $(NH_4)_2 SO_4$. The bacteria, which gave the largest zone of clearing around the colony, were selected for further investigation.

2.The organism and growth condition

Stock culture of the selected isolate was maintained at room temperature on nutrient agar slants supplemented with 1% casein and transferred to fresh medium every two

weeks. The medium used for growth and enzyme production was composed of (g/ml) 0.5% casein, 0.2% peptone, 0.1% K_2HPO_4, 0.5% NaCl, 0.02 $MgSO_4$. $7H_2O$, 0.01% $CaCl_2$,1% traces and 4% Na_2CO_3. Sodium carbonate was sterilized separately and added to the rest of medium after cooling. One hundred milliliters of the above medium in 500 ml baffled flasks was inoculated with 5 ml of inoculum culture grown in the same medium for 24 hr, and incubated at 35^0C with rotary shaking. After 48h. the culture was harvested and centrifuged. The cell free culture supernatant was used as crude enzyme preparation to quantify the protease activity and to study properties of the enzyme.

3.Characterization of the isolate

Biochemical and morphological characterizations such as Gram reaction, cell shape and size, spore shape and location, catalase production etc. were carried out following procedures described by Claus and Berkeley (1986) to identify the isolate to the genus level.

4.Enzyme assay

Protease activity was measured using casein as substrate. To 1 ml of 2% casein in 50 mM glycine/NaOH buffer, pH 10, 1 ml 1:5 diluted crude enzyme was added and incubated at 50^0C for 20 min. After 20 min the reaction was terminated by adding 2 ml of 10% TCA solution and centrifuged at 5000g for 10 min. To 0.5 ml of

supernatant 2.5 ml of 0.5 M sodium carbonate was added, followed by 0.5 ml, 1:10 diluted Phenol reagent. After 30-min incubation at room temperature, absorbency was measured spectrophotometrically at 660 nm against an appropriate reagent blank using spectronic 1001 spectrophotometer. One unit of protease, activity was defined as the amount of crude enzyme which released 1 mg of amino acid equivalent to tyrosine per min, under the above assay condition.

5.Growth and protease production

One hundred ml of the basal medium was inoculated with 4 ml of an overnight culture. Cultures were shaken at 110 rpm in an orbital shaker at 35^0C. Biomass was determined by measuring optical density (OD) at 600 ηm using spectronic 1001 spectrophotometer (Miltonroy Comp. UK) .The pH of the culture was determined using corning pH meter (England). Time course of growth and enzyme production studies were made by withdrawing samples at intervals of 6 h aseptically.

6. Effect of pH on growth

The optimum pH for growth was determined by adjusting the pH values of the nutrient broth supplemented with 0.5 casein and 4% Na_2CO_3 at an interval of 1 from pH 7 to 12.

7. Effect of temperature on growth

The effect of temperature on the growth was determined by incubating cultures at 25 -40°c. Biomass was estimated by measuring the absorbance or turbidity of the growth medium at 600ηm, after a specified incubation period.

8.Growth and protease production using natural substrate

To examine the potential uses of the organisms and/or their enzymes, 1gm dry weight of feather or nug was introduced to separate flasks of the basal medium instead of casein and autoclaved. After adjusting the final pH of the medium to 10, a standard inoculum of cells was inoculated to these flasks and the flasks were incubated at 35^{0}C. After 48 h the culture was harvested and assay was carried out by the standard assay procedure.

9.Study of conditions on enzyme's activity

9.1. Effect of temperature on enzyme's activity

The temperature profile of the isolate's protease was determined by assaying enzyme activity at different temperature (between 30-70°C).

9.2. Effect of temperature on enzyme's stability

The temperature stability of the enzyme was tested by incubating the enzyme at 60°C for 5 h in the presence or absence of 5 mM Ca^{++} and 10 mM EDTA. Residual activity was measured at an interval of 30 min.

9.3. Effect of assay pH on enzyme's activity

The pH profile of the enzyme was determined by assaying the enzyme activity at different pH values using different buffers of varying pH values. The buffers used were acetate (pH 4-6), phosphate (pH 6-8), Tris/HCl (pH 7.5-9) and Glycine/ NaOH (pH 8.5-12).

9.4. Effect of pH on enzyme's stability

The pH stabilities were determined by measuring relative activity after incubation of proteases for 30 min. at 50°C in buffers described in 9.3.

9.5 Effect of inhibitors on enzyme's activity

The effect of inhibitors on activity of the CH-W1 protease was tested by incubating the enzyme with different inhibitors at 30° C for 1 h. Then, residual activity was measured following the standard assay procedure.

9.6. Effect of cations on enzyme's activity

The effect of different metal ions on the activity of the CH-W1 protease was tested using assay mixture containing 5-mM of different ions. The residual activity was assayed at 50^0C.

III. Results

1. Isolation and screening

A total of 200 strains were isolated from samples collected out of which 22 were proteolytic. From 22 proteolytic isolates 14 were found be protease positive on agar plate. Among these three isolates which had shown wider clearing zone were selected and tested for protease production in liquid medium. On the basis of high productivity and better pH and temperature stability, one isolate coded as CH-W_1 from lake Chitu water sample; was selected for further study.

2.Characterization of the isolate

Isolate CH-W_1 was Gram variable, aerobic rod with a terminal endospore, cells in single or two to three cells chain motile and catalase positive.

3.Growth and protease production

The isolate CH-W1 was a slow grower reaching to a stationary phase around 42 h. The pH of the culture dropped from 10.95 to 8.93 within 12 h, and after 18 h, it began to rise up. Protease production was detected shortly after inoculation (at 6 h.) and reached to maximum after 48 h. (Fig 1).

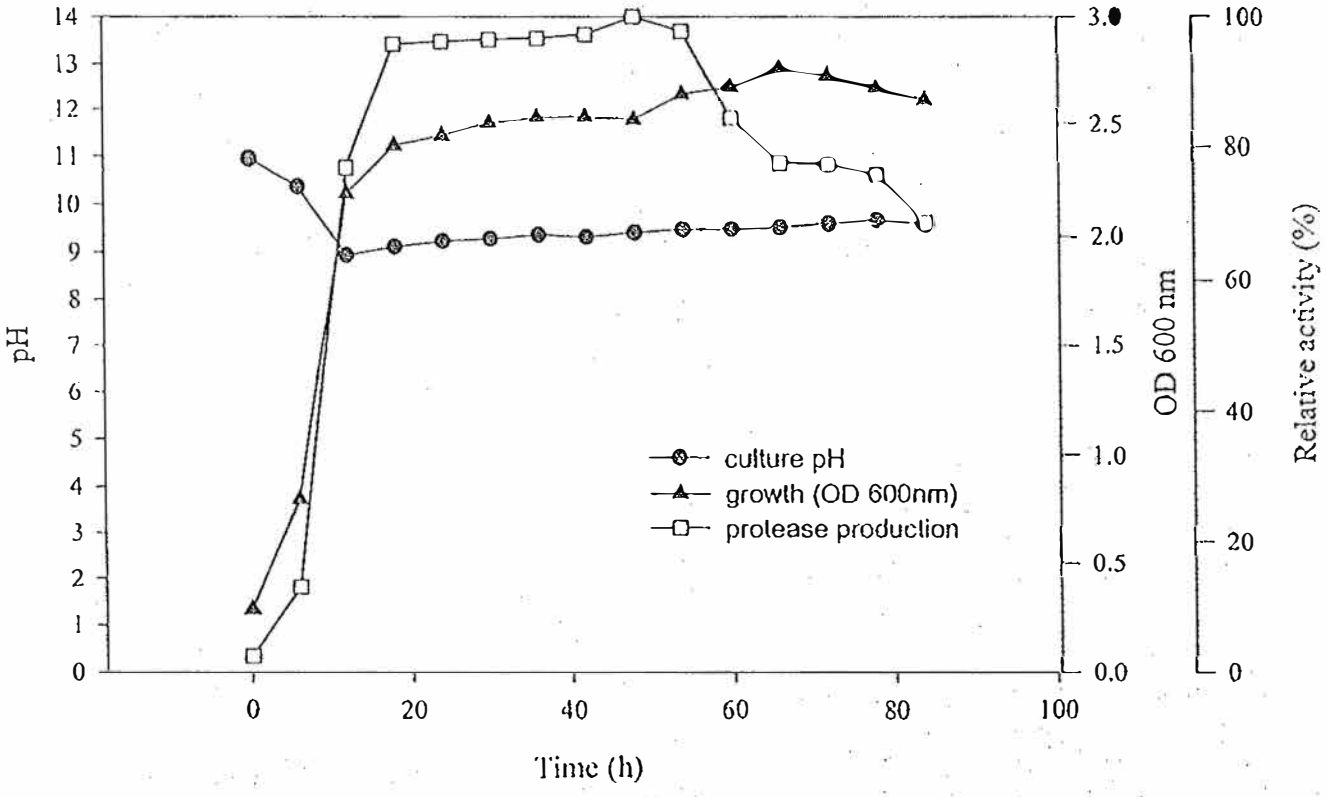


Fig. 1. Time course of growth and enzyme production by CH-W_1

● - culture pH

▲- growth (OD at 600ηm)

□- Protease production

4. Effect of temperature on CH-W_1 enzyme's activity

The temperature profile of CH-W_1 protease was determined by assaying enzyme activity at different temperature. Enzyme activity was detectable between 30 to 70^{0}C. However, maximum activity was observed when the enzyme was assayed at 55^{0}C. (Fig 2).

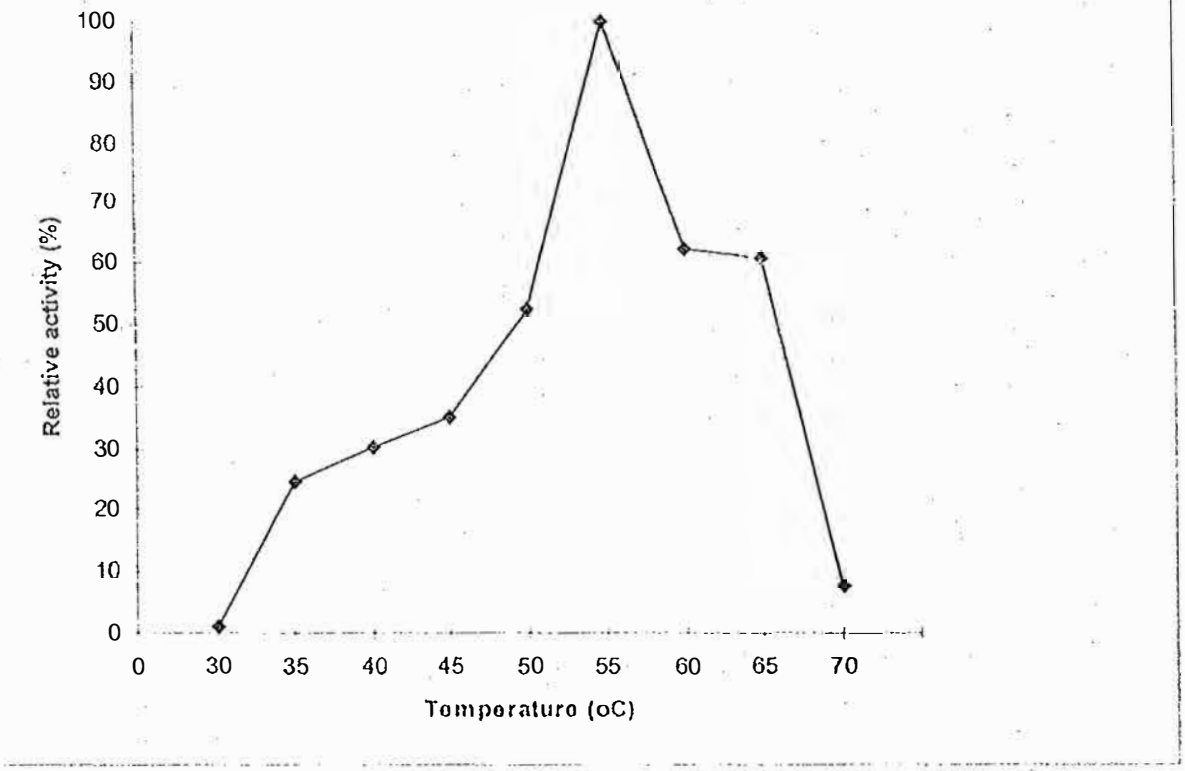


Fig. 2 Temperature profile of CH-W_1 protease activity on casein

5.Temperature stability of CH-W1 protease

The temperature stability of the enzyme was tested by incubating the enzyme at 60^0C for 5 h. in the presence or absence of 5 mM Ca^{++} and 10 mM EDTA. Residual activity was measured at an interval of 30 min at 60^0C. In the absence of Ca^{++} the enzyme lost 50%, 80% of its original activity after 30 min and 1 h incubation at 60^0c, and pH 10.0 respectively. However in the presence of Ca^{++} it lost 40%, 50%, and 60% of its original activity after 30 min, 1 h and 5 h. incubation at 60^0C

respectively. In the presence of EDTA, the enzyme lost 85% of its original activity right after 30 min of incubation (Fig 3).

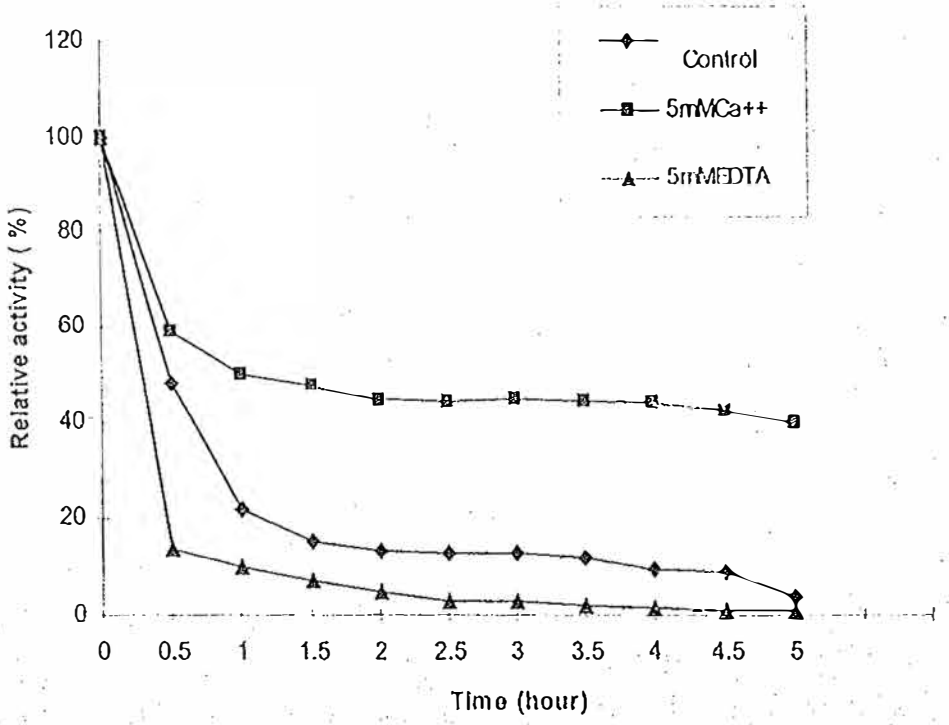


Fig. 3 Temperature stability of the CH-W_1 protease in the presence and absence of 5mM Calcium and 10 mM EDTA assayed at 60^0C.

6.The effect of pH on protease activity

Different buffers of 0.1M were used to determine the pH profile of the enzyme (Fig 4) . Activity though minimal was present at pH between 4 and 12 with the peak at pH 9.0.

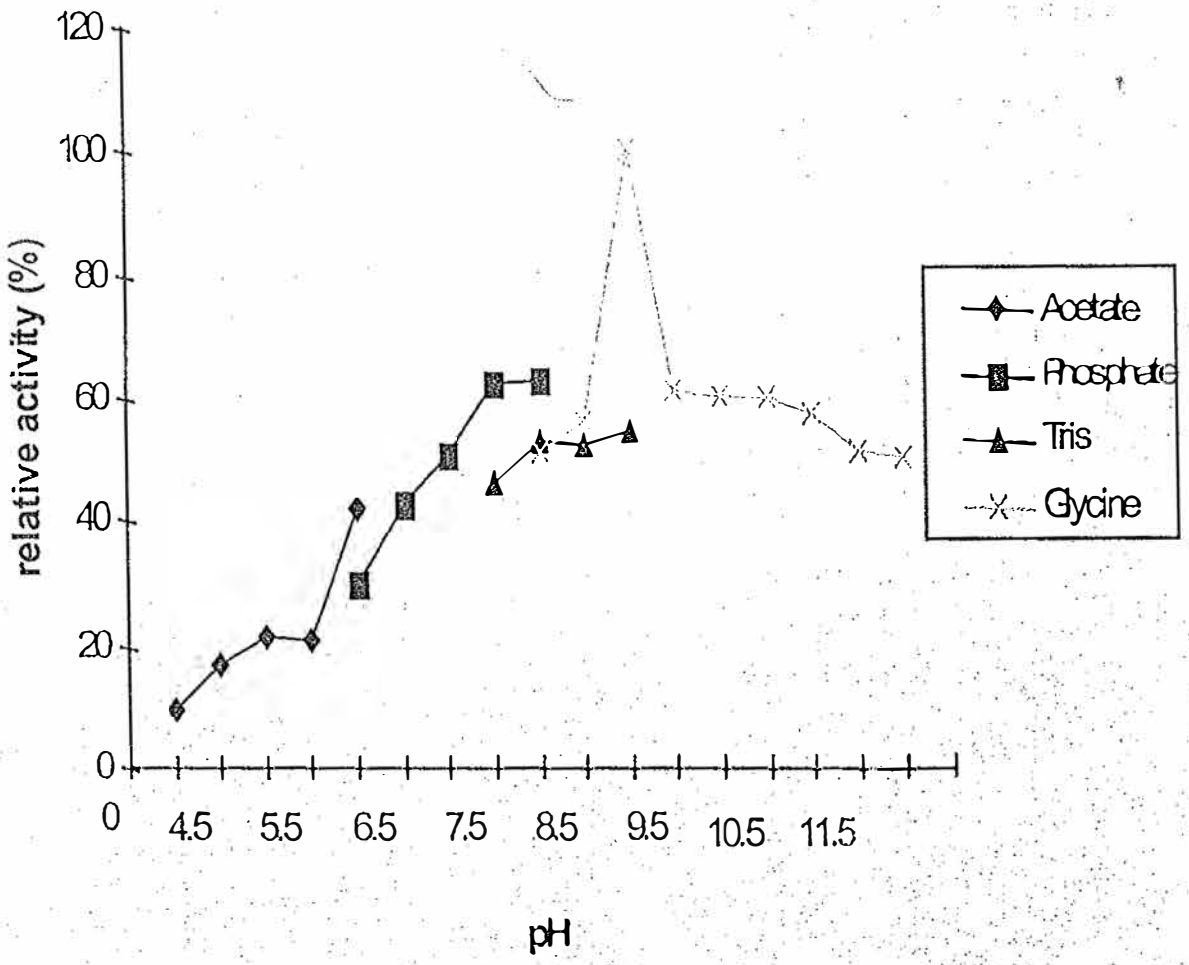


Figure 4 pH profile of CH-W_1 protease activity with different buffers assayed at 50^0C.
Acetate buffer (pH 4-6)

Phosphate (pH 6-8)

Tris / HCl (pH 7.5-9)

Glycine/ NaOH (pH 8-12)

7. The effect of pH on enzyme activity

The pH stability was determined after pre incubation of the protease for 30 min at 50^0C in the buffers described earlier. As presented in Fig 5, over 80% of the initial enzyme activity was retained in the pH range 7.5-10.5.

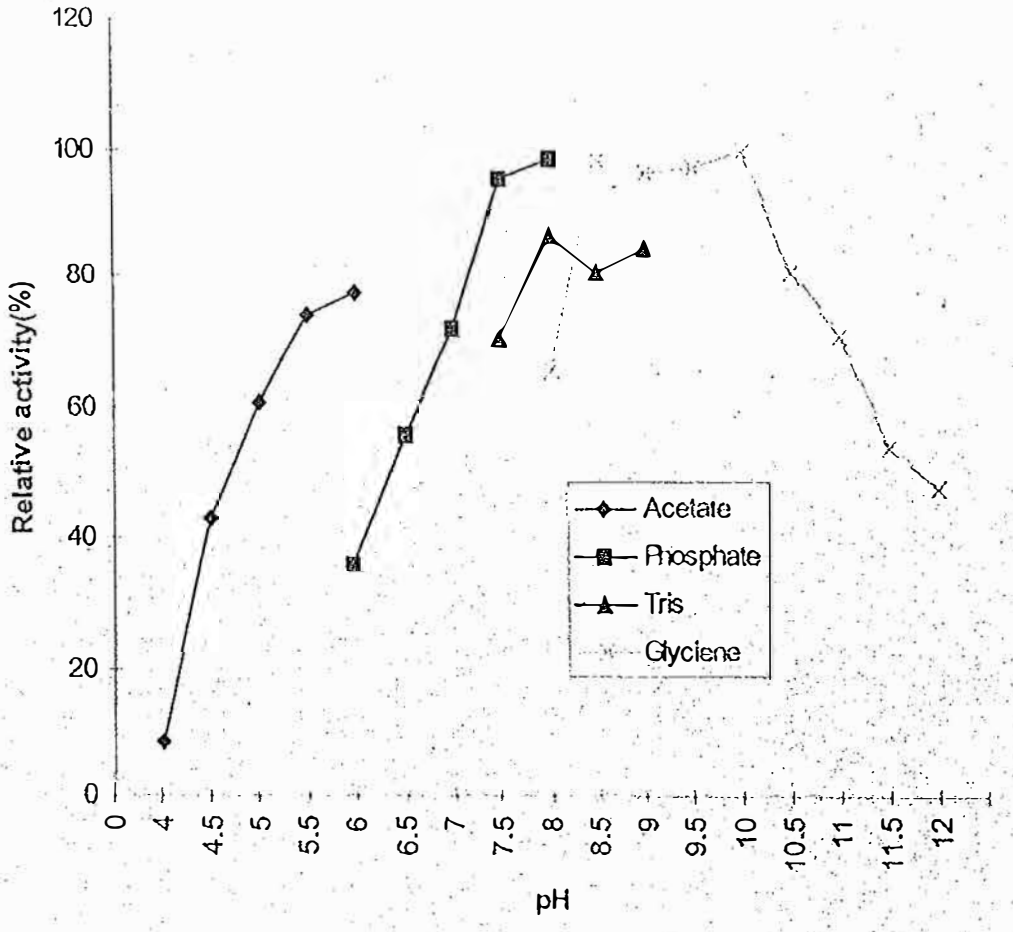


Fig.5 The pH stability of CH-W_1 protease incubated at 50^0C for 30 min. in buffers - Acetate (pH 4-6) phosphate (pH 6-8) Tris/HCl (pH 7.5-9)Glycine /NaOH (pH 8-12)

8. Effect of cations on enzyme's activity

Effects of different metal ions on protease activity were tested using assay mixture containing 5 mM of different ions. At 5 mM concentration $CuSO_4$ was slightly stimulatory where as the rest of the metalic ions had no significant effect on activity of the CH-W1 protease (Table 1).

Table 1- Effect of different metal ions on activity of the CH-W1 protease in concentration of 5 mM of each cations.

Ions	Activity (%)
None	100
$CaCl_2$	59.4
$BaCl_2$	88.9
$ZnCl_2$	89.9
$CoCl_2$	83.4
$MgCl_2$	81.5
$MnCl_2$	72.06
$FeCl_3$	75.9
$CuSO_4$	107.5
NaCl	72.8
KCl	84.3

9. Effect of inhibitors on protease activity

The effect of inhibitors was tested by incubating the enzyme with different inhibitors at 30^0C for 1 h 10 mM EDTA and 1mM PMSF were found to inhibit activity while addition of other inhibitors had little or no effect (Table 2) .

Table 2- Effect of Different inhibitors on CH-W_1 protease

Inhibitor	Concentration (mM)	Residual activity (%)
None	—	100
EDTA	10	48
Iodo acetamide	10	93.6
PMSF	1	30.4
P.CMBA	10	79.2
1:10 Phenanthroline	10	93.2

10.Natural substrate degradation

The protease from the isolate was capable of degrading feather and Nug (Table 3)

Table 3. Activity of protease of the CH-W1 on natural substrate.

Substrate	E. Assay	E. Blank	Net	% activity
Casein	1.506	0.074	1.432	100
Feather	1.157	0.105	1. 052	73.4
Nug meal	1.369	0.070	1.299	90.2

IV. Discussion

The isolate was aerobic, Gram variable, terminal endospore forming, catalase positive motile, and rod-shaped bacterium. On the basis of these properties it was classified as a strain of the genus *Bacillus* (Claus and Berkely, 1986). The isolate was grown at a pH range of 7 to 11. This suggests that it is alkaliphilic *Bacillus*. This range was similar to other *Bacillus* species that has been isolated which is from pH 5-10.5 (Priest, 1977).

The optimum rate of growth in nutrient broth occurred at temperature near 40^0C. No growth occurred at temperature in excess of 45^0c. This implies that the isolate is mesophjilic.

The growth study of this organism has shown that it entered to stationary phase around 42 h (Fig 1). This indicate that *Bacillus* sp. CH-W_1 is a slow growing organisms. There is no measurable decline in the OD of the culture even after 48 h. This discrepancy may be due to i. Accumulation of spores, which can diffract light and possibly increase OD reading. ii. Evaporation of liquid component of the culture and / or iii. Interference of dead cells in the OD measurement.

The pH of the culture dropped from 10.95 to 8.93 within 12 h (Fig 1). This drop in pH might be due to formation of acids like formic acid at the early stage of

fermentation. The pH rise after 18 h may be accounted to ammonification and cell autolysis as described by Mao, *et al.* (1992) and Gabriele *et al.* (1991).

There was little formation of protease during the earlier exponential phase. A rapid increase in protease activity began at the middle stage of exponential growth to stationary phase. Protease production by the isolate indicates low rate of secretion during active growth followed by increased synthesis in the late exponential and stationary phases of growth (Fig. 1). Schaeffer (1969) has reported the maximum synthesis of extracellular enzymes normally to occur before sporulation in the late exponential and early stationary phase of growth. Similar observations were also reported for other strains of *Bacillus* (Mao. *et al,* 1992; Atalo and Gashe, 1993; Macfarlane and Macfarlane 1992; Amare, 1997).

The production of extracellular protease by exponential phase cultures indicate that the enzymes have an important nutritional function, in making peptides and amino acids available for growth (Priest, 1977; Macfarlane and Macfarlane, 1992). On the other hand the production of extracellular protease during the stationary pHase is correlated with the onset of a high rate of protein turnover(Mandelstam, 1960; Munro, 1961) and of sporulation in certain bacilli (Bernlohr, 1964; Levisonhn and Aronson, 1967). During sporulation in the absence of exogenous nutrients, it is assumed that the turnover of vegetative cell proteins provides sufficient quantities of the free amino acids for the biosyntheses of spore proteins (Bernlohr, 1967).

The regulation of exoprotein synthesis in the genus *Bacillus* has been extensively studied (Ulrich *et al.*, 1991; Gabriele *et al.*, 1991; Bernlohr, 1964). Although the exact molecular mechanisms of regulation have not yet been clarified, it has been shown that the biosynthesis of alkaline serine protease is dependent on the growth rate of the culture and concentration of nitrogen and carbon sources in the medium (McConn *et al.*, 1964; Doi, 1972; Heineken and O'connor, 1972; Hanlon and Hodges, 1982; Frankena *et al.*, 1986; Ulrich *et al.*, 1991 Gabriele, 1994;).

The temperature optimum (55^0C) for the activity of CH-W_1 protease (Fig 2) is comparable to protease from *Bacillus thermoruber* (Manachini *et al.* 1988) , *Bacillus licheniformis* (Keay *et al.* 1970; Zudiweg *et al.* 1972). Atalo and Gashe (1993) and Amare (1997) have described protease from *Bacillus* sp. P001A and AR 009, isolated from Wendo-Genet hot spring and Lake Arenguade respectively, producing maximum activity at 55^0C.

At present, there is considerable interest, in the identification of alkaline protease, which are effective as detergent enzymes when used at lower rather than at elevated temperature. This is essentially due to the energy cost involved in heating the water for washing, In developing countries like India, detergents are commonly used at room temperature (Sanigata, *et al.* 1993). Another deriving force for low temperature washing is the development of synthetic and semi- synthetic fabrics that need to be washed only at low temperature (Kalisz, 1988; Grant and Horikoshli,

1992). Hence, the protease of CH-W_1could be promising for this application in spite of its being less heat stable

The observed effect of temperature on the enzyme activity might be related to the disturbance of the molecular structure (conformation) of the protein, which resulted to loss or decreased biological activity (Veronse, *et al,* 1974).

The strain CH-W_1 has a half- life of 30 min at 60^0C without calcium and half-life of 1 hr at 60^0C in the presence of Ca^{++}. The half-life of the isolate was greater than that of *Bacillus subtilis* reported by Cowan and co-workers (1985), which was 10 min at 60^0C. But the current protease resembles protease from *B. lichineformis* (half-life of 50 min at 65^0C) (Keay *et al,* 1970; Zudiweg *et al*, 1972).

The stabilization of proteins against thermal denaturation is generally conferred by small changes in the aminoacid sequences. Such changes can affect stability, without any obvious structural alternation, by giving rise to the relatively small number of additional intramolecular interactions. In proteases specific binding of metal ions (particularly of calcium) further enhances molecular stability (Cowan, *etal*, 1985).

Calcium was found to be important for enzyme stability. The requirement of the protease for Ca^{++} was reflected in a decrease in activity, when the chelating agent 10 mM EDTA was used (Fig 3). Many studies indicate that calcium ions are required to keep the enzyme in a conformation necessary for its catalytic functions.

The elevation of optimum temperature and heat stability in the presence of Ca^{++} is a general aspect of alkaline proteases (Horikoshi, 1971; Kelly and Fogarly, 1976; Strongin *et al.*, 1979; Manachine, 1988; Takami, *et al.*, 1989;).

The protease of the isolate has optimum pH of 9 and there is rapid decline above and below this pH value (Fig. 4). The pH profile of the enzyme showed the presence of alkaline protease. The alkaline protease of *B. subtilis* showed optimum activity between pH 8-10, while the neutral protease showed a single peak around pH-7 (Millet, 1970). The pH range of activity of the isolate proteases (4-12) coincides with other alkaline protease. The pH range for alkaline protease is said to fall between these ranges (Cowan and Daniel, 1982).

From the alkaline protease, the protease of CHW1 resembled subtilisin A_1 *of B. licheniformis* (Kacy *et al,* 1970; Zudiweg *et al,* 1972), proteases of *B.thuringienisis* (Kuniatale, *et al.,* 1989) and *B. thermoruber* (Manachini *et al.,* 1988.) All have an optimum pH value of 9.0.

The pH profile of the isolate's protease was fairly broad. This broad profile may reflect the conformational change of the substrate at high pH (Taguchi, *et al.,* 1983). Extreme pH tends to throw a high net charge on the protein, that will lead to repulsion between various parts of the molecule and might easily lead to expansion (unfolding) (Levisohn and Aronson, 1967).

The pH of the assay condition and the buffers used seem to have influenced the activities of the enzymes (Hwang and Hseu, 1980) . The buffers might have hindered the reaction products for the reasons like insolubility of substrates, unfavorable thermodynamic equilibrium, difficult product recovery, etc (McConn *et al.,* 1964;Barros, *et al.*, 1992). A different result could have been observed if organic solvent and purified enzyme were used (Janssen, *et al,* 1991).

The protease from the isolate maintained more than 80% of its peak activity in the pH range of 7.5-11.0 after incubation for 30 min at 50^0C (Fig 5). This implies that the study organism appears to be alkaliphilic.

According to McDonaled and Chambers (1966) protease from alkaliphiles are generally stable in the high alkaline regions, but they are not very stable to heat. Almost 100% of the enzyme's original activity were retained in the pH range 8-10. Amare (1997) has also described a protease from AR 009 showing good stability and activity in this range. The protease produced by different *Bacillus* species, vary not only in type but also in the pH and temperature necessary for optimal activity (Priest, 1977). The ambient pH and temperature for optimal activity and stability of our isolate is as comparable to the subtilisin A_1 of *B. licheniformis*, which had the optimum pH of 9 and temperature of 60^0C (Keay, *et al*, 1970, Zudiweg *et al*, 1972). It has half-life of 50 min at 65^0C.

With a very few exceptions, the extracellular proteases of the genus *Bacillus* are either serine or metal enzymes (Priest, 1977). The protease of CH-W_1 is a serine protease as its activity is strongly inhibited by 1 mM PMSF (Morihara, 1974, 1992). As with most proteases the stability is reduced in the absence of Ca^{++}, which probably explains the slight inhibition, observed in the presence of 10 mM EDTA. The concentrations of inhibitors used were in excess of the amount usually used (Table 2). *Bacillus*-derived alkaline proteases reported so far belong to the class of serine protease (Horikoshi and Akiba, 1982, Kalisz, 1988). Generally, the serine protease are not inhibited by metal chelating agents: however there are examples of serine protease that are affected by EDTA (Kato, *et al.*. 1974;Gnosspetius, 1978; Strongin *et al.*, 1979; Izstova *et al.*, 1983).

The metal ions tested so far did not greatly affect the enzyme activity (Table 1). However Cu^{++} slightly enhanced the activity, whereas Ca^{++} at the same concentration showed a less positive effect. Berhlohr (1964) reported lack of inhibition of gross proteolysis by *Bacillus licheniformis* protease with Cu^{++}, Hg^{++} and Zn^{++} ions. This data suggest that the enzyme does not require metal co-factors and that it does not contain an essential sulfhydryl group. At the assay temperature (50^0C) addition of 5 mM Ca^{++} had no effect on protease activity i.e. the enzyme does not require Ca^{++} for activation. This property is advantageous to be used as the detergent enzyme, since it would not required Ca^{++} for activation, which may precipitate by sequestering agents of the detergent additives.

Larger scale commercial producers use such cheaper natural nutrient sources which usually have high C: N ratios. This may be especially important in developing countries where the cost of the enzyme could be a factor limiting the industrial application of alkaline proteases.

The isolate grows well on feather and Nug meal as the primary organic substrate for supplying carbon, nitrogen and energy. About 30-40% of the production cost of many industrial enzymes is estimated to be accounted for by the cost of the growth substrate (Hinman, 1994). Since feather and Nug meals; are cheap and readily available substrates, their use for the production of alkaline protease may substantially lower the production cost of the enzyme. In Ethiopia Nug seed production is estimated to be over 45,000 tons (Central Statistics Authority, 1995). Most of the produce is used locally for oil extraction while the left over could serve as a substrate for the growth of such organisms.

The protease produced by CH-W_1 showed properties that make it suitable for a number of applications such as in leather tanning, and detergent additive for low temperature washing. On the other, hand the ability of the isolate to produce the enzyme on a low cost substrates, Nug meal and feather could greatly reduce the production cost of the enzyme.

V. References

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