1.2.1. MATERIALS

1.2.1.1. Organism:

The strain STS 1 was taken from the stock maintained at the Microbiology Unit, School of Life Sciences, Jawaharlal Nehru University, New Delhi. This strain was isolated from live termite infested mound soils from the virgin soils of Jawaharlal Nehru University.

1.2.1.2. Chemicals:

Coomassie brilliant blue, Crystal violet, Safranin, Malachite green, Bovine serum albumin, Amino acids, Uranyl acetate, Sugars, Creatine, Carboxymethyl cellulose (low viscosity), Lysozyme, Polyethylene glycol (6000), PNPG, PNPX and Casein were obtained from Sigma Chemical Company, St. Louis, USA. Yeast extract, Beef extract, Tryptone and Peptone were from Diffco, Michigan, USA. Dinitrosalicylic acid and Xylan (oat spelt) were obtained from Fluka, Switzerland. All other chemicals used were of analytical grade.
1.2.2. MICROBIOLOGICAL METHODS

1.2.2.1. Maintenance of the culture:

The strain was maintained in liquid medium as well as in solid medium on slants and was stored at 4°C.

1.2.2.2. Basal medium for growth, maintenance and preservation

Ref: Paul et al. (1985).

<table>
<thead>
<tr>
<th>Medium</th>
<th>(g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃ COONa</td>
<td>3.0</td>
</tr>
<tr>
<td>Na₂ SO₄</td>
<td>0.4</td>
</tr>
<tr>
<td>KCl</td>
<td>0.8</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.2</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.3</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.3</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>0.01</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.03</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0</td>
</tr>
<tr>
<td>pH</td>
<td>9.0</td>
</tr>
</tbody>
</table>

The culture was grown in this basal medium for all characterization studies.

1.2.2.3. Sterilization

Glassware used was thoroughly washed successively in detergent water, acid water, running tap water followed by rinsing in single and double distilled water. The flasks used were tightly plugged with cotton gauge. Further, the cotton plugs were covered with aluminium foils. Glassware
was then kept in an oven at 180°C for 4-5 hours. Liquid media and distilled water were sterilized in an autoclave at 122.4 kpa for 20 minutes. Laminar air flow chamber was sterilized by ultraviolet irradiation for 15 minutes.

1.2.2.4. Preparation of inoculum

A 20 hour old culture at the rate of 1 percent was used as inoculum in all experiments.

1.2.2.5. Culture conditions

The bacterium was grown aerobically at 60°C in a thermostatically controlled water bath shaker at 200 rpm. Petri plates were incubated in an incubator maintained at 55°C.

1.2.2.6. Preparation of samples
1.2.2.6.1. Cells: Cultures at required phase of growth were centrifuged at 9,000g for 20 minutes at 4°C. The cell pellet was collected and used as per the experimental procedure.
1.2.2.6.2. Cell free supernatant (CFS): The supernatant from 1.2.2.6.1. served as the cell free supernatant.

1.2.2.7. Measurement of growth:
1.2.2.7.1. Turbidity: Growth was monitored by measuring the change in absorbance of cells in medium at 550 nm using
uninoculated medium as blank. The growth rate was determined from the slope of the growth curve for the strain during logarithmic growth phase. Generation time was calculated from the slope using the equation:

\[ g = \frac{\log 2 \times T}{\log O.D_f - \log O.D_i} \]

where

- \( g \) = generation time
- \( O.D_i \) = initial absorbance
- \( O.D_f \) = final absorbance
- \( T \) = time in hours

The specific growth rate (\( \mu \)) was determined from the generation time using the formula:

\[ \mu = \frac{\ln 2}{g} \]

1.2.2.7.2. **Dry weight**: A known volume of culture was centrifuged at 9,000 g for 20 minutes at 4°C. The cell pellet was transferred to an aluminium dish and placed in an oven at 200°C till the material was dried to a constant weight.

A standard plot was made between the absorbance and dry weight of known volume (Appendix 1).

1.2.2.7.3. **Soluble protein**: Cells, from a known volume of culture obtained as per 1.2.2.6.1. were washed twice in 50 mM potassium phosphate buffer (pH 7.2). The washed
cells were resuspended in the same buffer and sonicated for 3 minutes with one minute burst followed by 30 seconds gap to avoid heating at an amplitude of 12 kHz fitted with a titanium probe. The above sample was centrifuged at 15,000g for 20 minutes to obtain a clear supernatant leaving cell wall debris and unbroken cells as pellet. The soluble protein from the supernatant, on quantification was another method of measurement of growth.

1.2.2.7.3.1. Estimation of protein
Ref: Bradford (1976).

Reagent:

<table>
<thead>
<tr>
<th>Bradford reagent</th>
<th>Coomassie blue</th>
<th>100 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (90%)</td>
<td>50 ml</td>
<td></td>
</tr>
<tr>
<td>Phosphoric acid (85%)</td>
<td>100 ml</td>
<td></td>
</tr>
</tbody>
</table>

Coomassie brilliant blue was dissolved in 90% ethanol and mixed with phosphoric acid. The solution was diluted to one litre with distilled water and filtered through Whatman filter paper No 1.

Procedure:

To 0.1ml of protein sample 5ml of Bradford reagent was added and mixed. Absorbance at 595nm was taken after 20 minutes of addition.

The protein content in the sample was calibrated from a standard plot of 0-60 μg/ml (Appendix 2).
1.2.3. METHODS FOR IDENTIFICATION AND TAXONOMIC NOMENCLATURE

1.2.3.1. Morphological preparations

1.2.3.1.1. Gram's stain

Ref: Jacob and Gerstein (1965).

Reagents:

1. Crystal violet solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
<th>Unit</th>
<th>Solution A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal violet</td>
<td>2 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol (95%)</td>
<td>20 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium oxalate</td>
<td>0.8 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>80 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Solution A and B were mixed and stored at room temperature for 24 hours and passed through filter paper into staining bottles. The solution could be used till no precipitation was observed.

2. Gram's Iodine solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine</td>
<td>1 g</td>
<td></td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>2 g</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>300 ml</td>
<td></td>
</tr>
</tbody>
</table>

Iodine and potassium iodide were mixed and ground to a smooth paste with water in a mortar. The remaining water was added and the resulting solution was filtered and stored in a dark bottle.

3. Acetone-alcohol

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute alcohol</td>
<td>50 ml</td>
</tr>
<tr>
<td>Acetone</td>
<td>50 ml</td>
</tr>
</tbody>
</table>
The two solvents were mixed just before use.

4. Counter stain

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safranin</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Absolute alcohol</td>
<td>10 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>90 ml</td>
</tr>
</tbody>
</table>

Safranin was first dissolved in absolute alcohol and then water was added. The solution was stored at room temperature.

Procedure:

Cells were suspended in physiological saline to give a thin suspension. On a clean grease free glass slide a loop full of suspension was taken and a thin even smear was made. The smear was let to air dry and then heat fixed. Crystal violet solution was flooded onto the smear and after one minute washed off with excess of distilled water and drained. Smear was flooded with Gram's iodine for one minute. Slides were washed with distilled water and destained with acetone-alcohol solution for one minute. After washing in distilled water the smear was flooded with the counter stain for 30 seconds before the final washing in distilled water. The slide was dried and viewed under light microscope.

The cells appearing red were considered Gram negative and those seen as violet were Gram positive.
1.2.3.1.2. Spore stain

Ref: Tanner (1944).

Reagents:

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Malachite green</td>
<td>5% (w/v) in water</td>
</tr>
<tr>
<td>2</td>
<td>Safranin</td>
<td>0.5% (w/v)</td>
</tr>
</tbody>
</table>

Procedure:

Heat fixed smears of the culture were made as in the case of Gram's stain (1.2.3.1.1.). Aqueous malachite green was flooded on the smear and steamed for 10 minutes. Care was taken not to allow the smear to dry. After the prescribed time the slide was washed with distilled water and counter stained with safranin for 30 seconds. The slide was washed and blotted dry.

The refractile spores absorbed the green colour while the vegetative cells were red.

1.2.3.1.3. Scanning electron microscopy

A thin smear of culture was air dried on glass cover slip and fixed to solid metal stubs. The specimen was silver coated to an approximate thickness of 500 Å, by means of vacuum evaporation. The silver coated cells were observed in an scanning electron microscope.
1.2.3.1.4. **Negative staining for transmission electron microscopy**

20 μl of culture was placed on a copper grid for 5 minutes and washed several times with cold sterile distilled water. Later 50 μl of 2% aqueous uranyl acetate was placed over the thin film of cells for 5 minutes. Excess stain was removed and grid was observed in a transmission electron microscope.

1.2.3.1.5. **Motility**

Ref: Skerman (1969)

Motility was observed by the hanging drop method. A loopful of an young culture was placed on a clean coverslip. This was overturned and placed on the concave portion of the slide and sealed. This was observed under the microscope. The drop should hang and not touch the sides of the slide.

1.2.4. **BIOCHEMICAL CHARACTERIZATION**

1.2.4.1. **Catalase activity**

Ref: Lewis and Reltger (1940)

With a loop, a smear of the organism was made on a clean glass slide. To this smear few drops of 3% H₂O₂ was added and observed.
Continuous bubbles indicated a positive reaction while no bubbles indicated a negative reaction.

1.2.4.2. **Urease activity**

Ref: Holdman and Moore (1975)

**Media:**

Peptone yeast extract medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1 g</td>
</tr>
<tr>
<td>Basal salt solution</td>
<td>100 ml</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.0003 g</td>
</tr>
</tbody>
</table>

The above ingredients were mixed and the pH adjusted to 6.8.

Peptone yeast Urea medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter sterilised urea</td>
<td>6 ml (20%)</td>
</tr>
<tr>
<td>Peptone yeast extract broth</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

The above two solutions were mixed after sterilization.

**Procedure**

To both the media the test organism was inoculated and incubated for three days. After the prescribed incubation the resultant pH of the media was monitored either by the change in colour of indicator used or a pH meter.

Increase in pH of the peptone yeast urea broth indicated a positive test for urease activity.
1.2.4.3. **Indole production**

Ref: Holdman and Moore (1975).

**Reagent:**

1. Ehrlich's reagent

- p-dimethyl amino benzaldehyde: 2 g
- Amyl alcohol (95%): 190 ml
- Hydrochloric acid: 40 ml

The above three chemicals were mixed together to make Ehrlich's reagent.

2. Xylene

**Procedure:**

To 2 ml of young culture broth one ml of xylene was added and vortexted thoroughly. The mixture was allowed to stand for two minutes and 0.5 ml of Ehrlich's reagent was added slowly along the sides of the test tube.

Appearance of a pink ring between the two layers was a positive test. An yellow ring was considered as a negative reaction.

1.2.4.4. **Hydrogen sulphide production**

Ref: Skerman (1969)

**Medium:**

- Peptone: 10 g
- Cysteine: 0.1 g
- Na₂SO₄: 0.5 g
- Agar: 15 g

The ingredients were dissolved in water and the pH adjusted to 7.0. After sterilization, slants were made. The organism was stabbed and incubated for 3 days.
Black colouration of the agar was a positive reaction for hydrogen sulphide production.

1.2.4.5. Nitrate reduction test

Ref: Skerman (1969)

Reagents:
1. Starch Iodine solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>0.4 g</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>2.0 g</td>
</tr>
<tr>
<td>KI</td>
<td>0.2% aqueous</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

ZnCl₂ was dissolved and boiled in 10 ml of distilled water, to which starch was added and diluted to 100 ml. This solution was allowed to stand for one week and then filtered. An equal volume of KI solution was added and mixed gently.

2. Hydrochloric Acid 16%

Medium:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal medium</td>
<td>100 ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5 g</td>
</tr>
<tr>
<td>KNO₃</td>
<td>0.02 g</td>
</tr>
</tbody>
</table>

Culture was grown on the sterilized medium for 24 hours.

Procedure:

On a clean slide one drop each of starch iodine solution and 16% HCl were mixed. To the above mixture one drop of culture was added and mixed with a toothpick.

A blue colour indicated the presence of nitrite.
1.2.4.6. **Voges Proskauer (V-P) test**

Ref: O'Mears (1931).

**Reagents:**

1. Creatine a pinch
2. NaOH (40%) a few drops

**Procedure:**

To an old culture (48 hours) a pinch of creatine was added and mixed well. Few drops of NaOH were added and allowed to stand.

Appearance of pink colour indicated a positive test.

1.2.4.7. **Sugar cleavage**

Ref: Hayward (1964).

**Medium:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5 g</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>3 g</td>
<td></td>
</tr>
<tr>
<td>Phenol red</td>
<td>3 mg</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
<td></td>
</tr>
</tbody>
</table>

The medium was dispensed into test tubes before autoclaving. Filter sterilized sugars were added at the rate of one percent.

**Procedure:**

Tubes were inoculated and incubated for three days. The tubes were observed daily for change in colour.
Decrease in pH of the medium was indicated by change in colour of indicator from red to yellow which in turn showed the production of acid due to sugar utilization.

1.2.4.8. **Gas production**

Ref: Hayward (1964).

Medium: Same as in 1.2.4.7.

Durham tubes were incorporated into the tubes along with the medium and sugar.

Procedure:

The tubes were inoculated and incubated. The tubes were daily observed for gas production.

Gas accumulated in the Durhams tube indicates a positive reaction for gas production.

1.2.4.9. **Tween hydrolysis**

Ref: Holdman and Moore (1975).

Media:

1. Peptone yeast tween medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1 g</td>
</tr>
<tr>
<td>Basal salt solution</td>
<td>100 ml</td>
</tr>
<tr>
<td>Tween 80</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

2. Peptone yeast medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1 g</td>
</tr>
<tr>
<td>Basal salt solution</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

33
Both media were sterilized, inoculated and incubated identically.

Better growth in Tween enriched medium was considered as a positive test.

1.2.4.10 Gelatin hydrolysis

Ref: Holdman and Moore (1975).

Medium:

Peptone yeast extract gelatin medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1 g</td>
</tr>
<tr>
<td>Basal salt solution</td>
<td>100 ml</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Agar</td>
<td>1.5 g</td>
</tr>
</tbody>
</table>

Procedure:

Culture was plated on the peptone yeast extract gelatin agar and incubated for ten days. Saturated ammonium sulfate solution was flooded on the plates.

Clearing zone around the colonies indicated a positive test for gelatin hydrolysis.

1.2.4.11. Starch hydrolysis

Ref: Holdman and Moore (1975)

Reagents:

1. Gram Iodine solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine</td>
<td>1 g</td>
</tr>
<tr>
<td>KI</td>
<td>2 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>300 ml</td>
</tr>
</tbody>
</table>
Medium:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1 g</td>
</tr>
<tr>
<td>Basal salt solution</td>
<td>100 ml</td>
</tr>
<tr>
<td>Starch</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>1.5 g</td>
</tr>
</tbody>
</table>

Procedure:

Starch agar plates were made and inoculated with culture. After 24 hours of incubation, plates were flooded with Gram's iodine and washed immediately.

Clear zones around the colony was an indication of starch hydrolysis.

1.2.4.12. Carboxymethyl cellulose hydrolysis

Ref: Teather and Wood (1982).

Reagents:

1. Congo red solution 0.1% (aqueous)
2. NaCl 1 N

Medium:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>1 g</td>
</tr>
<tr>
<td>Basal salt solution</td>
<td>100 ml</td>
</tr>
<tr>
<td>Carboxymethyl cellulose</td>
<td>1 g</td>
</tr>
</tbody>
</table>

Procedure:

CMC agar plates were made, inoculated and incubated. After three days of incubation, the plates were flooded with Congo red solution and left for 10 min. The dye was removed and washed thrice with NaCl solution.
Yellow zones around the colony was a positive test for CMC hydrolysis.

1.2.4.13. DNA base composition

A. DNA isolation

Ref: Grant and Carr (1984).

Reagents:

1. TES buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>50 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>100 mM</td>
</tr>
<tr>
<td>Sucrose</td>
<td>25%</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>5 mg/ml</td>
</tr>
</tbody>
</table>

2. TE buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>40 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>2 mM</td>
</tr>
<tr>
<td>pH</td>
<td>7.9</td>
</tr>
</tbody>
</table>

3. NaCl 5 M

4. SDS 10%

5. CsCl₂

6. Poly ethylene glycol (6000)

Procedure:

Cells obtained from four litres of culture broth were incubated in 20 ml of TES buffer at 37°C for 1 hour. After the incubation 3 ml of SDS was added and incubated for another one hour. 6 ml of 5M NaCl was added, mixed
thoroughly and kept overnight at 4°C. The volume of the supernatant was reduced to 7 ml in TE buffer using polyethylene glycol (10%). The whole material was centrifuged at 15,000g for 30 minutes at room temperature. To the supernatant 8 g CsCl₂ and 5mg/ml of ethidium bromide was added and ultracentrifuged at 100,000g for 60 hours (15°C). The upper fluorescent band was collected and reprecipitated with ethanol after removing the EtBr by butanol extraction.

B. DNA base composition

Ref: Marmur and Doty (1962)

The DNA base composition was determined by the melting temperature profile. The purity of the chromosomal DNA was checked by the 260 nm : 280 nm ratio. Using a spectrophotometer with a thermosensor and recording channel (for calibration of temperature inside the quartz cuvetts) the thermal denaturation midpoint was calibrated. A blank was run using saline citrate buffer. Guanine plus cytosine values in mole percent was calculated from the formula:

\[ G + C = (T_m - 69.3) \times 2.44. \]

where, \( T_m \) = thermal denaturation mid point.
1.2.5. **OPTIMIZATION OF GROWTH CONDITIONS**

1.2.5.1. **Temperature for growth**

Growth curves were drawn growing the culture at various temperatures. The 'g' and 'μ' values were found and optimum temperature for growth was maintained in all further experiments.

1.2.5.2. **pH for growth**

The medium for growth was adjusted to various pH values using 1 N NaOH or 1 N HCl as the case may be. The pH suited for maximum growth was maintained in all experiments.

1.2.5.3. **Nitrogen substrate for growth**

Different nitrogenous substrates both organic as well as inorganic were substituted in the basal medium and growth of bacterium in different sources was checked.

1.2.5.4. **Concentration of yeast extract for growth**

Various concentrations of yeast extract were added to the basal medium and growth was monitored as soluble cellular protein per ml of culture. Optimum concentration for good growth was maintained in all experiments.
1.2.5.5. **Salt tolerance**

NaCl at various concentrations was added to the basal medium before autoclaving. The extent of growth was monitored by the amount of soluble protein produced per ml of culture.

1.2.6. **Extracellular enzyme estimations**

1.2.6.1. **Reducing sugar**

Ref: Miller (1959).

**Reagents:**

1. DNSA reagent

Dinitrosalicylic acid  40 g
Phenol              8 g
Na$_2$SO$_4$        2 g
Sođ. pot. tartarate 800 g

The above components were mixed in 2 liters of 2% NaOH. The volume was increased to four litres and filtered. The reagent was stored in dark coloured bottles.

**Procedure:**

To the sample of sugars 3 ml of reagent was added and vortexed thoroughly. The tubes were kept in a boiling water bath for 5 minutes and the absorbance was taken at 540 nm.

1.2.6.2. **Carboxymethyl cellulose**

Ref: Mandels et al. (1976)
Reagents:

1. Carboxymethyl cellulose 1% in buffer
2. Potassium phosphate buffer 50 mM (pH 7.0)

Procedure:

To 1 ml of substrate 50 μl of enzyme (CFS) was added and incubated at 60°C for 30 minutes. The reaction was stopped by adding 3 ml of DNSA reagent and reducing sugars liberated were calculated as per 1.2.6.1.

The amount of sugars were estimated from a calibration curve of glucose (0 to 500 μg - Appendix 3).

One unit of CMCase activity was defined as the amount of enzyme required to release one micromole of reducing sugar as equivalent glucose in one minute under standard incubation conditions.

1.2.6.3. Xylanase activity

Ref: Rickard and Laughlin (1980).

Reagents:

1. Xylan (oat spelt) 1% in buffer
2. Potassium citrate buffer 50 mM (pH 7.0)

Procedure:

To one ml of substrate 50 μl of enzyme was added and incubated at 60°C for 30 minutes. The liberated sugars were estimated as in 1.2.6.1.
Calibrations were made using a standard curve plotted for xylose (0-1000 μg; Appendix 4).

One unit of xylanase activity was defined as the amount of enzyme required to release one micromole of reducing sugar as equivalent xylose in one minute under standard incubation conditions.

1.2.6.4. Amylase activity
Ref: Bernfeld (1951).

Reagents:
1. Starch 1% in buffer
2. Potassium phosphate buffer 50 mM (pH 7.0)

Procedure:
To one ml of substrate 50 μl of enzyme was added and incubated at 60°C for 30 minutes. The sugar liberated was quantified as in 1.2.6.1.

A standard curve plotted for maltose (0-500μg) was used for quantification (Appendix 5).

One unit of amylase activity was defined as the amount of enzyme required to release one micromole of reducing sugar as equivalent maltose in 1 minute under standard incubation conditions.
1.2.6.5 **Protease**

Ref: Drapeau (1976).

**Reagents:**

1. Casein solution 1% (aqueous)
2. TCA solution 10% (aqueous)

**Procedure:**

To five ml of substrate ten ml of enzyme was added and contents were incubated at 60°C for 20 minutes. The reaction was stopped by adding five ml of TCA. The tubes were allowed to stand for 30 minutes and filtered. Absorbance of the filtrate at 280 nm was measured.

One unit of enzyme was defined as the amount of enzyme which liberated acid soluble fragments equivalent to 0.001 A at 280 nm per minute under defined conditions.

\[
\text{Units/mg} = \frac{\text{O.D.sample-O.D.blank} \times 1000}{20 \text{ min} \times \text{Protein (mg)}}
\]

1.2.6.6 **β-glucosidase**

Ref: Deshpande and Eriksson (1988)

**Reagents:**

1. PNPG 1mM
2. Potassium phosphate buffer 50mM
3. Na₂CO₃ 2M
Procedure:

To 0.9 ml of substrate 0.1 ml of enzyme was added and incubated at 60°C for 30 minutes. The reaction was stopped by adding 1ml of 2M Na₂CO₃. The absorbance was taken at 420 nm.

One unit of β-glucosidase activity was defined as the amount of enzyme that catalyzes the formation of 1 micromole of p-nitrophenol per min under standard assay conditions.

\[
\text{Unit/ml} = \frac{\text{O.D.sample}-\text{O.D.blank} \times \text{Volume of reaction mixture}}{18.5 \times \text{time}}
\]

1.2.6.6. β-xylosidase

Ref: Lachke (1988).

Reagents:

1. PNPX 1 mM
2. Potassium phosphate buffer 50 mM
3. Na₂CO₃ 2 M

Procedure:

To 0.9 ml of substrate 0.1 ml of enzyme was added and incubated at 60°C for 30 minutes. The reaction was stopped by adding 1 ml of 2 M Na₂CO₃ and absorbance taken at 420 nm.

One unit of enzyme activity was defined as in 1.2.6.6.
1.2.7 DETERMINATION OF RESIDUAL CELLULOSE

The procedure involved acidifying the culture with 2 N HCl and filtering through a sintered glass filter which retained the insoluble cellulose but not the cells. Cells bound to the fibers were removed by washing with 100 ml each of 12% HCl, distilled water, 5% NH₄OH, distilled water, 95% ethanol and ether consequently. The residual cellulose was dried in an oven at 110°C for two hours, cooled to room temperature in a desiccator and weighed. (Taylor et al., 1986).