CHAPTER VI

ENZYMATIC STUDIES
VI. ENZYMATIC STUDIES

(A) INTRODUCTION

Catalysis may be defined as the acceleration of a reaction produced by the presence of a substance known as a catalyst. A catalyst is an agent that accelerates a chemical reaction without itself being destroyed or used up. It may be recovered practically unchanged at the end of the reaction. An enzyme or ferment may be defined as a thermolabile organic catalyst elaborated by a living cell and is capable of functioning independently of the cell.

Word Enzymes was coined in 1878 by Kuhne from a Greek term meaning "in yeast". Earlier enzymes were referred to as ferments because their action was similar to yeast fermentation. It can also be defined as any substance, protein in whole or in part that regulates the rate of a specific biochemical reaction in living organisms. It is capable of catalysing a reaction in which substrates are converted to products. Various microorganisms like bacteria, fungi and protozoa produce different kinds of enzymes. They produce extracellular enzyme to break those organic and inorganic compounds which have high molecular weight.

Enzymes possess the properties of proteins. They form colloidal solutions, dialyse through membranes either very slowly or not at all, are amphoteric, form opalescent solutions, are precipitated from solutions by the same agents which precipitate proteins, and have large molecular weights when treated with acids, alkalies, or proteolytic enzymes.
they yield mixtures of amino acids. Since enzymes are protein complexes, they are sensitive to all the various precipitating and coagulating (denaturing) factors that affect proteins in general, i.e., temperature over $80^\circ C$, excessive concentration ions of heavy metals or of hydrogen ($H^+$) or hydroxyl ($OH^-$) ions.

Available evidences indicate that enzyme combine with the substrate upon which they act and whether this union is physical or chemical is not clearly understood. After the substrate is decomposed, free enzyme is regenerated and becomes available to react with more substrate. The substrate is believed to bind itself to the enzyme at specific sites, usually one or a few, per molecule of enzyme. These are referred to as the active sites of the enzyme.

Enzymes are proteinaceous in nature which they can be separated from living tissues, purified and even crystallized. Under controlled conditions of isolation, they retain their original level of activity and in some cases even exhibit an increased activity. Enzymes are unstable molecules with a definite physico-chemical organization. Even a slight change in this organization reduces the activity of enzymes and sometimes the enzyme is totally inactivated. Therefore, the enzymes have to be isolated under controlled conditions of pH, ionic strength and temperature.

Enzymes of bacteria may be divided into two groups depending upon whether they are secreted into the surrounding culture medium or remain confined within the cell. The enzymes that belong to
the first group are known as extracellular and those classified in the second group are called intracellular enzymes.

The enzyme extract is centrifuged to remove cell debris, cell organelles and sometimes other molecule aggregates, leading to partial purification of enzymes. It also helps in characterization of an enzyme, since, depending upon its mass and shape the enzyme will move through a solution at a definite speed and occupy a characteristic position in the centrifuge tube.

**Location of enzymes in bacteria are by:**

(i) **Fractionation** - The cell wall of bacteria may be removed by hydrolysis in hypertonic solution to form protoplasts which may be emptied off cytoplasmic contents by subsequent osmotic shock. The method has proved successful in isolating components of certain cells.

(ii) **Centrifugation** - The individual sub-cellular structures may be separated by centrifugation. By this procedure the extract may be resolved into three fractions:

(a) Structures which are large enough to be observed with a light microscope sedimenting at 10,000 x g for 15 min.

(b) Submicroscopic particles which sediment at 100,000 x g for 60-120 min.

(c) The soluble fraction which contain substances that remain in solution in the supernatant that is separated from the sub-microscopic particles.

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Proteases are the single class of enzymes which occupy a pivotal position due to their wide applications in detergents, pharmaceuticals, brewing, leather, food and agricultural industries. The current estimated value of the world wide sales of industrial enzymes is $1 billion and proteases account for about 60% of the total sales (Rao et al., 1998).

Proteases regulate intracellular phenomena. Proteases and proteolysis play vital role in the biology of all cells (Wolf, 1992). The action of proteases can be divided into two categories, (1) limited proteolysis in which they split only a small number of peptide bonds of a target protein and (2) non-limited which is complete degradation of target protein. Proteases catalyse the splitting of peptide bonds. They are present in all living organisms in which they display many physiological functions ranging from generalised protein degradation to more specific and regulated processes such as blood coagulation, hormone activation or transport of secretory proteins across membranes.

Bacteria synthesize a large variety of proteases. The growth of any organism depends on the function of most of the enzymes which it contains. The environment influences cell growth primarily by altering the enzyme activity. The optimum temperature, pH, and salt concentration for the growth of any cell represent the best average for the function of all its cellular enzymes. The rates of enzyme reactions are influenced by a number of factors, the most important being (1) temperature (2) pH of the selection (3) ultraviolet light (4) concentration of enzyme (5) concentration
of substrate (6) presence of activators and (7) presence of inhibitors. The same factors which affect the growth and multiplication of bacteria also affect the action of enzymes. However, enzymes are generally more resistant to unfavourable environmental conditions than the cells producing them. Other factors of importance in enzyme functioning are hydrostatic and osmotic pressure, ultraviolet light and other radiations.
(B) MATERIALS AND METHODS

SCREENING OF BACTERIAL ISOLATE FOR PROTEASE PRODUCTION ON SOLID SUBSTRATE (GGYA) MEDIUM

Screening is a highly selective procedure which allows the detection and isolation of only those microorganisms which are of interest out of the available microbial population. Effective screening involves one or few steps for discarding unwanted bacterial colonies and for the detection and quantification of the useful microorganisms. In the present study the total number (120) of isolated bacterial strains were screened for their proteolytic activity on 1% Glucose Gelatin Yeast Extract agar (GGYA) medium. Identifications were carried out after the screening was completed and the proteolytic strains were separated from the non-producers.

1 % GGYA medium was prepared and 15 - 20 mL of the media was poured into sterile petriplates. After the agar solidification the bacterial isolate was streaked on this media and incubated at 37 ± 1°C for 24 h. After 24 h 1% mercuric chloride reagent was flooded on the plates and allowed to stand for 10 min and later decanted off. The formation of clear transparent zone/halo around the colony indicated hydrolysis of gelatin by extracellular proteolytic enzymes. Due to addition of HgCl₂ reagent on the plates, gelatin was denatured and precipitated whereby the plate becomes opaque. The clear zone was seen around the bacterial
colonies where gelatin was hydrolysed. The clear zone had polypeptides and amino acids which did not react with the reagent.

The radius of the clearing zone was measured and twice the radius (the diam) was considered as a criteria for extracellular protease activity. The diam of halo was determined for each case, as the growth rate of each bacteria was different from one another and so was their proteolytic activity. The greater the ratio of the halo the greater was the proteolytic activity. The experiment was done in triplicate and the diameter was measured in centimetres. All the bacterial strains screened positive for protease production were maintained on Nutrient Agar (NA) as well as GGYA slants in triplicate and incubated at 37 ± 1°C for 24 h.

Preparation of reagents and media

**Gelatin Glucose Yeast Extract agar (GGYA) : (g/mL)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin</td>
<td>1</td>
</tr>
<tr>
<td>Glucose</td>
<td>1</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.2</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.02</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.05</td>
</tr>
<tr>
<td>Di pot. Hydrogen phosphate</td>
<td>0.3</td>
</tr>
<tr>
<td>Potassium Di hydrogen phosphate</td>
<td>0.1</td>
</tr>
<tr>
<td>Agar</td>
<td>2</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100</td>
</tr>
<tr>
<td>pH</td>
<td>7.0 ± 2</td>
</tr>
</tbody>
</table>

**(ii) Mercuric Chloride Reagent (g/mL)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercuric Chloride</td>
<td>15</td>
</tr>
<tr>
<td>Con. Hydrochloric acid</td>
<td>20</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100</td>
</tr>
</tbody>
</table>
SCREENING FOR THERMOTOLERANT ISOLATES

The pure isolates were streaked on Nutrient Agar medium and incubated at 55°C for 24 h. The isolates which could tolerate this temperature were classified as thermotolerant (Wong et al., 1990).

SCREENING OF PROTEOLYTIC BACTERIA IN LIQUID MEDIUM FOR THE PRODUCTION OF EXTRACELLULAR PROTEASE ENZYME

To screen out the best proteolytic bacteria and the enzyme activity the experiment was done on production medium (Sen and Satyanarayana, 1993). Fifty mL of production medium was poured in each 150 mL Erlenmeyer flask. The flasks were sterilized for 15 min. Inoculum culture (2%) was introduced into the flasks containing 50 mL of the production medium. After inoculating the flasks with the bacteria the flasks were incubated at 37 ± 1°C without shaking. Triplicate flasks were withdrawn on 0 - 10 d of incubation. 5 mL broth from each flask was centrifuged at 8000 rpm for 15 min. The cell free culture supernatant was used as a source of extracellular protease or otherwise known as crude enzyme extract.

Preparation of Media

<table>
<thead>
<tr>
<th>(l)</th>
<th>Protease Production Media (g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin</td>
<td>1</td>
</tr>
<tr>
<td>Glucose</td>
<td>1</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.2</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.02</td>
</tr>
</tbody>
</table>

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MgSO₄·7H₂O  
Di potassium hydrogen phosphate  
Potassium di hydrogen phosphate  
Distilled water  
pH  

(ii) 2% of Inoculum

2 loopful of 24 hour old culture isolate was dissolved in 100 mL of distilled water. 2 mL of this suspension was taken as 2% of inoculum.

MEASUREMENT OF PROTEASE ACTIVITY/ENZYME ASSAY

Enzyme activity was determined directly in the sample of growth media. Protease activity was determined by the method of casein hydrolysis (Meyers and Ahrean, 1977). The reaction mixture contained 1 mL casein solution incubated with 0.5 mL of 0.05 M sodium citrate buffer and with 0.5 mL of crude enzyme extract. The reaction mixture was incubated for 20 min at 37±1°C. The reaction was stopped by adding 4.0 mL of 5% trichloroacetic acid (TCA) after the period of incubation. This solution was allowed to stand for 1 hour and then passed through Whatman filter paper No.1. One mL of the filtrate was mixed with 2.5 mL of 0.4 M Na₂CO₃ and 0.5 mL of dilute folin ciocalteau reagent. Blank was prepared by mixing 1mL of distilled water with 2.5 mL sodium carbonate and 0.5 mL dilute folin ciocalteau reagent. Liberation of Tyrosine was determined using spectrophotometer at 660 nm against a control prepared
by adding TCA prior to casein solution and compared with standard curve of tyrosine. One unit of activity is defined as the amount of enzyme liberating mg / min / mL of tyrosine under defined conditions.

Preparation of reagents for Enzyme Assay

(i)  **Sodium Citrate Buffer**

Stock Solutions:  
A: 0.1M solution of citric acid  
= 21.01 (g/L)

B: 0.1M solution of sodium citrate  
= 29.4 (g/L)

Mix A and B in ratio 24.4 mL and 73.6 mL respectively to obtain pH 6.2.

(ii) **Casein Solution (g/mL)**

Casein  
- 1

Citrate Buffer  
- 100

(iii) **5% Trichloroacetic acid (mL)**

Trichloroacetic acid  
- 5

Distilled water  
- 95

(iv) **0.4 M Sodium Carbonate (g/mL)**

Sodium carbonate  
- 10.6

Distilled water  
- 250

(v)  **Folin Ciocalteau Reagent (mL)**

Folin ciocalteau (available as lab reagent)  
50

Distilled water  
- 50
PARTIAL CHARACTERISATION OF PROTEASES OF THE SELECTED BACTERIAL ISOLATES

In order to study the effect of the cultural factors, the crude enzyme extract was used. On the basis of the result obtained from the enzyme activity experiments 3 of the 10 isolates were found to have greater enzyme activity (Table-10, Fig.21). It was clear from Table-10 Fig.21 that maximum activity was on the 4 d of incubation for *Planococcus sp.*¹ (DH-2A) and *Planococcus sp.*² (DH-5A) while 6 d of incubation for *Micrococcus lylae* DH-8C. These three isolates showed greater activity than the other isolates and hence these three isolates were selected for further studies. (Table – 11, Fig. 22). The crude enzyme extracts of these bacterial isolates were obtained for the respective days by centrifuging 10.0 mL of production medium at 8000 rpm for 15 min. The filtrate or the crude enzyme extract obtained was stored at 4°C for further studies.

(a) Determination of extracellular protease activity at different incubation time

Activity of the protease was studied at different incubation time using the reaction mixture which contained 1 mL of buffered casein solution and 0.5 mL crude enzyme extract and incubated at 37 ± 1°C in BOD incubator for 15, 30, 45, 60, 75, 90, 105 min. The mouth of the test tubes were covered with aluminium foil. Later the routine enzyme assay method was carried out. After the incubation time the enzyme terminator 5% (trichloroacetic acid solution) was added. Enzyme activity was determined by studying the amount of tyrosine residues liberated at 660 nm against control and absorbance was compared with standard curve of tyrosine.
(b) Effect of pH on protease activity (4 – 8 pH)

Culture supernatants of selected isolates were evaporated to half of its original volume. The reaction mixture contained 1 mL casein solution, 0.5 mL McIlvaine's buffer ranging from pH 4.0 – 8.0 and 0.5 mL of concentrated culture supernatant was incubated at 37±1°C for 20 min. Reaction was stopped with 4 mL of 5% TCA and absorbancy of TCA soluble peptides were measured at 660 nm against control.

Table – 8
Preparation of McIlvaine Buffer
Chemicals - Disodium hydrogen phosphate
- Citric Acid

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>MW (g)</th>
<th>g/mL</th>
<th>Mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄</td>
<td>141.96</td>
<td>14.20</td>
<td>7.10</td>
</tr>
<tr>
<td>Na₂HPO₄. 2H₂O</td>
<td>177.99</td>
<td>17.80</td>
<td>8.90</td>
</tr>
<tr>
<td>Na₂HPO₄. 7H₂O</td>
<td>268.07</td>
<td>26.81</td>
<td>13.40</td>
</tr>
<tr>
<td>Na₂HPO₄. 12H₂O</td>
<td>358.14</td>
<td>35.81</td>
<td>17.91</td>
</tr>
<tr>
<td>C₅H₈O₇. H₂O</td>
<td>210.14</td>
<td>21.01</td>
<td>10.50</td>
</tr>
</tbody>
</table>
The desired pH was obtained by adding 0.2M Na$_2$HPO$_4$ and 0.1M citric acid in the following ratio.

<table>
<thead>
<tr>
<th>Desired pH</th>
<th>0.2M Na$_2$HPO$_4$</th>
<th>0.1M Citric Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>38.5</td>
<td>61.5</td>
</tr>
<tr>
<td>4.4</td>
<td>44.1</td>
<td>55.9</td>
</tr>
<tr>
<td>4.8</td>
<td>49.3</td>
<td>50.7</td>
</tr>
<tr>
<td>5.0</td>
<td>51.5</td>
<td>48.5</td>
</tr>
<tr>
<td>5.6</td>
<td>58.0</td>
<td>42.0</td>
</tr>
<tr>
<td>6.0</td>
<td>63.1</td>
<td>36.9</td>
</tr>
<tr>
<td>6.6</td>
<td>72.7</td>
<td>23.3</td>
</tr>
<tr>
<td>7.0</td>
<td>82.3</td>
<td>17.7</td>
</tr>
<tr>
<td>7.6</td>
<td>93.6</td>
<td>6.4</td>
</tr>
<tr>
<td>8.0</td>
<td>97.6</td>
<td>2.8</td>
</tr>
</tbody>
</table>

(c) Effect of pH on stability of proteases

Stability of proteases in the pH range 4.0 to 8.0 was determined by pre-incubating the concentrated culture supernatant with appropriate buffer 0.5 mL at 4°C for 24 h. Residual activity was subsequently measured as mentioned earlier.

(d) Effect of temperature on protease activity

Temperature optima was determined by incubating reaction mixture containing 1 mL casein solution and 0.5 mL culture supernatant at different temperatures (10, 20, 30, 40, 50 and 60°C) for 20 min. Reaction was stopped by adding 5% of TCA solution and routine enzyme assay was done and tyrosine residues liberated were determined at 660 nm against a control.
(e) Effect of temperature on stability of proteases

The effect of temperature on stability of proteases was determined by pre-incubating 0.5 mL culture supernatant at different temperatures (10, 20, 30, 40, 50 and 60°C) with 1 mL casein at for 90 min. After every incubation period the reaction was stopped by adding 4 mL of 5% TCA and the absorbancy of TCA soluble peptides was measured at 660 nm against a control.

(f) Effect of metal ions on enzyme activity

It was done by adding the salts of the following metals (CaCl₂, MgSO₄, CuSO₄, K₂HPO₄ and BaCl₂) of 1 mM and 5 mM concentration in the reaction mixture. To 1 mL of casein solution 0.5 mL of different concentrations of each metal and 0.5 mL of culture supernatant was added. After 30 min of incubation at 37±1°C the reaction was stopped by the addition of 4 mL of 5% TCA. The routine enzyme assay was carried out and absorbancy was measured at 660 nm against a control and compared with standard curve.

Preparation of Concentration of Various Metals

<table>
<thead>
<tr>
<th>Molecular weight of</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>110.99</td>
</tr>
<tr>
<td>BaCl₂.2H₂O</td>
<td>244.28</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>249.68</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>174.18</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>246.48</td>
</tr>
</tbody>
</table>

- 1 M solution is molecular weight dissolved in 1000 mL water.
- 1 mM is one mL of 1 M solution dissolved in 1000 mL water.
- 5 mM is 5 mL of 1M solution dissolved in 1000 mL water.
(g) Effect of inhibitors on enzyme activity

Different compounds were used to determine the effect of inhibitors on the protease activity of selected bacteria. 1 mM and 5 mM of mercuric chloride, EDTA, sodium azide dissolved in distilled water were the compounds used. 1 mL of casein solution, 0.5 mL of different concentrations of each inhibitor and 0.5 mL of concentrated culture supernatant were incubated at 37°1°C for 20 min. Rest of enzyme assay was subsequently followed as mentioned earlier to record enzyme activity.

Preparation of Concentration of Various Inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>312.24</td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>27.50</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>65.01</td>
</tr>
</tbody>
</table>

- 1 M was prepared by dissolving molecular weight of each inhibitor in 1000 mL water.
- 1 mM is 1 mL of 1M solution dissolved in 1000 mL of water.
- 5 mM is 5 mL of 1M solution dissolved in 1000 mL of water.
(C) RESULTS AND DISCUSSION

SCREENING OF BACTERIAL ISOLATES FOR PROTEASE PRODUCTION ON SOLID SUBSTRATE (GGYA) MEDIUM

In the present investigation all the isolates (120) were screened for protease activity. The plates were observed after 24 h later the plates were flooded with mercuric chloride solution. Clear zones developed around the colonies (Fig. 20a, 20b, 20c and 20d). The halo diam was measured in cms (Table-9, Fig.19). This gave the relative proteolytic activity of the bacterial isolates. The greater the value the greater the activity. Planococcus sp.\(^1\) and Planococcus sp.\(^2\) had a halo diam of 2.2 cm having greatest proteolytic activity while Planococcus sp.\(^3\), Micrococcus varians and Micrococcus sp.\(^1\) had a halo diam of 0.4 cm giving least proteolytic activity. Micrococcus lylae, Micrococcus sp.\(^2\), Planococcus sp.\(^4\), Vibrio sp. and Vibrio metschnikovii had halo diam between 2.2 cm and 0.4 cm. Effective screening on solid substrate (medium with Agar) involved one or few steps for discarding unwanted microorganisms, thereby detecting and maintaining only a meagre percentage of useful microorganisms.

SCREENING FOR THERMOTOLERANT ISOLATES

In the present studies the ten identified bacterial strains were also screened for their thermotolerant nature when grown on Nutrient agar medium, and incubated at 55\(^{\circ}\)C for 24 h. Six isolates were found to be thermotolerant while four bacterial isolates could not tolerate high temperature. In the present investigation six bacterial isolates were found
Table - 9: Proteolytic activity of bacterial isolates on 1% gelatin glucose yeast extract agar medium

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Isolate number</th>
<th>Isolate name</th>
<th>Proteolytic activity (halo diameter in cms.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DH-2A</td>
<td><em>Planococcus sp.</em> 1</td>
<td>2.2</td>
</tr>
<tr>
<td>2</td>
<td>DH-5A</td>
<td><em>Planococcus sp.</em> 2</td>
<td>2.2</td>
</tr>
<tr>
<td>3</td>
<td>DH-8A</td>
<td><em>Planococcus sp.</em> 3</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>DH-8B</td>
<td><em>Planococcus sp.</em> 4</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>DH-8C</td>
<td><em>Micrococcus lylae</em></td>
<td>1.8</td>
</tr>
<tr>
<td>6</td>
<td>DH-10B</td>
<td><em>Micrococcus varians</em></td>
<td>0.4</td>
</tr>
<tr>
<td>7</td>
<td>DH-6C</td>
<td><em>Micrococcus sp.</em> 1</td>
<td>0.4</td>
</tr>
<tr>
<td>8</td>
<td>DH-3B</td>
<td><em>Micrococcus sp.</em> 2</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>DH-3C</td>
<td><em>Vibrio sp.</em></td>
<td>1.1</td>
</tr>
<tr>
<td>10</td>
<td>DH-1C</td>
<td><em>Vibrio metschnikovii</em></td>
<td>1.1</td>
</tr>
</tbody>
</table>

DH - Daphrin Hospital
Site A: Sewage discharge point
B: Sewage mixing point
C: Lake water
Fig. 19: Proteolytic activity of Bacterial isolates on 1% gelatin glucose yeast extract agar medium
Fig. 20a: Screening of isolates for proteolysis on solid substrate
(GGYA) medium
Control
DH - 1B : Unidentified isolate
DH - 10B : Micrococcus varians

Fig. 20b: Screening of isolates for proteolysis on solid substrate
(GGYA) medium
DH - 5A : Planococcus sp.²
DH - 3B : Micrococcus sp.²
DH - 8A : Planococcus sp.²
Fig. 20c: Screening of isolates for proteolysis on solid substrate

(GGYA) medium

DH - 8B : Planococcus sp.\(^4\)
DH - 1C : Vibrio metschnikovii
DH - 6C : Micrococcus sp.\(^1\)

Fig. 20d: Screening of isolates for proteolysis on solid substrate

(GGYA) medium

DH - 2A : Planococcus sp.\(^1\)
DH - 8C : Micrococcus lylae
DH - 3C : Vibrio sp.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Isolate number</th>
<th>Isolate name</th>
<th>Nature of bacterial isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DH - 2A</td>
<td>*Planococcus sp.*¹</td>
<td>Mesophilic</td>
</tr>
<tr>
<td>2</td>
<td>DH - 5A</td>
<td>*Planococcus sp.*²</td>
<td>Thermotolerant</td>
</tr>
<tr>
<td>3</td>
<td>DH - 8A</td>
<td>*Planococcus sp.*³</td>
<td>Thermotolerant</td>
</tr>
<tr>
<td>4</td>
<td>DH - 8B</td>
<td>*Planococcus sp.*⁴</td>
<td>Thermotolerant</td>
</tr>
<tr>
<td>5</td>
<td>DH - 8C</td>
<td><em>Micrococcus lylae</em></td>
<td>Mesophilic</td>
</tr>
<tr>
<td>6</td>
<td>DH - 10B</td>
<td><em>Micrococcus varians</em></td>
<td>Thermotolerant</td>
</tr>
<tr>
<td>7</td>
<td>DH - 6C</td>
<td>*Micrococcus sp.*¹</td>
<td>Thermotolerant</td>
</tr>
<tr>
<td>8</td>
<td>DH - 3B</td>
<td>*Micrococcus sp.*²</td>
<td>Mesophilic</td>
</tr>
<tr>
<td>9</td>
<td>DH - 3C</td>
<td><em>Vibrio sp.</em></td>
<td>Thermotolerant</td>
</tr>
<tr>
<td>10</td>
<td>DH - 1C</td>
<td><em>Vibrio metschnikovii</em></td>
<td>Mesophilic</td>
</tr>
</tbody>
</table>

DH : Daphrin Hospital  
Site A : Sewage discharge point  
B : Sewage mixing point  
C : Lake water  
Temperature : $55 \pm 1^\circ C$ (thermotolerant)  
below $55 \pm 1^\circ C$ (mesophilic)  
Incubation : 24 hrs.
thermotolerant (*Planococcus sp.*, *Planococcus sp.*, *Planococcus sp.*, *Micrococcus sp.*, *Micrococcus varians* and *vibrio metschnikovi*) (Table-10).

Temperature is one of the most important physical factors affecting bacteria. Bacteria are different from higher plants and animals in the lack of homeostatic mechanism and cannot regulate heat generated by metabolism and are therefore, directly and readily affected by temperature. There is a two fold increase in the rate of enzyme catalysed reactions for every 10°C rise in temperature.

It is well known that living activities of organisms are conditioned by their environment. Any marked change in the environment produces a corresponding change in the morphological and physiological characters of organisms. Bacteria are able to withstand with great changes in the environment and quickly adapt themselves to the new conditions. Bacteria are able to survive wide limits of temperature but the range in which they can grow and carry on their activities generally falls between 0 and 90°C. Bacteria which grow best at high temperatures are called thermophilic organisms.

SCREENING OF PROTEOLYTIC BACTERIA IN LIQUID MEDIUM FOR THE PRODUCTION OF EXTRACELLULAR PROTEASE ENZYME

The results presented in Table-11, Fig.21 show that maximum protease enzyme activity was recorded with *Planococcus sp.* (DH-2A), *Planococcus sp.* (DH-5A), *Planococcus sp.* (DH-8A) and *Micrococcus varians* (DH-10B) on 4 d while for *Micrococcus lylae* (DH-8C)
and *Micrococcus sp.*\(^2\) (DH-3B) on 6 d and with *Micrococcus sp.*\(^2\) (DH-6C), *Planococcus sp.*\(^4\) (DH-8B) and *Vibrio metschnikovii* (DH-1C) on 8d. With regard to incubation period, all the bacterial isolates vary from each other, for the production of extracellular proteases.

As the greatest enzyme activity was recorded in *Planococcus sp.*\(^7\) (DH-2A), *Planococcus sp.*\(^2\) (DH-5A) and *Micrococcus lylae* (DH-8C), these three isolates were selected for further studies (Table-12, Fig.22).

An increase in enzyme activity with increase in incubation time was recorded in all the ten isolates. After peak value there was a gradual decrease in the activity. Activity was very less on 10 d which may be due to the denaturation of enzymes with increased incubation period. For *Planococcus sp.*\(^4\) (DH-8B), *Planococcus sp.*\(^7\) (DH-2A) and *Planococcus sp.*\(^2\) (DH-5A) there were two peak values recorded on 4 and 8 d with decrease of enzyme activity on the 6 d of incubation.

All the ten bacterial isolates were also assayed on liquid medium to record protease activity. The majority of enzyme kinetic studies and experiments have been performed *in vitro*, under isothermal, homogenous and closed systems. Closed system means 150 mL Erlenmeyer flask containing 50 mL sterilized production media, (Sen and Satyanarayana, 1993) inoculated with 2% of bacterial culture. In closed systems there is no exchange of materials with the surroundings and in homogenous solutions more precise measurements and theoretical analysis can be made with the concomitant absence of concentration gradients. Steady state measurements are often tremendous assays for enzyme
Table - 11: Protease activity (EU/mL) of bacterial isolates from the effluents of Daphrin hospital into Sagar lake

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Isolate number</th>
<th>Name of isolate</th>
<th>Incubation period (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>DH-2A*</td>
<td>Planococcus sp. 7</td>
<td>0.002</td>
</tr>
<tr>
<td>2</td>
<td>DH-5A*</td>
<td>Planococcus sp. 2</td>
<td>0.003</td>
</tr>
<tr>
<td>3</td>
<td>DH-8A</td>
<td>Planococcus sp. 3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>DH-8B</td>
<td>Planococcus sp. 4</td>
<td>0.001</td>
</tr>
<tr>
<td>5</td>
<td>DH-8C*</td>
<td>Micrococcus lyiae</td>
<td>0.001</td>
</tr>
<tr>
<td>6</td>
<td>DH-10B</td>
<td>Micrococcus varians</td>
<td>0.001</td>
</tr>
<tr>
<td>7</td>
<td>DH-6C</td>
<td>Micrococcus sp. 1</td>
<td>0.001</td>
</tr>
<tr>
<td>8</td>
<td>DH-3B</td>
<td>Micrococcus sp. 2</td>
<td>0.001</td>
</tr>
<tr>
<td>10</td>
<td>DH-3C</td>
<td>Vibrio sp.</td>
<td>0.001</td>
</tr>
<tr>
<td>11</td>
<td>DH-1C</td>
<td>Vibrio metschnikovii</td>
<td>-</td>
</tr>
</tbody>
</table>

DH : Daphrin Hospital
Site A : Sewage discharge point
B : Sewage mixing point
C : Lake water
EU : mg/min/mL tyrosine liberated
Fig. 21: Protease activity (EU/mL) of bacterial isolates from the effluents of Daphrin hospital into Sagar Lake.
activity. In the simplest form steady state kinetics may be performed by commonly available laboratory equipments and has constituted the bulk, performed throughout the development history of enzymology.

Not much information is available on regulation of protease production but studies indicate that it is not a constitutive system. All extra-cellular protease requires the presence of organic nitrogen sources. Every organism is very specific with respect to the nature of inducer. Mckeller (1986) reported in *Pseudomonas*, that 1.5% peptone in the medium gives maximum synthesis while higher concentrations of peptone repressed, the synthesis which is probably due to the feed back inhibition. He has also reported the inducing ability of low molecular weight fraction of skimmed milk in *Pseudomonas*. Long *et al.*, (1981) reported that protease synthesis was sensitive to repression by a number of carbon sources including glucose and amino acids.

Gajju *et al.*, (1996) observed that 4% inoculum of 24 h old culture proved better for enzyme production with 0.75% casein in the production medium. The higher concentration of casein in the medium decreased the enzyme production. Kole *et al.*, (1988) reported that higher concentration of nitrogen sources tend to suppress the protease production. Fujiwara and Yamamoto (1987) have found organic sources to enhance the production of enzyme. Gelatin, glycine and potassium nitrate (nitrogen sources) decreased the production of enzyme. Similar results have also been obtained with other *Bacillus species* (Moon and Parulekar, 1990; Razak *et al.*, 1994; Dhandapani and Vijayaragavan, 1994).
Tsuchinder et al., (1980) observed that alkaline protease production started at stationary phase. It was maximum on 4 d which gradually decreased up to 8 d of incubation. Sinha and Satyaranayana (1991) reported that Bacillus licheniformis strain N3 produced alkanine protease and starch soyameal medium supported better production of protease. The use of soyameal in the production medium for protease has been reported by many workers (Kimura and Horikoshi, 1988; Kroll, 1990; Horikoshi and Akiba, 1982).

Ohkoshi et al., (1985) reported inhibition of protease enzyme production by casamino acids. The production of an extracellular proteolytic enzyme during the stationary phase of growth is a characteristic of bacterial species (Honda et al., 1985; Durham et al., 1987 and Horikoshi, 1971).

Purva et al., (1998) observed the synthesis of protease in late log phase (after 24 h) which then increased throughout the stationary phase for the alkalophilic Bacillus sp. IS-3. They also observed maximum yield of protease after 72 h of incubation while further incubation resulted in sharp decline in protease activity which might be due to cessation of enzyme synthesis together with auto proteolysis. The enzyme production of UV mutant Bacillus polymyxa in modified Reese medium was investigated by Madan et al., (2000). They also reported that stationary and submerged conditions at 50°C supported maximum enzyme production after 72 h of incubation. Submerged conditions proved to be more effective for protease production than stationary conditions. Matta et al., (1994) while studying the effect of agitation on the production of extracellular protease enzyme
by *Pseudomonas* sp. AFT-36 found better protease production under continuous agitation (180 rpm) than under intermittent or no agitation.

Protease production by *B. licheniformis* S-40 was reduced to half its maximum level when glucose was present as the carbon source (Sen and Satyanarayana, 1993). This observation was also supported by Sonnleitner (1993). Battaglino *et al.*, (1991) reported that glucose repressed protease synthesis while Gomaa *et al.*, reported glucose to be the best carbon source for protease production by *B. subtilis*.

Kaur *et al.*, (1998) used different inducers (soyameal, gelatin and tryptone 0.5 %) and evaluated protease production in *Bacillus polymyxa* and reported that enzyme activity in presence of soyameal, casein, tryptone and gelatin was 739, 827, 510 and 600 U, respectively indicating soyameal and casein to be good inducers of enzyme activity. Casein concentration when varied from 0.25 to 1.0% resulted in enzyme activity ranging from 246 to 827 U. While casein (0.5%) was found to be optimum, higher or lower concentrations severely inhibited enzyme production.

It is known that due to hydrolysis of casein or any protein by extracellular protease, amino acids are liberated which stay in the medium and these are estimated by the method of Lowry *et al.*, (1951). The principle behind the test was that phenol reagent of folin and ciocalteau is essentially a phosphotungstic phosphomolybdic acid solution and is reduced by substances like aromatic amines. Glucose, urea and amino acids (specifically tyrosine, tryptophan react with folin ciocalteau reagent and reduce it) to molybdenum blue which is determined spectrophoto-
metrically. Other amino acids do not produce significant colouration. The colour produced was entirely due to content of tyrosine residue.

PARTIAL CHARACTERISATION OF PROTEASES OF THE SELECTED BACTERIAL ISOLATES

In the present investigation the various cultural factors on enzyme activity and stability were studied. Each factor and its influence on production of enzyme has been discussed below in separate headings.

We know that the growth of bacteria is influenced by a variety of physical and chemical conditions. Since enzymes are responsible for catalysing the reaction associated with life processes conditions like pH, temperature affect the enzyme and thereby altering growth responses. The rate of enzyme reactions are influenced by a number of factors (i) temperature (ii) pH (iii) inhibitors and (iv) activators. The same factors which influence the growth and multiplication of bacteria also affect the action of enzymes. Setting up an enzyme kinetics experiment, cultural factors (pH, temperature, incubation time, metals and inhibitors) affect the synthesis and activity of enzymes significantly. These factors can work independently or in a cumulative / joint way, therefore, making any independent conclusion about these factors would be difficult. It is now well known that an optimum temperature and pH is essential for maximum enzyme activity.
(a) Effect of incubation time on protease activity

The normal enzyme assays were carried out for 30 min of incubation time. An experiment with different incubation time (15, 30, 45, 60, 75, 90, 105, 120 min) was carried out to study effect on the enzyme activity.

Table-13 and Fig.23 show that for Planococcus sp.\(^1\) (DH-2A) and Micrococcus lylae (DH-8C), maximum enzyme activity was recorded at 45 min of incubation time while least enzyme activity was at 120 min. Planococcus sp.\(^2\) (DH-5A) showed maximum enzyme activity at 60 min of incubation time while least enzyme activity was at 120 min. In can be concluded from the records, that as incubation time increased, there was an increase in enzyme activity. At a particular incubation time isolates showed greatest enzyme activity and as incubation time was further increased, a decreased enzyme activity was recorded which may be due to denaturation of enzyme due to longer exposure of enzyme to incubation time.

Enzyme synthesis / secretion appeared to be strictly related to cell growth, incubation time and pH of the medium. Kaur et al., (1998) reported that incubation of isolate Bacillus polymyxa at 70\(^0\)C for 10 min in modified Reese medium gave about 3 times more protease enzyme activity compared to that in the seed medium. Gajju et al., (1996) characterized protease enzyme of thermophilic Bacillus coagulains PB-77 at different time intervals (10-50 min). Increase in incubation time denatures the enzyme. Results of the present investigation are also in accordance with the above findings.
### Table 12: Protease activity (EU/mL) of the three selected bacterial isolates

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Isolate number</th>
<th>Name of isolate</th>
<th>Incubation period (d)</th>
<th></th>
<th></th>
<th></th>
<th></th>
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<td>6</td>
<td>8</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>DH-2A</td>
<td><em>Planococcus sp.</em> ¹</td>
<td>0.002</td>
<td>0.023</td>
<td>0.041</td>
<td>0.021</td>
<td>0.031</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DH-5A</td>
<td><em>Planococcus sp.</em> ²</td>
<td>0.003</td>
<td>0.023</td>
<td>0.044</td>
<td>0.026</td>
<td>0.043</td>
<td>0.034</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>DH-8C</td>
<td><em>Micrococcus lylae</em></td>
<td>0.001</td>
<td>0.022</td>
<td>0.037</td>
<td>0.041</td>
<td>0.039</td>
<td>0.036</td>
<td></td>
</tr>
</tbody>
</table>

DH : Daphrin Hospital  
Site A : Sewage discharge point  
C : Lake water  
EU : mg/min/mL tyrosine liberated
Table - 13: Effect of Incubation period on protease activity (EU/mL) of the three selected bacterial isolates

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Isolate number</th>
<th>Name of isolate</th>
<th>Incubation Time (in min)</th>
</tr>
</thead>
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<td></td>
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<td>15</td>
</tr>
<tr>
<td>1</td>
<td>DH-2A</td>
<td>Planococcus sp.¹</td>
<td>0.041</td>
</tr>
<tr>
<td>2</td>
<td>DH-5A</td>
<td>Planococcus sp.²</td>
<td>0.022</td>
</tr>
<tr>
<td>3</td>
<td>DH-8C</td>
<td>Micrococcus lyiae</td>
<td>0.025</td>
</tr>
</tbody>
</table>

DH : Daphrin Hospital
Site A : Sewage discharge point
C : Lake water
EU : mg/min/mL tyrosine liberated
Fig. 22: Protease activity (EU/mL) of the three selected bacteria

![Graph showing protease activity over time for three different bacteria species.]

Fig. 23: Effect of incubation time on protease activity (EU/mL) of three selected bacteria

![Graph showing protease activity over time for three different bacteria species.]
(b) Effect of pH on enzyme activity

Normal enzyme assays were carried out at pH 6.2. To see the effect of pH on enzyme activity, different pH values (4, 5, 6, 7 and 8) were selected and the results have been presented in Table-14, Fig.24a.

It is clear from the data that as compared to the normal enzyme assay values, the protease of *Planococcus sp.*\(^1\) (DH-2A) showed maximum activity at pH 7.0 while *Planococcus sp.*\(^2\) (DH-5A) and *Micrococcus lylae* (DH-8C) showed maximum enzyme activity at pH 8.0. The protease of *Planococcus sp.*\(^1\) (DH-2A) was of neutral type while *Planococcus sp.*\(^2\) (DH-5A) and *Micrococcus lylae* (DH-8C) produced alkaline protease. Majority of organisms producing alkaline proteases show growth and enzyme production better under alkaline conditions.

There are now several proofs to show that the enzyme and substrate bind together to form a complex. This binding of the substrate to the enzyme takes place at a specific site on the enzyme called the 'active centre', and the nature of binding is mainly electrostatic depending on the charges available on the substrate and the active centre. Enzymes are markedly influenced by the hydrogen ion concentration of the solution. Some enzymes act best in acid solutions while others require alkaline solutions, still others do not function well unless their environments are neutral. There exists for every enzyme a maximum, optimum and minimum pH. The pH values vary with temperature, type and concentration of substrate, type of buffer, presence or absence of inhibitors or activators and period of action of enzyme. An enzyme can tolerate slight changes in
acidity or alkalinity without being destroyed. Readjustment of the reaction to optimum pH restore fully the activity of the enzyme.

Thermostable alkaline protease of *Bacillus* sp. NG – 27 when partially purified and characterized retained more than 50% activity over a broad pH range of 8-12 while optimum pH was 9.2 (Sumandep et al., 1999). The purified enzyme of thermostable *Bacillus polymyxa* was found to be optimally active at pH 9.5 (Kaur et al., 1998). For *B. steareothermophilus* optimum pH of 9.5 has been recorded for the protease enzyme (Dhandapani and Vijayaraghavan, 1994). Thermophilic Bacilli which grow and produce enzyme at 50 – 60°C and pH 6-7 have been found to produce protease enzymes which prefer alkaline pH for enzyme activity (Sonnleitner, 1983). Joshi and Ball (1993) reported that *Bacillus brevis* II isolated from Kilns near Jabalpur was a best protease producer at pH 10.

Enzymes have a characteristic optimum pH at which their activity is maximal. The pH activity profiles of enzymes reflect the pH at which important proton donating or proton accepting groups in the enzyme catalytic site are in their required state of colonization. The optimum pH of an enzyme is not necessarily identical with the pH of the normal surroundings but may be just above or below the optimum pH. The catalytic activity of enzymes in cells may thus be regulated in part by changes in the pH of the surrounding medium. The pH changes the ionisation of the side chains in the enzymes and those in the active site which contribute to the enzyme activity. The three dimensional integrity of the enzymes is lost due to drastic pH changes.
(c) Effect of pH on stability of protease

The effect of pH on the stability of the enzyme was studied over a pH range of 4-8 using different buffers. The enzyme activity was maximum at pH 7.0 for *Planococcus sp.*\(^1\) (DH 2A) and the enzyme was quite stable at this pH. *Planococcus sp.*\(^2\) (DH-5A) and *Micrococcus lylae* (DH-8C) showed maximum activity of enzyme at pH 8.0 and it was quite stable at this pH (Table-15, Fig. 24b). These two isolates were also found to produce alkaline protease. Alkaline protease generally have broad pH optima in range of 8 – 12 (Madan, 2002). Sharma *et al.*, (1996) reported that the protease enzyme from *B. laterosporus* was stable from pH 6 to 9 while from *Flavobacterium sp.* it was stable from pH 7-9. Sinha and Satyanarayan (1991) reported that the strain N\(_3\) of *Bacillus sp.* showed high stability at pH 10.0, although a small peak at pH 6.0 was also observed, suggesting the presence of neutral and alkaline proteases. Multiple proteolytic enzymes of *B. licheniformis* were reported by Hall *et al.*, (1966); Bernhor and Novelli (1963) and Vitkovic and Sadoff (1977).

Madan *et al.*, (2000) reported that upto pH 9.5 protease enzyme was quite stable and retained full activity for the UV mutant of *Bacillus polymyxa*. Protease from *B. lentus* exhibited stability in the pH range 7-10 (Betze! *et al.*, 1992). A broader pH stability in the range pH 6-13 was reported for protease from *B. amyoliquefaciens*, *B. licheniformis* and *Bacillus sp.* No. AH 101 (Sinha and Satyanarayana; 1991; El-Beih *et al.*, 1991; Takami *et al.*, 1992).
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Isolate number</th>
<th>Name of isolate</th>
<th>pH</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.0</td>
<td>5.0</td>
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<td>7.0</td>
</tr>
<tr>
<td>1</td>
<td>DH-2A</td>
<td><em>Planococcus sp.</em></td>
<td>0.014</td>
<td>0.016</td>
<td>0.008</td>
<td>0.020</td>
</tr>
<tr>
<td>2</td>
<td>DH-5A</td>
<td><em>Planococcus sp.</em></td>
<td>0.017</td>
<td>0.011</td>
<td>0.015</td>
<td>0.024</td>
</tr>
<tr>
<td>3</td>
<td>DH-8C</td>
<td><em>Micrococcus lylae</em></td>
<td>0.019</td>
<td>0.019</td>
<td>0.017</td>
<td>0.023</td>
</tr>
</tbody>
</table>

DH: Daphrin Hospital  
Site A: Sewage discharge point  
C: Lake water  
EU: mg/min/mL tyrosine liberated
Table - 15: Effect of pH on Stability of protease (EU/mL) of three selected bacterial isolates

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Isolate number</th>
<th>Name of isolate</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td>1</td>
<td>DH-2A</td>
<td><em>Planococcus sp.</em> ¹</td>
<td>0.017</td>
</tr>
<tr>
<td>2</td>
<td>DH-5A</td>
<td><em>Planococcus sp.</em> ²</td>
<td>0.015</td>
</tr>
<tr>
<td>3</td>
<td>DH-8C</td>
<td><em>Micrococcus lylae</em></td>
<td>0.020</td>
</tr>
</tbody>
</table>

DH : Daphrin Hospital  
Site A : Sewage discharge point  
C : Lake water  
EU : mg/min/mL tyrosine liberated
Fig. 24a: Effect of pH on protease activity (EU/mL) of three selected bacteria

Fig. 24b: Effect of pH on stability of protease (EU/mL) of three selected bacteria
(d) Effect of temperature on protease activity

The culture filtrate of *Planococcus sp.* (DH-2A), *Planococcus sp.* (DH-5A) and *Micrococcus lylae* (DH-8C) were assayed at different temperatures (Table-16, Fig.25a). The results indicate that maximum activity for *Planococcus sp.* (DH-2A) and *Micrococcus lylae* (DH-8C) was at 40°C, whereas the maximum activity for *Planococcus sp.* (DH-5A) was at 50°C. The values subsided on both the lower and upper ends of variations in temperature.

*Planococcus sp.* (DH-5A) is a thermotolerant isolate (Table-9) which shows significantly an increase in enzyme activity at and above 40°C whereas *Planococcus sp.* (DH-2A) and *Micrococcus lylae* (DH-8C) showed a decrease in enzyme activity above 40°C, signifying them as not being thermotolerant. More decrease in enzyme activity with increase in temperature may be due to partial degradation of the enzymes.

Like any chemical reaction, the velocity of an enzymic reaction also increases with temperature but only upto a certain point. Beyond this point, phenomena like denaturation of proteins takes over. But a study of the response of reaction velocity to temperature helps us to calculate a very important parameter, namely the activation energy of the reaction.

Each enzyme functions optimally at a particular pH and temperature. Extreme variations in temperature, boiling for a few min will denature most enzymes. Extremely low temperatures, for all practical purposes stop enzyme activity but do not destroy the enzymes. Many enzymes can be preserved by holding them at temperature around 0°C or
lower. Temperature is an important parameter which affects the enzyme activity by altering the rate constant in accordance to Arrhenius law, by inactivating or denaturing the enzyme, thereby causing temperature dependant conformational changes and the mechanism of catalytic reaction.

A number of alkaline proteases isolated from *Bacillus* sp. have high optimal temperature for their activity. This is very important characteristic for the use of alkaline protease as detergent additive (Purva et al., 1998). Sharma et al., (1996) reported that *Bacillus laterosporus* and *Flavobacterium* sp. isolated from gelatin factory effluents showed maximum enzyme activity at 60°C after 90 min of incubation. Sumandeep et al., (1999) reported that protease of thermostable alkaline protease of *Bacillus* sp. NG-27 retained more than 50% activity over a temperature range of 25°C - 80°C with an optimum of 40°C. The purified enzyme of thermophilic *Bacillus coagulans* PB-77 was found to be optimally active at temperature 70°C. A temperature of 70°C has also been reported for alkaline protease from some thermophilic *Bacillus* sp. (Takami et al., 1989). Thermophilic *Bacilli* which grow and produce enzyme at 50 – 55°C have been found to produce enzymes which prefer alkaline pH and higher temperature (Sonnleitner, 1983). Sen and Satyanarayana (1993) reported that *Bacillus licheniformis* S-40 had 45°C as optimum temperature for the production of protease. Similar observation have also been reported by Manachini et al., (1988).
(e) Effect of temperature on stability of protease activity

The results of the present investigation reveal that the activity of crude enzyme increased progressively when assayed at different temperatures. The results recorded in Table-17, Fig.25b show that when the reaction mixture was exposed to various temperatures 10\(^0\)C– 60\(^0\)C for 90 min, the protease enzyme of Planococcus sp.\(^1\) (DH-2A) was stable at temperature below 45\(^0\)C whereas Planococcus sp.\(^2\) (DH-5A) and Micrococcus lylae (DH-8C) were stable at 30 to 50\(^0\)C. Most enzymes in solution are more or less stable at temperature below 45\(^0\)C but at 50\(^0\)C and above inactivation increases rapidly. Majority of enzymes are irreversibly destroyed at a temperature range of 70 to 80\(^0\)C.

Purva et al., 1998 reported that a number of alkaline proteases isolated from Bacillus sp. have high optimal temperature for their activity. This is very important characteristic for the use of alkaline protease as detergent additive. Effect of temperature on activity and stability of an alkalophilic Bacillus sp. IS – 3 reveals that the activity of partially purified enzyme increased progressively upto 60\(^0\)C. On further increasing the temperature to 70\(^0\)C protease activity declined and only 60\% of the maximum activity remained. Madan et al. (2002) found that the enzyme of UV mutant Bacillus polymyxa was fully stable and showed 100 \% enzyme activity at 50\(^0\)C. Thereafter, the activity declined and was 61\% and 40\% of the original activity at 60\(^0\)C and 70\(^0\)C respectively. However, the enzyme was completely inactivated at 80\(^0\)C. Sumandeep et al., (1999) found that enzyme of Bacillus sp. NG – 27 was stable at 80\(^0\)C for more than 1 h and
had half life of 55 min and 50 min at 90°C and 99°C respectively. This thermostability was comparatively higher in many available reports (Chu et al., 1992; Kobayashi et al., 1995 and Codbear et al., 1937). Kaur et al., (1998) studied the stability of enzyme of *Bacillus polymyxa* at three temperatures (4, 25 and 50°C) for different periods of time. There was no loss in enzyme activity upto 4 d at 25 and 40°C. At 4°C the enzyme was quite stable for one month while at 50°C the enzyme was stable only for 24 h which retained 50% of its original activity after 4 d and after one week the enzyme activity was completely lost.

Thermostability studies of proteases in the published literature show that the enzyme has half-life of 10 min (Horikoshi, 1990; Chu et al., 1992) and 23 min at 60°C (Kwan et al., 1994). Kobayashi et al., (1996) reported that alkaline serine protease of *Bacillus sp*. KSM – K16 remained stable at 50 and 60°C for 10 min. Ferrero et al., (1996) also reported thermostable protease from *B. licheniformis* MIR 29 which showed 100% stability upto 70°C for 10 min. The velocity of enzyme reaction is accelerated by an increase in temperature. This continues until a maximum is reached after which the velocity gradually decreases resulting finally in the destruction of the enzyme. Each enzyme has its own characteristic minimum, optimum and maximum temperatures. An enzyme displays its maximum activity at the optimum temperature. The temperature above which an enzyme is no longer active is known as maximum temperature and that temperature below which it cannot function is known as minimum temperature.
Table - 16: Effect of Temperature on protease activity (EU/mL) of three selected bacterial isolates

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Isolate number</th>
<th>Name of isolate</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>10</td>
</tr>
<tr>
<td>1</td>
<td>DH-2A</td>
<td><em>Planococcus sp.</em> ¹</td>
<td>0.010</td>
</tr>
<tr>
<td>2</td>
<td>DH-5A</td>
<td><em>Planococcus sp.</em> ²</td>
<td>0.015</td>
</tr>
<tr>
<td>3</td>
<td>DH-8C</td>
<td><em>Micrococcus lylae</em></td>
<td>0.009</td>
</tr>
</tbody>
</table>

DH : Daphrin Hospital  
Site A : Sewage discharge point  
C : Lake water  
EU : mg/min/mL tyrosine liberated
### Table - 17: Effect of Temperature on Stability of protease activity (EU/mL) of three selected bacterial isolates

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Isolate number</th>
<th>Name of isolate</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>DH-2A</td>
<td>*Planococcus sp.*¹</td>
<td>0.013</td>
</tr>
<tr>
<td>2</td>
<td>DH-5A</td>
<td>*Planococcus sp.*²</td>
<td>0.015</td>
</tr>
<tr>
<td>3</td>
<td>DH-8C</td>
<td><em>Micrococcus lylae</em></td>
<td>0.019</td>
</tr>
</tbody>
</table>

DH : Daprin Hospital  
Site A : Sewage discharge point  
C : Lake water  
EU : mg/min/mL tyrosine liberated
Fig. 25a: Effect of Temperature on protease activity (EU/mL) of three selected bacteria

Fig. 25b: Effect of Temperature on stability of protease (EU/mL) of three selected bacteria
(f) Effect of metal ions on enzyme activity

In the present study, Planococcus sp.\(^1\) (DH-2A), on addition of calcium ions showed an enhanced enzyme activity while other metals like MgSO\(_4\), K\(_2\)HPO\(_4\) and BaCl\(_2\) showed inhibitory effect.

In Planococcus sp.\(^2\) (DH-5A) the addition of divalent cations CaCl\(_2\) at 1 mM concentration enhanced enzyme activity while other cations showed slight inhibitory effect. The extracellular alkaline proteases are known to increase at high temperature with the addition of Ca\(^{++}\) (Prtest et al., 1977). Planococcus sp.\(^2\) (DH-5A) produced thermotolerant alkaline protease. Micrococcus lylae (DH-8C) also showed enhanced enzyme activity with addition of Ca and Mg metals. Slight inhibitory effect was also seen with addition of the other metals (Table-18, Fig.26a and 26b).

Some substances are capable of restoring the activity of enzymes. These are called activators. Many enzymes are activated by heavy metals (silver, zinc, mercury, calcium and copper). A large number of enzymes need metal ions for activation. Such enzymes are called metallo enzymes. The binding of metal ions to enzymatic protein also plays a structural role by stabilizing an effective conformation of the active centre. While some enzymes use zinc, but Mg\(^{++}\), Ca\(^{++}\), Na\(^{++}\) and K\(^{++}\) are also used by a large number of other enzymes for activation. Metallo enzymes which contain iron or copper in their active centre are most frequently used for catalysis in the oxidation-reduction processes.
The present study deals with various metals (CaCl₂, MgSO₄, CuSO₄, K₂HPO₄ and BaCl₂) to record the percentage of its effect on enzyme activity. For all the three isolates the metals CaCl₂, MgSO₄ and CuSO₄ showed increased activity at 1 mM concentration and decreased activity at 5 mM concentration, whereas some metals (K₂HPO₄ and BaCl₂.2H₂O) showed increased activity at 5 mM concentration and decreased activity at 1 mM concentration. This shows that the concentration of the metal ions also influence the increase or decrease in enzyme activity.

Thermopholic *Bacillus* strains showed enhanced proteolytic activity with Fe²⁺, Fe³⁺ and Co²⁺ metal ions significantly, while Ca²⁺, Zn²⁺ metal ions could not do so. The addition of divalent cations Cu²⁺ and Co²⁺ enhanced the thermostability and stimulated the enzyme activity. While other divalent cations like Ca²⁺ and Zn²⁺ had slight inhibitory effect, Mg²⁺ had no effect (Sinha and Satyanarayana, 1991). Sharma et al., (1996) studied that Ba²⁺ and Ca²⁺ stimulated the activity of *Bacillus* protease, whereas inhibition was recorded with Fe²⁺, Mg²⁺ and Zn²⁺. For *Flavobacterium* protease Ba²⁺, Ca²⁺ and Fe²⁺ were found to stimulate the enzyme activity while Mg³⁺, Mn²⁺ and Zn²⁺ were found inhibitory. Sumandeep et al., (1999) reported that protease from *Bacillus* sp. NG-27 was found stimulated by addition of 10 mM Ca²⁺ and Ni³⁺ ions. Kaur et al., (1998) studied the influence of a number of metal ions such as Mg²⁺, Mn²⁺, Co²⁺ and Hg²⁺ on enzyme activity of *Bacillus polymyxa* and observed that Cu²⁺ and Hg²⁺ at 1 mM concentration were found as the
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Metal</th>
<th>Concentration (mM)</th>
<th>Planococcus sp.¹ (DH - 2A)</th>
<th>Planococcus sp.² (DH - 5A)</th>
<th>Micrococcus lylae (DH - 8C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>EA</td>
<td>Effect (%)</td>
<td>EA</td>
</tr>
<tr>
<td>1</td>
<td>CaCl₂</td>
<td>1</td>
<td>0.021</td>
<td>+61.54</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.009</td>
<td>-30.77</td>
<td>0.021</td>
</tr>
<tr>
<td>2</td>
<td>MgSO₄</td>
<td>1</td>
<td>0.020</td>
<td>+53.85</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.015</td>
<td>+15.38</td>
<td>0.023</td>
</tr>
<tr>
<td>3</td>
<td>K₂HPO₄</td>
<td>1</td>
<td>0.009</td>
<td>-30.77</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.012</td>
<td>-7.69</td>
<td>0.021</td>
</tr>
<tr>
<td>4</td>
<td>CuSO₄</td>
<td>1</td>
<td>0.011</td>
<td>-15.38</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.010</td>
<td>-23.08</td>
<td>0.019</td>
</tr>
<tr>
<td>5</td>
<td>BaCl₂</td>
<td>1</td>
<td>0.012</td>
<td>-7.69</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.017</td>
<td>+30.76</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>Control (C)</td>
<td></td>
<td>0.013</td>
<td>-</td>
<td>0.025</td>
</tr>
</tbody>
</table>

EA : Enzyme activity
Effect (%) : C - EA x 100 / C
Compared to control increased activity = +
Compared to control decreased activity = -
Fig. 26a: Effect of Calcium Chloride on protease activity (EU/mL) of three selected bacteria

![Bar chart showing inhibition of enzyme activity (%) against concentration (mM) for different bacteria species.]

Fig. 26b: Effect of Magnesium sulphate on protease activity (EU/mL) of three selected bacteria

![Bar chart showing inhibition of enzyme activity (%) against concentration (mM) for different bacteria species.]

Legend:
- Planococcus sp.¹
- Planococcus sp.²
- Micrococcus lylae
Fig. 26c: Effect of Dipotassium hydrogen phosphate on protease activity (EU/mL) of three selected bacteria

Fig. 26d: Effect of Copper sulphate on protease activity (EU/mL) of three selected bacteria
Fig. 26e: Effect of Barium chloride on protease activity (EU/mL) of three selected bacteria
most potent inhibitors. Gajju et al., (1996) reported that protease activity of thermophilic Bacillus coagulans PB – 77 was greatly enhanced by the presence of 2mM and 5mM concentration of Fe^{2+} and Fe^{3+}, whereas Hg^{2+} severely inhibited this enzyme activity. A psychrophilic strain of B. coagulans have been reported to require 4mM of Fe^{2+} to stimulate the protease activity (Sai Ram et al., 1994). Hg^{2+} suppresses the enzyme activity in most of the thermophilic bacteria (Rahman et al., 1994 and Kabayashi et al., 1995). The extracellular alkaline proteases are known to be stabilized by Ca^{++} at high temperature (Vitkovic et al., 1977; Keay et al., 1970; Ottensen and Svendsen, 1971; and Prtest, 1977). Sinha and Satyanarayana (1991) observed that Ca^{++} caused a slight inhibition of the enzyme from Bacillus licheniformis.

Janssen et al., (1994) reported that the addition of Fe^{2+} and Mg^{2+} enhanced and stabilized the enzyme production. Ferrero et al., (1996) reported the use of trisodium citrate along with MgSO_4, CaCl_2, MnSO_4, ZnSO_4 for protease production by B. licheniformis MIR.29. Thus from the above studies a conclusion can be drawn that the presence of metal ions may increase or decrease particular enzyme activity.

(g) Effect of inhibitors on enzyme activity

Results recorded in Table-19 and Fig.27b shows that Planococcus sp.¹ (DH-2A) and Planococcus sp.² (DH-5A) when tested with inhibitors (EDTA, mercuric chloride and sodium azide), only EDTA could inhibit the protease enzyme activity upto a certain level whereas sodium
azide showed slight or insignificant inhibition on enzyme activity (Fig. 27c). *Micrococcus lylae* (DH-8C) showed less percentage of inhibition when tested with all the inhibitors. It does not show inhibition with EDTA therefore it is not regarded as serine protease. The inhibition of protease by EDTA is due to its binding with Ca$^{2+}$ ions, thereby rendering the Ca$^{2+}$ ions inaccessible to the enzyme and making it less active. The activity can be restored by using high concentration of Ca$^{2+}$ ions.

It is also known that if protease activity was not inhibited by EDTA, it suggests that metal ion as co-factor is not required for its catalytic function (Sinha and Satyanarayana, 1991). Rahman *et al.*, (1994) and Kobayashi *et al.*, (1995) also studied that Hg suppresses the enzyme activity in most of the thermophilic bacteria. Purva *et al.*, (1998) reported that enzyme synthesis of *Bacillus* sp. IS-3 was significantly enhanced (51.54%) when sodium acetate was used in combination with KH$_2$PO$_4$, MgSO$_4$ and CaCl$_2$.2H$_2$O.

Concentration of 1 mM of inhibitors (EDTA, PMSF and sodium azide) were tested on enzyme activity of *Bacillus polymyxa* resulting in 63% and 55% inhibition with PMSF and sodium azide respectively. EDTA was found as the most potent inhibitor. Kaur *et al.*, (1998) used 10 mM concentration of EDTA and SDS to determine enzyme activity of *Bacillus polymyxa*. Gajju (1996) reported that EDTA inhibited about 60% of enzyme activity in thermophilic *Bacillus coagulans* indicating the partial requirement of metal ions. Sharma *et al.* (1996) found that the activity of *Bacillus* and *Flavobacterium* protease...
Table - 19: Effect of Inhibitors on the protease activity (EU/mL) of three selected bacterial isolates

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Inhibitors</th>
<th>Concentration (mM)</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Planococcus sp.¹ (DH - 2A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EA Effect (%)</td>
</tr>
<tr>
<td>1</td>
<td>Mercuric Chloride</td>
<td>1</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.009</td>
</tr>
<tr>
<td>2</td>
<td>EDTA</td>
<td>1</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.006</td>
</tr>
<tr>
<td>3</td>
<td>Sodium Azide</td>
<td>1</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Control (C)</td>
<td></td>
<td>0.011</td>
</tr>
</tbody>
</table>

EA: Enzyme activity
Effect (%): C - EA x 100 / C
Compared to control increased activity = +
Compared to control decreased activity = -
Fig. 27a: Effect of Mercuric chloride on protease activity (EU/mL) of three selected bacteria

Fig. 27b: Effect of EDTA on protease activity (EU/mL) of three selected bacteria
Fig. 27c: Effect of Sodium azide on protease activity (EU/mL) of three selected bacteria

Inhibition of enzyme activity (%) vs. Concentration (mM)

- *Planococcus sp.*
- *Planococcus sp.*
- *Micrococcus lylae*
was affected by EDTA. Kaur et al., (1998) observed that the protease enzyme from thermostable *Bacillus polymyxa* was completely ignited by metal chelator EDTA and also by an active-site inhibitor of serine protease PMSF, hence it appears to be a metal ion dependent alkaline serine protease. Similar results were also observed by North (1982).

The normal state of living matter is a delicately balanced, spatially and temporally co-ordinated organisation. If a substance causes an adverse effect on this balance, it is usually termed as a poison, alternatively if it redresses a pathophysiological imbalance it is regarded as a drug. Both act as enzyme inhibitors. An enzyme inhibitor decreases the activity of an enzyme without significantly disrupting its three dimensional macromolecular structure. Inhibition is therefore distinct from denaturation and is the result of a specific action by a reagent directed or transmitted to the active site region. Studies on enzyme inhibition can yield much information on the mechanisation of enzyme catalysis.

Inhibition of enzymic activity is subject to both chemical and physical factors. A large number of reagents that bind themselves to sulphhydryl groups of proteins will inhibit the enzyme. Strong acids generally inhibit enzymes by hydrolysing the protein. Besides, many drugs also inhibit enzymes. Most enzymes can be poisoned or inhibited by certain chemical reagents. From the study of enzyme inhibitors valuable information has been obtained about the substrate specificity of enzymes, the nature of the functional groups at the active site and the mechanism of the catalytic activity. Enzyme inhibitors are also very useful in

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elucidating metabolic pathways in cells moreover, some drugs useful in medicine appear to function as they can inhibit certain enzymes in malfunctioning of the cells.

Irreversible inhibitors of some enzyme inhibit the hydroxyl groups of serine. Thiol groups of cysteine have been identified as participating in the catalytic activity of different classes of enzymes. Certain compounds (drugs and poisons) combine with an enzyme but do not serve as substrate. Thus they block catalysis and function as inhibitors. The inhibitors thus closely resemble with the substrate in structure. The enzyme and inhibitor form an enzyme inhibitor complex which is inactive of the inhibitor complex and when sufficient in high concentration displaces the substrate molecules.