Chapter 1  Isolation and characterization of Bacillus sp for polyhydroxyalkanoate production

1.0 INTRODUCTION

A wide variety of microorganisms are capable of synthesizing polyhydroxyalkanoates (PHAs). The amount of PHA accumulated by microorganisms vary from 20-80% of their cellular dry weight and form intracellular granules in the cytoplasm as inclusion which can be observed under light microscope as refractive bodies. PHA was first discovered by Lemogine (1926), who reported the presence of 3-hydroxybutyric acid in the cells of Bacillus megaterium. The similarity in material properties of PHA with polypropylene has enabled the use of the polymer in several applications, such as packaging, pharmaceuticals, agriculture and food industry, or as raw materials for the synthesis of enantiomerically pure chemicals and the production of paints (Anderson and Dawes, 1990).

PHAs are synthesized as intracellular energy and carbon storage material, which enable the bacteria to survive under certain adverse conditions. They are biodegradable, insoluble in water, non-toxic, biocompatible, thermophilic and elastomeric. The bacterial origins of the PHAs make these polyesters a natural material, and many microorganisms have evolved the ability to degrade these macromolecules.

It took a long time to achieve the commercial production of PHA and it was produced in 1982, by ICI at commercial level under the trade name Biopol®. Among the various microorganisms identified as PHA producers, only a few have been exploited commercially for PHA production due to their higher efficiency to accumulate PHA. Some of the commercially important strains are Ralstonia eutropha, Alcaligens latus, Azotobacter vinelandii, Pseudomonas spp etc. Various Bacillus spp are known to produce PHA (Valappil et al 2007a; Shamala et al, 2003). Generally Bacilli produce scl - PHA, which comprises of PHA of C4-C6 and a few strains are reported to the ability of some members in the genera to produce mcl –PHA also (Caballero et al, 1995; Tajima et al, 2003).
There are advantages using *Bacillus* spp as PHA production hosts as they grow fast, can produce copolymers from single carbon substrate and they can utilize cheaper carbon sources for polymer synthesis (Table 6). The fermentation can result in the release of certain industrially important enzymes such as proteases and amylases, they lack lipopolysachharide which is present in gram-negative organisms that co purify with PHA and causes immunogenic reactions in certain individuals. The genome data for some *Bacilli* are available so it is easier to manipulate them genetically. A vast number of *Bacillus* spp can be isolated from the environment, which may produce varied quantity and quality of the polymer. The present chapter describes the isolation and screening of some PHA producing *Bacillus* spp, identification and characterization of a potent PHA producing strain using morphological, physiological and molecular techniques.
1.1 MATERIALS AND METHODS

Media used for maintenance of *Bacillus spp* and production of polymer using the isolated strains are describe below. *E. coli* DH5α was grown in LB medium and the composition of which is detailed under general materials and methods (page 53).

1.1.1 Nutrient Agar (Maintenance medium)

<table>
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<td>NaCl</td>
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<tr>
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<tr>
<td>Yeast extract</td>
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<td>Agar</td>
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<td>pH</td>
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1.1.2 PHA production medium

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</thead>
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<tr>
<td>KH₂PO₄</td>
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</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1.5</td>
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<tr>
<td>MgSO₄ 7 H₂O</td>
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</tr>
<tr>
<td>Sucrose</td>
<td>20.0</td>
</tr>
<tr>
<td>pH 7.0</td>
<td></td>
</tr>
</tbody>
</table>

1.1.3 Isolation of *Bacillus spp*.

Soil samples were collected from different parts of the country and stored at room temperature. The dry soil samples were heated at 60°C for 30 min, serially diluted and plated on nutrient agar plates. The plates were incubated at 30 °C for 24-48 h. In the identification process the *Bacillus spp* were initially selected based on the Gram reaction, morphology and catalase test. The screening of PHA producing *Bacilli* was performed by Sudan black B staining method (materials and methods page 52). The *Bacilli* showing
PHA granules were selected for further studies. The selected strains were used further for molecular detection of PHA synthase gene by polymerase chain reaction (PCR).

1.1.3.1 Inoculum
Inoculum was prepared by transferring 1-2 loop full of 24 h old slant culture in to 5 ml of nutrient broth. The cultures were allowed to grow at 30°C, 250 rpm for 18-20 h. The inoculum hence obtained contained 2 x 10³ viable cells / ml as assayed on nutrient agar plates.

1.1.3.2 PHA production in shake flasks
The isolated strains were cultivated in sterile PHA production medium (50 ml) in triplicate that was contained in 250 ml capacity Erlenmeyer flasks. The above-mentioned inoculum was transferred in to production medium and the flasks were incubated at 30°C, 250 rpm for 72 h.

1.1.3.3 Estimation of biomass and extraction of PHA
Culture broth was centrifuged (7000 rpm, 20 min) and the washed cell sediment was dried to a constant weight at 70 °C. The dried cells were extracted by sodium hypochlorite extraction method, which is described under the materials and methods section (Page 56).

1.1.4 Characterization of  Bacillus sp. 256
Taxonomic characteristics of the Bacillus sp 256 was studied by following biochemical tests (Reddy and Reddy, 2000).
1.1.4.1 Gram staining

Reagents

- **Crystal violet**
  
  **Solution A**
  - Crystal violet (90% dye content) 2 g
  - Ethyl alcohol (95%) 20 ml

  **Solution B**
  - Ammonium oxalate 0.8 g
  - Distilled water 80 ml
  - Mix the solutions A and B

- **Gram’s iodine**
  - Iodine 1 g
  - Potassium iodide 2 g
  - Distilled water 300 ml

- **Ethyl alcohol 95%**

- **Safranin**
  - Safranin 0.25 g
  - Ethyl alcohol 95% 10 ml
  - Distilled water 100 ml

A thin smear of the culture was made on a glass slide and heat fixed. Crystal violet reagent was added to the smear and allowed for 30 sec. Excess of stain was removed by rinsing with distilled water. Iodine solution was added and the slide was left for 30 sec and rinsed again with distilled water. Ethanol was added to remove excess stain and the slide was rinsed with water and finally safranin solution was added and allowed to act for 30 sec. The slide was then washed and then observed under the microscope. Retention of crystal violet indicated that the bacterium was gram positive and uptake of pinkish safranin counter stain by cells was considered gram negative.
1.4.1.2 Endospore staining

- A smear of *Bacillus sp* 256 was made on a clean slide, the cells were air dried and heat fixed
- A few drops of 5% malachite green solution were placed on the smear and the slides were heated by steaming for 5 minutes. More stain was poured on to the smear from time to time.
- The slides were washed under slow running water
- Samples were air dried and counter stained with 0.5% safranin and allowed for staining for 30 second and excess stain was removed using water and blotted to remove the moisture and dried
- The slides were viewed under microscope. Bluish green spores in the cells indicated presence of endospore.

1.1.4.3 Cell size

Cell size was measured using ocular micrometer (one division = 1/10mm) in the microscope. The ocular micrometer was calibrated by using the stage micrometer (1 scale division = 1/100 mm). The cell size was calculated on the basis of 10 values recorded for length and breadth.

1.1.4.4 Catalase test

NA slants were inoculated with the culture and incubated overnight at 30°C. 1ml of 3%H₂O₂ was pipetted on to the slant. The slant was examined for evolution of bubbles, the presence of which indicated a positive test.

1.1.4.5 Oxidase test

A filter paper strip was moistened in 1% solution of N,N,N₁,N₁-tetramethyl-p-phenylenediamine-hydrochloride. Cells from the test culture were transferred on to the paper. Development of purple colour indicated that the test was positive for oxidase.
1.1.4.6 Nitrate reduction

The basal medium was supplemented with 0.1% potassium nitrate and 0.17% agar and inoculated with fresh culture of Bacillus sp 256 and incubated for 24 h. To 24h old culture 1 ml of solution A (1-naphthyl ethylenediamine (0.02 g) dissolved in 100 ml of 1.5N HCl) and 1 ml of solution B (1 g of sulfanilic acid in 100 ml of 1.5N HCl) were added. The development of pink/ red colour indicated the presence of nitrite in the medium.

1.1.4.7 Hydrolysis of gelatin

12% gelatin was added to nutrient agar medium and the Bacillus was inoculated and incubated at 30\(^{0}\)C for 24h. The tubes were observed for hydrolysis or loosening of the solid medium.

1.1.4.8 Hydrolysis of casein

Skimmed milk agar was prepared by mixing sterile skimmed milk with double strength nutrient agar medium at 50\(^{0}\)C and plated. The plate was streaked with test culture and incubated. The clear zone around the colony indicated the hydrolysis of casein.

1.1.4.9 Hydrolysis of starch

Nutrient agar medium containing 2% starch was prepared in petriplates. The plates were streaked with the test organism and incubated at 30\(^{0}\)C for 24h. The plates were treated with iodine solution and observed for the clearance zone around the colony.

1.1.4.10 Acid production from sugars

Nutrient agar medium containing 5mg% of bromo cresol purple and 2g% of sugar was prepared. Various sugars tested included arabinose, mannitol, lactose, xylose, rhamnose, cellobiose, sucrose, glucose and maltose. The bacterial culture was inoculated in the medium and incubated. The colour change from purple to yellow indicated acid production by bacteria.
1.1.4.11 MR-VP test

**Requirements** Nutrient broth culture, MR-VP broth tubes, methyl red, pH indicator, Barrit’s reagent

**Procedure**

1) Took 5 ml of MR-VP broth in each tube and sterilized by autoclaving.
2) Inoculated two MR-VP broth with *Bacillus* 256, incubated at 37°C for 48 h.
3) At the end of incubation period, added 1-2 drops methyl red and 2-3 drops of Barrit’s reagent mixed thoroughly after removing the cap to expose optimum amount of oxygen.
4) Allowed the reaction to complete for 15-30 minutes.

The results were analysed on the basis of presence of red and yellow color (+ve and –ve for MR respectively); presence of pink colour (VP positive and colourless for VP negative).

1.1.4.12 Growth in 3% NaCl and anaerobic growth in the presence of glucose

The strain was inoculated into the nutrient broth and nutrient agar media containing 3 % NaCl and incubated overnight at 30°C.

The strain was stabbed into nutrient agar medium containing 1 % glucose, the culture was overlaid with sterile glycerol and incubated overnight at 30°C.

1.1.4.13 Confirmatory tests for *B. endophyticus*

Tubes containing the culture were incubated at 28°C for 3-4 days on PHA agar medium. Anaerobic growth was tested in the fermentative medium under sterile mineral oil. Growth in the presence of ampicillin (100µg/ml) and lysozyme (1 mg/ml) were tested in nutrient broth for 3-4 days at 28°C.

1.1.5 Molecular characterization

The molecular level characterization of the *Bacillus sp* 256 was carried out by 16srRNA gene sequence homology study. One portion of the 16srRNA gene was amplified from
genomic DNA of *Bacillus sp* 256 by PCR. The primer used for PCR amplification is given below (Table 11).

**Table 11: 16SrRNA gene primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>rRNAF</td>
<td>GCT CTA GAG CGA TTA CTA GCG ATT CCG ACT TCG</td>
<td>1324–1353</td>
</tr>
<tr>
<td>rRNAR</td>
<td>CGA CGT CGG CTC AGG ATG AAC GTC GGC GGC</td>
<td>15-43</td>
</tr>
</tbody>
</table>

**1.1.5.1 Polymerase chain reaction**

The PCR reaction was carried out by combining the following components in 25μl reaction volume:

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (μl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
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<td></td>
</tr>
<tr>
<td>10 X Reaction Buffer</td>
<td>2.5</td>
<td>1 x</td>
</tr>
<tr>
<td>dNTP mix (10 mM)</td>
<td>0.5</td>
<td>0.2 mM</td>
</tr>
<tr>
<td><em>Taq</em> polymerase (3U/μl)</td>
<td>0.3</td>
<td>0.03U/μl</td>
</tr>
<tr>
<td>Primer B1F (Forward)</td>
<td>1.0</td>
<td>0.2 μM</td>
</tr>
<tr>
<td>Primer B1R (Reverse)</td>
<td>1.0</td>
<td>0.2 μM</td>
</tr>
<tr>
<td>Template (~100 ng)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>25.0</td>
<td></td>
</tr>
</tbody>
</table>

10-x reaction buffer for *Taq* polymerase (Bangalore Genie, India) contained 15 mM MgCl₂ and 0.1% gelatin. The contents of the tube were mixed by a brief spin in a micro centrifuge. The reaction was carried out in a thermocycler GeneAmp PCR System 9700 (Perkin-Elmer, USA) and the reaction parameters were as follows:
Initial denaturation: 94\(^0\)C for 3 min
Denaturation: 94\(^0\)C for 1 min
Annealing: 46\(^0\)C for 1 min
Extension: 72\(^0\)C for 2 min
Final extension: 72\(^0\)C for 10 min

The 1.2 kb PCR product was cloned in sequencing vector pTZ57R/T and sequenced partially by dideoxy method at Bangalore Genie, Bangalore, India. The sequence data was taken for further analysis.

1.1.6 Construction of phylogenetic tree
The phylogenetic relationship of the Bacillus sp 256 was studied by deriving phylogenetic tree from BLAST results.

1.1.7 Detection of PHA producing Bacillus sp by PCR
Genomic DNA was isolated from all the Bacillus isolates and subjected to PCR using phaC specific primers (Table 12).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon size</th>
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</thead>
<tbody>
<tr>
<td>B1F</td>
<td>AACTCCTGGGCTTGAAGACA</td>
<td>600 bp</td>
</tr>
<tr>
<td>B1R</td>
<td>TCGCAATATGATCACGGCTA</td>
<td></td>
</tr>
</tbody>
</table>

1.1.7.1 Methodology
Various PCR conditions were optimized to obtain the 600 bp amplicons for phaC gene. Primer combination of B1F and B1R resulted in amplification of a product of 600 bp in size. The PCR was carried out by the following PCR parameters: Initial denaturation at 94\(^0\)C for 2 min, annealing at 56\(^0\)C for 1.5 min and the extension temperature at 72\(^0\)C for 2 min and a final extension step at 72\(^0\)C for 10 min. The PCR amplifications were performed using Taq DNA polymerase (Bangalore Genei, India). The PCR reactions were conducted in 25 \(\mu\)l volumes containing 50 ng of Bacillus genomic DNA, 10 mM of
dNTP mix, 10 μM each of forward and reverse primer and 1x Reaction Buffer (10 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM MgCl$_2$, 0.01% gelatin)

### 1.1.7.2 Analysis of PCR product

After the reaction, 10 μl of the sample was run on 1% agarose gel electrophoresis as described in chapter III (page 124). The size of the *Bacillus phaC* gene amplicon was checked in comparison with a 100 bp DNA ladder (Bangalore Genei, India).
1.2 RESULTS

1.2.1 Isolation of Bacillus sp and PHA production.
Different Bacillus sp were isolated from various soil samples. PHA production was carried out using 38 isolated strains. Growth and accumulation of PHA among these strains varied in the medium containing sucrose as carbon source (Table 13). Accumulation of PHA varied from 12-55% of dry biomass weight. Maximum concentration of PHA was synthesized by Bacillus sp 256 compared to others. This culture gave optimum growth on nutrient agar slant and dense growth in synthetic medium and was used for further studies (Figs. 14 & 15). Amongst the cultures isolated many of them gave poor yields of PHA ranging from 12% to 20% of biomass dry weight (No. 2, 3, 4, 8, 9, 11, 14, 17, 18, 19, 22, 18, 25, 31, and 36), while in others the yields ranged from 20-55%.

1.2.2 PHA production by Bacillus sp 256
The Bacillus sp 256 grew efficiently in PHA production media, containing sucrose as the carbon source, and accumulated PHA at 18-20 h of incubation. Maximum PHA accumulation occurred during 65 to 70 h. The polymer concentration decreased after 72 h of the growth due to intracellular degradation of the polymer. The isolated strain produced 55% PHA of dry biomass weight.

1.2.3 Characterization of Bacillus sp 256
Bacillus sp 256 was characterized by morphological, biochemical (Table 14) as well as at molecular level. Bacillus 256 was gram positive, rod shaped (Fig. 16A) and non motile. The bacterium produced endospores at apical position (Fig. 16B). The cells were found in chains. The size of the cell was determined and it was 6 x 1.5μ. The Bacillus colonies were circular, off white dry and translucent. It showed positive reaction for catalase, nitrate reduction, and oxidase tests. It could not hydrolyse starch, gelatin and casein.
Table 13: Accumulation of PHA by various *Bacillus* isolates

<table>
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<th>Culture No.</th>
<th>Biomass (g/l)</th>
<th>PHA% of dry biomass</th>
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<td>1</td>
<td>1.8</td>
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<tr>
<td>2</td>
<td>2.2</td>
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<td>3</td>
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<td>5</td>
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<tr>
<td>6</td>
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<td>16</td>
</tr>
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<td>37</td>
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<td>26</td>
</tr>
<tr>
<td>256</td>
<td>2.8</td>
<td>55</td>
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</table>
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Fig. 14: Slant culture of *Bacillus sp 256*

Fig. 15: Shake flask culture of *Bacillus sp 256*
The ability of the strain to utilize sugars was tested and it was found that the bacterium was able to produce acids from all the sugars provided for growth except lactose. The results of the morphological studies suggested that the isolated bacterium belonged to the genus *Bacillus*. The strain showed similarities with *B. endophyticus* because: it had ellipsoidal spore situated at terminal position (Fig. 16B); nonmotile, absence of anaerobic growth; Voges-Proskauer negative; oxidase positive; not able to hydrolyze casein, gelatin and starch; acid production from arabinose, glucose, manitol, maltose, mannose, rhamnose and xylose; etc. The strain produced pale pink pigmentation on PHA agar slants and the pigment produced was not diffusible. On nutrient agar the colonies were slimy; the cells were resistant to ampicillin and also grew in the presence of lysozyme. Further the bacterium was subjected to molecular phylogeny characterization.

### 1.2.4 Molecular phylogeny of *Bacillus sp 256*

This was carried out by studying the 16SrRNA sequence homology. One portion of 16SrDNA was amplified from the genomic DNA of *Bacillus sp 256* by PCR (Fig. 17). The DNA fragment was cloned into sequencing vector and sequenced partially (Table 15). The DNA sequence was analysed using various online softwares. The BLAST analysis of the 16SrDNA sequence (Table 16) showed DNA sequence similarity with *Bacillus endophyticus* (99%), *Bacillus sp*. 19490 (99%), *Bacillus sp*. GB02-16/18/20 (97%), *Bacillus sp*. MSSRF (96%) etc. Using the *Bacillus sp* 256 sequence the phylogenetic tree of *Bacillus sp 256* was derived from BLAST results (Fig. 18). The test strain was closely related to *B. endophyticus* than with *B. megaterium* which is known to produce PHA.
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Fig. 16:  
(A) Gram stained cells of *Bacillus sp* 256  
(B) Endospore of *Bacillus sp* 256 (malachite green stained cells)
Table 14: Characterization of Bacillus sp 256

<table>
<thead>
<tr>
<th>Morphological characters</th>
</tr>
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<tbody>
<tr>
<td>Gram staining</td>
</tr>
<tr>
<td>Presence of endospore</td>
</tr>
<tr>
<td>Cell size</td>
</tr>
<tr>
<td>Cell shape</td>
</tr>
<tr>
<td>Motility</td>
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<table>
<thead>
<tr>
<th>Morphological characters of the colony</th>
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<tbody>
<tr>
<td>Shape</td>
</tr>
<tr>
<td>Colour</td>
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<tr>
<td>Surface</td>
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<table>
<thead>
<tr>
<th>Biochemical characters</th>
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<tr>
<td>Catalase</td>
</tr>
<tr>
<td>Nitrate reduction</td>
</tr>
<tr>
<td>Anaerobic growth with 1% glucose</td>
</tr>
<tr>
<td>M.R.test</td>
</tr>
<tr>
<td>V.P test</td>
</tr>
<tr>
<td>Oxidase</td>
</tr>
<tr>
<td>Growth in 3% NaCl</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
</tr>
<tr>
<td>Hydrolysis of casein</td>
</tr>
<tr>
<td>Hydrolysis of gelatin</td>
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<tr>
<td>Anaerobic growth at 50°C</td>
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<table>
<thead>
<tr>
<th>Acid production</th>
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<tbody>
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<td>Arabinose</td>
</tr>
<tr>
<td>Mannitol</td>
</tr>
<tr>
<td>Lactose</td>
</tr>
<tr>
<td>Xylose</td>
</tr>
<tr>
<td>Rhamnose</td>
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<tr>
<td>Cellobiose</td>
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<td>Sucrose</td>
</tr>
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<td>Glucose</td>
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<td>Maltose</td>
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</table>
Fig. 17: PCR amplification of 16S rDNA gene of Bacillus sp 256

Table 15: 16S rDNA sequence from Bacillus sp 256

GATTCGAGCTCGGTACCTCGCAGATGCATCTAGATTGCTCTAGACGATTACTAGCGATTTCCGACTTCTG

GGCGAGTTTGAGCTAGCTAGCAGATTTTATGGGATTTTGCTCAGCTCCTACGTTTTGCGAGCCCT

TGTTACATTTAGTGACAGCTGTCGCCAGCTAAAGGCTATGATTGTTGACCTCCACCATCCACCT

TCCTCCTGGTTACGGCAGTGACCTACTAGTGGCCAACACTGAACTGCTGCAAGACTCGGAGAGACA

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1 kb
Table 16: BLAST results of 16SrDNA sequence

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- Bacillus endophyticus
- Bacillus sp 256
- Bacillus endophyticus
- Bacillus sp 1949016SrRNA
- Bacillus endophyticus 16 SrRNA
- Bacillus sp SCD 2001
- Bacillus sp GB 0230 16SrRNA
- Bacillus sp GB02-161820
- Bacillus spMSSRF1 16 SrRNA

Fig.18: Phylogenetic tree of Bacillus sp 256
1.2.4. Molecular detection of PHA producing *Bacillus* spp.

PCR reaction was carried out for molecular detection of PHA producing *Bacillus* spp. The genomic DNA was isolated from all the *Bacillus* isolates and using it as a template one portion of *PhaC* gene was amplified, using *PhaC* specific primers. A 600 bp fragment of *phaC* gene was amplified from the genomic DNA of some *Bacillus* isolates by PCR. The size of the fragment was confirmed by agarose gel electrophoresis using 100 bp DNA ladder (Fig. 19).
Fig. 19: PCR amplification of \textit{phaC} fragment from various \textit{Bacillus} isolates: Lane 8: 100 bp marker; lanes: 1-6 isolate numbers 5, 6, 13, 21 and 32 (as in Table 12), respectively; Lane 7: \textit{B. megaterium}
1.3 DISCUSSION

Bacteria belonging to the genus *Bacillus* are Gram-positive rods that produce heat resistant endospores during their growth cycle. Morphological groups have been formed based on the shape and position of the endospores and shape of the sporangium or mother cell (Gordon *et al.*, 1973). Genus *Bacillus* are widespread and can be isolated from various habitats such as marine and aquatic regions, thermal or Antarctic areas and from soil. They are isolated from acidic and alkaline environments. *Bacillus* species are also found in the inner tissues of various plants such as cotton, sweet corn (Misaghi and Donndelinger, 1990; McInroy and Kloeppe, 1995) etc, where they are known to protect the plants from pathogenic fungi and support growth promotion and enhance the plants natural resistance (Emmert and Handelsman, 1999). These endophytic bacteria are known to occur as free-living soil bacteria and this includes *B. cereus* (Pleban *et al.*, 1997), *B. megaterium* (McInroy and Kloeppe, 1995) and *B. pumilus* (McInroy and Kloeppe, 1995; Benhamou *et al.*, 1998). Out of 78 strains of bacteria isolated and characterized from the inner tissues of healthy cotton plants (*Gossypium sp*) majority of isolates were identified as *Bacillus amyloliquifaciens, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus* and *Bacillus subtilis* but four strains could not be assigned to any known species of *Bacillus* or the related genera of the aerobic, endospore forming bacteria. Further the authors identified these as the novel species of the genus *Bacillus*, for which the name *Bacillus endophyticus sp* nov was proposed (Reva *et al.*, 2002). Phylogenetic analyses indicated that the strain belonged to the genus *Bacillus* and was most closely related to *Bacillus sporothermodurans* with a sequence similarity of 98% (Fig. 20).

In the present study, 38 *Bacillus spp* were isolated from different dry soil samples collected from various parts of the country. The soil samples were heated at 60°C to eliminate the non-spore forming bacteria. All the isolates possessed catalase activity. Among the different isolates of *Bacillus, Bacillus sp* 256 showed the highest growth and PHA accumulation. This culture was isolated from soil sample collected from a very hot and dry area in the northern region of the country. The strain was characterized by
morphological, biochemical as well as molecular methods. The distinguishing phenotypic characters for this isolate as identified by morphological and biochemical characters are shown in Table 17. The strain isolated was Gram-positive rods and the cells occurred singly and in short chains (Fig. 16A). Growth on PHA agar slants occasionally produced pale pink colour that was not diffusable or water-soluble. The strain was examined further by 16S rRNA to determine its relationship at the genomic level. The sequence was aligned with about 80 published and unpublished Bacillus related 16S rRNA gene sequences and was found to match by 99% with that of Bacillus endophyticus. This species has been isolated only recently (Reva et al, 2002), and hence only the biochemical and morphological characterization and consultation of earlier identification data may lead to its identification as B. licheniformis. Differences between these species as reported in the literature are shown in Table 17. The phylogenetic tree constructed by Reva et al., (2002), indicates that B. licheniformis is closer to B. endophyticus in the evolutionary position (Fig. 20). Based on these factors the isolated culture has been identified as B. endophyticus. This is the first report, which indicates that the strain can exist outside the host plant tissue as a soil bacterium and has the ability to produce higher quantity of PHA. The amount of PHA produced is relatively high (55% of biomass) and it appeared encouraging to study various aspects of PHA production and characterize the PHA synthesis genes using this new strain.
Table 17: Distinguishing characters of *Bacillus sp* 256 isolated from soil compared to known endophytic isolates of *B. licheniformis* and *B. endophyticus*

<table>
<thead>
<tr>
<th>Characters</th>
<th><em>B. licheniformis</em></th>
<th><em>B. endophyticus</em></th>
<th>Current soil isolate</th>
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<tr>
<td>Position of the spore</td>
<td>Central</td>
<td>Terminal</td>
<td>Terminal</td>
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<tr>
<td>Spore shape</td>
<td>Oval</td>
<td>Ellipsoidal</td>
<td>Ellipsoidal</td>
</tr>
<tr>
<td>Cell width &gt;1.0μm</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
<td>Non motile</td>
<td>Non motile</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
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<td>-</td>
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<tr>
<td>Hydrolysis of Starch</td>
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<td>-</td>
</tr>
<tr>
<td>&quot; Gelatin</td>
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<td>-</td>
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</tr>
<tr>
<td>&quot; Casein</td>
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<td>-</td>
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<tr>
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<td>Growth at 50ºC</td>
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* Reva *et al*, 2002
Fig. 20: Phylogenetic tree showing the evolutionary position of *B. endophyticus* in the family of Bacillaceae (Reva et al., 2002). This indicates the proximate relationship of the species with that of other Bacilli with special reference to *B. licheniformis*.
1.4 CONCLUSIONS

To date, *B. endophyticus* has been isolated from the inner tissues of cotton plants, and this is the first report to show that it exists outside this habitat similar to other endophytic bacteria that have been isolated as free living forms from the soil. Importance of *Bacillus* in food fermentation has been known since long time. The genus is industrially important for the production of extracellular amylases, proteases. The genus also includes several species that are pest control agents. This is the first report to show that *Bacillus endophyticus* can exist in the soil habitat as a free living form and can produce industrially important polymer such as polyhydroxyalkanoate.