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REVIEW

# Advance on the production of polyhydroxyalkanoates by mixed cultures

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**Abstract** Polyhydroxyalkanoates (PHAs) are the polymers of hydroxyalkanoates that accumulate as carbon/energy or reducing-power storage material in various microorganisms. PHAs have attracted considerable attention as biodegradable substitutes for conventional polymers. Until now, however, industrial production of PHAs has encountered only limited success. The main barrier to the replacement of synthetic plastics by PHAs has been the higher cost. The use of mixed cultures and renewable sources obtained from waste organic carbon can substantially decrease the cost of PHA and increase their market potential. This work reviews two main methods of PHA production by mixed cultures, anaerobic–aerobic processing and aerobic transient feeding processing, and analyzed the metabolic and effective factors.

**Keywords** polyhydroxyalkanoates (PHAs), mixed cultures, anaerobic–aerobic processing, aerobic transient feeding processing

## 1 Introduction

In the latest decades, polyhydroxyalkanoates (PHAs) have attracted increasing interest as an alternative to petroleum-derived plastics. PHAs are polyesters produced by bacteria or plants that undergo complete biodegradation, meanwhile sharing similar physical properties with most petroleum-derived plastics (Davide et al., 2005). Until now, however, industrial production of PHAs has encountered only limited success. The main barrier to the replacement of synthetic plastics by PHAs has been the cost difference (€9/kg for PHAs vs. €1/kg for synthetic plastics) (Biby, 2002 Degradable plastics: http://www.icma.com /info/polymers).

Most processes for PHA production are based on pure cultures grown in well-defined nutrient-deficient synthetic media and single substrates. The high cost of the substrate and of the equipment required for aseptic operation is the main factor responsible for the high selling price of PHA. The use of open mixed cultures and waste materials can substantially decrease the cost of PHA and increase their market potential (Satoh et al., 1998). An economic evaluation showed that the price of PHA can be reduced to €4/kg if low-cost substrates and mixed cultures are used (Meesters, 1998). This value is still higher than the price of polypropylene, but this disadvantage should be partially offset by PHA's complete biodegradability versus the environmental problems resulting from the non-biodegrability of polypropylene.

The idea of PHA production using mixed culture arose from the recognition of PHA's role as a metabolic intermediate in microbial processes for wastewater treatment. Biological wastewater treatment usually occurs under dynamic conditions (Van Loosdrecht et al., 1997). Activated sludge, a well-known mixed culture, is able to store PHA as carbon and energy storage material under unsteady conditions arising from an intermittent feeding regime and variation in the presence of an electron acceptor. Microorganisms which are able to quickly store available substrate and consume the storage to achieve a more balanced growth have a strong competitive advantage over organisms without the capacity of substrate storage (Van Loosdrecht et al., 1997).

In this paper, the metabolism and production process of anaerobic–aerobic processing and aerobic transient feeding processing, which are the main methods of PHA production by mixed cultures, were discussed. Meanwhile, the factors affecting the production of PHA by mixed cultures in the two processes were also discussed.

## 2 Anaerobic–aerobic processing

The anaerobic–aerobic processing was invented in the middle of the 1970s (Spector, 1971). It was a modified activated sludge process widely used for the removal of phosphorus

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from wastewaters, often referred to as "enhanced biological phosphorus removal process" or EBPR process.

### 2.1 Metabolism

During the anaerobic–aerobic processing, where electron donor and acceptor availability are separated, PHA plays a specific role in the ecophysiology of a certain group of bacteria, the most well-known being the polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) (Cech and Hartman, 1990; Hesselmann et al., 2000).

PAOs are probably the most widely recognized for producing storage polymers (PHA, glycogen, and polyphosphates) (Salehizadeh and Van Loosdrecht, 2004). The whole competitive advantage for these organisms is based on their capacity to utilize the energy stored as poly-P to store exogenous substrate in the form of PHA when no electron acceptor (oxygen or nitrate) is available for energy generation. GAOs were recognized recently as competitors of PAOs. Effectively, these organisms rely on substrates which can be fermented (e.g., glucose), and they store the fermentation products inside the cell rather than excreting them. These organisms can also use internal stored glycogen for fermentation to PHB. The energy released in the glycolysis process is subsequently used to accumulate fermentation products (e.g., acetate) in the form of PHB. PAOs and GAOs proliferate in systems where the substrate is present regularly while an electron acceptor is absent (Cech and Hartman, 1993).

Acetate is commonly used as the model substrate for culture stability and the use of open fermentation metabolic studies of PAO and GAO cultures. There are some similarities in the metabolic pathways for the production of the reserve polymers (Salehizadeh and Van Loosdrecht, 2004) (Fig. 1). Under anaerobic conditions both microbial groups can take up acetate at the expense of energy (ATP), transport it over the membrane, and activate to acetyl-CoA. Acetyl-CoA is then drifted to the synthesis of PHB by condensation to acetoacetyl-CoA and reduction to hydroxybutyryl-CoA (consuming NADPH), which is finally incorporated in the growing chain of PHB (Reis et al., 2003).

### 2.2 Production process

The production process contains two stages: anaerobic stage and aerobic stage. The biomass in activated sludge is fed with organic wastewater which was hydrolyzed to short-chain fatty acids (SCFAs), like acetate, during the anaerobic phase. In this phase, PAOs activate the metabolic tools to take up acetate and to store it as polyhydroxyalkanoates (PHAs), mainly polyhydroxybutyrate (PHB). PHA storage proceeds using the internal polyphosphate (poly-P) pool as energy source resulting in a release of orthophosphate (Pi) (Nevin et al., 2003). In the subsequent aerobic stage, part of sludge was taken for PHAs extraction, and the other was returned to the anaerobic stage.

Ueno et al. (1993) and Saito et al. (1995) applied acetate to laboratory-acclimatized anaerobic–aerobic activated sludge, and reported that the sludge accumulated more PHB under aerobic conditions than they did under anaerobic conditions. Ueno et al. (1993) reported that PHB accumulated under aerobic conditions reached 36% of the sludge dry weight, while it was 17% under anaerobic conditions. The cause of the enhancement is attributed to the difference in the availability of energy for PHA accumulation; anaerobic substrate uptake is stopped when the stock of energy such as polyphosphate and glycogen are exhausted, while more energy can be produced by oxidative degradation of substrates under aerobic conditions.

## 3 Aerobic transient feeding processing

In recent years, many studies refer to the production of PHA by mixed cultures when exposed to transient carbon supply.

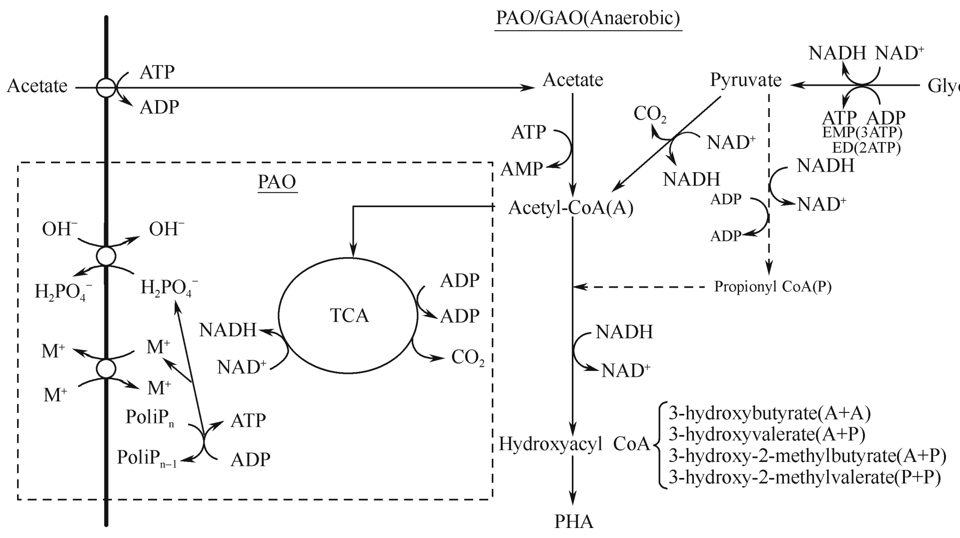


**Fig. 1** PHA production metabolism in PAO/GAO system (Salehizadeh and Van Loosdrecht, 2004)

It is well known that sludge submitted to consecutive periods of external substrate accessibility (feast) and unavailability (famine) generates a so-called unbalanced growth.

3.1 Metabolism

Under these dynamic conditions, during excess external carbon substrate, the uptake is driven to simultaneous growth of biomass and polymer storage, and after substrate exhaustion, stored polymer can be used as energy and as a carbon source (Salehizadeh and Van Loosdrecht, 2004) (Fig. 2). In these cases storage polymers are formed under conditions that do not limit growth. The storage phenomenon is usually dominant (70%) over growth, but under conditions in which substrate is present for a long time, physiological adaptation occurs and growth becomes more important (Van Aalst-Van Leeuwen et al., 1997; Dionisi et al., 2001). The ability to store internal reserves gives these microorganisms a competitive advantage over those without this possibility, when facing transient substrate supply (Reis et al., 2003).

3.2 Process and production

A new process (Fig. 3) (Majone et al., 1999b) has been proposed for the production of biodegradable PHAs from wastes, involving a combination of anaerobic (acidogenic fermentation) and aerobic (activated sludge enrichment) stages. The process uses mixed cultures with high storage response, which are enriched and produced from activated sludges under aerobic periodic feeding of volatile fatty acids (from the acidogenic fermentation stage).

A previous experiment (Luisa et al., 2004) showed that when 180 mmol/L of acetate was supplied in three pulses, a very high PHB content was achieved during aerobic transient feeding processing. The value obtained, 78.5% of the cell dry weight, was similar to the values reported in the literature for commercial PHB pure producer strains. Moreover, the specific PHB production rate was one order of magnitude higher than that reported for pure cultures.

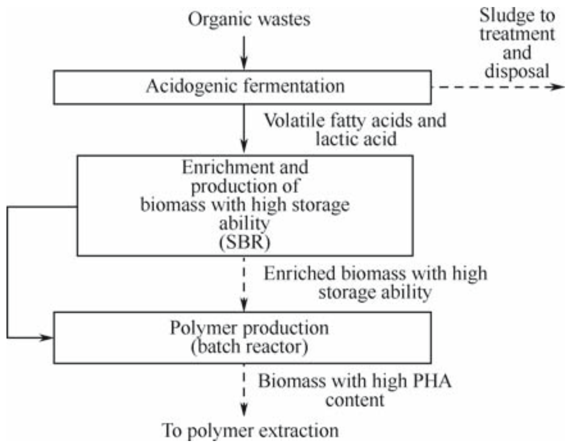


**Fig. 3** Flowsheet of the proposed process for PHA production from organic wastes (solid lines refer to substrate fluxes, dotted lines to biomass fluxes) (Majone et al., 1999)

## 4 Comparison of the two methods

The two methods are different in metabolism and the accumulated PHAs content. During the anaerobic–aerobic processing the electron donor and acceptor availability are separated, thus PAOs and GAOs play a main role in the production of PHAs. According to the reference, activated sludge could accumulate PHA to around 20% of dry weight under anaerobic conditions (Salehizadeh and Van Loosdrecht, 2004).

During the aerobic transient feeding processing, a long period of lack of substrate is alternated with a short time of excess of substrate. The microorganisms able to store internal reserves have a competitive advantage over those that do not

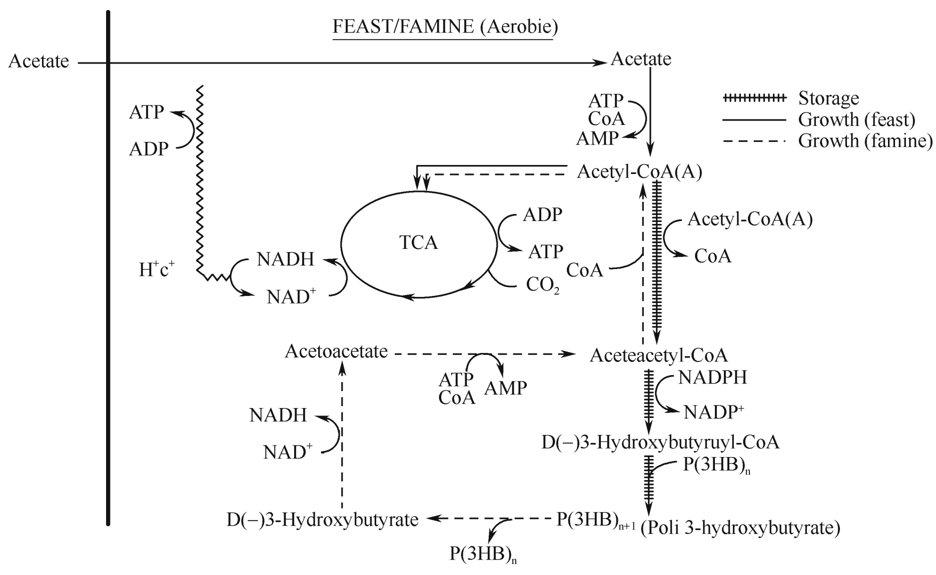


**Fig. 2** PHA production pathways in feast/famine conditions (Salehizadeh and Van Loosdrecht, 2004)

have this capacity, thereby becoming dominant in systems submitted to transient conditions (Majone et al., 1996). According to the reference, activated sludge could accumulate PHA to 78.5% of the cell dry weight under aerobic transient feeding (Luisa et al., 2004).

## 5 Factors affecting the production of PHAs by mixed cultures

### 5.1 Effect of carbon source

The nature of the substrate not only determines the PHA content but also its composition, which subsequently affects the final polymer properties (Satoh et al., 1998a). Lemos et al. (1998) demonstrated that acetate uptake by PAOs leads to the production of a copolymer of hydroxybutyrate (HB) and hydroxyvalerate (HV), with the HB units being dominant (69%–100% HB; 0%–31% HV). With propionate, HV units are mainly incorporated in the polymer. The yield of polymer (YP/S) was found to diminish from acetate (0.97) to propionate (0.61) to butyrate (0.21). A previous experimentation (Davide et al., 2004) showed that took acetic, lactic, and propionic acid as single carbon sources under the aerobic transient feeding conditions, the three substrates were stored in the form of homopolymers (PHB from acetic or lactic acid, PHV from propionic acid), whereas the copolymer P (HB/HV) was formed when propionic acid was present along with lactic and/or acetic acid.

Furthermore, raw materials may account for 40%–50% of the total operating costs (Van Wegen et al., 1998). The price of PHAs can be substantially reduced if cheap organic substrates, such as waste materials from agriculture and food industry (e.g., whey and molasses) are used. Because an open-culture system would be used, not all substrate would be equally suitable. For example, starch and cellulose hydrolysates could lead to the growth of glycogen-accumulating organisms (Mino et al., 1996). This problem can be easily overcome by acidification of sugar, starch, and cellulose hydrolysates with a mixture of volatile fatty acids (VFAs) such as acetic, propionic, butyric, etc. Such a mixture can be easily converted to PHAs.

### 5.2 Effect of pH

For PAOs, stoichiometry, glycogen consumption, and PHA accumulation are independent of pH over the pH range of 6.5–8.0. The amount of phosphorus released per mole of acetate taken up (P/HAc ratio) is linearly dependent on pH, because of the additional energy requirement for acetate transport at higher pH (Filipe et al., 2001b). Filipe et al. (2001b) showed that high pH values in anaerobic phase are bad for GAOs, suggesting that pH may be manipulated to minimize the presence of GAOs in EBPR (Filipe et al., 2001a; Filipe et al., 2001b).

A previous experimentation (Luisa et al., 2004) showed that during the aerobic dynamic feeding process, PHB production was higher when pH was not controlled than when controlled at 7.0 or 8.3, because when the pH was not controlled only 12% of the substrate was used for energy and the highest fraction (88%) was used for storage and growth, whereas at pH 7.0, 44% of the substrate was used for cell energy and about 56% was diverted for polymer storage and growth. It was also reported that biomass activity and corresponding storage rates were maximal in the pH range 7.5–8.5, but were significantly high (in the range 70%–80% of the rate at pH 7.5) also at pH 6.5 and 9.5 (Davide et al., 2005). Meanwhile, polymer composition was strongly affected by the pH change: the HV content in the stored polymer increased from about 10% mol at pH 5.5 to about 30% mol at pH 9.5. This can be attributed to an increase in the fraction of the removed propionic acid which was converted into the stored polymer (Davide et al., 2005).

### 5.3 Effect of C/N

In a conventional nitrifying/denitrifying system with 66% of the time under aerobic condition, storage of PHB increases the COD need by 30% (Beun et al., 2000b). During the PHA production under aerobic dynamic feeding conditions, it was reported (Luisa et al., 2004) that a lower ammonia concentration originated in a higher proportion of carbon for PHB storage and the higher the carbon/ammonia ratio, the higher the PHB content. But, it was also reported (Davide et al., 2005) that with reference to the influence of nitrogen limitation (i.e., absence of growth) on polymer production, no significant increase in polymer production rate was observed in comparison with conditions with nitrogen excess. This demonstrates that storage induction by feast and famine conditions is substantially different from the selection promoted in industrial processes, where storage is switched through the starvation for an essential nutrient.

### 5.4 Effect of temperature

The influence of temperature on the accumulation of PHB in sequencing batch reactor cultures fed with acetate has been studied by Krishna et al. (1999) under conditions when the acetate was rate limiting. The PHB formation rate was shown to decrease with increasing temperature because of an increase in the anabolic rate at higher temperatures (Salehizadeh and Van Loosdrecht, 2004).

## 6 Conclusions

Although the PHAs content in biomass is lower in the production of PHAs by mixed cultures (activated sludge), compared with the production of PHAs by pure cultures, which has been used for industrial production, the former can substantially decrease the cost of PHA and reuse waste. Especially, the aerobic transient feeding processing is the most promising because of high PHA accumulation.

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