# Poly(3-Hydroxyalkanoates) from pseudomonas putida

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Poly(3-hydroxyalkanoate) or PHA is the general name for a group of biodegradable polyesters which are produced by a range of different bacteria. One type of PHA, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHB/V) is now produced at an industrial scale by Imperial Chemical Industries, Ltd. using a strain of *Alcaligenes eutrophus*. A different class of PHAs is composed of monomers with longer side chains and is produced by *Pseudomonas oleovorans* when grown on aliphatic hydrocarbons. In these PHAs the monomers range from 3-hydroxyhexanoate ($C_6$) to 3-hydroxydodecanoate ($C_{12}$) and the type and number of monomers in such PHAs is dependent on the growth substrate.

PHAs are formed by microorganisms as a reserve material for storage of energy. They are accumulated under conditions where the growth rate is slow due to the exhaustion of a nutrient such as nitrogen and may be used at later times when the carbon source becomes limiting. The biosynthetic and regulatory pathways which result in bacterial PHA synthesis are tuned to specific environmental conditions and are different from the conditions which permit efficient synthesis for production purposes. An understanding of these metabolic routes and the ability to influence them will facilitate the controlled synthesis of these compounds for future industrial applications. The experimental work described in this thesis aimed at an understanding of the cellular processes which determine the chemical characteristics of PHAs.

**Abundance of PHA accumulating bacteria.**

Initial studies on PHAs composed of medium chain length 3-hydroxy fatty acids ($C_6$ to $C_{12}$) were performed with *Pseudomonas oleovorans*. This microorganism is able to grow on aliphatic hydrocarbons due to the presence of a large extrachromosomal piece of DNA, namely the OCT plasmid. *P. oleovorans* is classified as a member of the fluorescent pseudomonads which do not accumulate PHB. Since our studies have shown that *P. oleovorans* accumulates PHA, we initiated a study on different related bacteria to evaluate the generality of PHA synthesis in the fluorescent pseudomonads. We found that different *P. putida*, *P. aeruginosa* and *P. fluorescens* strains were able to synthesize these polyesters, and this characteristic can therefore be regarded as a common feature of fluorescent pseudomonads. Furthermore, these studies indicated that there are strains other than *P. oleovorans* which have an even greater capacity to produce PHAs than *P. oleovorans* and one such strain, *P. putida* KT2442, was used in genetic studies.

**Genetics of PHA metabolism.**

The present best PHA producer, *P. putida* KT2442, was mutagenized to generate mutant strains unable to accumulate PHA. *P. putida* GPp104 is such a PHA-negative mutant. The genetic defect responsible for the absence of PHA in this strain was restored by the introduction of a specific piece of DNA. This DNA contained a 6.4 kb *Eco*RI fragment which complemented both PHA-negative mutants and mutants which were defective in the utilization of intracellularly stored PHA. By definition, this DNA fragment was named the *pha* locus. Analysis of the *pha* locus by nucleotide sequencing revealed that three polypeptides were encoded on this fragment. Two of these polypeptides showed a 35 to 40 % identity to PHB polymerase from *Alcaligenes eutrophus*. This enzyme transfers 3-hydroxybutyrate from coenzyme A to the growing polymer chain in this strain. Subsequently, it was conluded that the corresponding *pha* genes (*pha*A and *pha*C) encode PHA polymerases. In between these genes, a third enzyme was encoded by the *pha*B gene. This gene complemented the PHA degradation mutant and based on an amino acid sequence characteristic for lipases, the encoded protein was named PHA depolymerase. Further *in computo* analysis of the primary structure of the depolymerase and its comparison with a range of other bacterial enzymes indicated that this enzyme belongs to a class of hydrolytic enzymes with a common tertiary structure.

**Chemical and physical characteristics of PHAs.**

Having at our disposal several *Pseudomonas* mutants in PHA biosynthesis and degradation and the *pha* genes, we examined the polyesters produced by these different strains with respect to chemical and physical characteristics. The monomeric composition of PHAs was known to depend on the growth substrate. In our studies with different recombinant strains we found that both the specificity and the activity of the PHA polymerases determines the composition of the polymer. The substrate specificity of the two PHA polymerases was studied by cloning the respective genes separately on broad-host range vectors followed by analysis of the polyester formed by recombinants in which either one was introduced. We found that PHA polymerase 1 has a slightly higher affinity for the 3-hydroxyhexanoate monomer compared to PHA polymerase 2. A more profound change in polymer composition was obtained in recombinant strains grown on decanoate. The polymer produced by the wild-type strain on this compound consists of 3-hydroxydecanoate, 3-hydroxyoctanoate and 3-hydroxyhexanoate in a 7 to 12 to 1 ratio.

Upon introduction of plasmids which encode PHA polymerases, this ratio changes to 9 to 9 to 1. These results were interpreted to mean that the increased levels of PHA polymerases resulted in an improved channeling of 3-hydroxydecanoate into PHA synthesis.

Analysis of the physical characteristics of several PHAs, taught us that only the molecular weight was affected by the introduction of recombinant plasmids expressing PHA polymerases. The glass transition temperature, melting temperature and the heat of fusion were similar for all PHAs isolated from different octanoate grown strains. The average molecular weight of recombinant polymers was significantly lower compared to the wild-type PHAs. Since recombinant strains accumulate similar amounts of PHA, the number of individual PHA chains has increased. Increased polymerase levels resulting in an increased rate of PHA initiation explain these findings. As a consequence it turns out that the average molecular weight is inversely proportinal to the polymerase activity.

**PHA production.**

The potential of any production process not only depends on the versatility of its products, but, very importantly, also on its efficiency. *P. oleovorans* produces PHA when growth has stopped and consequently, PHA production by this strain demands two stages. In the first stage only biomass is produced, followed by a second stage in which the cells accumulate PHA. Unfortunately, a third stage is present in *P. oleovorans* in which the accumulated PHA is degraded. Two mutant strains were studied with respect to their PHA accumulation profile. *P. putida* KT2442 was used to generate PHA negative mutants, because it accumulated large amounts of PHA when grown on solid media. Further analysis revealed that this strain synthesizes PHA preferentially during the exponential growth phase in which PHA is accumulated up to 50 % of the cell dry weight. A mutant strain of *P. oleovorans* unable to degrade its intracellularly stored PHA retained this inability when it was grown in large volumes in computer controlled bioreactors. Throughout the cultivation of this strain, no polymer degradation was observed. Although the isolated polymer of this strain did not show an increased average molecular weigth, the finding that the material is not degraded may prove useful for future applications.

It is not unlikely that in the future these biological polyesters will be produced by the individual, isolated enzymes. A first characterization of the PHA polymerases was

achieved by identification of these enzymes as the major protein constituent of recombinant PHA granules. The enzymes are tightly bound to the PHA granule and may be dissociated from them by the use of hydrophobic agents such as SDS or Triton-X100. Extensive experimentation is needed to analyze these isolated enzymes with respect to activity, stability and specificity.

**Conclusion.**

PHA synthesis in *Pseudomonas putida* requires one of two PHA polymerases and possibly some further unidentified factors. The availability of different mutant and recombinant strains allows the rational synthesis of biodegradable polyesters with different monomeric compositions and molecular weigths. The relations evident from this work between strain, polymer composition and polymer characteristics are expected to support a future production of these polyesters.