This chapter includes methods for isolation and identification of bacteria and results obtained for characterisation of the isolate were presented and discussed. The chapter was organized under the following subheadings.

5.1 Methodology
5.1.1 Isolation
5.1.1.1 Sample collection
5.1.1.2 Enrichment in liquid medium
5.1.1.3 Isolation using solid media
5.1.1.4 Test for tolerance of ammonia
5.1.1.5 Culture preservation

5.1.2 Characterization of the isolate
5.1.2.1 Morphological identification
5.1.2.2 Biochemical identification
   a. Enteric tests
   b. Tests for extracellular enzyme production
   c. Amino acid tests
   d. Sugar fermentation tests
5.1.2.3 Genetic identification
   i. 16s rRNA sequencing
   ii. Phylogenetic analysis
5.1.1 ISOLATION

Visakhapatnam city was surveyed for selecting a suitable source for the isolation of heterotrophic bacteria, which has the ability to remove ammonia. Wastewater treatment plant located in Appughar area was selected as a suitable source based on the analyzed ammonia concentrations in the previous study (Khasim Beebi, 2005).

5.1.1.1 SAMPLE COLLECTION:

A 1 litre sewage sample was collected from sewage treatment plant located at Appughar in Visakhapatnam. Collection of sample was done by using a polythene bottle. This is sewage treatment plant has a capacity of 28million liters per day and is operated by the Greater Visakhapatnam municipal corporation, Visakhapatnam. Immediately after collection of the sample, the bottle was sealed and transported to GITAM laboratory. In the laboratory, the sample was acidified using concentrated sulphuric acid and stored at 4°C for further studies.

5.1.1.2 ENRICHMENT IN LIQUID MEDIUM

Wastewaters contain a variety of microorganisms. Some of the microorganisms become dominant under favourable environmental conditions. Enrichment with specific nutrients provides favourable conditions for the growth of the desired organisms. Ammonium sulphate, a source of ammonical nitrogen and energy, was added to the basal inorganic media (Table 10) as given by Brierley. E.D.R and Wood. M., 2001. The ability of heterotrophic bacteria in utilizing the inorganic nitrogen was adopted for enriching the desired organism.
Medium composition for enrichment:

Table 10 Medium composition (Brierley and wood, 2001)

<table>
<thead>
<tr>
<th>Constituent in g/L</th>
<th>Trace element solution g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>H$_3$BO$_3$        - 2.86</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>ZnSO$_4$.7H$_2$O - 0.22</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>CuSO$_5$.7H$_2$O - 0.08</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>MnSO$_4$.4H$_2$O - 2.03</td>
</tr>
<tr>
<td>Trace element solution - 1ml</td>
<td>Na$_2$MoO$_4$.2H$_2$O - 1.26</td>
</tr>
</tbody>
</table>

i. 1$^{st}$ stage enrichment

100ml of this medium with 1Kg/m$^3$ of ammonium sulphate was prepared and sterilized in an autoclave at 121$^0$C at 15lb pressure. After sterilization, media was cooled to room temperature. 3ml of the wastewater sample collected from Appughar sewage treatment plant (STP) was used to inoculate the media for enrichment. After inoculation, the media was incubated in an orbital shaking incubator (ORBITEK, Scigenics Biotech) at 30$^0$C and 140rpm. After 4 days of incubation, growth of the microorganisms was assessed by measuring optical density at 610nm using colorimeter (Photoelectronic colorimeter 113, Systronics). For identifying the nitrification ability of the isolated microbe, 1ml of the enrichment sample was taken in a sterile condition in a clean petridish. To this, 2drops of sulphanalimide and 2 drops of NEDE (N-1-Naphthylethylene diamine dihydrochloride, C$_{12}$H$_{14}$N$_2$.2HCl, AR) were added. The products of the reaction produce color to the medium.
ii. 2nd stage enrichment

In 2nd stage of enrichment, 100ml of basal inorganic medium with same composition and pH was prepared and sterilized at 121°C and 15lbs pressure. After cooling to room temperature, media was inoculated with 2ml of previously enriched media. This inoculated media was incubated at 30°C and 140rpm in a shaking incubator. After 7 days of incubation, the growth of the organism was assessed by taking optical density at 610 nm.

iii. 3rd stage enrichment

The enrichment was carried out with increasing concentration of ammonia in the mineral medium. In the 3rd phase of enrichment, the mineral inorganic medium was incorporated with 2Kg/m³ of ammonium sulphate and incubated for 4 days and 1ml of previously enriched culture was transferred into freshly prepared medium with same initial ammonium nitrogen concentration. In 4th stage, ammonium sulphate concentration was increased to 3Kg/m³. After enrichment, from the medium, 1ml of culture was transferred into freshly prepared medium containing same ammonical nitrogen concentration.

5.1.1.3 ISOLATION USING SOLID MEDIA

100ml of basal inorganic medium with 2Kg/m³ of ammonium sulphate was prepared and 1.5g of purified agar (Himedia) was added for solidification of the medium. Phenol red indicator was incorporated into the media (Grunditz. C and Dalhammer.G, 2001). The pH of the media was adjusted by using Elico pH meter. The prepared media was sterilized at 121°C and 15lb pressure. After sterilization, 1ml of the enriched culture was taken and serially diluted from 10⁻¹ to 10⁻⁷ dilutions; 0.1ml of each dilution was used for spreading with L-shaped rod on to the petriplates containing inorganic solid media. These inoculated plates were incubated at 30°C for
72hrs. Six colonies showing nitrification zones, were selected and subjected to repeated streaking on solid basal inorganic media to obtain pure colonies. This process was repeated for several times until the isolates were grown into pure visible colonies on the media.

5.1.1.4 TEST FOR TOLERANCE OF AMMONIA

In order to test the isolated bacterial species for maximum tolerance of ammonia concentration, the organisms were grown in the medium containing higher concentration of ammonia ranging from 1 to 10Kg/m³ of ammonium sulphate, keeping all other chemical constituents constant.

Medium preparation: 100ml of the basal inorganic media with varying concentrations of ammonium sulphate, 1 to 10Kg/m³ was prepared and for each type of media concentration, 1.5g of purified agar (Himedia) was added for solidification of the media. pH of the medium was adjusted to 8 and the medium was sterilized at 121°C and 15 lbs pressure. After cooling to room temperature, the medium was transferred to petridishes and allowed to solidify the medium. Six selected isolates were taken from enriched culture and were inoculated onto prepared petriplates with increasing concentrations of ammonia. All the inoculated plates were incubated at 30°C for 48 to 96hrs in an incubator. After incubation, the plates were observed for colony formation. The bacterial isolate, which showed maximum tolerance for higher concentrations of ammonia, was selected and further subjected to identification tests following standard microbiological procedures.
5.1.1.5 CULTURE PRESERVATION

100ml of basal inorganic medium with pH 8, after the addition of 1.5g of purified agar was taken in 250ml conical flask, this medium was dispensed into test tubes of 5ml each and the test tube opening was closed with non-adsorbant cotton plugs and sterilized at 121°C and 15 lbs for 15minutes. After sterilization, test tubes were kept in slanting position and allowed for solidification. Inoculation of the slants were carried out with flame sterilized inoculation loop in sterile condition and incubated at 30°C for 72 hrs. The slants with cultures were labelled mentioning date and enclosed in a polythene bag. This bag is stored at 4°C in refrigerator. The slants with culture were preserved for a period of one month and again inoculated in to fresh media to avoid contamination and starvation of cells (Gopal reddy, 2008).

5.1.2 CHARACTERIZATION OF THE ISOLATE

The isolated bacteria was characterized morphologically, biochemically and genetically.

5.1.2.1 Morphological identification

The isolated bacterial colony’s morphology was observed and the culture was stained by Gram staining for identifying the morphological characteristics of the isolate. The motility of the isolate was also observed.

i. Colony morphology

The colonies of the isolate were grown on petriplate after incubation was observed using a magnifying lens and colony morphology was recorded.
ii. Gram staining of the isolate

Reagents:
95% alcohol, Gram’s iodine, Crystal violet and Safranin

Procedure:
Bacterial smear was prepared and heat fixed on a clean grease free slide. To this smear, crystal violet stain was added and retained for 1 minute. After 1 min, crystal violet stain was drained and Gram’s iodine was added and allowed to retain it for 1 minute. Later Gram’s iodine solution was drained and slide was washed under tap water. This slide was washed with 90% alcohol and again washed under tap water. After washing with 90% alcohol, counter stain safranin was added. After 1 minute, safranin was washed under tap water. The stained slide was air-dried and observed using objective lens of 100X in oil immersion (Cappuccino and Sherman, 2004).

iii. Motility test by agar dip method

Medium composition:
Beef extract-0.5g, Peptone-0.5g, Sodium chloride-0.5g, Agar-0.06g, Distilled water-100ml and pH -7.2

Procedure:
100ml of nutrient media was prepared and distributed into test tubes and tube opening was closed tightly with non-adsorbant cotton plugs and sterilized at 121°C and 15lb. After cooling to room temperature, inoculation was done with a sterile inoculation needle from top to the centre of the media. This media was incubated at 30°C for 24hrs (Gopal reddy et al., 2008). A control test was used for reference.
5.1.2.2 Biochemical identification

Various biochemical tests were carried out with freshly grown 24hr old cultures for understanding the characteristics of the isolate.

a. Enteric tests

i. Indole test

Medium composition:

Peptone-0.2g, Sodium chloride-0.5g, Distilled water-100ml and pH- 7.4

Procedure:

100ml media was prepared and distributed into test tubes and test tube opening was tightly closed with cotton plugs and sterilized in an autoclave at 121°C and 15lbs pressure for 15 minutes. After cooling to room temperature, inoculation was done with a sterile inoculation loop and incubated at 30°C for 98hr. After incubation, cotton plugs were removed and 0.5ml of Kovac’s reagent was added and allowed for 10 minutes (Gopal reddy et al., 2008). A control test was used for reference.

ii. Methyl red test

Medium composition:

Glucose-0.5g, Peptone-0.7g, di-potassium hydrogen phosphate (K$_2$HPO$_4$)-0.38g, Distilled water-100ml and pH-7.4

Procedure:

For methyl red test, 100ml media was prepared and distributed into test tubes and test tube openings were closed with cotton plugs and sterilized in an autoclave at 121°C and 15lb pressure for 15 minutes. After cooling to room temperature, medium was inoculated with flame sterilized inoculation loop and incubated at 30°C for 48hr. After
incubation, cotton plugs were removed and 0.5ml of methyl red indicator was added to the medium and left for 30 minutes (Gopal reddy et al., 2008). A control test was used for reference.

iii. **Voges proskauer test**

Medium composition:

Glucose-0.5g, Peptone-0.7g, di-potassium hydrogen phosphate (K₂HPO₄)-0.38g, Distilled water-100ml and pH-7.4

**Procedure:**

For methyl red test, 100ml media was prepared and the pH of the medium was adjusted to 7.4. This medium was distributed into test tubes and test tube openings were closed with cotton plugs. The entire medium was sterilized at 121°C and 15lb pressure for 15 minutes. After cooling to room temperature, medium was inoculated with fresh 24 hr old cultures with a sterilization loop and incubated at 30°C for 48hr. After incubation, cotton plugs were removed and 1ml of 40% KOH, 3ml of 5% α-napthol was added with vigorous shaking and allowed for 30minutes for colour development. (Gopal reddy et al., 2008). A control test was used for reference.

iv. **Citrate test**

Medium composition:

Magnesium sulphate (MgSO₄.H₂O)-0.02g, Ammonium di-hydrogen phosphate (NH₄H₂PO₄)-0.1g, Potassium di-hydrogen phosphate KH₂PO₄-0.1g, Sodium citrate-0.5g, Agar-1.5g, Bromothymol blue indicator-4ml, Distilled water-100ml and pH-6.8

**Procedure:**

For Citrate test, Simmons’s citrate agar (Himedia, pvt, Ltd) was used. For identification of citrate utilization by the organism as a sole source of carbon, medium
was prepared and test tubes openings were closed with non-absorbent cotton plugs. The prepared media was sterilized at 121°C and 15lb pressure for fifteen minutes. The sterilized test tubes were cooled to room temperature in a slanting position. After solidification, slants were stabbed with heat sterilized stabbing needle. The slants were incubated at 30°C for 24hr in a bacteriological incubator (Gopal reddy et al., 2008). A control test was used for comparison of results.

v. Nitrate reduction test

Medium composition:
Potassium nitrate-0.1g, Disodium phosphate-0.2g, Dextrose-0.1g, Trypticase-2g, Distilled water-100ml and pH-7.2.

Procedure

5ml of the medium was dispensed in each test tube and all test tube openings were closed with non-adsorbent cotton plugs. Medium was autoclaved at 121°C for 15 minutes and cooled to room temperature in sterile chamber and inoculation was carried out by using sterile inoculation loop. After inoculation, tubes were incubated at 30°C for 48hr (cappuccino and Sherman, 2004). A control test was used for reference.

vi. Urease test

Medium composition:
Peptone-0.1g, Sodium chloride-0.5g, Potassium di-hydrogen phosphate-0.2g, Phenol red (0.2%)-0.6ml, Glucose (filter sterilized)-0.1g, Urea (filter sterilized 20%)-10ml, Distilled water-100ml and pH-7.2
Procedure:

80ml of the media except glucose and urea was prepared. The pH of the medium was adjusted to 7.2 and sterilized at 121°C and 15lb pressure. Glucose was dissolved in 10ml of distilled water and filter sterilized with 0.42µm Millipore membrane by using filtration unit. The urea solution was filtered through 0.42µm membrane filter and stored in sterile condition. When the sterilized media was cooled to room temperature, 10ml of glucose and 10ml of filter sterilized urea solution were added and this media was distributed into sterile test tubes closed with non-adsorbent cotton plugs. All the tubes were kept in slanting position. When slants were solidified, active cells of 24hr old culture was used for inoculation of slants. Stabbing of the media done with a heat sterilized inoculation needle and the tubes were incubated at 37°C for 5days (Gopal reddy et al., 2008). A control plate was used for comparison of results.

Vii Catalase test

Reagents:

Hydrogen peroxide

Procedure:

Culture was grown on nutrient agar (Himedia) plates for 24hr and one drop of hydrogen peroxide was added on the surface of the colony with a dropper. (Gopal reddy et al., 2008). A control plate was used for comparison of results.
b. Tests for Extracellular enzyme production

i. Starch hydrolysis

Medium composition:
Peptone-0.5g, Beef extract-0.3g, Starch-0.2g, Agar-1.5g, Distilled water-100ml and pH-7

Procedure
Starch hydrolysis test determines the extracellular activity of the organism. 100ml of the medium was prepared in 250ml conical flask. This medium was sterilized at 121°C and 15 lb pressure for 15 minutes. The medium was allowed to cool to room temperature, poured into sterile petriplates, and allowed to solidify. By using heat, sterilized inoculation loop a straight streak was made and these plates were incubated at 30°C for 96hr. After incubation, plates were flooded with Gram’s iodine and excess iodine solution was discarded form the plate (Gopal reddy et al., 2008). A control plate was used for comparison of results.

ii. Gelatin liquefaction

Medium composition:
Bacto-gelatin-12g, Peptone-0.5g, Beef extract-0.3g, Sodium chloride-0.5g, Distilled water-100ml and pH-7.2

Procedure:
For gelatin liquefaction test, 100ml media was prepared and was heated on Bunsen burner until all the gelatin is dissolved and uniformly distributed throughout the flask. When the gelatin is completely dissolved, media was distributed in test tubes and
openings of test tubes were closed with non-absorbant cotton plugs. Media was sterilized at 121°C and 15lb pressure for fifteen minutes. The sterilized media was cooled to room temperature and inoculation was done with a heat sterilized inoculation loop and incubated at 37°C for 5 days. After 5 days of incubation, the inoculated test tubes were kept in deep freezer at 4°C for 20 minutes. These tubes were taken out and kept in a slanting position for identification of gelatin liquefaction (Gopal reddy et al., 2008). A control tube was used for reference.

iii. **ONPG test**

Ortho nitro-phenyl-β galactoside test was done on biochemical strip KB013 (Himedia) for *Bacillus* identification.

c. **Test for utilization of Amino acid**

i. **Arginine test**

Arginine test was done on biochemical strip KB013 (Himedia) for *Bacillus* identification.

d. **Sugar fermentation tests**

i. **Sucrose fermentation test**

Sucrose fermentation test was done on biochemical strip KB013 (Himedia) for *Bacillus* identification.
ii. Mannitol fermentation test

Mannitol fermentation test was done on biochemical strip KB013 (Himedia) for \textit{Bacillus} identification.

iii. Glucose fermentation test

Glucose fermentation test was done on biochemical strip KB013 (Himedia) for \textit{Bacillus} identification.

iv. Arabinose fermentation test

Arabinose test was done on biochemical strip KB013 (Himedia) for \textit{Bacillus} identification.

v. Trihalose fermentation test

Trihalose test was done on biochemical strip KB013 (Himedia) for \textit{Bacillus} identification.

5.1.2.3 Genetic characterization

i. 16S rRNA sequencing

The isolated culture was ice packed carefully and sent to Bioaxis DNA research centre, Hyderabad for 16s rRNA sequencing. The sequencing was carried out by
using forward and reverse bacterial universal primers named 27F (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1429R (5’-GGTTACCTTGTTACGACTT-3’) (ZhangQ.L et al., 2012). Pure cultures of the isolated bacteria were subjected to DNA extraction and purification. After the DNA was quantified by using forward and reverse universal bacterial primers, amplification of DNA was carried out. After amplification of DNA, sequencing was carried out on ABI PRISM 377DNA sequencer. The obtained sequence was compared with sequences available in NCBI gene bank for bacterial identification.

ii. Phylogenetic analysis

Similar 16s rRNA sequences were retrieved from gene bank database of NCBI.nlm.nih.gov using query sequence and multiple sequence alignment with CLUSTAL W was carried out. After alignment, the sequences were analyzed by maximum likelihood method with MEGA 6.06 for phylogenetic tree construction. Statistical analysis was performed using Bootstrap method with 1000 replicates for tree construction.

The query sequence was submitted to NCBI.gov.nlm.in to retrieve similar sequences from gene bank database, which were aligned pair wise, and 13 sequences with maximum similarity of 95-100% were selected. These were used for CLUSTAL W nucleotide alignment. After multiple alignment of the query sequence, Phylogenetic tree construction was carried out by using maximum likelihood method (Saitou.N and Nei.M, 1987) following Kimura 2 parameter for distance calculation (Kimura.M, 1980). Bootstrap analysis of 1000 replicates was performed for Phylogenetic tree construction and Statistical nodes are placed near to the branches in the tree (Felsenstein.J, 1985). For construction of Phylogenetic tree MEGA software version 6.06 was used (Tamura et al., 2013).
RESULTS AND DISCUSSION

5.2 CHARACTERIZATION OF THE ISOLATE

5.2.1 MORPHOLOGICAL IDENTIFICATION

i. Colony morphology:

The colonies of the isolated bacterial species were analyzed for colony morphology and their description for further distinction between the species is given in Table 11. A petriplate showing colony morphology of the isolate was shown in Figure 7.

Table 11: Morphological features of isolated bacterial colonies

<table>
<thead>
<tr>
<th>S.No</th>
<th>Isolate code</th>
<th>Colony morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A1</td>
<td>Round small in size, blue green pigmented colonies</td>
</tr>
<tr>
<td>2</td>
<td>A2</td>
<td>Round smooth, entire, mucoid colonies</td>
</tr>
<tr>
<td>3</td>
<td>A3</td>
<td>Small, round, entire, mucoid colonies.</td>
</tr>
<tr>
<td>4</td>
<td>A4</td>
<td>Small, shiny, round, entire, sticky colonies.</td>
</tr>
<tr>
<td>5</td>
<td>A5</td>
<td>Small, mucoid, entire, round colonies</td>
</tr>
<tr>
<td>6</td>
<td>A6</td>
<td>Large, opaque, dense with irregular margins on the edges</td>
</tr>
</tbody>
</table>

Figure 7: Colony morphology of the bacterial isolate
ii. **Gram staining:**

All the isolates were subjected to Gram staining for morphological observation. The isolates were given codes for convenience as follows A1, A2, A3, A4, A5, and A6. Out of all the 6 isolates, A6 was found to be Gram positive rod (Figure 8) and remaining isolates were found to be Gram negative rods. Gram staining of the isolates revealed that all the isolates were of rod shaped bacteria. The staining results were tabulated in Table 12.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Isolate code</th>
<th>Gram staining result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A1</td>
<td>NEGATIVE rods</td>
</tr>
<tr>
<td>2</td>
<td>A2</td>
<td>NEGATIVE rods</td>
</tr>
<tr>
<td>3</td>
<td>A3</td>
<td>NEGATIVE rods</td>
</tr>
<tr>
<td>4</td>
<td>A4</td>
<td>NEGATIVE rod</td>
</tr>
<tr>
<td>5</td>
<td>A5</td>
<td>NEGATIVE rods</td>
</tr>
<tr>
<td>6</td>
<td>A6</td>
<td>POSITIVE rods</td>
</tr>
</tbody>
</table>

Figure 8: Gram staining of the isolate, A6
iii. **Motility test:**

The motility of the isolate was tested by Agar dip test, which constitutes 0.6% of agar in the medium, allowing the organism’s movement in the medium. After incubation, the growth of the isolate reached to the surface of the inoculated medium to understand that the isolate is motile in nature. If the organism is motile, the movement of the growth can be observed around the inoculated place and if the organism is non-motile, the growth of the isolate will be restricted to the inoculated site in the medium. Based on this test results, the motility of the organism was confirmed.

### 5.2.2 BIOCHEMICAL IDENTIFICATION

The results for the biochemical identification of the isolates were discussed in this section. All the biochemical identifications were followed according to Bergey’s manual of determinative bacteriology 9th Edition, 1994.

#### 5.2.2.1 Enteric tests:

Enteric tests or IMViC tests are used for the identification of the isolate to know whether the bacteria belong to enteric group or not as it was isolated from a wastewater sample collected from sewage treatment plant, Visakhapatnam, India. Sewage water is contaminated with many enteric bacteria. IMViC tests were performed for the isolated bacterial isolate.

i. **Indole test:**

Indole test is used for the identification of microbes. Indole test detects the presence of an enzyme *tryptophanase*, oxidizes of tryptophan present in the medium. The
ability to oxidize tryptophan with the production of indole is not a characteristic of every organism. The result for the isolate was found to be indole negative as the isolated organism A6 was unable to produce indole from tryptophan due to the absence of enzyme \textit{tryptophanase}. The result of the test is shown in Figure 9.

\textbf{ii. Methyl red test:}

Monosaccharide, glucose is the major substrate for all enteric bacteria for energy production. The end product will vary depending on specific enzymatic pathways present in the bacteria. Bacterial metabolism produces acidic products like 2, 3-butanediol and acetoin (acetyl-methylcarbinol) and reduces the pH of the medium. \textit{E.coli} bacteria will reduce the pH of the medium to approximately 4 and the indicator turns red. This test is specific for \textit{E.coli}. The result of the methyl red test for the isolate A6 was found to be negative. The result of the test is shown in Figure 12, indicates that the given isolate is not \textit{E.coli}.

\textbf{iii. Voges-proskauer test:}

The Voges-proskauer test determines the capability of organisms to produce non-acidic neutral end product, acetyl methyl carbinol, from organic acids that are produced from glucose metabolism. Barritt’s reagent (mixture of alcoholic $\alpha$-napthol and 40%, KOH solution) reacts with guanidine group of acetyl methyl carbinol, in presence of $\alpha$-naphthol, changes the medium to pink colour. The result of VP test for the isolate A6 was found to be positive. The result of the test is shown in Figure 15.

\textbf{iv. Citrate test:}

The citrate utilization test determines the capability of microbe to utilize citrate as a sole carbon source in the medium. This ability is due to the presence of \textit{citrate}
permease enzyme that facilitates citrate transport into the cell. Citrate is the first major intermediate in the Kerb’s cycle and is produced by the condensation of active acetyl with oxaloacetic acid. *Citrate permease* enzyme acts on citrate producing oxaloacetic acid and acetate and further converted to pyruvic acid and carbon dioxide. The carbon dioxide generated in the above reaction produces alkaline environment due to the formation of sodium carbonate, by combining with sodium and water. The presence of sodium carbonate changes the bromothymol blue indicator incorporated in the medium from green to deep Prussian blue. The result for the test is shown in Figure 15 and found to be positive for the isolate A6 indicating the presence of *citrate permease* enzyme activity.

v. Urease test:

Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bonds of amide compounds such as urea and forms alkaline product ammonia. The presence of *Urease* enzyme is detected by growing the microbe in presence of urea incorporated with phenol red indicator. Presence of alkaline end product changes medium into deep pink. The result for *Urease* test was shown in Figure 11 and was found to be negative indicating the absence of *Urease* enzyme in the isolated bacterial species.

vi. Nitrate reduction:

The reduction of nitrate to nitrite is an anaerobic process carried out by nitrate reducing bacteria. Anaerobic process of respiration is actually oxidative process in which molecular oxygen is taken from NO$_3^-$ and is transferred to final hydrogen acceptor by enzyme *nitrate reductase* to form water. Reduced nitrites are detected using sulfanilic acid and $\alpha$-napthyl amine in the medium with the production of magenta colour. Further, conversion of nitrite to N$_2$ gas is limited to few genera of microbes and can be detected with the addition of Zinc powder. Due to the absence of nitrites, the medium colour changes to colourless. The result of nitrate reduction by
the isolated bacteria A6 is shown in Figure 15 and was found to be positive indicating that the presence of enzyme nitrate reductase.

vii. Catalase test:

During aerobic respiration, all microorganisms produce hydrogen peroxide in excess quantities and is lethal to cells. To breakdown hydrogen peroxide, microbes produce catalase enzyme which will catalyse H$_2$O$_2$ releasing water and molecular oxygen. Presence or absence of this enzyme is tested for microbial identification. The result of this catalase test of the isolated bacteria A6 is shown in Figure 15 and was found to be positive.

5.2.2.2 Extracellular enzyme production tests

i. Starch hydrolysis test:

Amylase hydrolytic activity can be detected by performing starch test. Starch, in presence of iodine imparts dark blue colour indicating the absence of amylase enzyme and a clear zone around the colony indicates the presence of amylase. The result of starch hydrolysis test is shown in Figure 13 and is positive for isolate A6 suggesting the presence of amylase activity in the isolate.

ii. Gelatin liquefaction test:

Microbes produce proteolytic extracellular enzyme known as gelatinase which can hydrolyze gelatin and breakdown to amino acids. After hydrolysis, the gel characteristic of gelatin cannot be restored even at 4°C. The gelatin test result for the isolate was shown in Figure 14 and was positive indicating presence of gelatinase enzyme.
iii. **Ortho nitro phenyl β-galactoside:**

Bacteria that lack *permeases* come under entereobacteriaceae family and are detected by ONPG test. For metabolizing lactose, bacteria requires two types of enzymes *permeases*, which facilitates the entry of lactose molecule into the cell and the other enzyme *β-galactosidase*, which breakdown lactose molecule in to simple monosaccharide glucose molecules. ONPG is synthetic colourless compound, structurally similar to lactose molecule. When a bacterium cleaves ONPG, galactoside and O-nitrophenyl, yellow coloured compound are formed. This test determines the presence of *β-galactosidase* enzyme by changing the medium colourless. The result of ONPG test for the isolate was shown in Figure 15 and was found to be positive indicating the presence of *β-galactosidase* enzyme.

### 5.2.2.2 Amino acid utilization tests

i. **Arginine test:**

Bacteria utilize arginine from the medium as a source of carbon and energy with the help of enzyme *Arginine dihydrolase*. Utilization of arginine and production of *citrulinate* releases CO$_2$ changing the medium alkaline (Washington Winn.Jr *et al.*, 1997). Indicator Bromo cresol purple, changes the colour to yellow in alkaline environment. The result of arginine dihydrolase test for the isolated bacteria species A6 was shown in Figure 15 and was positive indicating the presence of *arginine dihydrolase* enzyme. The results were tabulated in Table 13.

### 5.2.2.3 Sugar fermentation tests:

Microbes use different carbohydrates in different enzymatic mechanisms. Some microbes use glucose anaerobically while some other bacteria use aerobic pathway producing acids and gases as end products. Using these metabolic mechanisms for sugar fermentation, bacteria are categorized. Carbohydrates including sucrose, glucose, trehalose, arabinose and malonate are used for the identification bacterial species. The results were tabulated in Table 13.
Table 13: Results of biochemical tests for the isolated bacterial species

<table>
<thead>
<tr>
<th>Test</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>A5</th>
<th>A6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enteric tests</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl red</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>Nitrate reduction</td>
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<td><strong>Extracellular Enzyme production tests</strong></td>
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<td>Gelatin liquefaction</td>
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<td><strong>Pigment production test for Serratia species</strong></td>
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<td>Pigment production at 25°C</td>
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<td><strong>Lactose fermentation test (β-Galactosidase activity)</strong></td>
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<td>Sugar fermentation tests</td>
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<td>Malonate</td>
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<td>Sucrose</td>
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<td>Mannitol</td>
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<td>Arabinose</td>
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<td>Glucose</td>
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<td>Trehalose</td>
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Biochemical identification resulted in naming of the isolates

After considering the results of biochemical tests, the isolated bacterial species are identified and are given in Table 14.

Table 14: Summary of isolated cultures biochemical characterisation

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<thead>
<tr>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>A5</th>
<th>A6</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas</em> species</td>
<td><em>Klebsiella</em> species</td>
<td><em>E.coli</em> species</td>
<td><em>Enterobacter</em> species</td>
<td><em>Serretia</em> species</td>
<td><em>Bacillus cereus</em> species</td>
</tr>
</tbody>
</table>

Results were confirmed by following Bergey’s manual of determinative bacteriology, 9th edition.

![Figure 9: Result of Indole test](image1)

![Figure 10: Motility test on semisolid dip](image2)
Figure 11: Urease test

Figures 12: Methyl red tests

Figure 13: Starch agar test

Figure 14: Gelatin liquefaction
These tests revealed that the isolated bacterial species belongs to enteric group. The bacteria belonging to enteric group are *E.coli, Bacillus cereus, Salmonella enteric, Clostridium difficile, Helicobacter pylori, campylobacter* etc. These bacteria colonize in the gut of animals; humans are capable of escaping as bile secretions and gastric enzymes create highly acidic environments and hence tolerant.

### 5.2.3 Genetic identification

#### i. Molecular sequencing (16s rRNA)

A molecular technique carried out in the study is nucleic acid sequencing especially rRNA which is highly conserved and is involved in translation. The bacteria are the least structurally complex microbes, which offer great metabolic activity and diversity. Based on 16s rRNA sequence, more than 50 bacterial Phyla are identified (Schloss and Handelsman, 2004).
The importance of 16s rRNA is illustrated by the highly conserved regions that encode for rRNA. 16s rDNA contains both conserved and versatile genes that encodes for 16s rRNA of smaller subunit that is currently used for classification of bacteria and Archaea. The length of the 16s rRNA is 1500 basepairs and contains most conserved regions so that the scientists have taken advantage in classification and identification of bacteria based on this conserved sequence (Maier.R.M et al., 2009). By using molecular markers, the sequence similarities were recognized for unknown organisms and identified at molecular level. Newly identified 16s rRNA gene sequences from worldwide deposit their sequences in gene bank databases like NCBI and Silvia. The sequences from these gene bank data bases can be retrieved through BLAST (Basic Local Alignment Search Tool) and Phylogenetic analysis can be done using software like MVSP (Kouki.S et al., 2011), BLAST(Chen.P et al., 2012), phylip and MEGA(Zhang.Q.L et al., 2012) etc.

**ii. 16s rRNA Sequence of isolated Bacillus cereus SB1**

The partial sequence of 16s ribosomal RNA gene of the isolated bacteria *Bacillus cereus* SB1 obtained from DNA Bioaxis research centre, Hyderabad was given below. The sequence consists of 471 base pairs.

```
CGCCAACTTAAGGGCCGCCATATAATGGCAAGTCGAGCGGATGGATT
AAAGAGCTTGCTCTTTATAGTTAGCAGGCGGACCGGTAGTAAACACG
TGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTA
ATACCCGGATAACATTTTGAACCCGCATGGTTCTGAATTTGAAAGGC
CGCTCAGCTGACACTTTGATGACACGCTGCTGCCGCTGCTTGCATTAG
CTAGTATGGTTGCTCAGCTGACACTTTGATGACACGCTGCTGCCG
```

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Amplicon of gel analysis for PCR product showing partial sequence of 16s rRNA of the isolated bacteria compared with ladder sequence. In Figure 16, it can be observed that at 1541 base pairs, sample sequence was showing band with ladder sequence.

![Sample vs Ladder](image)

**Figure 16:** Gel electrophoresis of the isolated 16s rRNA sequence of isolate

After obtaining sequence form, Bioaxis DNA research centre, the sequence was used for phylogenetic analysis and as a template for sequence alignment in NCBI blast. The sequence similarities of 98% were only considered for phylogenetic analysis and 13 sequences were retrieved from NCBI database. These sequences from NCBI database can be depicted in the Figure 17.
The results obtained after the phylogenetic analysis were that the isolated bacteria query covers 100% coverage of the sequences *Bacillus cereus* species and sequence similarity was found to be only 99% and there is 1% variation in sequence similarity. This might be due to place of isolation of the species. Bacteria show high diversity in their genetic characters when exposed to different environmental conditions. This can be the fact for variation in similarity matching.
Figure 18: Construction of phylogenetic tree using maximum likelihood method with 1000 bootstrap analysis

It was not showing 100% similarity with any of the given query sequence and this can be confirmed that the isolated organism was found to be new *Bacillus cereus* strain which matches with *Bacillus cereus* genetic sequence up to 99% only. Because it is not matching 100% similarity with any of the sequences from NCBI gene bank, the organism was denoted with new strain name SB1 (Figure 18). Further, the obtained sequence was submitted to gene bank database, and an accession number **KJ 804254** was allotted by NCBI gene bank for future reference.

--------------------------------------End of the Chapter-------------------------------------