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The biodegradation of poly-3-hydroxyalkanoates, PHAs, with long alkyl substituents by *Pseudomonas maculicola*

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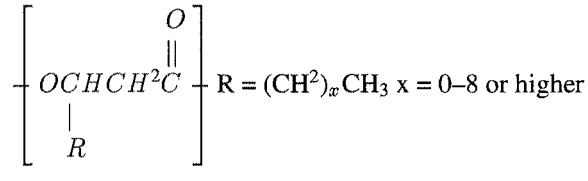
Abstract

Utilizing a quantitative clear zone technique, the activity of an extracellular depolymerase system from *Pseudomonas maculicola* was investigated. Polymer degradation was influenced by the amount and availability of secondary carbon sources, with a simultaneous utilization of both sources. The initial carbon source in the liquid preculture also affected the eventual colony growth and polymer degradation. The enzyme solution was determined to readily degrade poly-3-hydroxyalkanoates (PHAs) with relatively 'long' alkyl substituents at the 3 position: poly-3-hydroxyoctanoate (PHO), poly-3-hydroxynonanoate (PHN), and their copolymers (P[HO-*co*-HN]) and poly-3-hydroxyundecanoate (PHU). However, the system was unable to degrade either PHAs with shorter alkyl groups, including poly-3-hydroxybutyrate (PHB) and the copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P[HB-co-HV]) or PHAs with unusual substituents such as poly(3-hydroxy-5-phenylvaleric acid) (PHPV). It is proposed that degradation of these more bulky side chain polymers was prevented by the inability of the bacteria to assimilate their monomeric components, which inhibited the successful utilization of secondary carbon sources and thus inhibited colony growth.

Introduction

Since its discovery by Lemoigne in 1925 (Lemoigne 1925, 1926), poly-3-hydroxybutyrate (PHB) and its copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(HB-*co*-HV) have been the focus of much attention as potentially biodegradable, biocompatible thermoplastic polymers (Holland et al. 1990; Yasin & Tighe 1992 & 1993; Kanesawa & Doi 1990; Brandl & Püchner 1990; Mergaert et al. 1992). Recently, there has been an increase in the synthesis and development of a number of more novel poly-3-hydroxyalkanoates (PHAs) (Fritzsche et al. 1990; Kim et al. 1992; Brandl et al. 1988; Lenz et al. 1992) shown below. Very little attention has been paid to the biodegradability of these unusual polymers.



The extracellular biodegradability of PHB, and the inherent biodegradability of these novel long-side chain microbially produced polyesters, lends credence to their potential for extracellular degradation within the macroenvironment, but research pertaining to PHAs has tended to emphasize their synthesis and production (Brandl et al. 1988; Lenz et al. 1992).

In this report we discuss our investigations regarding the biodegradation of a number of the more novel biopolymers by the bacterium *Pseudomonas maculicola*, utilizing an adaptation of a method for the production of a colloidal polymer suspension or 'latex' of poly-3-hydroxyoctanoate (PHO) recently reported by Ramsey et al. (1993).

Materials and methods

PHAs were produced by growing the bacterium, *Pseudomonas oleovorans*, on various carbon sources (Fritzsche et al. 1990; Kim et al. 1992; Brandl et al. 1988; Lenz et al. 1992). P(HB-*co*-HV) was purchased commercially.

Cultures of *P. maculicola* were grown in 150 mls modified E* media with 20 mM glucose as a carbon source, using a shake flask at 30 °C. These cells were then used to inoculate 1 liter precultures of modified E* media and 20 mM of a desired carbon source (Brandl et al. 1988). Cell growth was monitored by measuring the change in optical density at 660 nm in quartz cuvets (OD660). At the desired time, samples were diluted to an OD660 of 0.50 and 5 μl utilized to inoculate the culture plates.

10 mls of sterilized modified E* media with or without a carbon source and 2% agar were pipetted into the base of a sterile petri dish and allowed to solidify by standing (base layer). A polymer latex of known concentration was produced according to a method of Ramsey et al. (1993). To a known volume of this latex, a five fold dilution of agar solution was added with stirring, and 6 mls of the sterilized mixture pipetted on the surface of the base layer and allowed to solidify (top layer or overlay) (Fig. 1). In cases of low polymer availability, the clear zone was enhanced by the addition of Sudan Red dye during the polymer latex preparation. This dye stained the polymer in the top layer orange/red, and thus, the clear zone was more distinguishable from the background.

Degradation was expressed as the weight loss of polymer with time and calculated according to the equation:

$$\text{Polymer weight loss} = \frac{(\text{Clear zone area} \times \text{total weight of polymer top layer})}{\text{Total area of petri dish}}$$

Results and discussion

P. maculicola colonies exhibited similar growth in the liquid precultures using either 20 mM glucose, sodium octanoate, octanoic acid or nonanoic acid as the sole source of carbon. No intracellular PHA inclusions were observed. Inoculation of the plates occurred at the onset of the stationary growth phase with similar OD660's, thus ensuring similar live cell quantities in each inoculum. Figure 1 illustrates the increase in colony and clear zone size for *P. maculicola* grown on PHO with 20 mM glucose as a secondary carbon source. A small piece of this clear zone agar was removed and placed on a fresh overlay plate. Within 30 h of incubation at 30 °C, a region of clearing around the agar piece was observed, with the noticeable absence of colonies. This confirmed the presence of an extracellular depolymerase and may be important in its isolation.

Figures 2A to 2D illustrate the change in colony and clear zone size and the degradation of PHO by an extracellular depolymerase secreted by *P. maculicola*, with a variation in the concentration of a secondary carbon source in the base layer. In all cases there was a noticeable increase in clear zone and colony size. As the concentration of the secondary carbon source increased, there was an increase in the colony size and density but a decrease in clear zone size and, therefore, in polymer degradation. The *P. maculicola* colony with 10 mM glucose as a secondary carbon source showed little growth, but exhibited a continuous secretion of depolymerase with an overall PHO degradation rate of approximately 6.7 μgHr^{-1} at 360 h after inoculation. In contrast, *P. maculicola* grown with 60 mM glucose as secondary carbon source exhibited a noticeable colony growth but little increase in the clear zone. The gradual increase in the clear zone size for the colonies with 20 and 40 mM glucose as secondary carbon sources tends to indicate that the depolymerase was continuously secreted by the colonies, with the simultaneous utilization of the PHO and glucose. Secretion of the depolymerase and, therefore, polymer degradation was limited by the amount of secondary carbon present.

P. maculicola colonies exhibited similar growth with 20 mM glucose or sodium octanoate as secondary carbon sources (Figs. 3B and 3C). The PHO degradation for the colony with glucose as a secondary carbon source was comparatively more gradual than the colony with sodium octanoate as a source of secondary carbon, which revealed a short lag phase prior to the initial synthesis of enzyme, followed by the secretion of more depolymerase and a sudden surge in the PHO degradation rate after approximately 270 h. This second region of depolymerase activity might have been due to a number of factors, including: (1) sodium octanoate required a longer time period for its incorporation into the cells than glucose, and (2) the greater chain length of the octanoate compared to the glucose may have facilitated an initial reduction in enzyme secretion by exceeding a threshold value in a similar way that was observed with the plate having 60 mM glucose as a secondary carbon source. Therefore, polymer degradation

Top Layer or Overlay: Polymer latex in agar.

Base Layer: Modified E* media,
Secondary carbon source, (optional).

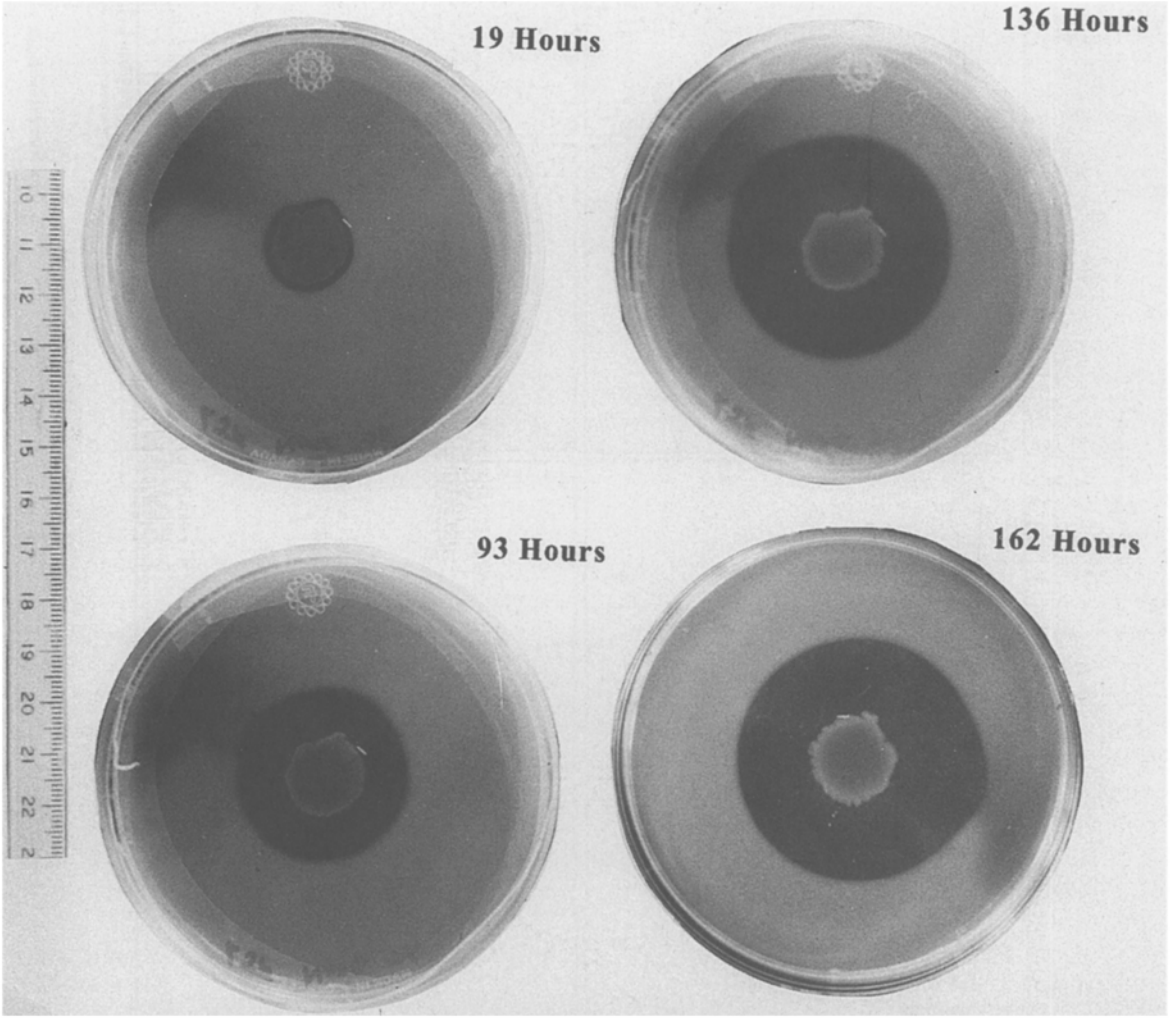


Fig. 1. Diagrammatic representation of sterile petri dish illustrating the two agar layers and photograph illustrating the gradual increase in colony and clear zone size of *P. maculicola* grown on PHO with 20 mM glucose as a secondary carbon source.

was influenced by the availability of the secondary carbon source to the cells.

There was a noticeable difference in PHO degradation by *P. maculicola* colonies inoculated from liquid precultures possessing different carbon sources. In all cases there was a noticeable lag period of approximately 22 h before any depolymerase activity was detected. It was observed that *P. maculicola* inoculated from a preculture with 20 mM nonanoic acid as a carbon source and grown on plates with sodium octanoate as a secondary carbon source, exhibited a comparatively greater colony growth and PHO depolymerase activity than its counterparts with glucose and nonanoic acid as secondary carbon sources. This differs from the trend observed for the colonies initially grown with 20 mM sodium octanoate in the preculture. Differences in PHO degradation by *P. maculicola* colonies inoculated from precultures with different carbon sources may be due to the influence of these different carbon sources and to using inocula from different times in their growth curves.

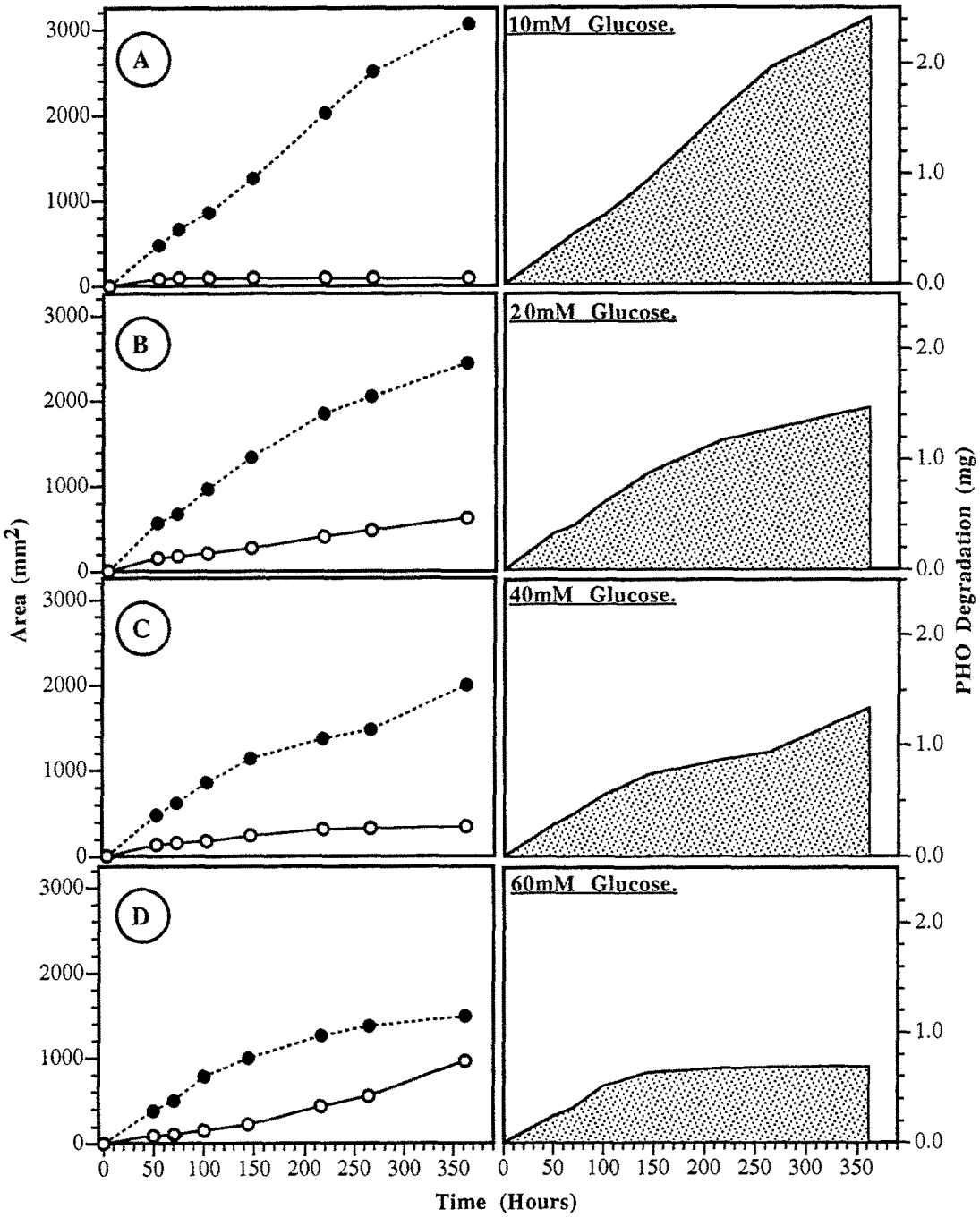


Fig. 2. Change in colony (o), clear zone (•) size and PHO degradation by *P. maculicola* with time and variation in concentration of glucose as a secondary carbon source.

Figure 4A demonstrates that the depolymerase from *P. maculicola* was also capable of degrading poly-3-hydroxynonanoate (PHN). The colony grown on PHN with no secondary carbon source exhibited a similar growth as that of *P. maculicola* grown on PHO with no secondary carbon source, but exhibited a considerably smaller clear zone. This was due to the relatively greater proportion of carbon present in the PHN compared to the PHO. The colony grown on a PHN overlay with glucose as a source of secondary carbon showed a similar growth as its PHO counterpart but a reduced clear zone. *P. maculicola* grown

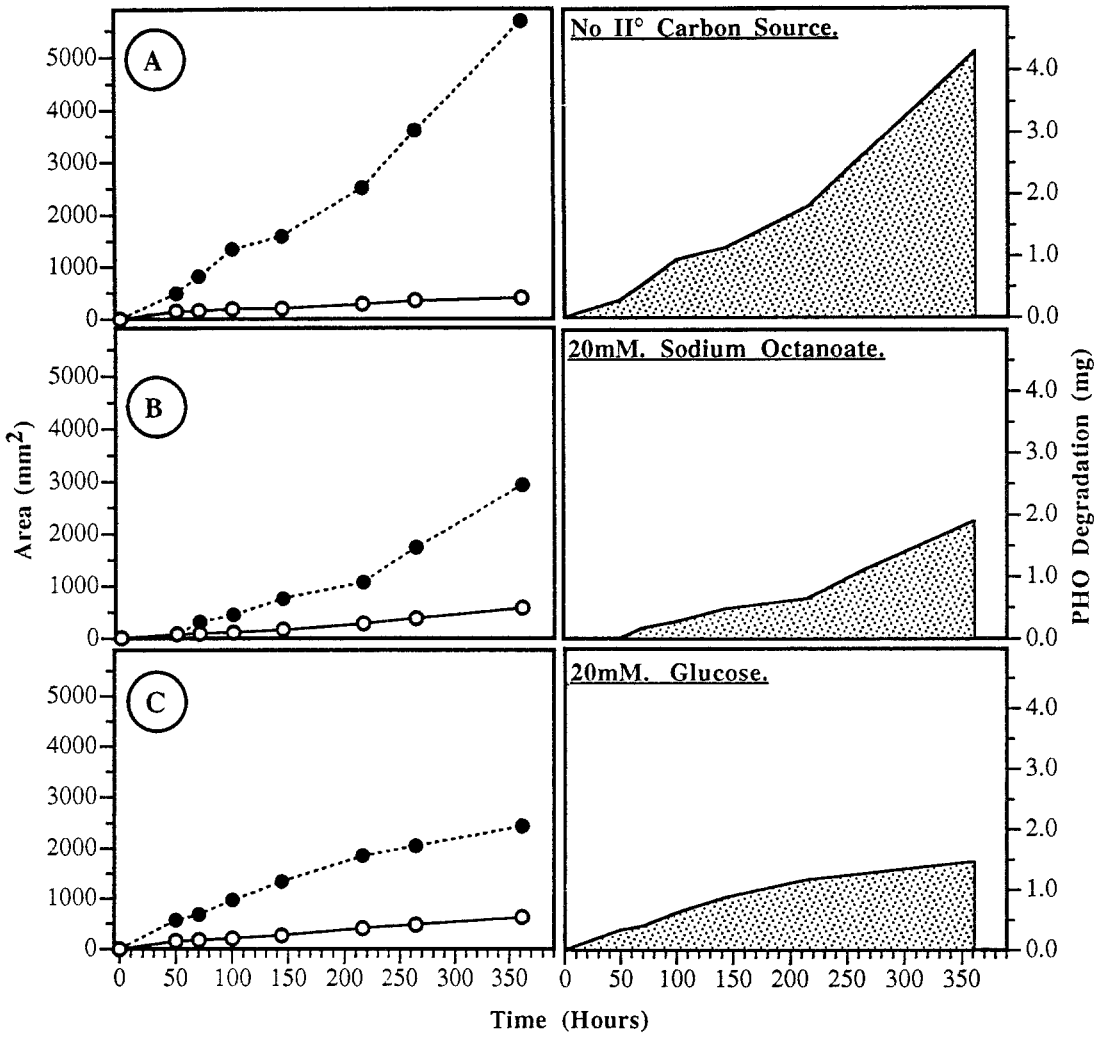


Fig. 3. Change in colony (o), clear zone (•) size and PHO degradation by *P. maculicola* with time and variation in type of secondary carbon source, 20 mM sodium octanoate as initial preculture carbon source.

on a PHN overlay with nonanoic acid as the secondary carbon source also exhibited similar growth and degradation patterns. However, the colony grown using 20 mM sodium octanoate as a secondary carbon source exhibited a noticeable difference from the PHO degradation data, with a lag time of approximately 70 h, before achieving a rapid degradation rate of approximately 4.1 μgHr^{-1} which was coupled with a noticeable increase in colony growth. As anticipated, the *P. maculicola* depolymerase system was also quite capable of degrading a copolymer of 3-hydroxyoctanoate and 3-hydroxynonanoate (Fig. 4B).

The extra CH_2 in the side group of nonanoate compared to octanoate may have influenced the molecular architecture of the polymer helix and the uptake of the substrates into the cell and their utilization in the synthetic pathway. This suggestion could explain why polymer producing microbes have comparatively low polymer yields when grown on substrates with bulky groups and the apparent lack of the extracellular degradation of their polymers, such as for poly(3-hydroxy-5-phenylvalerate) (PHPV) which was synthesized by *P. oleovorans* in small quantities and was not degraded by *P. maculicola*, (Fig. 5).

Figure 5 also illustrates that the extracellular depolymerase was unable to degrade P(HB-*co*-HV). It is interesting to note, however, that the colony growth for the P(HB-*co*-HV) plate was substantially larger than that of its PHPV counterpart. It is suggested that *P. maculicola* was unable to degrade the P(HB-*co*-HV)

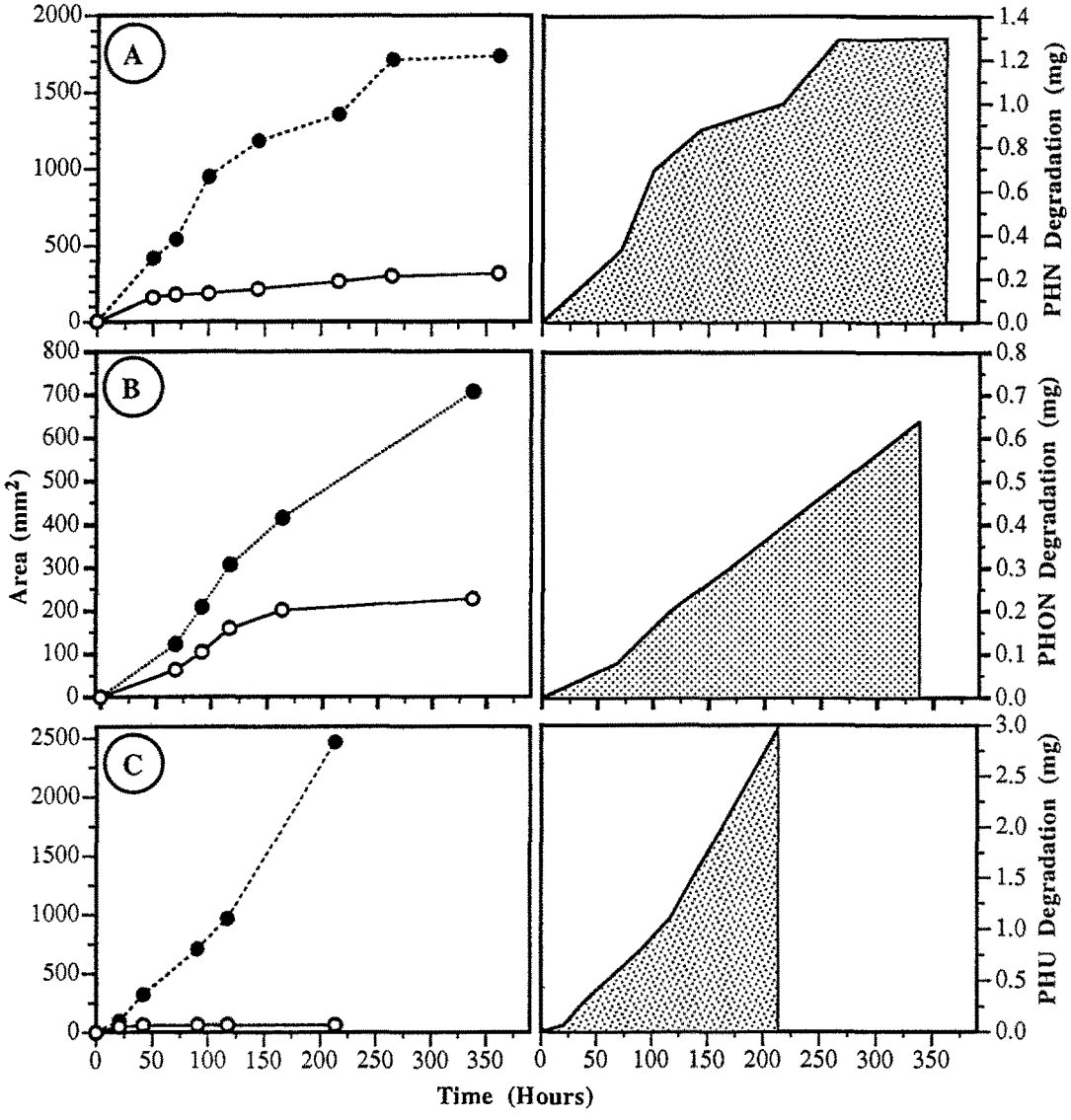


Fig. 4. Change in colony (o), clear zone (•) size and degradation of PHN, P(HO-co-HN) and PHU by *P. maculicola* With time, no secondary carbon source and 20 mM Sodium Octanoate as initial preculture carbon source.

due to an inaccessibility of the active site by its extracellular enzyme system. In the case of the *P. maculicola* grown with the PHPV overlay, the enzyme system could attack, but the bulky phenyl group inhibited the uptake of the substrate into the cell and this prevented the utilization of the glucose base layer. It is clear, however, that the architecture and biochemical influence of the monomeric units in polymer synthesis must be investigated further in order to improve polymer yields and degradation potential.

The degradation of unsaturated poly-3-hydroxyundecanoate (PHU) by *P. maculicola*, grown on 20 mM sodium octanoate in the preculture, is illustrated in Fig. 4C. As anticipated, the colonies grown on PHU plates with no secondary carbon source exhibited less growth than those grown with the 20 mM glucose or sodium octanoate cosubstrates, but showed a comparatively greater rate of PHU degradation.

Thus we have shown that the method of Ramsey et al. (1993) for the preparation of a polymer latex, originally used for PHO, can also be used to produce latex's of PHN, P(HO-*co*-HN), PHU and PHPV, and perhaps many others. This technique provides optimum utilization of limited polymer weights and, as such, is valuable for the detection, isolation and identification of organisms capable of degrading these more novel

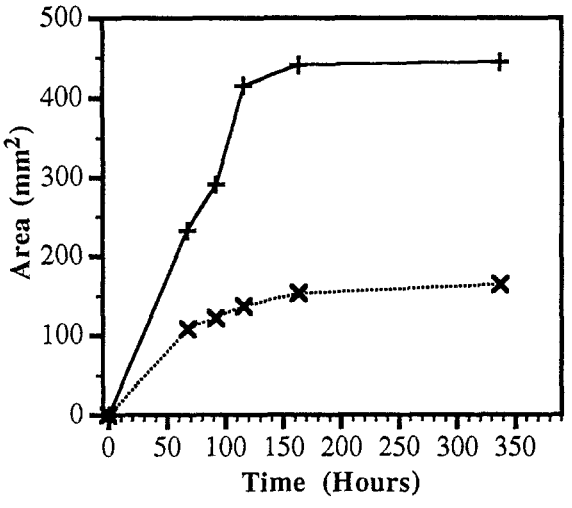


Fig. 5. Growth of *P. maculicola* colonies, on PHB (+) and PHPV (×) biopolymers and 20 mM Glucose as a secondary carbon source with time.

biopolymers. The use of clear zones to demonstrate polymer degradation and isolate polymer degrading microbes, as reported by Ramsey et al. (1993), can also be adapted to quantitatively monitor the depolymerase activity and investigate other factors affecting the rates of polymer degradation.

The extracellular depolymerase system secreted by *P. maculicola* is capable of degrading a variety of PHAs with relatively large substituents at the 3 position including: PHO, PHN, P(HO-*co*-HN) and PHU. However, it was unable to degrade PHAs with shorter substituents, including PHB and P(HB-*co*-HV), or the unusual PHA with a large phenyl group as the substituent, PHPV. The rate of polymer degradation was affected by the presence of secondary carbon sources, with a simultaneous utilization of both sources. Generally, there was an increase in colony growth with an increasing degree of total carbon and a decrease in clear zone activity. In all cases there was a noticeably greater colony growth and depolymerase activity for *P. maculicola* with sodium octanoate as a secondary carbon source.

In conclusion, the rate of polymer degradation was influenced by the degree and availability of secondary carbon and by the initial carbon source in the liquid preculture. It is proposed that polymer degradation, as well as synthesis, may be limited by the nature of the monomeric units and the relative ease or difficulty of their uptake into the cell, and consequent utilization in biochemical pathways.

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