Production of Proteases by Alkalophilic Bacteria From A Hot Spring At Wondo Genet

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Atalo Kassa
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ABSTRACT:

Two organisms with high proteolytic activity on casein, gelatin or haemoglobin were selected out of the bacterial isolates obtained from a hot spring at Wondo Genet. The test organisms were Gram positive, rod-shaped, spore-forming with chains of two or more cells, motile bacteria. Based on the biochemical and morphological tests, it has been confirmed that they belong to the genus Bacillus. Growth of the two isolates was observed in the pH and temperature ranges of between 5-10, and 30-55°C, respectively. Their optimum pH and temperature for growth were 10 and 55°C. Glucose 0.5% and peptone at 0.5%, 0.4%, respectively resulted better yield of biomass and protease production. 0.5mM Ca^{+2}, 0.1mM Mg^{+2}, and 1mM $PO4^{-4}$ increased biomass and enzyme production of both isolates. High enzyme production was observed in the presence of 1mM Cu^{+2} and 5mM Mn^{+2} for Bacillus sp-A, and 0.1mM Zn^{+2} and 5mM Mn^{+2} for Bacillus sp-B. The highest enzyme activity of both isolates was observed when assayed at 55°C and pH 9.5. The protease of Baccilus sp-A showed higher hydrolytic activity on casein than on gelatin or haemoglobin. The protease of Bacillus sp-B have been found to hydrolyse only gelatin. However, both isolates failed to hydrolyse egg - albumin. The two organisms were more efficient in degrading skin when supplied in the growth medium than feather, horn or hair. The least weight lose was recorded on hair. Bacillus sp-A showed

high degrading activity on feather than Bacillus sp-B. On the other hand Bacillus sp-B showed high degrading activity on horn than Bacillus sp-A. The protease of Bacillus sp-A was thermally stable between 60-70°C, with a half life of 50 min at 60°C and 65°C, and 40 min at 70°C. The protease of Bacillus sp-B was thermally stable at 60 and 65°C, and the half life was 50 min at these temperatures. However, the enzyme activity of both isolates ceased after 60 min of incubation at the temperatures examined.

PART I

INTRODUCTION

The synthesis and secretion of extracellular proteases by bacteria has long been of interest to scientists in various disciplines. The physician, the food, pharmaceutical, and textile technologists, the microbiologist, and biochemists have all displayed interest in this area; albeit for different reasons.

Microbial proteases have been utilized in varied facets of industries over the years, but it was only recently that attention was diverted to industrial production from thermophilic microorganisms. The findings of Baross _et al_., (1982) that bacterial communities sampled from super-heated, deep sea waters (temperature of up to 330°C) could form methane at 100°C (and grow under high pressure up to 300°C) overthrew the previous notions about the growth temperature range of bacteria. At present biotechnologists are trying to isolate thermophilic microorganisms for industrial production from extreme environments.

The major attractive attribute of thermophiles for the microbial technology is that, they produce enzymes that are heat stable themselves and many of them do not require cofactors for stabilization (Tanaka _et al_., 1981). Because their growth temperature is generally much higher than that of mesophiles, industrial processes can take place with less fermenter contamination and low cooling costs (Johri et al., 1985; Taguchi et al., 1983).

Although quantitative data are not available, in Ethiopia proteases are crucially required in many industries, like dairy, leather, etc. Therefore the need for proteolytic enzymes from thermophilic organisms inhabiting hot spring was responsible for the initiation of this research project. The objectives of this thesis project were:

a. to isolate and characterize protease producing thermophilic and alkalophilic bacteria from hot spring;

b. to optimize cultivation conditions for maximum growth and enzyme production;

c. to asses the potential application of these microorganisms and their enzymes to convert waste like horn, feather, hair and skin.

PART II

LITERATURE REVIEW

2.1 PROTEASE(S)

Protease is a frequently used synonym for the large class of enzymes that hydrolyse the peptide links of proteins, and peptides known as proteolytic enzymes or proteinases (Haurowitz, 1963).

The action of proteases on proteins occur by addition of water in the peptide linkage, resulting in protein hydrolysis, i.e. the disruption of the molecule into diffusible fractions. It is fascinating to note how bacterial cells synthesize and secrete often vast amount of potentially lethal enzymes (Swoden et al. 1989).

2.1.1 Importance.

Proteases are conventionally the most important group of industrial enzymes and form the higher portion of world-wide enzyme sales and production (about 530 tons annually) (Eveleigh, 1981). As a single class, proteolytic enzymes constitute about 60% (US $236 million) and 67% of the total industrial enzyme market in 1981 and 1989, respectively (Thomas and Kenealy, 1989). For instance in Chile proteases account for more than 70% of the total industrial enzyme sale (Gonzalez et al., 1992).

Microbial proteases are considerably important enzymes for practical uses in industries.

2.1.1.1 **Dairy industry:** In dairy industry they are used to produce cheeses of different flavour, firmness and drainage. In cheese manufacturing process the proteolytic system of the starter bacteria hydrolyses casein and produces amino acid or/and peptides which result in the development of flavour during ripening (Hugenholtz et al.,1987).

2.1.1.2 **Leather factories:** In leather factories alkaline proteases are effectively utilized in the soaking, dehairing, dewooling process to produce bright or clean hides and skin that will dye more or less even (Dalev and Simenova, 1992).

2.1.1.3 **In biological insect control:** the degenerative changes they bring about in insect integuments have made them to be candidates for commercialized microbial insecticide (Hockenhull, 1971; Schimidt et al., 1988).

2.1.1.4 **Brewing industries:** in brewing process, proteinases degrade haze-forming proteins during the course of fermentation, hence would be of considerable economic value in chill-proofing beers (Bilinski et al., 1986).

2.1.1.5 **Detergent industries:** in detergent industries proteases are added to remove binder proteins that fix other soil components to fabric and make them difficult to remove the stain from fabrics (Eveleigh, 1983).

2.1.1.6 **Food processing industries:** in these industries the value and availability of proteins is raised through enzymatic modification of proteases (Boven et al., 1988).

2.1.1.7 **Feed processing:** ranchers and farmers have come to realize much of the gross significance of protein feeding and the contribution made by the proteases in the preparation of animal feeds. The complete dissolution of the feather keratin (which accounts for 90% of the feather material, (Dalev, 1990), by thermophilic organisms from the feather waste generated in large quantities as a by-product of commercial poultry is currently on trial to be used as dietary protein supplement for animal feed stuff. The feather concentrate significantly exceeds that of cereal fodder (Williams et al., 1990) on the other hand the degradative activity of proteolytic enzymes is known to affect greatly the availability and how efficiently a ruminant animal is able to use dietary protein (Wallace et al., 1987).

In some countries extracellular protease producing thermophiles are used in the disposal of sewage to digest incompletely oxidized foul smelling compounds to eliminate offensive odours (Sale, 1973). This method of treatment is more preferable because it increases reaction rate and decreases retention time. Besides it will help to destroy pathogenic microbes and lowers the viscosity of the waste. (Burt et al., 1990).

The intrinsic stability of proteases renders almost all of them to have some potential applications in industry. Even though proteases have such a wide application their commercialization depends on several attributes. An 'effective' industrial protease must be easily recoverable preferably extracellularly, and must be produced in large quantity by the microorganisms with low cultivation costs. However the possible advantages of proteases are heavily outweighed by the necessary development cost (Wallace et al., 1987). At present Alcalase /alkaline protease-A), which is produced by a strain of B. licheniformis appears to be a widely used protease as additive in detergents (Chen et al., 1991).

Thermostable proteases are the most preferred proteases for many biotechnological applications.They are preferred for various reasons. The major attribute of these enzymes is that the reaction carried out at high temperature improve the mass transfer rate, lower the viscosity, and minimize the risk of contamination. They are most resistant to most chemical and physical denaturants (Taguchi et al., 1983). In general their selection mostly depends on process constraints and/or cost effectiveness.

Thermophilic bacteria which inhabit thermal environments are often good producers of thermostable industrial proteases, such as thermolysin from B. thermoproteolyticus; aqualysin and coldolysin from Thermus aquaticus; subtilisin from B.subtilis (Khoo et al., 1984). The most thermostable protease so far reported is archealysin, an extracellular serine protease producded by Desulfurococcus sp, with a reported half-life of 75-90min at 95°C, its optimum temperature is 98°C (Blumentals et al., 1990).

2.1.2 TYPES OF MICROBIAL PROTEASES:

Microbial proteases are grouped in various ways, but for purpose of convenience, in this review they are treated into two large groups, namely intracellular and extracellular proteases.

2.1.2.1 Intracellular proteases:

These proteases are implicated to be used in protein elimination or transformation. They might be especially important for the organism which undergo complex changes in physiology and morphology leading to formation of dormant spores under nutrient deprivation (Strongin et al., 1978).

Schaefer (1969) and Piggot and Coole (1976), assumed that, interacellular enzymes play a direct role in degrading some inhibitors of sporulation. Glen (1976), hypothesized that, the enzyme may cleave the beta-subunit of RNA polymerase to produce the enzyme responsible for sporulation.

2.1.2.2 Extracellular Proteases.

An extracellular protease is the one which exists in the medium around the cell, having originated from the cell without alteration to the cell structure greater than the maximum cmpatible with the cell's normal processes of growth and reproduction (Polloc and Richmond, 1962). In the main Gram positive organisms produce true extracellular proteases, and Gram negative bacteria do not (Gleen, 1976).

The mechanism of protease production involves a signal transduction pathway, which informs the cell about its nutritional status and thus influences exoprotein production (Levisohn and Aronson, 1976).

The excretion of proteases (proteins) especially during nutrient limited occasion would not seem beneficial for microorganisms. But the formation and release of exoproteinases seem to be an efficient substrate-scavenging tactic over a large area during growth and non-growth conditions. These microbial proteases are the ones which are used in industries. Hence it is worthwhile to discuss the most common features of these enzymes.

2.1.2.3 Site of Synthesis:

The exact mechanisms responsible for cellular control of protease synthesis are unknown. Moreover the site of synthesis have been a point of dispute among many scholars.

Glen (1976) traces the cytoplasmic origin of extracellular proteinases. Nevertheless his hypothesis fails to explain why cells that are actively producing proteases do show either a total absence or trace amounts of exocellular proteases and some times mutants have elevated levels of intracellular proteases.

Based on the presence of small portion of protease on the cell membrane, Polloc and Richmond (1962), forwarded the membrane associated synthesis theory. However their argument about the problem of spatial separation on exoproteins mRNA transcription and translation is extremely feeble and can not be proved experimentally. As can be seen neither of the above assumptions produce plausible evidence about the origin of extracellular proteases. Thus one can temporarily understand the presence of multiple site of protease production.

Speculations about excretion of these enzymes are based on the structural flexibility of proteases and on active pumping mechanisms. Most extracellular proteases so far reported from bacteria contain few or no cysteine, hence have low or no disulphide linkage (Johri et al., 1985). A protein molecule lacking this bond would have more than the usual degree of flexibility and will be capable of more easily unfolding and refolding than proteins which contain S-Sbond. Possibly these bacterial enzymes might pass more freely through a rigid cell-wall mesh work by evolving a degree of flexibility through elimination of disulphide bonds (Kealy et al., 1970).

2.1.2.4 Classification.

Extracellular proteases differ in such characteristics like, thermal stability, substrate specificity, sensitivity to inhibitors, nature of active site, complete amino acid sequence, etc (Gleen, 1976). Extracellular proteases are classified in various ways. Johri et al. (1985), have classified microbial proteases as follows:

Table 1

Classification of proteases according to Johri _et al_., (1985)

Name	Source	pH Range Max. activity	 Max. stability
Acid	Mainly fungi	2 - 5	2 - 6
Neutral	Fungi and bacteria	7 - 8	7 - 9
Alkaline	Fungi and bacteria	9 - 10	5 - 11

The scheme for differentiating between groups of proteases based on pH activity profiles, amino acid composition, esterase/protease activity ratios and immunological cross reactions appear to be most satisfactory (Kealy _et al_, 1970). However at present there is no further differentiation possible to show whether or not the enzymes with in each group are identical.

2.1.2.5 Factors Affecting Protease Activity and Production

Enzymes posses demonstrable biological activity, but an array of physical and chemical factors interferes in their function by affecting their water solubility and exposure of reactive groups.

(i) pH

Factors like pH and temperature initiate gross changes in proteins often referred to as denaturation which results in opening up of a highly folded peptide chain in which the reactive groups would be entirely exposed (McGilvery and Goldstein, 1983).

The rate of enzymatic reactions has a range of pH of maximum activity which may be narrow or broad depending on the enzyme. The actual value of optimal pH is usually near the pH at which the enzyme function in nature and to some extent on the nature of the substrate (Albertson et al., 1990). Extremes of pH tend 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 of the structure) (Levisohn and Aronson, 1967).

(ii) Temperature

The rate of most chemical reactions increases by factor of two or three for a rise in temperature of 10°, but the rate of denaturation may increase by as much as 600 fold (Fox and Foster, 1957). So marked is the effect of temperature on the growth, enzyme production and enzyme activity of organisms.

Proteases are extracted from a number of thermophilic bacteria inhabiting geothermic terrestrial and marine environments. Among the many interesting features associated with these bacteria and their enzymes are their ability to grow and carry out biological function at normally denaturing temperature.

The combined effect of hydrogen bonding, ionic interactions, metal binding and disulphide bridges are the main thermal stabilizing forces of enzymes (Taguchi et al., 1983). Nevertheless the extent of these interaction is greatly affected by purity of the sample, pH and the ionic species found in the solutions of the protease (Kealy, et al., 1970). But Brock (1985), considered thermostability an inherent function of the structure of macromolecules and is not due to the presence of stabilizing substances.

(iii) Other factors

The action and synthesis of proteases is influenced by chemical factors such as, addition of an exogenous carbon and nitrogen sources, by catabolic products, substrates, ionic concentration etc. via the mechanisms of induction, repression and catabolic repression (Hisano et al., 1989). For instance in the presence of glutamate and alpha-ketoglutarate the amount of the proteinase produced per unit growth was greater in B. cereus, but succinate, malate and fumarate allowed growth but did not support proteinase formation, while the presence of maltose resulted in partial suppression (Neumark and Citrin, 1962).

In Pseudomonas maltophilia the rate of exoenzyme (protease) production was increased by several fold, when the cells were grown in minimal medium which contains only yeast extract and no additional carbon sources (Gottesman and Maurizi, 1992).

On the other hand inorganic substances like Ca, Zn, Cu, Co Mg ions have been found to have role in enhancing enzyme activity or stability (Ikegaya et al., 1992).

PART III

MATERIALS AND METHODS

3.1 SAMPLING AND ISOLATION

3.1.1 Sampling: Hot water samples were collected from Wondo Genet Hot spring (270 km to the south of Addis Ababa, between Shashemene and Awassa) at seven different sites. The pH and the temperature of the hot spring at the sampling site was 8%, and 53°C, respectively.

3.1.2 Enrichment and Isolation: Water samples (10ml) were introduced into duplicate flasks containing 100ml of enrichment media. The enrichment medium contained Nutrient broth (Oxoid, Basingstoke, England) supplemented with 1% gelatin (Oxoid). The pH was adjusted to 8 using sterile 0.1M NaOH. They were incubated in an orbital shaker Gallenkamp, UK), 180rpm for 24 hour at 55°C. A loopful of the cultures from each flask was transferred several times into nutrient agar plates supplemented with 1% gelatin (pH8) and incubated at 55°C for 48 hours until pure colonies were observed.

3.1.3 Screening for proteolytic activity: among the bacterial isolates, organisms with proteolytic activity were selected by observing a clear zone formation around

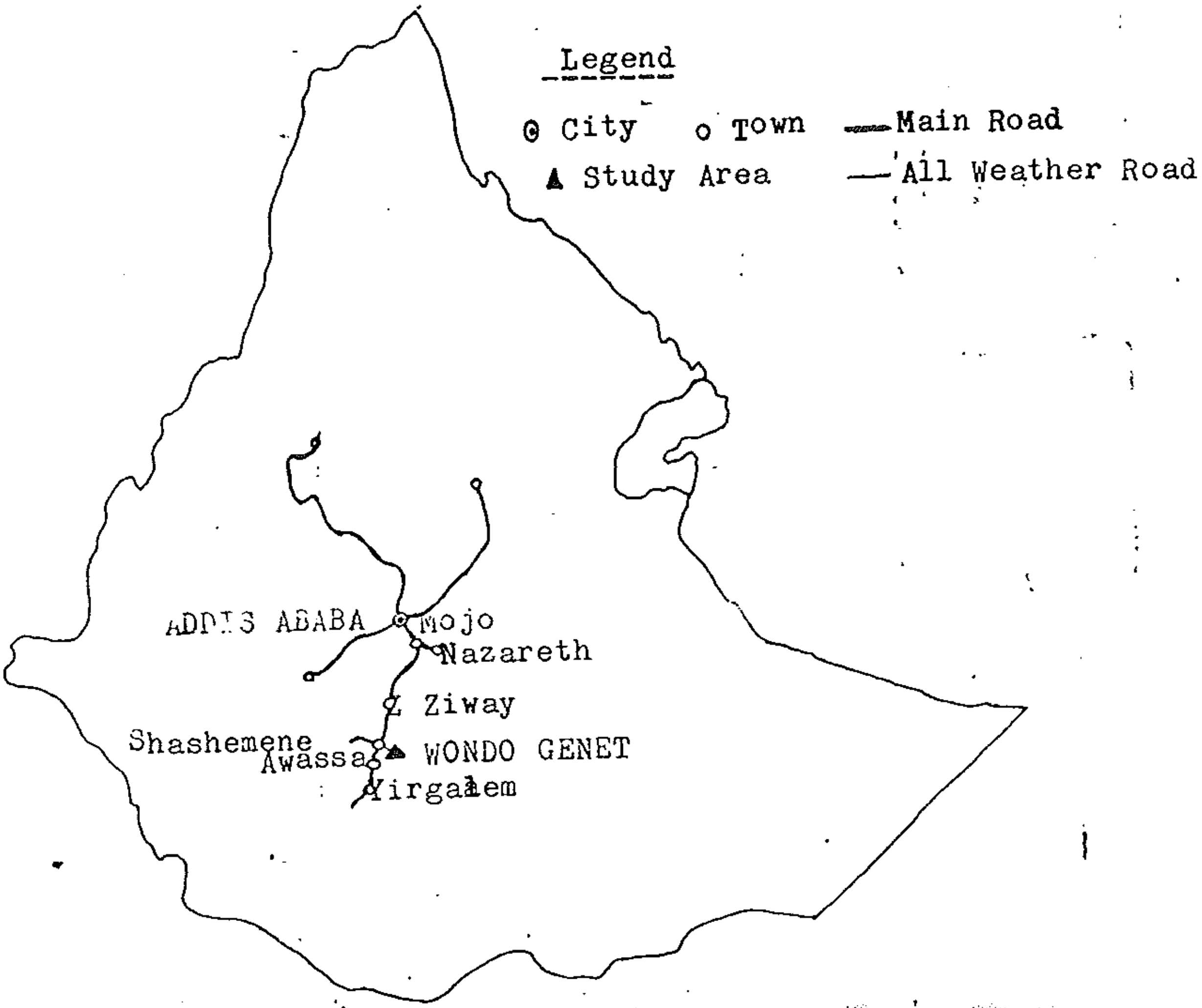

Fig-1. Location Map of the Study Area.

the colonies on nutrient agar plates containing 1% gelatin. Detection was carried out by flooding the medium with few drops of $HgCl_2$ solution (15g $HgCl_2$ and 20% HCl in 100ml of aqueous solution).

Further screening of the positive isolates was carried by comparing the ratio of their zone of hydrolysis to colony size on nutrient agar plates containing 1% of haemoglobin, casein, egg-albumin (allSIGMA, USA) or gelatin.

3.2 Media and Culture Conditions

Stock cultures of the selected organisms were maintained at room temperature on nutrient agar slants supplemented with 1% gelatin, and transferred to fresh medium every two week.

A standard inoculum was always prepared from a 24 hr culture by scraping and washing the surface of agar slant with physiological saline. The optical density of the cells of the inoculum was adjusted to give an absorbance of 0.3 at 540 nm. Following aseptic techniques one ml of the cell suspension was transferred into 100 ml of growth medium contained in 250 ml capacity erlenmeyer flasks. The flasks were incubated at 55° C in an orbital shaker (180 rpm), unless otherwise specified.

The growth medium (M) was composed of (gm/100 ml unless otherwise noted):

$K2HPO_4$	0.1	Yeast extract	0.1
$CaCl_2$	0.05	Glucose	0.1
$MgSO_4.7H_2O$	0.01	peptone	0.1

The organic and the inorganic constituents of the medium were sterilized (autoclaved) separately at 121°C and 15lb for 15min. The two components were mixed after the temperature dropped to 45-50°C. The measured medium pH was adjusted by adding known quantity of sterile 0.1M NaOH or HCl.

3.3 Identification Procedures.

Morphological: Microscopic examination of cells from broth or agar plates and morphological characteristics of colonies on solid medium were considered for characterization of the test organisms. Gram-stain character, cell shape, spore shape and location, etc. was considered in the process of morphological identification.

Biochemical and Physical Tests: all the biochemical and physical tests described in Table-1 were mainly carried out based on Bergey's Manual of Systematic Bacteriology Vol. 2 (1986).

3.4 Enzyme Source and Protease Cssay Conditions:

3.4.1 Growth of cultures and isolation of enzyme.

Enzyme preparation:

Ten ml of culture was removed and centrifuged at 7000 4pm for 15 min using clinical centrifuge. The cell-free supernatant thus obtained served as source of crude enzyme.

3.4.2 Protease assay method:

The Remerdes and Klostermeyer (1976) method was used with some modifications. Briefly, One ml of properly diluted enzyme solution was mixed with 2.5ml of 0.5% substrate solution in 0.1M borate buffer, pH 9 (unless otherwise noted). This was incubated at 55°C for 30 min with shaking (180 rpm). At the end of incubation period the reaction was terminated by adding 2.5ml of 10% Tri-Chloroacetic acid. After it was allowed to stand at room temperature for 10 min, it was then centrifuged at 6500 rpm for 5min.

3.4.3 Estimation of TCA soluble amino acids:-

The ninhydrin method (Reimerdes and Klostermeyer, 1976) with some modifications was used to estimate the soluble amino acids. One ml aliquot from the clear supernatant was mixed with 1ml of ninhydrin solution in test tubes and immersed in boiling water bath for 10 min. After adding 2.5 ml of distilled water the absorbance or intensity of colour was measured spectrophotometrically (Spectronic-21, Bauch and Lomb) at 570 nm.

One unit of protease activity (U) was expressed as ug of amino acids released/ml of enzyme solution/minute under the stated assay condition. A standard curve was constructed using aspartic acid.

3.5 Studies on Conditions for growth and enzyme production

3.5.1 The effect of pH on growth and enzyme production

The optimum pH for growth and enzyme production was determined by adjusting the pH values of the growth medium at intervals of 0.5 from 4.5-10.5 pH.

Biomass was estimated by measuring the absorbance or turbidity of the growth medium at 540 nm, after a specified incubation period. Enzyme production in the same culture was measured from the cell free supernatant by enzyme assay procedure.

3.5.2 The influence of glucose and peptone concentrations on growth and protease production

In order to determine the optimum glucose and peptone concentrations for growth and enzyme production, their concentration was varied in the basal medium (0.1, 0.3, 0.5 and 1% for glucose and 0-4% for peptone) one at a time. The pH of the basal medium was adjusted to 10 and growth was allowed for 36hr at 55°C before measuring the biomass and protease production. Both biomass and protease production was determined by the method described above (3.5.1).

3.5.3 The Effect of Temperature on Growth and Protease Production.

The effect of temperature on the growth and protease production was determined by incubating cultures at 25-60°C. The growth and enzyme production of the cultures was measured by the method describe in 3.5.1 above.

3.5.4 The Effect of Detergents.

Sodium lauryl sulphate, Triton X-100, polyoxyethylene sorbital, Tirgitol-7 (all purchased from SIGMA, USA) and Tween-80 (BDH, Poole, England) were used at 0.05, 0.1, 0.2 and 0.3% concentrations in the growth medium. Growth and enzyme production was determined after 36hr of incubation at 55°C.

3.5.5 The Effect of Ions on Protease Production

A volume of 0.1 ,1, or 5mM of the ionic compounds described in Table-5 was introduced separately to the growth medium after sterilization. The growth of cells and protease production in each flask containing a particular ionic species of defined concentration was measured at the end of incubation period (36 hour), at 55°C.

3.6 Study on Conditions for Enzyme Activity.

3.6.1 The Effect of Assay Temperature on Protease Activity

The clear supernatant harvested after 36 hr of incubation at 55°C was mixed with the substrate solution and left to react for 30min in an orbital shaker (180 rpm) at different temperature (between 30-70°C). At the end of incubation aliquots were removed from the reaction mixture which was incubated at specified temperature and enzyme activity was measured spectrophotometrically (570nm), after mixing and boiling with 1ml of ninhydrin solution.

3.6.2 The Effect of Assay pH on Enzyme Activity

The test organisms were allowed to grow for 36 hour in the basal medium (pH 10) at 55°C. The proteolytic activity of the protease harvested from the cultures was assayed by mixing with the substrate which is dissolved in buffers between pH 4 and 11.5. The buffers used were citric acid - Na_2HPO4 buffer solution (pH 4-5), citric acid - sodium citrate buffer solution (pH 5.5 and 6.5),Tris-HCl buffer (pH 7-8.5),borate buffer solution (pH 9-10.5) and phosphate buffer (pH 11 and 11.5).

3.6.3 The Study of Protease Activity on Different Substrates

Supernatant obtained after growing cells for 36hr was used for determination of enzyme activity on 0.5% egg albumin, casein, haemoglobin (all from SIGMA, USA) and gelatine (Oxoid, England).

Assay was carried out by the standard assay procedure (3.5.1).

3.6.4 The Study of Growth Using Feather, Hair, Skin and Horn

To examine the potential uses of the organisms and /or their enzymes, 1gm dry weight of hair, skin, feather (all were cut to small pieces) or scraps of horn was introduced to separate flasks of the basal medium, and autoclaved at 121°C and 15 lb for 15 min. After adjusting the final pH of the medium to 10, a standard inoculum of cells was inoculated to these flasks and was incubated at 55°C. Observations were made twice a day on the dissolution of the materials in each flask. When the natural substrate in most flasks was dissolved the content of each flask was filtered out using a filter paper (Whatman No.1). After rinsing for several times using distilled water it was oven dried at 80°C. The percent of weight loss was calculated by considering the changes which occurred on the material in the controls.

3.6.5 Tests of Thermal Stability

The thermal stability of the protease was carried out by placing undiluted enzyme solution in water bath at 65-70°C. At time intervals aliquots were withdrawn and the residual activity was assayed under the optimal conditions.

PART IV

RESULTS

4.1 Screening for Proteolytic Activity

Among the bacterial isolates from samples of the hot spring 57 organisms (out of 150 isolates) showed proteolytic activities. Two organisms with relatively high zone of hydrolysis to colony size on nutrient agar supplemented with 1% gelatine, haemoglobin or casein were selected for further studies (Table-2).

Table 2

Zone of hydrolysis to colony size ratio on different substrates.

	Zone of hydrolysis:colony size	
Substrate	*Bacillus* sp-A	*Bacillus* sp-B
Casein	2.1	2.0
Gelatin	1.9	2.3
Haemoglobin	1.4	1.2
Egg albumin	-	-

4.2 Identification and Characterization of Isolates

4.2.1 Morphological Studies

Microscopic examination of samples from cultures of the two isolates at different levels of growth revealed the presence of Gm rods, forming chains of two or more cells, and were found to be spore forming and motile. The spores of both strains were ellipsoidal and centrally located. The sporangia of Sp-B was swollen and spore formation occurred within 18 hour of growth. But, for that of Sp-A, spore formation was observed after 30 hr of growth and the cells were not distended.

When grown on nutrient agar plates, both isolates produced creamy colonies. Species -A differed from Sp-B by the presence of hairy like outgrowth or extensions from the colonies. In nutrient broth they produced dull, wrinkled, tough pellicles with little turbidity.

On glucose agar plates both species produced waxy colonies having brown to yellow colour at the bottom. Species-A showed abundant growth with whip like projections. Whereas Sp-B produced waxy colonies with glistening droplets and growth was not abundant.

4.2.2 Biochemical and Physical Tests

The biochemical tests (acid production from sugars and polyhydroxy alcohols, indole formation, utilization of certain compounds like glycerol, etc.) together with the morphological studies indicated that the two isolates differ in some characteristics (Table-3).

Based on the results obtained from the physical and biochemical tests (Table-3) the two isolates appeared to show close similarities to two *Bacillus* species out of the species listed in Bergey's Manual of Systematic Bacteriology Vol-2 (1986).

Except for the difference in acid production from arabinose, sp-A resembled *B.licheniformis* more than any of the species described in the manual (Claus and Berkeley, 1986). Nevertheless, tests of acid production from galactose, maltose, mannose and fructose for *B.licheniformis* was stated as undetermined in the manual. Hence it was difficult to say they were the same species. Species-B resembled *B.cereus* in most characteristics including the *bility to use glycerol. However results for propionate utilization, dihydroxy acetone formation, growth at 2-10% NaCl for *B.cereus* were not available in the manual. Therefore there characteristics were not used for classification of sp-B.

The morphological, biochemical and physico-chemical tests did not help to identify the 2 isolates to a species level. Hence Sp-A and Sp-B were tentatively named as *Bacillus* sp-A and *Bacillus* sp-B, respectively.

Table 3

Biochemical and Physical characteristics of the isolates.

Biochemical tests. Test (Description)	Species A	Vspecies-b	Remark
Catalase	+	+	
Starch hydrolysis	+	+	
Casein hydrolysis	+	+	
Gelatin liquidification	+	+	
Gas production in glucose broth	-	-	
Gas production in lactose broth	-	-	
Gas production in glucose in NH_4SO_4 broth	-	-	
Final pH of glucose broth	5.23	5.53	with NH_4HPO_4 5.23, 5.2
Final pH lactose broth	8.39	8.56	
Final pH of Arabinose broth	8.71	8.71	
Gas production in Arabinose broth	-	-	
Acid production from glucose	+	+	
Acid production from lactose	+	-	
Acid production from xylose	+	+	
Acid production from arabinose	-	-	
Acid production from maltose	+	+	
Acid production from Galactose	+	-	
Acid production from sucrose	+	+	
Acid production from Fructose	+	+	
Acid production from Raffinose	+	+	

Acid production from Manose	+	+
Acid production on Mannitol salt agar	+	+
Citrate utilization	+	+
Propionate utilization	+	+
Nitrate reduction	+	+
Methy red test	+	+
VP test	+	+
Final pH of MRVP medium (5th day)	6.62	6.34
Final pH of MRVP medium (7th day)	7.05	7.14
Final pH of VP medium 3 day	5.27	5.23
Final pH of VP medium 5 day	5.34	5.33
Final pH of VP medium 7 day	5.37	5.38
PH of 24 hrs N.broth culture (after adjusting to 7)	8.43	8.22
pH of 24 hrs N.broth culture (without adjusting to 7)	8.74	8.78
Indole formation from tryptone	-	+(weakly)
Glycerol utilization	-	+
Acid production from mannitol	+	+
Physico-chemical tests		
Growth in 2% NaCl	+	+
Growth in 5% NaCl	+	+
Growth in 7% NaCl	+	+
Growth in 10% NaCl	+	+
Growth at pH 6.8	+	+
Growth at pH 5.7	+	+
Growth at 10°C	-	-
Growth at 25-30°C	+	+
Growth at 45°C	+	+
Growth at 55°C	+	+
Growth at 65-70°C	-	-

4.3 Biomass and protease production in nutrient broth.

Studies were carried out using nutrient broth whose PH was adjusted from 4.5-10.5. Growth of both isolates was inhibited below pH5.0 and above pH 10. Both isolates showed higher biomass production at or above neutral PH values (Fig 2a and 2b). As can be seen on Fig. 2a and 2b both isolates showed a longer lag-phase in the acidic and neutral conditions, whereas in the alkaline conditions there was a very short lag-phase. Regardless of PH, the organisms reached the stationary phase at about 30-36 hour of growth. This was followed by a decline in growth between 42 and 48 hours.

Enzyme production occurred in wide range of pH conditions (Fig. 3a and 3b). Enzyme production for *Bacillus* sp. A and *Bacillus* sp-B started after 6 and 12 hours of growth in the alkaline and acidic medium, respectively. However, maximum enzyme production occurred at 30-36 hour of growth.

These series of studies on biomass and enzyme production and the influence of medium pH on these properties helped to

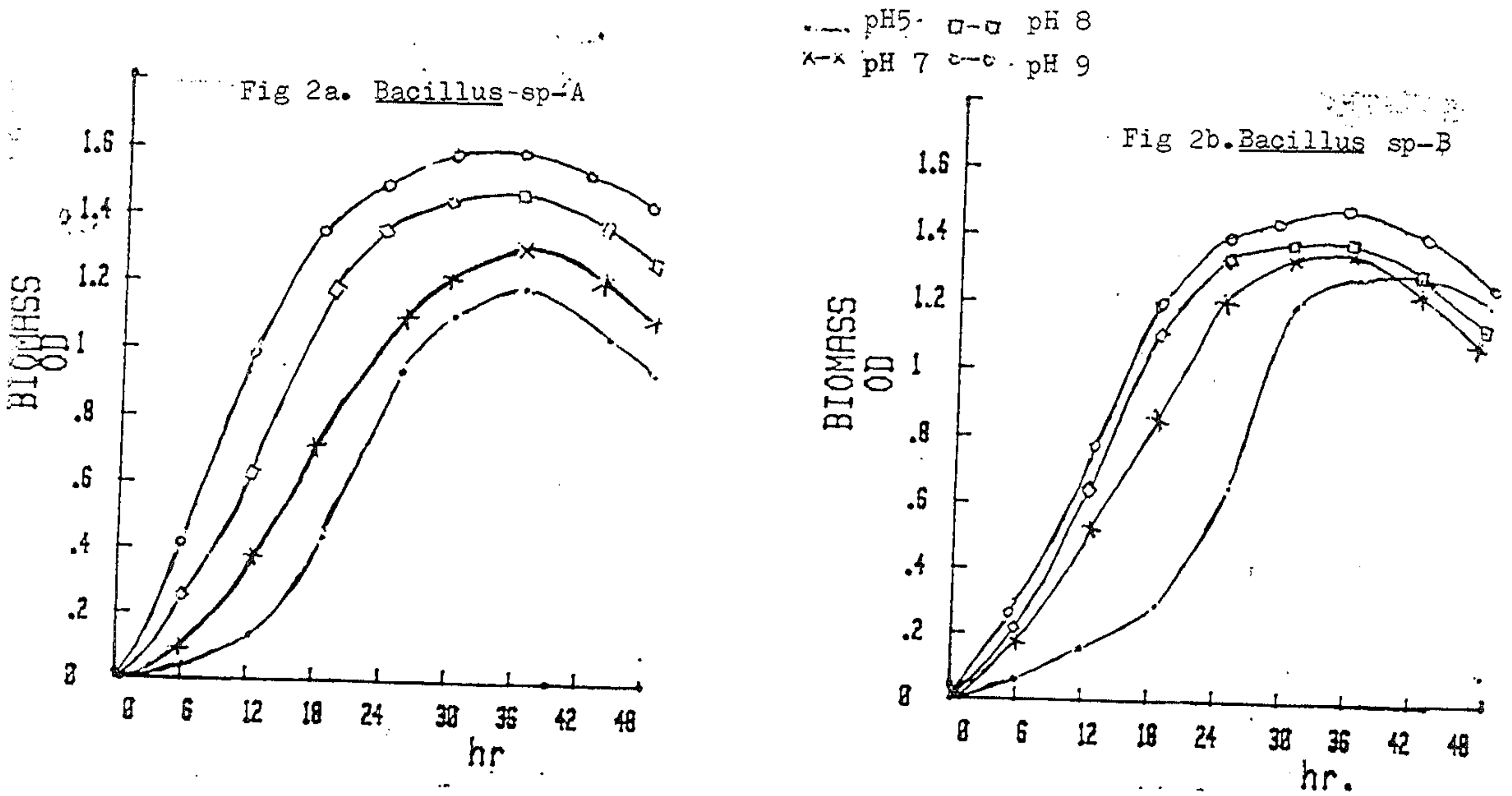

Fig 2a. Bacillus-sp-A

Fig 2b. Bacillus sp-B

Fig 2a and 2b. The study of biomass at different pH (representative) in nutrient broth. Growth temperature was 55 °C and enzyme assay was carried out at 55 °C using borate buffer (pH9).

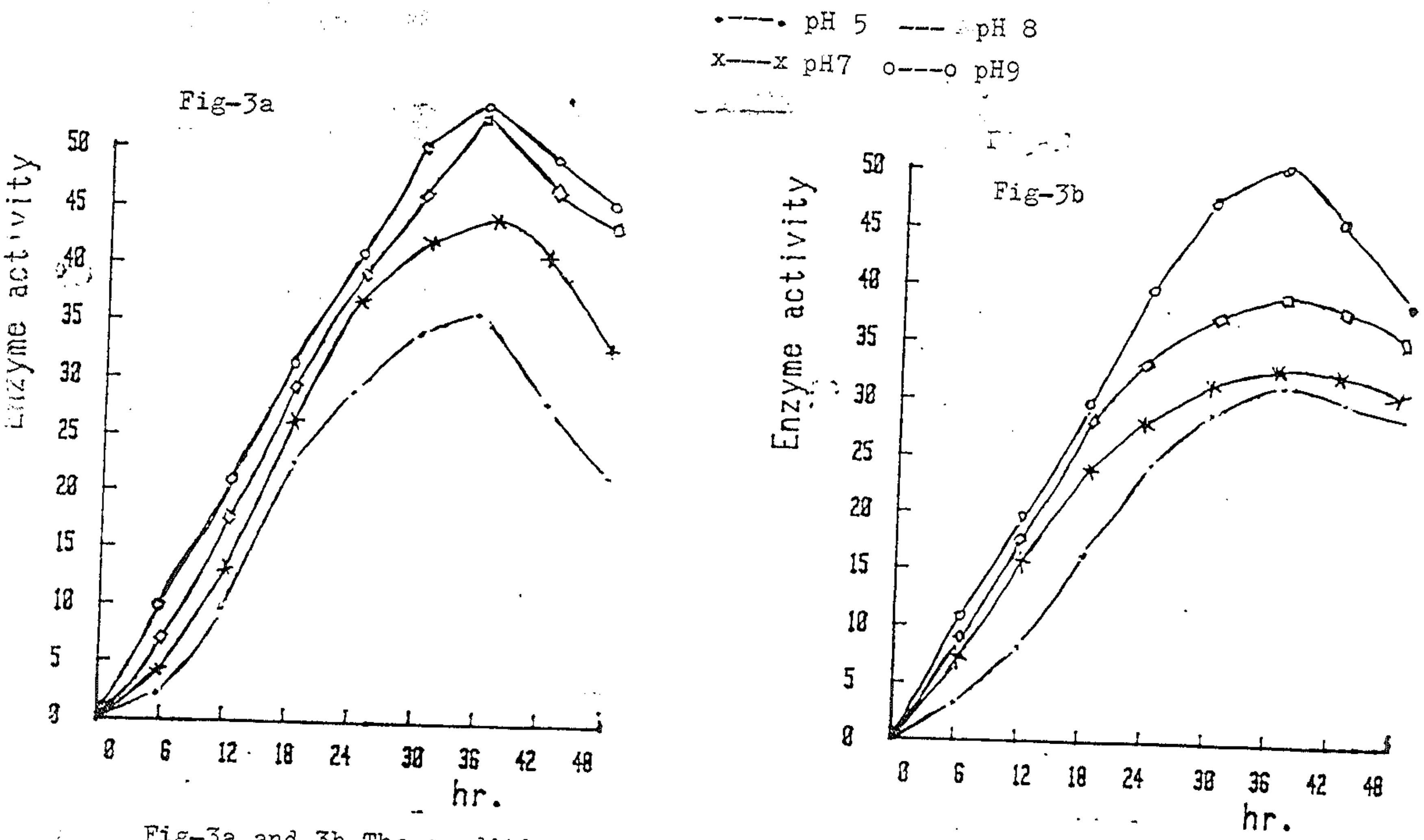

Fig-3a and 3b. The condition of protease production at different pH in nutrient broth for Bacillus sp-A and Bacillus sp-B, respectively.

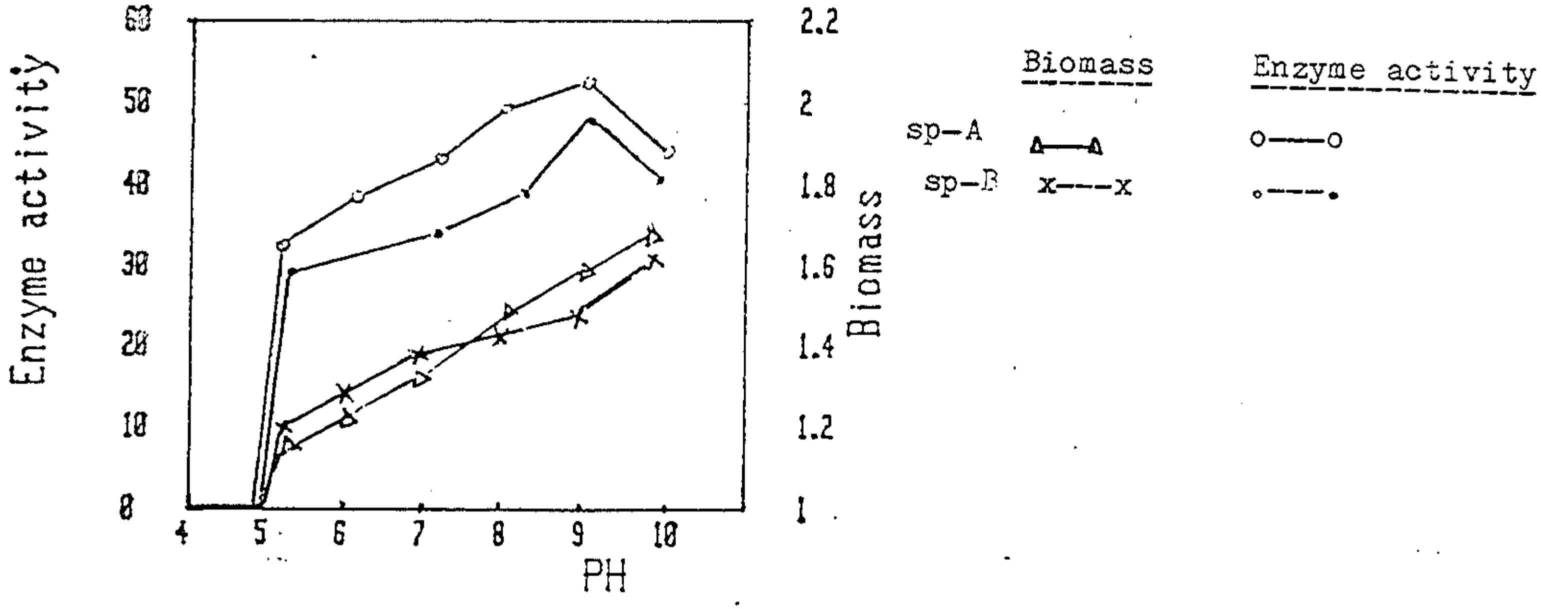

Fig-3c.Growth in nutrient broth at different pH.Enzyme assay was carried out at 55°C in borate buffer (pH9).

determine the appropriate medium pH and time of maximum enzyme production. These information were then used during studies on a basal medium which was formulated in the laboratory.

4.4. Growth and enzyme production on formulated medium.

Before measuring the effect of physico-chemical parameters on the growth and protease production of Bacillus sp-A and sp-B efforts were made to study once again biomass and enzyme production using the formulated medium whose pH was adjusted to 10 (Fig. 4). As compared to growth in nutrient broth (Fig. 2a and 2b) there was low production of biomass, but enzyme production was higher in the formulated medium (Fig. 4). Bacillus sp-A grew better than Bacillus sp-B in the formulated medium. Both strains required 3-6 hour to overcome the effect of the lag phase. The exponential growth phase continued from 6 - 33 hours of growth. The stationary phase lasted from about 33 to 39 hr of growth. Thereafter growth decreased (39 - 48 hour).

Enzyme production was observed at the exponential and stationary growth phase. Bacillus sp-A showed higher enzyme production than Bacillus sp-B. The highest enzyme production for both strains was observed in the late stationary phase at about 36 hour of growth. There was, however enzyme activity during the death phase of both isolates.

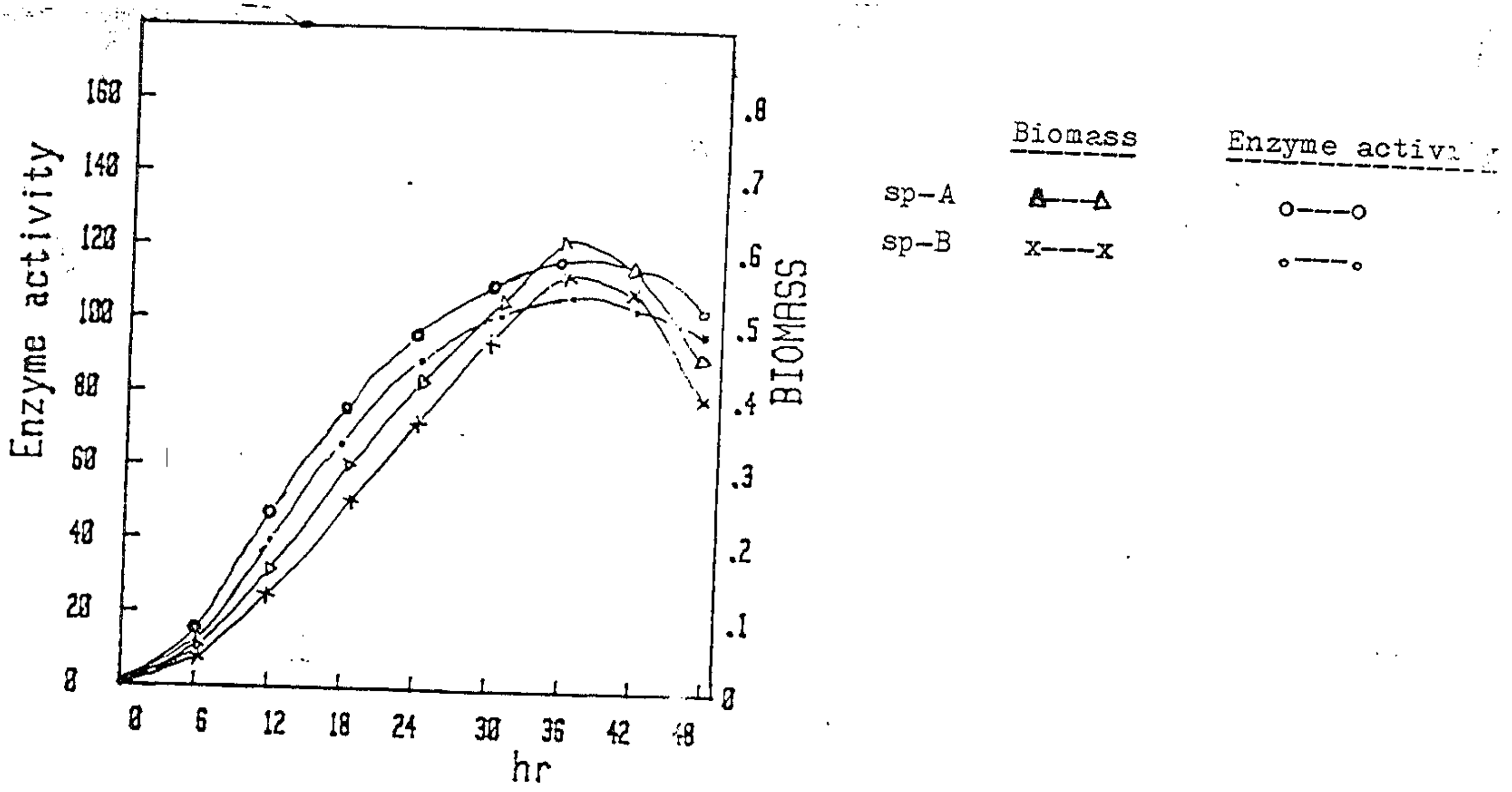

Fig-4. Growth study in synthetic medium (pH10). Enzyme was assayed in borate buffer (pH9).

4.5 The influence of different concentrations of glucose and peptone on growth and protease production

The response of both isolates to glucose concentration appeared to be similar (Fig. 5). For both strains high initial glucose concentration (1%) resulted in decreased biomass and protease production. The highest protease and biomass production was observed in the medium containing 0.5% glucose. Bacillus sp-A showed higher biomass and enzyme production at all concentrations of glucose used. Based on these findings the concentration of glucose was changed from 0.1% to 0.5% during formulation of growth medium-1 (M-1).

To study the effect of peptone as a nitrogen source on growth and enzyme production, the tests organisms were allowed to grow for 36 hr in growth medium-1 (M-1). The concentration of peptone in the medium varied from 0.1 - 0.4%. After the end of the incubation period the information generated for both isolates revealed the presence of higher biomass and enzyme production in medium containing 0.4% peptone. Therefore the concentration of peptone in the growth medium for subsequent experiments was adjusted to 0.4% (growth medium-2 (M-2).

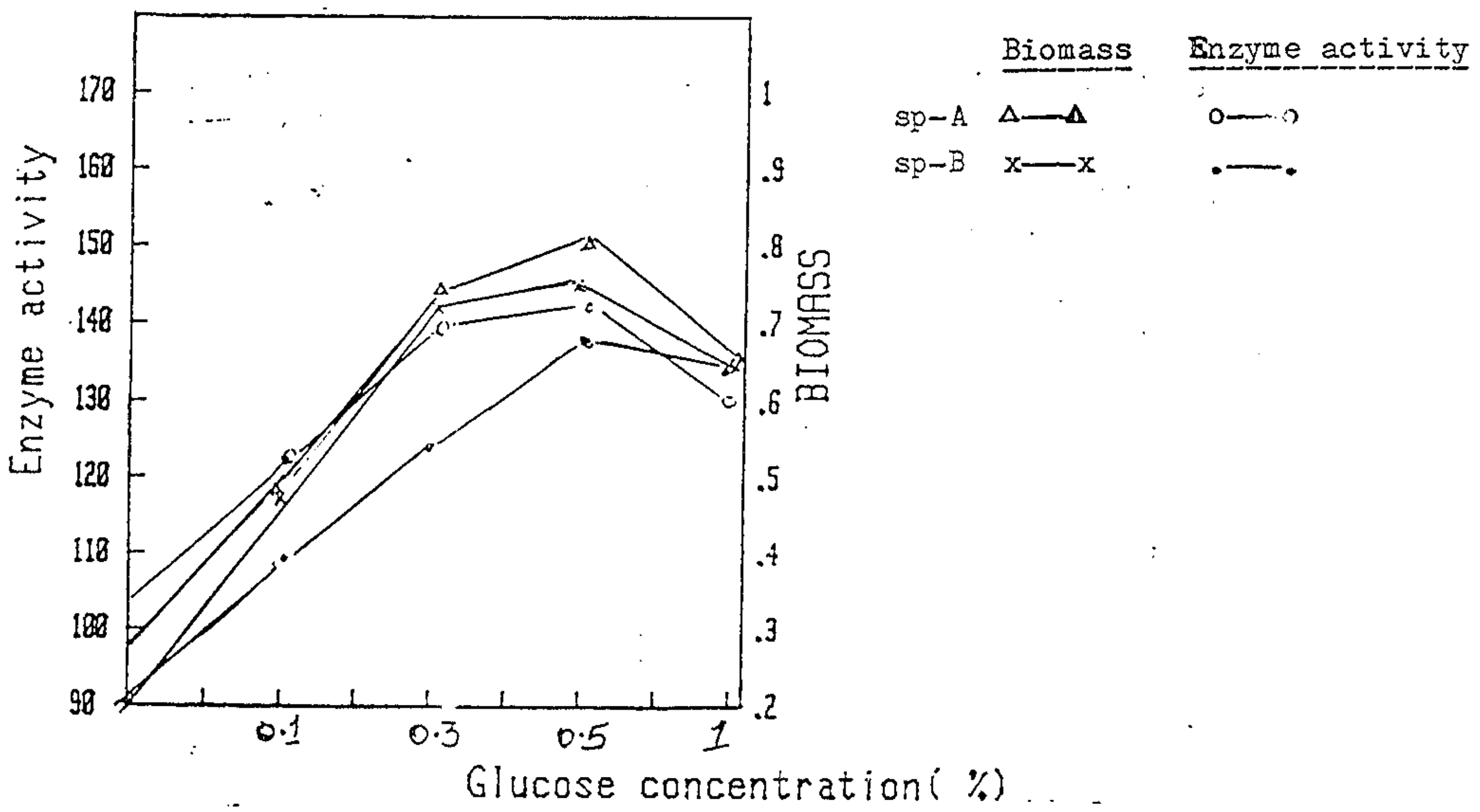

Fig-5 The effect of glucose concentration on biomass and enzyme production. Organisms were cultivated in growth medium(PH10). at 55 C°. Enzyme assay was carried at 55 °C in borate buffer(PH9)

4.6 The effect of detergents on protease production

Different detergents at various concentrations were included into the growth medium-2 (pH 10), which was supplemented with 0.1g K_2HPO_4, 0.05g $CaCl_2$ (anhydrous), 0.01g $MgSO_4$ $7H_2O$, 0.1g yeast extract (oxoid), 0.4g Peptone (oxoid) and 0.5g anhydrous D-glucose (BDH, poole, England) per 100 ml. Except Tween-80 the other surfactants did not allow growth of the test bacteria at all concentrations used. Tween-80 at 0.2% concentration increased both biomass and protease production of both isolates (Table 4) and inhibited growth at 0.3% concentration. Then in the above medium (M-2) 0.2% Tween-80 was included to grow the test bacteria. This medium was named as formulated medium-3 (M-3).

4.7 The effect of pH on biomass and protease production

The test bacteria were grown in the medium-3 (M-3), whose pH was adjusted from 7 to 10.5. Since protease production was found to be low for both isolates below pH 7 (Fig. 3a and 3b), analysis was not carried out using the formulated medium. Growth was allowed to proceed for 36 hours before biomass and protease activity were determined. Both strains failed to grow at pH 10.5. As can be seen in Fig. 6 the biomass of both isolates increased progressively as the pH of the medium was altered from neutral to alkaline condition and attained a maximum at pH 10. The biomass of Bacillus sp-A was lower than SP-B up to pH 8.5. But in most alkaline conditions (pH9 - 10) the biomass of strain-A was higher than strain-B.

Table 4

The effect of Tween - 80 on biomass and enzyme production. Enzyme activities was measured after 36hr of incubation at 55°C in borate buffer solution (pH 9). A unit of enzyme activity is expressed as ug amino acid/ml enzyme solution/minute.

Tween - 80 conc (%)	ORGANISM				
	Bacillus SP-A			Bacillus SP-B	
	Biomass OD	Enzyme activitiy		Biomass O.D	Enzyme activity
0	0.80	198		0.70	186
.05	0.80	198		0.75	188
0.1	0.85	211.3		0.80	188
0.2	1.1	235		1.0	196
0.3	No growth	--		No growth	--

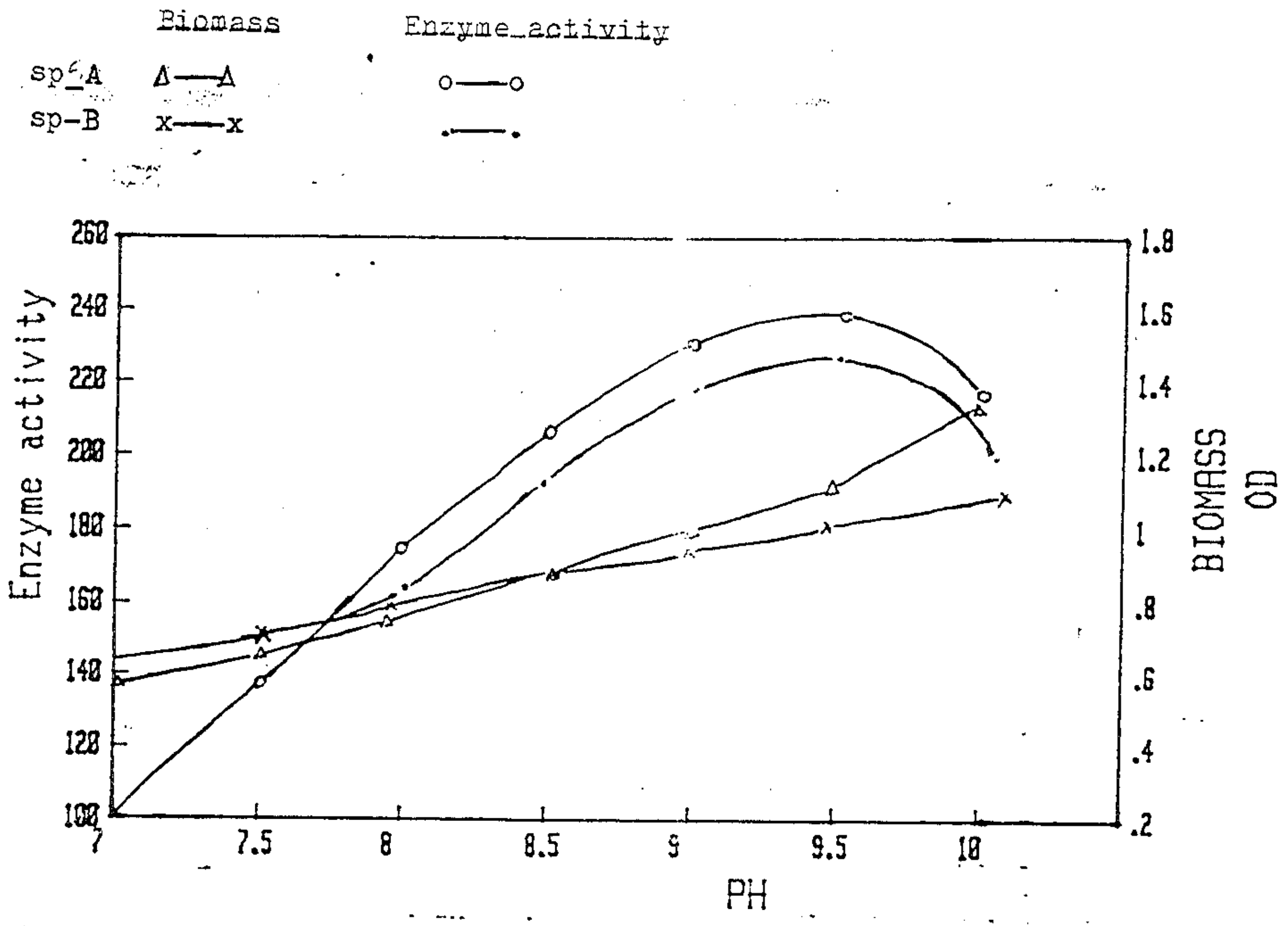

Fig-6.The effect of pH on biomass and enzyme production.Growth was allowed for 36 hr. Sampled cultures were assayed in borate buffer (pH9).

High enzyme production for both isolates was observed in cultures which were cultivated at pH 9.5. In the neutral conditions (pH 7 and 7.5) Bacillus sp-B showed a higher enzyme production than Bacillus sp-A. However from pH 8-10 Bacillus sp-A showed higher enzyme production than Bacillus sp-B.

4.8 The effect of temperature on growth and protease production

The two isolates were allowed to grow in medium-3 (M-3) for 36 hours at various temperatures between 25-60°C. Both isolates failed to grow below 30°C and above 55°C. The optimum temperature for growth of both isolates was 50°C (Fig. 7). The biomass of both strains increased from 30°C up to 50°C, but it decreased between 50°C-55°C. The growth of Bacillus sp-A was higher than Bacillus sp-B at all of the temperatures examined.

Protease production by Bacillus sp-A was not influenced by temperatures below 50°C. However, it showed a significant increase at 55°C. On the other hand, Bacillus sp-B showed constant enzyme production between 30-35°C. It then increased steadily from 35 to 45°C and then stabilized there after. The optimum temperature for protease production was found to be 55°C for Bacillus sp-A and 45-55°C for Bacillus sp-B.

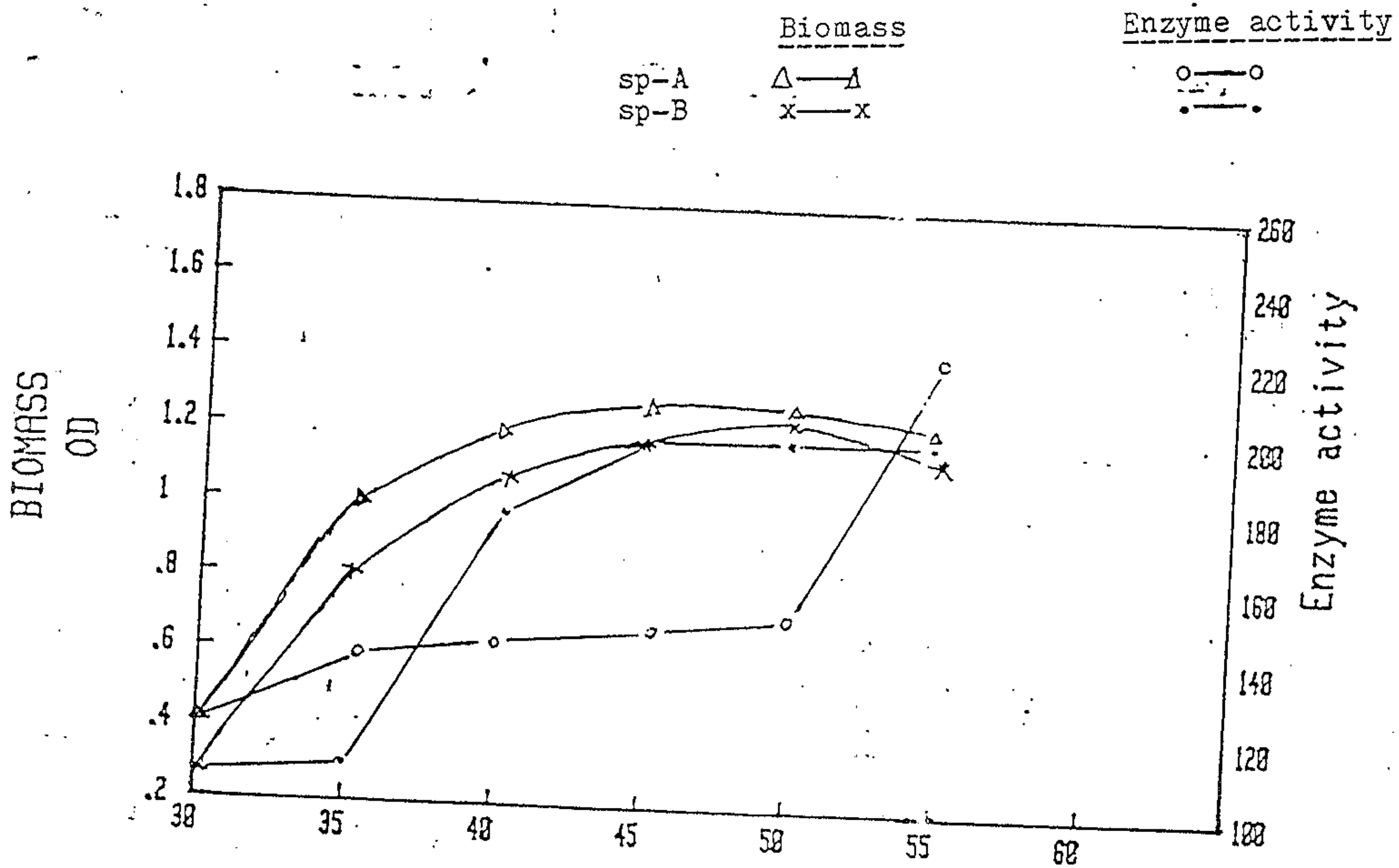

Fig. 7 The effect of temperature on growth and enzyme production. Growth was allowed for 36 hr. All enzymes assayed at 55 C° In borate buffer (PH9.5)

4.9 The effect of ions on biomass and protease production

In general higher protease production was obtained by including salts of divalent cations in the growth medium-3 (M-3) (Table 5). The protease production and/or activity of Bacillus sp-A was improved by about 270%, 226%, 170%, 130% and 35% (as compared to the untreated one) in the presence of 1mM Cu^{+2}, 5mM Ca^{+2}, 5mM Mn^{+2} or 0.1mM Zn^{+2}, 0.1mM Mg^{+2} and 0.1mM Co^{+2}, respectively. On the other hand Bacillus sp-B showed increments of 217%, 209%, 191% and 90% when 5mM Ca^{+2} and 1mM Zn^{+2}, 1mM Cu^{+2}, 5mM Mn^{+2} and 5mM Mg^{+} respectively were used. Mercuric ion inhibited growth completely at all concentrations tested. Fe^{+3}, Mg^{+2} and KI have decreased the enzyme production of Bacillus sp-A at the concentrations higher than 0.1mM of these cations only iron and cobalt created similar effects on Bacillus sp-B.

The biomass of Bacillus sp-A was unaffected by NaCl (at all concentrations), Ca^{+2} (0.1mM), Mg^{+2} (0.1mM) and KI (0.1 and 1mM). Na^{+} (0.1mM), Mn^{+2} (5mM), Ca^{+2} (0.1mM), Co^{+2} (0.1mM), Fe^{+3} (0.1mM), Mg^{+2} (0.1mM) and KI (0.1mM) showed no influence on the biomass production of Bacillus sp-B. The growth of Bacillus sp-A increased in the presence of 5mM KI, 5mM Ca^{+2} and 0.1mM of Fe^{+3}. It was extremely suppressed and inhibited in the presence of higher concentrations of Mn^{+2}, Cu^{+2}, Zn^{+2}, Co^{+2}, Fe^{+3}, Mg^{+2} ions. Inhibition was observed on Bacillus sp-B when high concentrations of Cu^{+2}, Zn^{+2}, Co^{+2} were used. Since 0.5mM Ca^{+2}, 0.1mM and 0.5mM

Mg^{+2} (for Bacillus sp-A and Bacillus sp-B, respectively), 1mM PO_4^{-4} was found to stimulate both biomass and protease production their concentration in the medium-3(M-3) was adjusted to the indicated mM concentration/ 100ml of the growth medium-4(M-4) in the next activities. On the other hand 1mM Cu^{+2} and 5mM Mn^{+2} was included in the buffer during assaying the enzyme activity of Bacillus sp-A. For Bacillus sp-B 1mM Zn^{+2} and 5mM Mn^{+2} was included in the assay buffer in the subsequent tests.

4.10 The influence of assay temperature on protease activity.

The least protease activity (at pH 9.5) for both isolates was found at 30°C. However, with increase in assay temperature protease activity also increased (Fig. 8). Bacillus sp-B had less activity than Bacillus sp-A. The highest activity was observed at 55°C for both test bacteria. No activity was detected at or above 65°C and 70°C for Bacillus sp-B and Bacillus sp-A, respectively.

4.11 The effect of assay pH on protease activity

A series of experiments were carried out from pH 4 - 11.5 to study the effect of assay pH on protease activity (Fig. 9). Activity was observed only in the range between 4.5 - 11.0 pH values. The protease from Bacillus sp-B was unaffected at pH 4.5 - 6.0 and showed rapid increment in the range between 6.5 - 7.5 pH values. On the other hand the protease from Bacillus sp-A

Table 5

The effect of ions on enzyme production and Biomass. Enzyme was harvested after 36hr of growth. Assay was carried out in borate buffer (pH9.5) at 55°C incubation temperature.

		SP-A		SP-B	
TYPE OF ION	mM conc	BIOMASS	ENZYME ACTIVITY	BIOMASS OD	ENZYME ACTIVITY
NaCl.	0.1	0.70	93	0.35	111
	1	0.70	106	0.45	142
	5	0.70	106	0.50	142
$MnSO_4.7H_2O$	0.1	0.14	100	0.20	126
	1.0	0.23	106	0.29	126
	5.0	0.20	124	0.33	134
$CuSO_4.4H_2O$	0.1	0.45	155	0.52	126
	1.0	0.31	170	0.13	142
	5.0	-	-	-	-
$ZnSO_4$	0.1	0.40	124	0.20	126
	1.0	-	-	0.15	146
	5.0	-	-	-	-
$CaCl_2$	0.1	0.20	62	0.35	46
	1.0	-	-	0.51	46
	5.0	-	-	0.64	146
$CoCl_2$	0.1	0.20	62	0.46	46
	1.0	-	-	0.28	30
	5.0	-	-	-	-
$FeCl_3$	0.1	0.75	77	0.47	46
	1.0	0.52	62	0.52	30
	5.0	0.34	46	0.59	-
$MgSO_4.7H_2O$	0.1	0.62	106	0.48	46
	1.0	0.52	62	0.58	62
	5.0	0.15	46	0.68	87
KI	0.1	0.75	77	0.48	80
	1.0	0.75	62	0.50	57
	5.0	1.00	46	0.52	103
K_2HPO_4	1.0	0.90	93	0.51	103
$HgCl_2$	.1,1 ,5	-	-	-	-
none	-	0.67	46	0.36	46

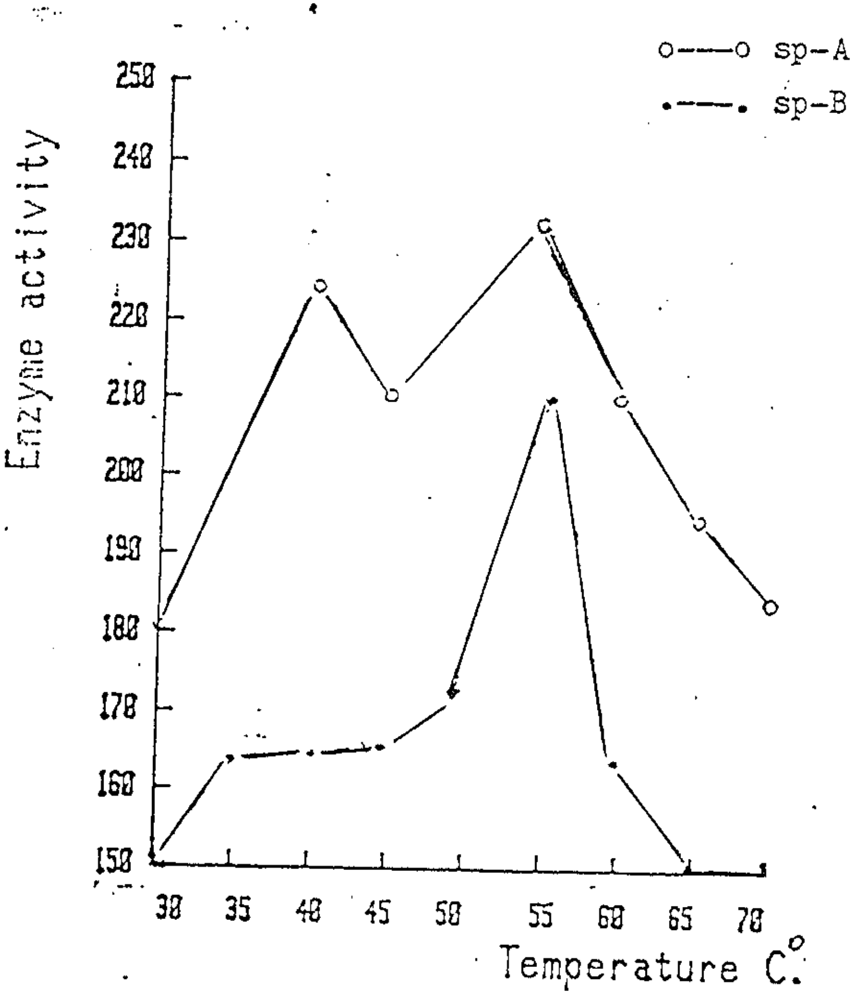

Fig. 8 The effect of assay temperature on enzyme activity. Assay was performed in borate buffer pH 9.5.Growth was allowed for 36 hr in growth medium whose PH was 10.

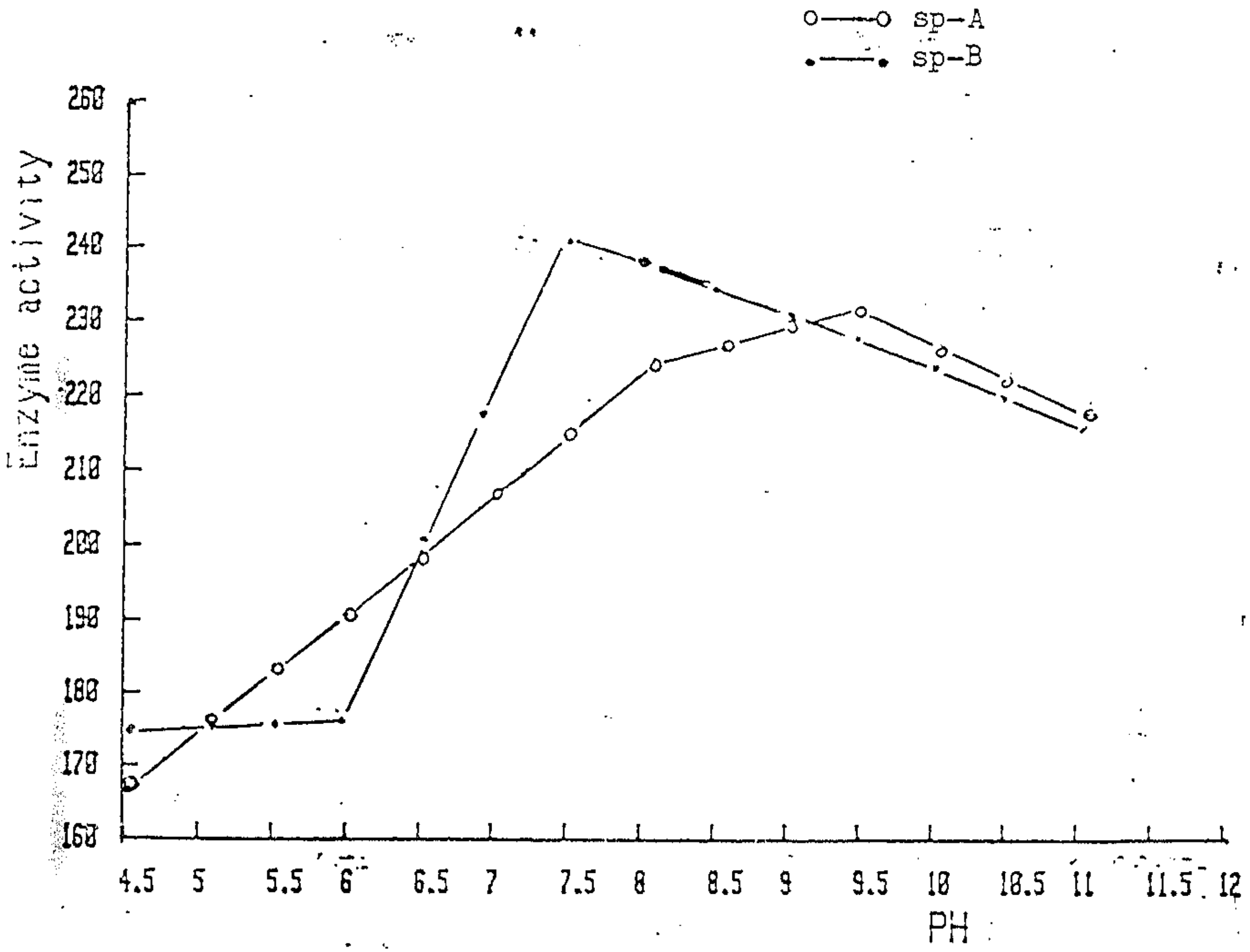

Fig. 9 The effect of assay PH on enzyme activity. Organisms were allowed to grow for 36 hr in growth medium whose PH was adjusted to PH 10.

showed increased activity as the assay pH was increased. Maximum activity was observed at pH 7.5 for Bacillus sp-B and at pH 9.5 for Bacillus sp-A. Protease activity decreased gradually above pH values of 7.5 and 9.5 for Bacillus sp-B and sp-A, respectively.

4.12 Assay of Protease activity on different substrates

The proteases from the two organisms showed marked substrate specificities. Casein was easily degraded by Bacillus sp-A than haemoglobin or gelatin. On the other hand gelatin was hydrolysed by Bacillus sp-A easily. The buffer used for enzyme assay was Tris-HCl (pH 7.5) and Borate buffer (pH 9.5) for enzyme solutions of Bacillus sp-B and Bacillus sp-A, respectively.

4.13 Growth and Protease production on feather, skin, horn and hair

As shown in Fig-9 the weight lose recorded on skin was much higher than the rest of the natural substrate. The least weight loss was observed on hair. It also seemed that both organisms were efficient in degrading horn than feather. However the degradative activity of Bacillus sp-B on horn was markdly higher than feather (29% increment). Comparatively speaking Bacillus sp-A showed high degrading activity on feather than Bacillus sp-B; conversely Bacillus sp-B performed high degrading activity on horn than Bacillus sp-A.

Table 6

Measurement of protease activity on different substrates. Proteases were obtained from 36hr cultures at 55°C. The growth medium was adjusted to pH 10.

Substrate	ORGANISM	
	Bacillus sp-A	*Bacillus* sp-B
	PROTEASE ACTIVITY	PROTEASE ACTIVITY
Egg albumin	-	-
Casein	62	4
Gelatin	20	60
Haemoglobin	42	8

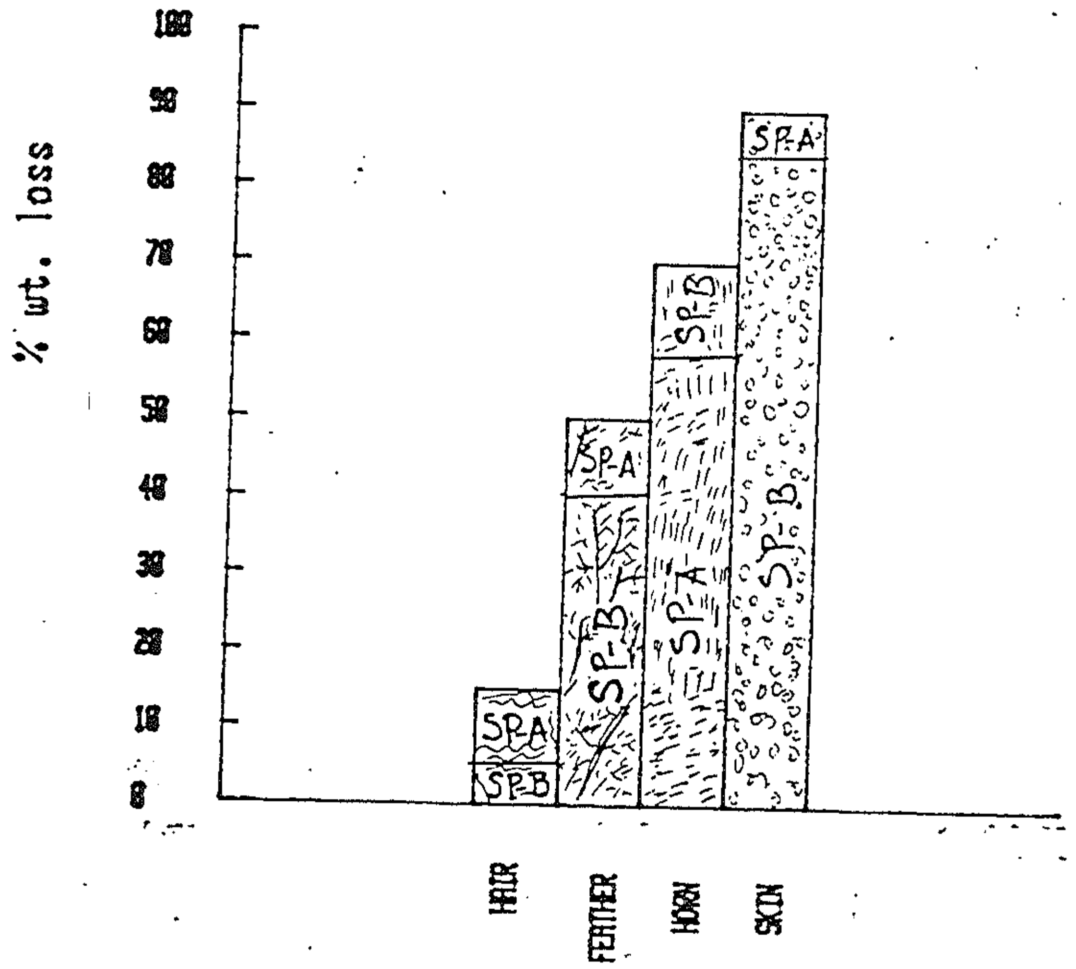

Fig.10 The action of the two organisms on natural substrates.Cultures were incubated at 55 C in growth medium(PH 10) supplemented with the natural substrates.

4.14 Thermal stability tests.

The results presented in Table - 5 show the effect of heat treatment on protease activity. The protease from Bacillus sp-A was thermally stable from 60-70°C and that of SP-B was stable only at 60 and 65°C. The protease activity was lost completely after 60 minutes of incubation at temperatures of 60-70°C. The half life of the protease from Bacillus sp-A was 50min at 60 and 65°C and 40 min at 70°C. For Bacillus SP-B, it was 50 minute at both 60 and 65°C.

Table 7

Examination of Thermal stability.

Temp. C°	min	Enzyme activity (U/ml)	
		<u>Bacillus sp</u>-A	<u>Bacillus sp</u>-B
	10	196	165
	20	196	157
60	30	165	134
	40	142	97.3
	50	86.2	86.2
	60	-	-
	10	165	111
	20	157	111
65	30	149	103
	40	118	72
	50	86.2	56.0
	60	-	-
	10	118	-
70	20	111	
	30	95.1	
	40	72.0	
	50	25.2	
	60	-	
75	10	-	

PART V

DISCUSSION

Morphological and biochemical tests (Table 3) showed that the two isolates (sp-A and sp-B) belonged to the genus Bacillus (Claus and Berkeley, 1986). The differences observed between our isolates and the two organisms described as B. licheniformis and B.cereus may have emerged due to strain variation. Thus what has been called Bacillus sp-A can be referred as a strain of B. licheniformis and Bacillus sp-B as a strain of B. cereus.

Growth and enzyme production occurred under a wide range of pH. Generally the pH range of growth was similar to other Bacillus sp, which is from pH 5 - 10.5 (Priest, 1970) Growth and enzyme production at the alkaline conditions was most probably due to similarities between the pH of the medium and the natural environment from which the organisms were isolated.

Many workers have used different growth media for the production of enzymes by different organisms (MacFarlane and MacFarlane, 1992). A growth medium which was formulated in the laboratory was used to study the effects of different factors on growth and enzyme production. Growth and enzyme production was influenced by the nature of the medium used for cultivating the test organisms. There was low biomass and high enzyme production in the formulated medium. Protease synthesis is known to be markedly influenced by the growth medium. This is due to the mechanisms of induction, repression or catabolite repression (McConn et al., 1964). A similar low rate of enzyme synthesis in cultures which had high cell biomass than in cultures with low biomass in media with different composition was also observed by Mosses and Sharp (1972).

Protease production occurred at the exponential and stationary phase of growth. Out of the two definite patterns of exoenzyme synthesis during the growth cycle of organisms described by Chalonpka and Kreckova (1965) the results in this study coincided with a very low rate of secretion during active growth followed by increased synthesis in the late exponential and stationary phase of growth. The presence of extracellular protease in cultures at the exponential phase may indicate the nutritional function of the enzymes (MacFarlane and MacFarlane, 1992). According to Arronson et al., (1971), enzyme production during the exponential growth in some species is associated with the presence of appropriate growth condition.

The use of defined medium is necessary to elucidate changes in cell growth and product formation. With this in mind the effect of varying concentration of glucose on growth and enzyme production of the organisms was examined. Higher protease production was observed in cultures supplemented with 0.5% glucose concentration than 1% and this showed that most of the initial glucose will make no contribution to the protease production. (Kole *et.al,* 1988) Also exocellular protease production is subjected to glucose repression above a certain specific growth rate (Bernlohr and Clark, 1971).

Although the specific regulatory mechanisms are not known, the production of protease, in most species is inhibited by high concentration of glucose (Kurowski and Donleary, 1976). Glucose inhibited formation of gamma-protease of Cl.welchii, but did not affect proteinase production of B.linens (Hayashi and Law, 1989). However it was required for high yields of B. subtilis protease production (Biduell, 1960). Other factors like growth temperature (Martely et al., 1991); carbohydrate/protein ratio (Votruba et al., 1991) are known to affect the utilization of available carbon source for growth and enzyme production.

The presence of peptone in the medium induced enzyme production. Peptone as a partially degraded protein, may induce protease production through signal transduction pathway to inform the cell about the presence of peptides bond in the growth medium and initiate extracellular protease production. It is also speculated that the smaller peptides may enter the cell and trigger, additional enzyme formation (Patterson-Curtis and Johnson, 1989).

The presence of high protease production in the presence of metabolizable carbon source, indicated that the primary function of the protease was to ensure a supply of amino acid for formulated process rather than carbon for growth (Guntelberg, 1964). The amount of biomass and proteolytic activity produced by the two organisms at various pH values may indicate that organisms have a particular optional condition for growth and protease production. The environmental pH is one of the conditions.

The observed effect of pH may have occurred either as a result of increased level of enzyme production or enhanced activity of the already existing enzyme(s).

For instance Sargent and Lampen (1970) found out that at neutral pH approximately half of penicillinase synthesized by *B. licheniformis* was secreted into the medium. The remaining 50% was released when the cells were incubated at pH 9; however, the two enzymes were different. Johnson *et al*. (1992) have noted a significant decrease (40-50%) in yield of biomass and lipid in yeasts when the pH was decreased from 3.45-5.7 range to pH 2.7.

The proteolytic activity difference observed between the two organisms in the neutral and alkaline conditions could be due to the ratio of neutral to alkaline protease production (Uehara *et* al., 1974). Nevertheless the mechanism which governs pH requirement for growth and enzyme production is still unclear (Aono et al. 1992).

The biomass of the two isolates increased as the growth temperature is cultured between 30-50°C and decreased at 55°C. According to Arrhenus' law, an increase in temperature speeds up microbial growth as long as the optimum temperature is not exceeded (Sonnleitner and Fiechter, 1983).

The strains of Bacillus sp. differ in their optimum temperature requirements for growth and metabolic product formation or secretion (Chalonpka and Kreckova, 1965). Generally proteases are excreted at relatively low levels by most thermophilic bacteria than mesophiles. For instance protease activity of 55, 51 and 35 ug amino acid/ml of enzyme solution/min at growth and assay temperature of 37, 55 and 75°C was observed for B subtilis, B.stearothermophilus and Themus aquaticus, respectively (Cown and Daniel, 1982). The equivalent activity of our isolates, Bacillus sp-A (on casein) and Bacillus sp-B (on gelatin at growth and assay temperature of 55°C was 62 and 60 ug amino acid/ml of enzyme solution/min, respectively.

The two isolates used in this study showed high biomass and enzyme production at 50 and 55°C, respectively. This may indicate that the former temperature (50°C) is suitable for growth and the later (55°C) promoted protease secretion. Even though the regulatory mechanism of temperature in the production of proteinase is not fully understood, it affects the synthesis of RNA polymerase subunits, and inactivates regulatory proteins (Votruba et al., 1991). Temperature also causes alterations in the physical properties of the cell membrane, which influence the secretion of protease by organisms (Gould et al., 1975, and Giesecke, et al. 1991).

The production of metabolite by microorganisms through industrial process needs altering the permeability of the plasma membrane. The increased enzyme production brought by Tween-80 might be due to lose of membrane phospholipid which results in facilitating the release of the product (Niven et al., 1988). It may also indirectly stimulate enzyme production through adhesion to substrate or through nutrient emulsification (Horowitz et al., 1990).

The two organisms had different requirements of ions. This is in line with the explanation of Dancer and Mandelstam (1975). Even though microorganisms require minerals for their growth and metabolic product formation their requirements vary with the type of organism as well as with the nature of basal medium under investigation. Ions also differ in stimulating enzyme production or enhancing enzyme activity (Hanion et al., 1982). Divalent cations like Cu^{+2}, Mg^{+2}, Zn^{+2}, Mn^{+2} are essential metals found in a number of enzymes and serve as prosthetic group, or co-factor to conform autolysis or in the formation of salt bridge among inter subunits (Hartely, 1960; Sheppard and Cooper, 1971; Hegered et al., 1972; Ohmizu et al., 1983). The high biomass and enzyme production initiated by Ca^{+2} might be related to its effect on a diverse array of cellular functions. Ca^{+2} functions to activate protein kinases (Leger et al., 1989); to protect autolysis by firmly bounding to the nascent chain of the enzyme and confer protection from protease attack (May and Elliot, 1968). In B.cereus 50% of the enzyme activity was lost in the absence of added Ca^{+2}, but in the presence of 0.05M $CaCl_2$ all of the activity was recovered (Feder et al., 1971).

The higher enzyme activity of Bacillus sp-B as compared to Bacillus sp-A in the presence of Zn^{+2} is most probably related to the presence of high neutral protease synthesis by the organism Zn ion is incorporated as prosthetic group in most neutral protease (Higerd et al., 1972; Down et al., 1990).

In general divalent ions produce charge imbalance which induces the shading of the cell surface components (Ohmizu et al., 1983). The inhibition caused by Hg^{+2} can be ascribed due to denaturing action proteins. It also competes with essential microelements, inhibits enzyme activity, binds to cell structures, precipitates nutrients, etc. (Abbas and Edward, 1990).

The presence of higher enzyme activity at 55°C for both organisms may indicate the exposure of the protease to a similar condition under which they function in their natural environment. The high protease activity at 55°C can be related to the presence of higher amount of inactivated protease. However the temperature of maximal activity is not necessarlly fixed to 55°C (Sonnleitner and Fiechter, 1983).

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 a lose or decreased biological activity (Veronse _et_ _al_., 1984).

The results of the effect of assay pH on enzyme activity show that, the two isolates (_Bacillus_ sp-A and _Bacillus_ sp-B) produced different protease. Sonnleitner and Fiechter (1983) have described the possibility of almost an infinite variation in the pattern of development of extra cellular protease formation by _Bacillus_ species. The pH profile of the protease of _Bacillus_ sp-A showed the presence of alkaline protease(s). The alkaline proteolytic enzymes 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 _Bacillus_ sp-A (4.5 - 11.5) coincides with other alkaline protease. Generally alkaline protease show activity in mildly alkaline condition i.e. less than pH11.5, but lose activity rapidly below pH4 (Cown and Daniel, 1982). From the alkaline protease the enzyme of Bacillus sp-A reassembled more to subtilin carlsberg or Bacillus pumulus proteases, which have an optimum pH value of 9.5 (Priest, 1970).

The presence of higher rate of hydrolysis at pH 7.5 for Bacillus sp-B (55%) as compared to sp-A may indicate the presence of more neutral proteinase(s). The observed protease activity below or above pH7.5 is not necessarily related to the presence of acid or alkaline protease. Tsuchiya et al. (1992), have improved the pH stability of protease from the range of pH6-Ph13 at 37°C to pH4-12.5 by including 5mM Cu^{+2} ion. The protease was even stable at 60°C in the range of pH5-12.5 when Ca^{+2} was present.

Casein and gelatin were efficiently hydrolysed by Bacillus sp-A and Bacillus sp-B, respectively. The ease with which the two substrates were hydrolysed could be due to their chemical composition and physical properties. Gelatin is a neutral protein with a unique amino acid sequence in which glycine is found in every third residue on the molecule (Takami et al., 1992); where as, casein is a phosphoglycoprotein in which the component parts are organized in micelles to form soluble complex (Smid et al., 1991). Therefore these structural and chemical differences may have contributed to the observed activity differences on the substrates.

The greater specificity of the enzyme of Bacillus sp-B towards gelatin complements previous studies made by Hartely (1960). He has described proteinases from Streptomyces sp which attack only gelatin, by splitting the peptide bonds of glycine on the N-terminal. On the other hand the coagulation of casein micelles as a result of K-casein hydrolysis (Smid et al., 1991) may have resulted the enzyme(s) of SP-B to be insensitive to the substrate. To the contrary the enzyme(s) of Bacillus sp-A may have a mechanism of adsorption on the new exposed surface of the coagulated casein micelles and split the remaining parts. A very close activity to Bacillus sp-A (66 ug amino acid/ml enzyme solution/min) on casein have been found for alkaline protease of B. subtilis (Millet, 1970).

Bacillus sp-A appeared to elaborate alkaline protease(s) than neutral ones. Hwang and Hseu (1980) have reported that, alkaline protease show extensive activities against casein and open a wide range of peptide bonds than neutral protease.

The other source of difference which sometimes if not always, is considered to be the molecular size of the substrates. Morihara et al. (1979) reported a considerably different behaviour of enzymes against peptide bonds of different molecular size.

When the enzyme(s) acted on the substrate it might have released some amino acids which may have a significantly inhibited enzyme activity. In B. megaterium, enzyme activity was reduced up to 2% by the presence of Threonine and Isoleucine. (May & Eliot, 1968).

The pH of the assay condition and the buffers influence the activities of the enzymes (Hwang and Hseu, 1980). The buffers may have hindered to get the reaction products for the reasons like insolubility of substrates, unfavourable thermodynamic equilibrium, difficult product recovery, etc (Barros et al., 1992, Mcconn et al., 1964). A different result could have been observed if we used organic solvent and purified enzyme (Janssen et al., 1991.)

The results in this study clearly showed the degrading activity of the test organisms on feather, skin, horn and hair. It seems that the nature and the structural configuration of the proteins in the natural substrates have restricted their action on the supplied materials.

Subtle conformational and/or chemical variations between protein molecules have been found to result in significant resistance differences of heat denaturation (Solomon and Balas, 1991). The thermal stability difference between the enzymes of the two organisms might be associated to this. According to the observation of Cown et al. (1985) the protease of B.licheniformis or B.subtilis (grown at 37°C) and B. stearothermophilus NCIB 8924 (grown at 55°C) showed half-life of 10min (for the former two) and 15 for B. stearothermophilus, at incubation temperature of 70, 60 and 74°C, respectively. The protease(s) of the study strains have certain similarities with the proteases of the above organisms. The protease of Bacillus sp-A (half-life of 40min at 70°C) reassembled to the protease of B. stearothermophilus NCIB 8924 and that of Bacillus sp-B (half-life of 50min at 60°C) reassembled the protease of B. subtilis.

Some ionic species have been found to have a role in conferring thermostabilization. Ohta (1963) have related the effect of divalent ions with high net charges and strong potential field around them to produce a highly rigid structural conformation of an enzyme which will make it insensitive to heat denaturation.

The study organisms appeared to be more alkalophilic than hyperthermophilic. According to McDonald and Chambers (1966) protease from alkalophiles are generally stable in the high alkaline regions, but they are not very stable to heat, while the vice versa is true for thermophiles. The absence of activity, after one hour, might be related to the presence of autolysis, because proteinases, according to Matsuzawa et al., (1988), have the disadvantage of attacking themselves.

VI. REFERENCES

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