A study on accumulation of PHB in native *Pseudomonas* isolates
LDC-5 and LDC-25

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Poly (β-hydroxyalkanoates) (PHAs) are natural polyesters produced by a variety of bacteria. They are represented most commonly by poly (β-hydroxybutyrate) (PHB), an intracellular storage biodegradable polymer material. The production costs of PHB are quite high compared with those of synthetic non-degradable plastics, hence search for potential strains with high PHB accumulating ability. Hundreds of indigenous bacterial strains were screened for the accumulation of PHB by Nile red, fluorescence microscopy (Nile blue A) and PCR. Three degenerate primers were used as PCR primers to detect PHA synthase genes. Among the tested isolates, 35 strains yielded a specific amplicon of 496 bp and 406 bp in colony PCR and seminested PCR, respectively. Among the 35 short chain length positive strains, only 2 isolates yielded a specific amplicon of 540 bp PCR product in medium chain length PCR, representing partial coding sequences of *phaC1/phaC2* genes. The mcl-PCR positive *Pseudomonas* indigenous isolates (LDC-5 and LDC-25) could be potential candidates for bioplastic production.

**Keywords**: polyhydroxyalkanoate; PHA synthase; colony PCR; seminested PCR; biodegradable plastics, *Pseudomonas*

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**Introduction**

Poly(β-hydroxybutyrate) (PHB) was first discovered in bacteria. It is an unique intracellular polymeric material accumulating under unbalanced growth conditions in a wide variety of bacteria, but with excess carbon source. It is regarded as a source of potentially useful biodegradable natural plastics since its physical characteristics are similar to those of petrochemical polyesters such as polypropylene.

Poly (β-hydroxyalkanoates) (PHAs) are generally grouped into two classes depending on the carbon chain length of the β-hydroxy ester monomers. Short-chain-length (scl) PHAs contain monomer repeat units of 3-5 carbon atoms, whereas medium-chain-length (mcl) PHAs are composed of monomer repeat units of 6-14 carbon atoms. The mcl-PHAs have interesting potential applications in coatings and in medical temporary implants, such as scaffolding for the regeneration of arteries and nerve axons. These are synthesized by the action of three enzymes, β-ketothiolase (encoded by PhaA), aceto acetyl (coA)-reductase (encoded by PhaB) and PHB synthase (encoded by PhaC). β-ketothiolase condenses two acetyl CoA molecules to aceto acetyl (coA)-reductase to D-()-β-hydroxybutyrl coA. Subsequent polymerization to PHB is catalyzed by PHA synthase, a crucial enzyme in all PHA synthesis pathways. There are many phenotypic detection methods for detecting intracellular PHA granules, which are applied to the screening of PHA producers including Sudan black B staining and Nile blue A staining which result in dark or fluorescent granules.

Much work has been done towards understanding and enhancing the production, material properties and biodegradability of PHAs. PHAs have been detected in many natural environments e.g. estuarine and intertidal sediments, ground water aquifers, sewage sludge, gypsum rich sands, rivers, root nodules but very few reports are available about PHB accumulating indigenous bacterial populations of natural ecosystems. Also, PHAs have been commercially developed and marketed. A major drawback to the commercialization of PHAs is their higher price as compared to conventional petrochemical based plastic materials. Alternative strategies for PHA production are imperative to boost its production in order to alleviate environmental pollution. Though a number of standard and patented

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strains are available in the market, the viable strategy is the identification of potent indigenous PHA producers with high PHB accumulating power. The present study was aimed to isolate potent PHA producers with high PHB accumulating ability.

**Materials and Methods**

**Standard Bacterial strains, Media and Growth Conditions**

*Ralstonia eutropha* formerly *Alcaligenes eutrophus* (CH34), Gifted by Dr Daniel van der Lelie, USA and *Bacillus megaterium* (received from *Bacillus* Genetics Stock Centre, USA, reported to produce PHB) were grown in nutrient broth medium and the tubes were incubated at 37°C overnight. Pure cultures were maintained on LB slants. The culture purity was ascertained periodically by standard microbiological methods. The PHB accumulation in these strains was determined by growing in RC growth medium, supplemented with filter sterilized carbon source (1% glucose)\(^{19}\). These cultures were incubated at 30°C on an orbital shaker with agitation at 100 rpm for 3 days.

**Isolation of Soil PHB Producers by Nile-red Staining**

Soil samples collected from different ecological niches in and around Madurai District (Table 1) were subjected to direct dilution and plating on LB medium supplemented with 2% glucose and 1% Nile red \(^{20}\) and the plates were incubated at 37°C for 48 hrs. Colonies with pinkish pigment were selected for further studies and maintained on LB-glucose slants.

**Screening for PHB Accumulation**

*Nile blue A staining*

PHB positive strains identified by Nile red staining were cultured in 10 ml LB medium containing appropriate carbon source (1% glucose) at 37°C with reciprocal shaking at 150 rpm for 3 days. The bacterial cells were subjected to centrifugation at 3000 rpm for 10 min at 4°C. The supernatant was discarded and the cell pellet resuspended in 1ml of sterile distilled water. Smears of these cell suspensions were heat fixed to glass microscope slides, stained with a 1% Nile blue A solution by the method described by Ostle and Holt\(^{14}\) and observed under Nikon microscope with an 12 filter (excitation wavelength of 460 nm). Nile blue A stained PHA granules in the cells fluoresce orange.

**Colony PCR (scl—Short Chain Length)**

The bacterial strains isolated from various ecosystems were grown in Nutrient broth (NB) medium (Bacto beef extract and Bacto peptone with 2% carbon source-glucose w/v) at 37°C, 250 rpm in 250 ml/1000 ml Erlenmeyer flask\(^{18},\ 21\). Genomic DNA was isolated as described by Sambrook *et al*\(^{22}\) and used as template. Colony PCR was performed following the procedure of Shen *et al*\(^{20}\). In each PCR analysis, 30 ng of genomic DNA of Nile red and Nile blue A positive strains with three degenerate primers PhaCF1 (5'-ATCAACAA-(GGG/A)T(TT/A)-CTAC(AA/G)TC(CC/T)-GACCT-3', PhaCF2 (5'GT(CCC/GG)-TTC(GGG/A-A)T(GGG/C-C)(AAA/GG)T(CC/G)-(T-T/A)(CCC/G-G)CTGGCGC-AACCC3') and Pha-CR4 (AGGTAGT-T-GT(TT/C)GAC(CCC/GG)-(AA-A/-CC)(AAA/CC)-(G-GG/A)TAG(TT/G)TCG-A-3'), were used to detect PHA synthase genes as previously described\(^{23}\). The cycling conditions were as follows: \(94°C \) for 10 min, \(51°C \) for 2 min, \(72°C \) for 2 min, and 35 cycles of \(94°C \) for 20 sec, \(57°C \) for 45 sec (decreased by 1sec per cycle), \(72°C \) for 1 min, and then incubation at \(72°C \) for 5 min, and a final incubation at \(4°C \). PCR amplified DNA fragments were observed by agarose gel electrophoresis in 1% agarose gel (Sigma). Ten microlitres of each amplification mixture and the molecular weight marker (Gene Ruler 100 bp DNA Ladder-Qiagen) were subjected to agarose gel electrophoresis and ethidium bromide staining. The amplified DNA fragments were visualized by UV illumination.

**mcl-PCR – Medium Chain Length PCR**

mcl-PCR was performed in an Eppendorf PCR system, primers have been designed based on the highly conserved sequences found in the coding regions of *Pseudomonas phaC1* and *phaC2* genes (I-179L(5'-ACAGATCAACAAAGTTCTACATCTTC-GG-AC-3') and I-179R (5'-GGTTGTGTTGCAGTAGGATGTC-3'))\(^{24}\). Purified genomic DNA as described\(^{22}\) was used as template for PCR reaction. The cycling conditions were \(94°C \) for 10 min, and 35 cycles of \(94°C \) for 20 sec, \(57°C \) for 45 sec, \(72°C \) for 1 min, incubation at \(72°C \) for 5 min and a final incubation at \(4°C \).

**Microbial Identification**

Identification of the selected microorganisms was done as described by Palleroni\(^{26}\).

**Results and Discussion**

**Isolation and Screening of Soil PHB Producers**

The PHB and related co-polymers can be used as biodegradable plastics on a large scale if the cost of
production is lesser than non-biodegradable plastics. The authentic potential strain and the substrate represent a major fraction of the manufacturing costs of PHB and is, therefore, a prime target for potential cost reduction\textsuperscript{25}.

The authors report here the natural, \textit{in situ}, occurrence of PHB accumulating microbes obtained from a variety of soil and water samples, tannery effluent and sewage sludge. A striking prevalence was observed in samples B and C (Table 1), some were also found in soil sample D. New isolates of LDC-5 and LDC-25 were selected based on the screening and isolation procedures described in this study. These isolates were further characterized by morphology, PHB granule formation, physiological and biochemical characteristics (Table 2). Results of morphological and biochemical tests indicate that the isolate has general biochemical characteristics such as gram negative small rods, motile, tested positive for both catalase and oxidase activity and does not form endospores similar to those of \textit{Pseudomonas} as described\textsuperscript{26,27}. It is unable to hydrolyze starch and casein but able to hydrolyze arginine and Tween 80. Phase contrast microscopy clearly showed the presence of poly beta granules.

Selection of the 65 cultures from 100 soil samples, showing strong evidence for the presence of pinkish pigments, in agreement with the earlier observations\textsuperscript{28-30}, were isolated in pure form. These 65 bacterial cultures were originated from soil samples B and D (Table 1) indicating that the diversity of PHB producers in the soil samples analyzed was comparatively low except those in tannery and sludge samples. Screening of all the 65 isolates by Nile blue A staining and observed under Phasecontrast and Fluorescence microscope (Figs 1a & b) revealed that 35 isolates possessed significant PHB accumulation ability. These results were similar to the earlier findings\textsuperscript{17,23,28}. This outcome may be either due to a low yield of PHB granule accumulation or the isolates harbour a non-functional PHA synthase gene resulting in negative detection by Nile blue A staining. The preliminary assay for quantification was carried out to determine the PHB accumulation in these two isolates and a reference strain (data are not given). The PHB accumulation in these two isolates was as like that of wild type \textit{Ralstonia eutropha} (CH34). The 35 isolates were characterized genotypically by PCR following the protocol\textsuperscript{23}.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Soil/Water sample} & \textbf{Description} & \textbf{No. of soil samples screened} & \textbf{No. of Nile red positive strains} \\
\hline
A & Garden soil & 25 & 2 \\
B & Tannery Effluent & 10 & 25 \\
C & Sewage sludge & 15 & 30 \\
D & Field soil & 45 & 8 \\
\hline
\end{tabular}
\caption{Origin and description of the samples used for the isolation of PHB producing microbes}
\end{table}

All samples from locations in and around Madurai District

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{S.No} & \textbf{Characteristics} & \textbf{LDC- 5} & \textbf{LDC-25} \\
\hline
1 & Gram staining & \textsuperscript{++} & \textsuperscript{--} \\
2 & Oxidase & \textsuperscript{++} & \textsuperscript{++} \\
3 & Catalase & \textsuperscript{++} & \textsuperscript{++} \\
4 & Formation of poly-\textit{β}-hydroxybutyrate granules & \textsuperscript{++} & \textsuperscript{++} \\
5 & Motility & \textsuperscript{++} & \textsuperscript{++} \\
6 & Hydrolysis of a)Starch & \textsuperscript{--} & \textsuperscript{--} \\
& b)Casein & \textsuperscript{--} & \textsuperscript{--} \\
& c)Arginine & \textsuperscript{++} & \textsuperscript{++} \\
7 & Levan formation & \textsuperscript{++} & \textsuperscript{+} \\
8 & Growth at 41ºC & \textsuperscript{++} & \textsuperscript{+} \\
9 & Growth in Hofer’s alkaline broth & \textsuperscript{++} & \textsuperscript{++} \\
10 & Hydrolysis of Tween 80 & \textsuperscript{++} & \textsuperscript{+} \\
11 & Utilization of Carbohydrates a)Glucose & \textsuperscript{++} & \textsuperscript{++} \\
& b)Fructose & \textsuperscript{++} & \textsuperscript{++} \\
& c)sucrose & \textsuperscript{++} & \textsuperscript{++} \\
& d)Raffinose & \textsuperscript{++} & \textsuperscript{++} \\
12 & Utilization of Citrate-utilization & \textsuperscript{++} & \textsuperscript{++} \\
13 & Egg-yolk lecithinase & \textsuperscript{++} & \textsuperscript{++} \\
14 & Phenazine-Pigment Production (Medium “A”) & \textsuperscript{--} & \textsuperscript{--} \\
15 & Pyoverdin-Pigment production & \textsuperscript{--} & \textsuperscript{--} \\
\hline
\end{tabular}
\caption{Biochemical characteristics of the isolates LDC-5 & LDC-25}
\end{table}

Symbols: \textsuperscript{+}-Positive; \textsuperscript{++}-Strong positive; \textsuperscript{--}Negative

\textbf{Colony PCR}

The PCR screening protocol described in this paper is important for the identification and verification of organisms that harbour scl-PHB and mcl-PHB biosynthesis genes. Although, dye-based screening procedures\textsuperscript{20,32} are useful for visualizing the presence of PHA inclusion bodies in microbes, these methods cannot differentiate scl-PHA, mcl-PHA and other lipid materials. Moreover, at the time of screening if the microbe is not accumulating inclusion bodies (PHB) staining methods would not be effective\textsuperscript{23,24}. Many bacteria accumulate PHAs as energy and
carbon reserves but the types of PHA produced are not clearly defined.

In this study, the 35 phenotypically PHB positive soil isolates were subjected to colony PCR of which, 30 strains produced predicted 496 bp amplicon and in 5 strains no amplicon was detected. Fig. 2 shows colony PCR of *Pseudomonas* LDC-5 and LDC-25. A very sensitive and time saving semi-nested PCR method was combined with colony PCR for the

![Fig. 1— Phase contrast micrographs of whole cells (A) and Fluorescence microscopic view of Nile blue A stained cells (B)](image)

specific and individual amplification of subgenomic fragments. Using the first round amplification reaction mixture as the source of DNA template in order to obtain the specific subgenomic sequences of the desired phaC gene by using the primers PhaCF2, PhaCR4. The results obtained from the semi-nested PCR reaction showed successful amplification of the intended fragments (Fig. 2) of all the 35 Nile blue A positive strains with the predicted size of 406 bp, even from the 5 PHA positive strains that did not produce a colony PCR product. These results suggested that colony PCR negative products still contain trace amounts of specifically amplified DNA fragments, which could not be detected by ethidium bromide staining. By semi-nested PCR, the unobserved signal was amplified and detected again. These results strongly support our indigenous isolates possessing PHB accumulation ability. The negative control *E. coli* XL-1 blue did not produce PCR product. The detection experiments were repeated thrice with the same results, confirming reproducibility. This strongly supports the accumulation of PHB inclusion in our isolates.

![Fig. 2—Analysis of colony PCR positive products and their confirmation by semi-nested PCR: Lane 1 Gene Ruler 100 bp DNA Ladder; Lane 2 Positive control (Ralstonia eutropha); Lane 3 Colony PCR of *Pseudomonas* LDC5; Lane 4 Semi-nested PCR of *Pseudomonas* LDC-5; Lane 5 Colony PCR of *Pseudomonas* LDC-25; Lane 6 Semi-nested PCR of *Pseudomonas* LDC-25; & Lane 7 Negative control (E.coli XL-1 Blue).](image)

Among the 35 scl-PCR positive strains only 2 strains yielded the characteristic 540 bp PCR product (Fig. 3). Size of the PCR products consistently matched the predicted results, further supporting biochemical observation that mcl-PHAs are mostly produced by *Pseudomonas* belonging to the rRNA-DNA homology group 1. The results showed that screening of 35 scl positive isolates subjected to mcl-
PCR, only 2 strains yielded expected amplicon in mcl-PCR and in others there was no apparent PCR product or longer than the expected amplicon was observed (Fig. 3). Strain variation is important in determining the PHA producing potential of a bacterial species. Pseudomonas strains, P. stutzeri ATCC 17588, P. andropogonis 27 and P. andropogonis 11 belonged to the rRNA-DNA homology group but did not harbour phaCl/phaC2 genes. These organisms may contain non-type II PHA synthase genes that would not be detected by the present PCR protocol. A multiplex PCR method that combines the current phaCl/phaC2-specific primer-pair with non-type II pha-specific PCR oligomers allows for simultaneous detection or verification of all classes of PHA synthase genes. The failure to detect the specific amplicon in other isolates may be indicative of strain variation. The present study revealed that Pseudomonas isolates LDC-5 and LDC-25 may be important prospective potential candidates for the production of PHB and need to be characterized further especially in terms of PHB quantitation.

Conclusion
Results from this study conclusively showed that our indigenous isolates of Pseudomonas (LDC-5 and LDC-25) are capable of producing mcl-PHB. Further studies are needed to characterize these strains. These indigenous strains may be important organisms for the production of mcl-biopolymer on large-scale.

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