Poly-β-hydroxybutyric acid (PHB) and similar bacterial polyesters are promising candidates for the development of environment-friendly, totally biodegradable plastics. The cost of these biopolymers is 25% more than the synthetic polymers that prevents their usage in wider range of applications. In order to reduce the cost, much effort has been made to screen the promising indigenous PHB producing strain in the present study. As a first step, among thirty scl (Short-Chain-Length) positive strains screened, the most promising mcl (Medium Chain Length) PHA positive indigenous isolate, Pseudomonas LDC-5 was selected for further characterization. The nucleotide sequence analysis of the specific PCR product revealed open-reading frames probably relevant for PHA biosynthesis. The similarity search for nucleotide sequence exhibited 92% homology with Pseudomonas sp. The amino acid sequences of the putative proteins deduced from these genes indicate that they encode a PHA synthase, which exhibited 96% amino acid identity with PHA synthase from Pseudomonas putida. Sequence alignment of the partial sequence of PHA synthase genes and putative proteins showed conserved signatures. Phylogenetic analysis further places the origin of indigenous isolate closer to P. putida. The partial sequence of PHA synthase was submitted in EMBL and obtained the accession number AJ586810. FT-IR spectral analysis confirmed the presence of strong characteristic ester carbonyl band at 1733cm⁻¹. The identification of this novel biopolymer producing strain reveals a capability for the synthesis of technically interesting biopolymers in future.

**Keywords:** Poly-β-Hydroxybutyrate, Cloning, Colony PCR, Pseudomonas sp., FT-IR analysis, phb Operon

**IPC Code:** Int.Cl.7: C08G63/00; C12N15/10; C12R1:38

**Introduction**

The past hundred and fifty years of this millennium have witnessed an unbelievable pace of scientific and technological development making this century seem like a sci-fi novel. Plastic is considered to be an invaluable gift of modern science and technology to mankind. This unique wonder material has some mutually exclusive qualities of being very light, yet strong and economical. The mammoth scale of use of plastics and their improper disposal has threatened natural environment the world over. Thus the need of the hour is to find alternative materials, having the physical and industrial properties similar to petrochemically derived plastics, which are biodegradable.

Biodegradable polymers have evolved significantly over the past fifteen years. Poly-hydroxy alkanoates (PHAs) are one such biopolymers, which they are synthesized by a broad range of bacteria as carbon and energy storage products. PHAs are of technological and commercial interest because the extracted materials are thermoplastics, which can be processed into a variety of consumer goods and medical devices. In contrast to petroleum-based plastics, these biologically produced polymers are synthesized from renewable resources and are completely biodegradable.

The genes responsible for PHA biosynthesis from acetoacetyl coA to PHB in three enzymatic steps, comprises a β-ketothiolase (phaA), and acetoacetyl coA reductase (phaB) and the PHA synthase (phaC) the key enzyme of PHA biosynthesis. These genes are clustered and are presumably organized in one operon. The gene encoding the PHA synthase (phbC) is adjacent to phbA and phbB enzymes closely linked to the biosynthesis of scl-PHA.

Cloning of PHB synthase genes provides the information to find out whether the indigenous isolate has the new type of PHA synthase exhibiting an unusual bioplastic production.

In this study, the authors report the cloning of partial phaC synthase gene of indigenous isolate...
Pseudomonas sp. LDC-5 and sequence analysis using Bioinformatics tools.

Materials and Methods

Bacterial Strains and Culture Conditions
PHA positive bacterial strains Ralstonia eutropha (CH34) and Bacillus megaterium were obtained as a gift from Dr Daniel van der Lelie, USA and Bacillus Gentic Stock Center, respectively and used as a reference strain. Escherichia coli XL-1 Blue was used as PHA negative strain. The PHB producer candidates were screened as previously reported by plating the serially diluted soil samples collected from various ecological niches in and around Madurai district on LB medium supplemented with 2% glucose and 1% Nile red and the plates were incubated at 37°C for 48 h. Colonies with pinkish pigment were selected for further studies and maintained on LB-Glucose slants at 4°C and sub cultured periodically. The PHB accumulation in these strains was determined by growing in RC growth medium, supplemented with filter sterilized carbon source (1% glucose) at 30°C for 3 days with an agitation speed of 100 rpm. Solid media were prepared by adding agar 1.5% (w/v) to the corresponding broth before autoclaving. Chloramphenicol was used at a final concentration of 20 μg/mL. Growth was monitored photometrically at 600 nm.

Molecular Biology Procedures
Colony-PCR (scl-Short Chain Length), mcl-PCR (Medium Chain Length) and semi-nested PCR were used to identify the PHA producers as previously reported. Based on our previous report, Pseudomonas sp. LDC-5 was selected for further characterization. Genomic DNA was isolated from the Pseudomonas sp. LDC-5 (colony with pinkish pigment) as described. In each colony-PCR reaction mixture contained 30 ng of genomic DNA, 1x buffer, 3 mM MgCl₂, 200 μM each deoxynucleotide triphosphate, 1U Taq DNA polymerase (Fermentas) and 2.5 μM each primer (designed based on conserved sequences deduced from a multiple alignment analysis of the phaC genes) as previously described. The thermal cycle programme run on an Eppendorf PCR system (Mastercycler Personal 5332). PCR products were analyzed by 1.3% agarose gel electrophoresis and ethidium bromide staining. The amplified DNA fragments were visualized by UV illumination. E.coli XL-1 Blue was used as a negative control.

PCR Cloning and Sequencing
scl-PCR amplified product from the indigenous isolate LDC-5 (mcl & scl positive) was eluted using PCR purification kit (Qiagen). The purified PCR fragment was cloned in the pDrive Cloning vector using Qiagen PCR cloning kit. White transformants (The genotype of Qiagen EZ competent cells is: F’::Tn10 (Te') proA+ B+ lacIq AM15] recA1 end A1 hsdR17 (fR- mK+) lac gln V44 thi-l gyrA96 relA1] were selected on solid Luria medium containing 100 μg ampicillin/mL and pre-spread with both 35 μL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (50 mg/mL) and 20 μl of 100 mM isopropyl-β-D-thiogalactopyranoside. Plasmid DNA was purified from the transformants using alkaline lysis method.

DNA Sequencing of the PCR Amplicon
The plasmid isolated from the white transformants was subjected to cycle sequencing by dideoxy termination method in automated DNA Sequencer (ABI Prism 310, School of Biotechnology, Madurai Kamaraj University) using universal and custom made primers.

Analysis of Sequence Data
Bioinformatic analysis of DNA sequences was performed with the Genbank and EMBL databases by using the BLAST programs. Multiple alignment sequences for comparison were obtained from Genbank. The alignment was generated by Clustal W program. Sequence Phylograms were constructed using PHYLIP package (Bootstrap analysis with 1000 replicates) and unrooted trees were generated using TREEVIEW software.

Polymer Isolation from Lyophilized Bacterial Cells
The indigenous isolate Pseudomonas sp. LDC-5 was grown in 1L Erlenmeyer flasks containing 300 mL of RC growth medium supplemented with 10g 1-1 of glucose. Cells were grown at 30°C with 200-250 rpm orbital shaking for 72 h. Control flasks were set up to test the aseptic condition. At the appropriate time the cells were precipitated by centrifugation from shake-flask cultures (8,000 g, 20 min, 4°C) and washed twice with deionized water. The distilled water washed cells were freeze-dried using iLSHIN Freeze dryer (iLSHIN model No: TFD 5505) to constant weight. All intracellular PHA polymers were extracted from lyophilized cells using the method with slight modifications as described. Freeze-dried cell mass was treated with acetone and the acetone
dried cell mass (2 g) was treated with a dispersion of chloroform (25 mL) and 30% sodium hypochlorite (25 mL) at 37°C for 90 min. The dispersion was centrifuged at 8,000 g for 20 min at 30°C. Three phases were obtained. The upper phase was hypochlorite solution, the middle phase contained non-PHB cell materials and undisrupted cells, and the bottom chloroform phase contained PHB. First the hypochlorite solution was removed with a pipette and then the chloroform phase was obtained by filtration. PHB was later recovered from the chloroform phase by non-solvent precipitation. Ice-cold methanol was used as the non-solvent (4-6 volumes). The white powdery polymer was again dissolved in chloroform used as the non-solvent (4-6 volumes). The white powdery polymer was again dissolved in chloroform and used for further studies.

FT-IR Spectroscopic Analysis
The IR spectra were taken with a Fourier Transform spectrometer (JASCO FT/IR – 410, Madurai Kamaraj University, Department of Chemistry, Madurai). A thin film was prepared from the chloroform solution of the polymer extracted from the lyophilized cells and was scanned between 400 and 4000 wavenumber (cm⁻¹) as per the method 17.

Nucleotide Sequence Accession Number
The nucleotide sequence data reported in this paper have been submitted to the EMBL databank under the accession number AJ586810.

Results and Discussion
The use of PHA as a substitute for non-degradable petroleum-derived plastics hinges on the ability to produce it at a cost, which is competitive with that incurred in the production of conventional plastics. From the studies on the design and economic evaluation of the processes used for the production of PHAs, it was found that PHA productivity is one of the most important factors determining overall production cost 18. Even though there are many reports on PHAs in nature, only a few bacteria can produce PHB to an extent that meets commercial interest 19. In this study the authors identified and characterized the indigenous PHA producer Pseudomonas sp. LDC-5.

PCR Cloning and Sequencing
The purified PCR fragment encoding phaC of the Pseudomonas sp. LDC-5 was cloned into pDrive cloning vector. Ten clones were identified that formed as white colonies after overnight incubation on SOC medium with ampicillin indicating the recombinant plasmid. Clones were confirmed for the presence of the insert by restriction digestion with EcoR1. The selected clone, which showed the characteristic PCR product, was used for further analysis. A similar approach of cloning and characterization of the phaC genes cloned from several Pseudomonas strains was from different countries 20-27. None of these strains has included samples from Madurai, India. The earlier reports confirm the author’s knowledge that this is the first report on phaC gene of indigenous isolate Pseudomonas sp. LDC-5, that has been cloned and sequenced. Studies like this kind of cloning and sequencing strategy will lead to specific identification of the existence of PHA synthase genes and their organization type 27. Further, an expanded molecular study is needed to confirm the pha locus of Pseudomonas sp. LDC-5. Such study could also provide an evaluation regarding the usefulness of this PCR based detection of pha gene in aiding the classification and verification of Pseudomonas.

Sequence Analysis of the Pseudomonas sp. LDC-5
Cloning of phaC was confirmed by the analysis of the nucleotide sequence, whose deduced protein sequence (Fig. 1) had a high degree of identity with those of other PHA synthases. The nucleotide sequence of LDC-5 (AJ586810) was analyzed using Bioinformatics Sequence Analysis tools. The primary structure deduced from Pseudomonas sp. LDC-5 revealed a high degree of amino acid identity with other PHA synthases (Table 2) BlastN search using the cloned sequence as query produced significant nucleotide sequence matches with more than 16 Poly-β-hydroxy alkanoate (PHA) and Poly-β-hydroxy butyrate (PHB) synthases (92% similarity). Blast N and Blast P results with significant identities were summarized (Tables 1 & 2). Pseudomonas sp. LDC-5 shows significant identity with P. putida at both protein and nucleic acid searches.

Alignments
Multiple Sequence alignments were generated using Clustal W. Clustal W alignment of the translated putative protein with 18 other PHA-specific synthases showed a high degree of homology among the sequences (Figs 2-5). The nucleotide and amino acid sequence of the isolates used for comparison are given in Tables 3 & 4 and their alignments shown (Figs 3 & 4). Phylogenetic relationship was constructed for taxa with more than 95% identity with the alignments of PHA synthase sequences (Figs 3 & 4). Pseudomonas sp. LDC-5 clustered together with P. putida (Figs 6 & 7).

PHA Characteristic Bands in FT-IR Spectra
Fourier-transform infrared spectroscopy (FT-IR) has been demonstrated to be a powerful tool for screening
and studying large amounts of bacteria for their abilities to synthesize various types of PHA in its intact as well as in purified form\(^ {26,29}\). Several methods have been developed for qualitative analysis of PHA including GC, nucleic magnetic resonance and pyrolysis\(^ {30-32}\). The FT-IR method is simplest, rapid and very useful technique for broad screening of PHA producing microorganisms. Purified PHB extracted from the strain *Pseudomonas* LDC-5 in IR spectrum reflects the presence of marked peaks at 1733 cm\(^{-1}\), 2925-2954 cm\(^{-1}\) in comparable with the reference
* Indicates - Conserved Regions
Fig. 2—Multiple alignment of the LDC-5 (AJ586810) nucleotide sequence
The alignment shows 88% Identity.

Fig. 3—Basic local alignment of the LDC-5 (AJ586810) translated sequence with P26494

* Indicates Conserved Regions
Fig. 4—Multiple alignment of the translated LDC-5 (AJ58610)
Fig. 6—Phylogenetic tree constructed using neighbour-joining method of Pseudomonas sp. LDC-5 and related bacteria based on a comparison of phaC nucleotide sequences. Accession numbers or references for each strain are given. Bootstrap values for branches are given at the nodes. Bar, 0.02 as substitutions.
Fig. 7—Phylogenetic tree constructed using neighbour-joining method of *Pseudomonas* sp. LDC-5 and related bacteria based on a comparison of phaC nucleotide sequences. Accession numbers or references for each strain are given. Bootstrap values for branches are given at the nodes. Bar, 0.01 as substitutions.

Fig. 8—Fourier-transform infrared (FT-IR) spectra of pure PHB extracted from *Pseudomonas* sp. LDC-5. Bands near 2925-2954, 1733 cm⁻¹ represent the presence of ester carbonyl bond.
strain, demonstrated the presence of PHA characteristic ester carbonyl band (Fig. 8). The FT-IR results of Pseudomonas sp. LDC-5 is in agreement with the earlier works\textsuperscript{28,33-34}. All absorptions due to the PHB moiety appeared in the spectrum and other characteristic bands were also observable in the spectra at different positions, indicating the possible accumulation of various PHA in cells. In E.coli (negative) strain, no band was observable near 1732 cm\(^{-1}\) further demonstrating that the band between 1728 cm\(^{-1}\) and 1744 cm\(^{-1}\) is characteristic of PHA. The IR spectroscopic analysis gave further insight into the chemical structure of the polymer. Further study is required to evaluate the accurate molecular mass, additional physical and technical properties of this polymer.

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References


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