Lampiran 3

# Biosynthesis and characterization of polyhydroxyalkanoate blends accumulated by *Pseudomonas* sp. USM 4-55

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**Abstrak:** *Pseudomonas* sp. USM 4-55 ialah satu bakteria yang telah dipencilkan dari sampel tanah ladang kelapa sawit yang diperolehi dari Chini, Pahang. Bakteria ini mampu menghasilkan polihidroksialkanoat (PHA) dari asid lemak bebas dan gula. Kebanyakan pseudomonad boleh menghasilkan PHA yang terdiri daripada monomer rantai sederhana (mcl) (6 hingga 14 atom karbon), manakala *Pseudomonas* sp. USM 4-55 pula mampu menghasilkan PHA yang terdiri daripada monomer rantai pendek (scl) (3 hingga 5 atom karbon) dan juga monomer mcl. PHA ini apabila diekstrak dari sel kering boleh dipisahkan kepada homopolimer poli(3-hidroksibutirat) [P(3HB)] dan mcl-PHA dengan menggunakan pelarut yang tertentu. Namun, ianya tidak diketahui samada kedua-dua PHA ini juga tersimpan dalam granul yang berasingan di dalam sitoplasma sel. Untuk memastikan perkara ini, kami telah menggunakan pengemparan-ultra kecerunan ketumpatan sukrosa untuk memencilkan granul PHA berdasarkan kepada ketumpatan. Analisis kromatografi gas terhadap granul PHA yang dipencilkan menunjukkan bahawa granul P(3HB) dan granul mcl-PHA wujud secara berasingan di dalam sitoplasma sel. Pemerhatian ini mencadangkan *Pseudomonas* sp. USM 4-55 mempunyai sekurang-kurangnya dua jenis PHA sintase dengan kespesifikan substrat yang berlainan. Mekanisme sintesis PHA dan pembentukan granul dalam *Pseudomonas* sp. USM 4-55 dibincangkan.

**Abstract:** *Pseudomonas* sp. USM 4-55 is a bacterium isolated from soil samples of oil palm plantation obtained in Chini, Pahang. The bacterium has the ability to produce polyhydroxyalkanoates (PHA) from free fatty acids and sugars. Unlike most common pseudomonads that produce PHAs consisting of medium-chain length (mcl) monomers (6 to 14 carbon atoms), *Pseudomonas* sp. USM 4-55 is capable of producing PHA consisting of both short-chain length (scl) (3 to 5 carbon atoms) and mcl monomers. The PHA when extracted from the dried cells can further be separated by solvent fractionation into poly(3-hydroxybutyrate) [P(3HB)] homopolymer and mcl-PHA. However, it is not known if both the P(3HB) and mcl-PHA were also stored in separate granules in the cell cytoplasm. To investigate this matter we have used sucrose density gradient ultracentrifugation to isolate the PHA granules based on their density. Gas chromatography analysis of the isolated PHA granules revealed the separate existence of P(3HB) granules and mcl-PHA granules in the cell cytoplasm. This observation strongly suggests that *Pseudomonas* sp. USM 4-55 has at least two types of PHA synthases with different substrate specificities. The mechanism of PHA biosynthesis and granule formation in *Pseudomonas* sp. USM 4-55 is discussed.

## INTRODUCTION

Recently, owing to mounting environmental concerns much effort is being directed towards the development of ecologically friendly polymeric materials. This is because the common petrochemical-based polymers that are widely used as commodity plastics such as polyethylene and polypropylene are non-biodegradable. Thus, they accumulate in landfill sites and cause environmental pollution. In order to minimize this problem, much research is being carried out by research groups all over the world to find alternative materials. Among the various potential substitutes for petrochemical-based plastics is the microbial polyhydroxyalkanoates (PHA) (for extensive review see Steinbüchel and Lütke-Eversloh, 2003; Sudesh *et al.* 2000a; Anderson and Dawes, 1990). PHA is a polyester synthesized and accumulated intracellularly by various microorganims. The accumulated PHA serves as carbon and energy storage material for the microorganism. In the cell cytoplasm, PHA exists as amorphous water insoluble granules (Barnard and Sanders, 1989). However, upon extraction from the cell, the PHA shows crystalline and thermoplastic behavior similar to petrochemical-based plastics (Doi, 1990). Depending on the monomer composition, the extracted PHA can have properties ranging from being highly crystalline and brittle to amorphous and sticky. High degrees of crystallinity is usually shown by PHA that contain short-chain length (scl) monomers (3 to 5 carbon atoms) while soft and sticky characteristics indicate the major presence of medium-chain length (mcl) monomers (6 to 14 carbon atoms), often with the occurrence of double bonds in the side-chain.

PHA synthase is the key enzyme in the biosynthesis of PHA. Generally, there are two types of PHA synthases based on the substrate specificity. The PHA synthase of *Ralstonia eutropha* is efficient in the polymerization of scl-PHA, while that of the pseudomonads specifically polymerizes mcl-PHA. Lately, PHA synthases with broader substrate specificities have been identified which can polymerize both scl- and mcl-PHA (Fukui and Doi 1997) Such PHA synthases are of great commercial interest because the types of PHA that they produce have improved physical properties. However, PHA synthases having broad substrate specificities are not common.

In this study, we report the identification of a *Pseudomonas* sp. USM 4-55 that is capable of producing PHA containing both scl and mcl monomers from palm oil free fatty acids as well as from sugars. The PHA produced by this bacterium can be fractionated into two components, i.e. poly(3-hydroxybutyrate) [P(3HB)] and mcl-PHA by solvent fractionation. The latter also contains small amounts of 3HB monomers. In addition, we have used sucrose density gradient ultracentrifugation to isolate the intact PHA granules from the cell cytoplasm. The results show that *Pseudomonas* sp. USM 4-55 stores both the P(3HB) and mcl-PHA in separate granules. This implies that *Pseudomonas* sp. USM 4-55 has at least two types of PHA synthases with specificity towards 3HB monomers and mcl monomers, respectively. The mechanism of PHA biosynthesis and granule formation in this interesting isolate is discussed.

## MATERIALS AND METHODS

### Bacterial strain

The *Pseudomonas* sp. USM 4-55 used in this study was isolated from soil samples obtained from an oil palm plantation in Chini, Pahang. The isolation technique and biochemical characterization of this bacterium is available elsewhere (Few, 2001).

**Media and culture conditions**

*Pseudomonas* sp. USM 4-55 was maintained on nutrient agar (NA) (nutrient broth, 16 g/L and bacteriological agar, 15 g/L) as well as in glycerol (25% v/v) stocks stored at –20 °C. For inoculum preparation, single colonies from NA plates were inoculated into nutrient rich (NR) medium (yeast extract, 5 g/L; peptone, 5 g/L; glucose, 10 g/L) and incubated at 37 °C, 200 rpm for 24 h.

For PHA biosynthesis, 3% (v/v) inoculum concentration of *Pseudomonas* sp. USM 4-55 was added to 50 mL mineral medium in a 250 mL flask. The mineral medium consisted of 3.7 g $KH_2PO_4$, 5.8 g $K_2HPO_4$ and 1.1 g $(NH_4)_2SO_4$ in 1 L of distilled water in addition to 0.1 M $MgSO_4 \cdot 7H_2O$ and 0.1 mL trace elements solution. The trace elements solution contained 2.78 g $FeSO_4 \cdot 7H_2O$, 1.98 g $MnCl_2 \cdot 4H_2O$, 2.81 g $CoSO_4 \cdot 7H_2O$, 1.67 g $CaCl_2 \cdot 2H_2O$, 0.17 g $CuCl_2 \cdot 2H_2O$ and 0.29 g $ZnSO_4$ in 1 L of 0.1 M HCl. Oleic acid (0.5% v/v) that was autoclaved separately was added as the sole carbon source for cell growth and PHA biosynthesis. The mixture was incubated at 37 °C, 200 rpm for 36 h.

**Analysis of PHA**

The accumulated PHA was subjected to compositional analysis by gas chromatography (GC). For this, both whole cells that have been freeze-dried and purified PHA were used. Cells at the end of 36 h incubation were harvested by centrifugation (4000 *g*, 4 °C, 10 min) and the pellet was washed twice with sterile distilled water to remove remaining media constituents that may be attached to the cell surface. The pellet was then frozen overnight at –20 °C and subsequently lyophilized. 25 mg of the freeze-dried cells was methanolyzed in a mixture of 2 mL methanolysis solution (methanol:sulphuric acid, 85:15, v/v) and 2 mL chloroform in a screw-cap test-tube at 100 °C for 140 min. Addition of 1 mL of water to the reaction mixture at room temperature induced phase separation. The resulting methyl esters in the chloroform layer were analyzed by GC using caproic acid methyl ester as the internal standard. The GC used is a Shimadzu GC-14B system equipped with a Fused Silica capillary column (SPB™-1; 30 m by 0.25 mm) and a flame ionization detector. For purified PHA, 6 mg of the polymer was subjected to methanolysis and GC analysis.

PHA was extracted from 1 g of freeze-dried cells by refluxing in 100 mL of chloroform at 60 °C for 4 h. Subsequently, the mixture was filtered to remove cell debris and the chloroform solution was concentrated to a final volume of about 10 mL in a rotary evaporator. This was then added drop-wise to 100 mL of rapidly stirred cold methanol to precipitate out the PHA. The extracted PHA was then filtered and allowed to dry at room temperature. In order to separate the P(3HB) component from mcl-PHA, the dried PHA was subjected to solvent fractionation. For this, the PHA was dissolved into hot propanol at 60 °C for 5 h. The P(3HB) component that do not dissolve was filtered out and the mcl-PHA component was obtained by evaporating the propanol.

**Isolation of intact PHA granules**

Harvested cells were resuspended in 0.1 M Tris-HCl buffer (pH 7.5) and then disrupted by sonication (150 W; 30 min). The resulting crude cell extract was loaded directly onto discontinuous sucrose density gradient consisting of 1 mL each of 2.00, 1.67, 1.33, and 1.00 M sucrose in 0.1 M Tris-HCl buffer. Following ultracentrifugation (210,000 *g*; 2 h; 4 °C), the intact PHA granules accumulated in the form of white bands at different

density levels. The different bands of PHA granules were then recovered and washed once in Tris-HCl buffer before lyophilization and subsequent GC analysis.

**Electron microscopy studies**

*Pseudomonas* sp. USM 4-55 cells were prefixed in McDowell-Trump's fixative (McDowell and Trump, 1976) prepared in 0.1 M sodium phosphate buffer (pH 7.2) and then fixed by 1% osmium tetroxide. After dehydration using a graded ethanol series, the cells were embedded in Spurr's low viscosity resin (Spurr, 1969). Ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with an electron microscope (Philip CM-12/STEM).

## RESULTS

**Biosynthesis of PHA containing both scl and mcl monomers by *Pseudomonas* sp. USM4-55**

*Pseudomonas* sp. USM 4-55 is capable of producing PHA from a variety of carbon sources such as palm oil, fatty acids and sugars. All the carbon sources were shown to produce PHA containing 3HB monomers as well as the mcl monomers with 6 to 14 carbon atoms (Few, 2001). In this study, we have used oleic acid (0.5% v/v) as the sole carbon source. Table 1 shows the PHA content and composition synthesized by *Pseudomonas* sp. USM 4-55 when cultivated in a one-stage culture for 36 h. The PHA is composed of both the scl and mcl monomers. The major mcl monomers were 3-hydroxyoctanoate (3HO, C8) and 3-hydroxydecanoate (3HD, C10). It must be noted that there is a double bond in the side-chain of some of the 3-hydroxydodecanoate (3HDD, C12) monomers. The presence of 3-hydroxy-5-*cis*-dodecenoate (3H5DD) monomers has been confirmed by nuclear magnetic resonance (NMR) spectrometry analysis of the purified PHA (Few, 2001).

**Solvent-fractionation of purified PHA by boiling in propanol for 5 h**

Previous studies have shown that some microorganisms can produce a blend of PHA. This means that the PHA when purified from the bacterial cells can further be fractionated into different components by solvent fractionation. In order to determine whether the PHA produced by *Pseudomonas* sp. USM 4-55 is a blend, we boiled the purified PHA in propanol for 5 h. The remaining insoluble component after 5 h of boiling was removed by filtration while the soluble component was recovered upon evaporation of the solvent. Upon drying, both the soluble and insoluble components differed in appearance. The soluble component was a viscous liquid that was yellowish in color. A sticky and transparent film that can be peeled off formed upon storage for 5 days at room temperature (24 °C). The film was less sticky at lower temperatures. On the other hand, the insoluble fraction formed solid particles that were brittle and white in color.

Table 2 shows the results of GC analysis on the soluble and insoluble components. The soluble fraction predominantly consisted of mcl monomers with a small portion of 3HB monomer. The ratio of the various mcl monomers in the PHA was the same as that in the whole dry cells. 3HO and 3HD were the major monomer constituents whereas 3-hydroxyhexanoate (3HHx, C6), 3HDD, 3H5DD and 3-hydroxytetradecanoate (3HTD, C14) were the minor constituents. The insoluble fraction consisted solely of 3HB monomers. The solvent fractionation result clearly shows that

the PHA produced by *Pseudomonas* sp. USM 4-55 is a blend or mixture of P(3HB) homopolymer and mcl-PHA. The latter consisted of small amounts of 3HB monomer that is most probably copolymerized with the mcl monomers.

**PHA granules of *Pseudomonas* sp. USM 4-55 observed by TEM**

Figure 1 shows the ultrastructure of *Pseudomonas* sp. USM 4-55 containing PHA granules in the cell cytoplasm. Both P(3HB) and mcl-PHA are hydrophobic materials that are stored in the form of water insoluble granules in the aqueous cell cytoplasm. The granules exist individually and are characterized by a sharp border that separates the hydrophobic material from not only the hydrophilic cytoplasm but also from other granules. It has been reported that in some cases the granules seem to coalesce, especially at high PHA contents (Barnard and Sanders, 1989). The TEM micrograph in Fig.2 shows that the PHA granules in *Pseudomonas* sp. USM 4-55 still existed as discrete particles that can be isolated in their native form. All PHA granules regardless of their monomer constituents appear the same when observed by TEM. Therefore it is not possible to determine if both the P(3HB) and mcl-PHA synthesized by *Pseudomonas* sp. USM 4-55 are stored in the same or separate granules. TEM of freeze-fracture replica has been used by some researchers to show the separate existence of P(3HB) and mcl-PHA granules in a single bacterial cell (Preusting *et al.* 1993; Fukui *et al.* 1998). This method relies on the different deformation pattern shown by both these granules. However, it must be noted that depending on the freeze-fracture temperature the same PHA granule can show different deformation behaviors (Sudesh *et al.* 2000b). It is also known that PHA granules with different monomer constituents have different densities. In order to characterize further the PHA granules, we have used sucrose density gradient to fractionate the native PHA granules based on their densities.

**Sucrose density gradient ultracentrifugation of PHA granules**

The isolation of PHA granules in its native form is crucial for the study of granule surface architecture and granule-associated proteins (GAP). For these purposes, the native PHA granules are isolated from crude cell extracts using sucrose or glycerol density gradient ultracentrifugation. Previous studies have shown that PHA granules with different monomer constituents have different densities. The densities of native PHA granules increase in the following order, mcl-PHA<P(3HB)<poly(4-hydroxybutyrate) (Sudesh and Doi, unpublished results). Based on this knowledge, we have prepared a discontinuous sucrose density gradient to fractionate the native PHA granules of *Pseudomonas* sp. USM 4-55 by ultracentrifugation. Figure 2 shows the resulting bands of PHA granules fractionated according to their densities. The bands were then carefully recovered and subjected to GC analysis. Table 3 shows that band I consisted of PHA granules that contain predominantly mcl-PHA monomers with a small portion of 3HB monomers. The ratio profile of monomers from band I was similar to that of monomers in the propanol-soluble fraction (Table 2). Band II on the other hand consisted solely of 3HB monomers, matching the monomer constituent of the propanol-insoluble fraction. This demonstrates that both the P(3HB) and mcl-PHA are stored in separate granules in the *Pseudomonas* sp. USM 4-55 cell cytoplasm. Band III contained trace amounts of 3HB monomers and cellular debris.

## DISCUSSION

An aspect of PHA biosynthesis that still remains unclear at present is the mechanism of granule formation and the role of granule-associated proteins (GAPs). GAPs include the PHA synthase, which is the enzyme involved in the polimerization of hydroxyalkanoate monomers to form high molecular weight PHA. The active form of PHA synthase is located on the granule surface. The PHA synthase is thought to be bound to the growing polymer chain. Besides the PHA synthase, other proteins known as phasins are also known to be attached to the granule surface. The direct observation of these proteins on the granule surface has recently been reported for the first time using atomic force microscopy (Sudesh *et al.* 2003). The GAPs are known to be important in PHA biosynthesis and granule formation (Maehara *et al.* 2002; York *et al.* 2002). However, the precise role of GAPs in the granule formation mechanism is not known. The results obtained in the present study cannot be explained using the widely accepted mechanism of PHA granule formation (Gerngross *et al.* 1993). We therefore have attempted to explain the PHA granule formation mechanism in *Pseudomonas* sp. USM 4-55 using a different mechanism.

*Pseudomonas* sp. USM 4-55 is an interesting isolate because of its ability to synthesize a blend of P(3HB) homopolymer and mcl-PHA that also contains small amounts of 3HB monomers. The latter 3HB monomers are most probably copolymerized with the mcl-PHA because they cannot be separated by solvent fractionation. In this study it has further been shown that both the types of PHA are stored in separate granules in the cell cytoplasm. This isolate therefore resembles the PHA biosynthesis ability shown by *Pseudomonas* sp. 61-3 reported by Doi and coworkers (Abe *et al.* 1994). The PHA synthesizing ability shown by *Pseudomonas* sp. USM 4-55 indicates that this bacterium possesses more than one type of PHA synthase based on the substrate specificity. Recent molecular studies on this bacterium had revealed the presence of two PHA synthase genes, *phaC1* and *phaC2* (Aida, 2002). Both the PHA synthase genes were separated by a PHA depolymerase gene (*phaZ*). The organization of the three genes and their putative amino acid sequences showed high similarities to the corresponding genes of *Pseudomonas* sp. 61-3. The two PHA synthases coded by *phaC1* and *phaC2* have been shown to have broad substrate specificity and can polymerize both scl and mcl monomers (Matsusaki *et al.* 1998).

Since *Pseudomonas* sp. USM 4-55 can also simultaneously synthesize P(3HB) homopolymer this bacterium must possess a third PHA synthase that is only specific towards scl monomers. The presence of a third PHA synthase has been demonstrated in *Pseudomonas* sp. 61-3 (Matsusaki *et al.* 1998). Attempt to clone and characterize this third PHA synthase from *Pseudomonas* sp. USM 4-55 is currently ongoing in our laboratory.

The fact that P(3HB) granules and mcl-PHA granules exist separately in the same cell suggest that the scl-PHA synthase and the mcl-PHA synthase with broad substrate specificity do not combine to form a single granule. This observation cannot be explained by the granule formation mechanism that is currently being used. The currently accepted model for granule formation was first proposed by Ellar *et al.* (1968) and Griebel *et al.* (1968) and later supported by Gerngross *et al.* (1993) with new information on the amorphous nature of native granules (Barnard and Sanders, 1989). According to that model, the PHA granule formation shows some similarities to the standard emulsion polymerization. Initially, the PHA synthase with the growing

polymer chain form micellar structures that continue to grow in size as more and more monomers are incorporated [see mechanism I(a) in Fig. 3]. In the micelle formation stage, hydrophobic polymer chains growing from more than one PHA synthase will aggregate and finally form the granule core. This model however cannot explain why P(3HB) chains and mcl-PHA chains growing from different PHA synthases are segregated into separate granules as is shown in mechanism I(b) (Fig. 3).

Segregation of the two types of PHA may take place if, i) the P(3HB) chains growing from different PHA synthases have the tendency to aggregate only with each other and likewise mcl-PHA chain aggregate only with another mcl-PHA chain; ii) the PHA synthase enzymes with similar substrate specificity form a complex and each complex results in a single granule as is shown in mechanism II (Fig. 3).

Since both P(3HB) and mcl-PHA are hydrophobic polymers, they would naturally tend to blend and form a single granule. So, the first explanation do not give satisfactory reason for the segregation of P(3HB) and mcl-PHA. Unless if, specific phasin proteins bind selectively to different types of PHA chains and promote the aggregation of similar PHA chains to form a single granule. This would imply that some phasins maybe trapped in the granule core. The presence of proteins inside a granule has not been reported. However, some phasins have been shown to bind to PHA single crystals (Sudesh *et al.* 2003), which means that phasins can directly interact with the hydrophobic PHA chains. It might also be worth noting that the presence of mcl monomers in a polymer chain that is consisted of predominantly 3HB monomers results in an unstable granule (Sudesh *et al.* 2002). This is probably because of hindrance to close packing of the 3HB-rich PHA chains imposed by mcl-monomers with long side chains. Therefore, the segregation of P(3HB) and mcl-PHA into separate granules maybe an essential requirement.

The second explanation of PHA synthase complex formation is based on the observation of a distinct lag phase in the polymerization reaction catalyzed by this enzyme. The enzymes with similar substrate specificity form complexes with each other (see mechanism II in Fig.3). The complex formation may involve the substrate of the PHA synthase. Each PHA synthase complex will then produce one granule. Mechanism II provides a simple explanation for the segregation of P(3HB) and mcl-PHA into separate granules in *Pseudomonas* sp. USM 4-55. Mechanism II will therefore result in granules that contain either the scl-PHA synthase or the mcl-PHA synthases with broad substrate specificity (that includes 3HB monomer). Recent studies have shown that the scl-PHA synthase was only located on the P(3HB) granules in *Pseudomonas* sp. 61-3 (Matsumoto *et al.* 2002) while the other two PHA synthases were located on the P(3HB-*co*-mcl PHA) granules. This finding further supports Mechanism II. Finally, it should be noted that Mechanism II is also in agreement with the theory of chain transfer reaction for the polimerization of PHA by the PHA synthase (Kawaguchi and Doi, 1992).

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**Table 1:** Biosynthesis of PHA by *Pseudomonas* sp. USM 4-55 in one stage culture

| Carbon source | Dry cell weight (g/L) | PHA content[a] (wt%) | PHA composition[b] (mol%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 3HB | 3HHx | 3HO | 3HD | 3HDD | 3H5DD | 3HTD |
| Oleic acid (5 g/L) | 2.1 | 38 | 35 | 3 | 19 | 23 | 10 | 2 | 8 |

The cells were harvested after cultivation for 36 h at 37 °C using oleic acid as the sole carbon source

[a]PHA content in dried cells

[b]PHA composition determined by GC analysis.

3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate; 3H5DD, 3-hydroxy-5-*cis*-dodecenoate; 3HTD, 3-hydroxytetradecanoate

**Table 2:** Fractionation of purified PHA by boiling in propanol for 5 h

| PHA | Fraction weight (mg) | PHA composition (mol%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 3HB | 3HHx | 3HO | 3HD | 3HDD | 3H5DD | 3HTD |
| Whole | 1330 | 35 | 3 | 19 | 23 | 10 | 2 | 8 |
| Soluble | 890 | 1 | 6 | 29 | 37 | 15 | 3 | 9 |
| Insoluble | 440 | 100 | - | - | - | - | - | - |

**Table 3:** GC analysis of intact PHA granules isolated from *Pseudomonas* sp. USM 4-55

| PHA granules | PHA composition (mol%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3HB | 3HHx | 3HO | 3HD | 3HDD | 3H5DD | 3HTD |
| Band I | 8 | nd | 43 | 49 | nd | nd | nd |
| Band II | 100 | - | - | - | - | - | - |

PHA granules were isolated from crude cell extract by sucrose density gradient ultracentrifugation. Band I, low density PHA granules recovered from upper layer between 1.33 M and 1 M sucrose gradient. Band II, high density PHA granules recovered from lower layer between 1.67 M and 1.33 M sucrose gradient.
Approximately 5 mg of freeze-dried PHA granules were used for GC analysis.
nd, not detected

## LEGENDS FOR FIGURES

**Figure 1.** Transmission electron micrograph of *Pseudomonas* sp. USM 4-55 cultivated on oleic acid (0.5% v/v) as the sole carbon source for 36 h. PHA granules appear as distinct electron transparent particles in the cell cytoplasm.

**Figure 2.** Discontinuous sucrose density gradient ultracentrifugation of PHA granules isolated from *Pseudomonas* sp. USM 4-55. Band I, II and III were recovered, washed with Tris-HCl buffer and freeze-dried for GC analysis. The diagram on the left shows the different density layers of sucrose solution.

**Figure 3.** Model of PHA granule formation in *Pseudomonas* sp. USM 4-55. PHA synthase molecules are indicated by the white and gray spheres. For simplicity, the white spheres represent the scl-PHA synthase while the gray spheres represent the two mcl-PHA synthases. The thin lines represent the growing scl-PHA chains while the thick lines represent the mcl-PHA chains that also contain small amounts of 3HB monomer. Mechanism I(a) shows the existing model for PHA granule formation. Mechanism 1(b) shows the observed phenomenon in *Pseudomonas* sp. USM 4-55. Mechanism II shows the proposed new model for PHA granule formation in *Pseudomonas* sp. USM 4-55.

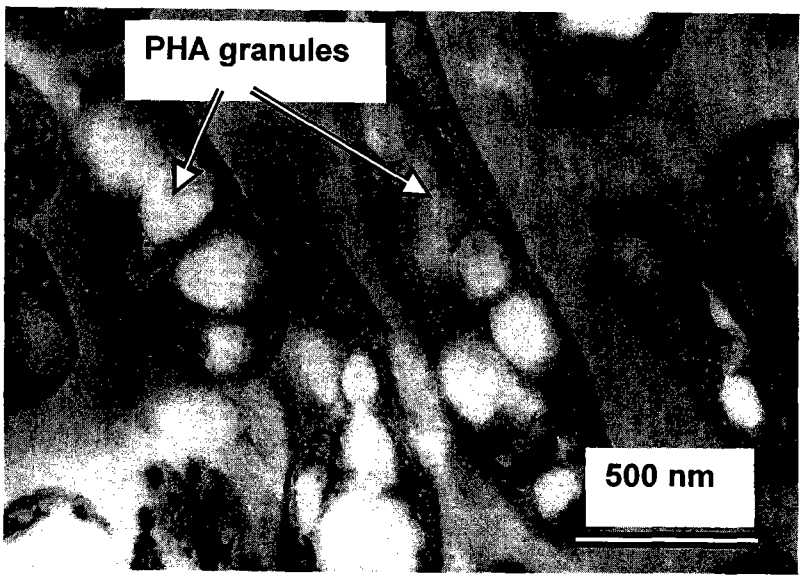



**Figure 1 Sudesh et al**

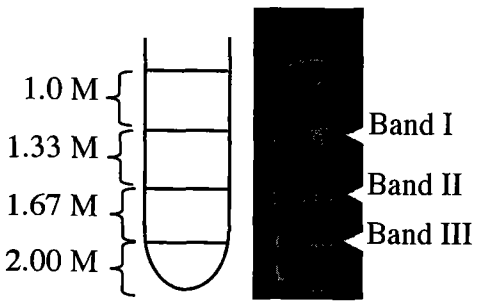
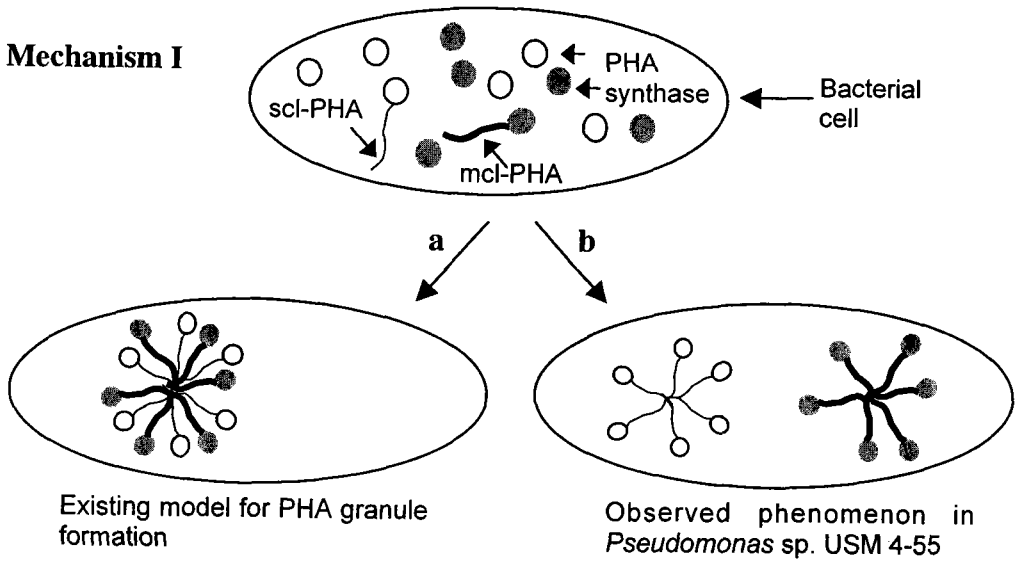



**Figure 2 Sudesh et al**



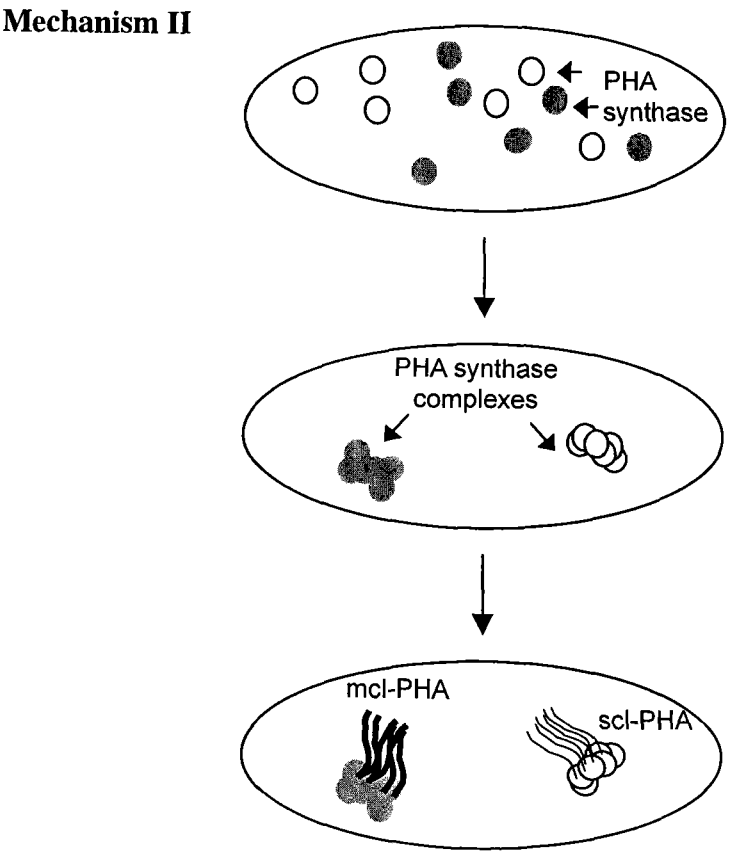




**Figure 3 Sudesh et al**