Production of PHAs from Agricultural Waste Material

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SUMMARY: Polyhydroxyalkanoates (PHAs) are biodegradable substitutes to fossil fuel plastics that can be produced from renewable raw materials such as saccharides, alcohols and low-molecular-weight fatty acids. They are completely degradable to carbon dioxide and water through natural microbiological mineralization. Consequently, neither their production nor their use or degradation have a negative ecological impact. By keeping closed the cycle of production and re-use, PHAs can enable at least part of the polymer-producing industry to switch from ecologically harmful end-of-the-pipe production methods towards sounder technologies. Up to now such polyesters have been produced biotechnologically from refined raw materials (e.g. glucose and sodium propionate), but they can as well be produced much cheaper from agricultural waste materials (e.g. molasses, maltose, glycerol phase from biodiesel production, whey), as long as these materials have a known composition and are available in appropriate quantities. Yield factors and specific rates for growth and PHA accumulation are shown for 3 strains of *Alcaligenes latus* for different agricultural waste carbon sources.

Introduction

Polyhydroxyalkanoates are biopolyesters stored as reserve polymers by a great number of prokaryotes [1,2]. Stored as intracellular granules, PHAs serve as reserve polymer for carbon and energy for the producing microorganism [3]. During normal growth, its content in the cells usually does not reach high concentrations; depending on the bacterial strain, typical concentrations are between 2 and 10 % of the cell dry weight. But PHB contents can reach more than 80 % of the dry biomass if growth is limited by depletion of an essential nutritional compound in the producer's environment [4]. When this effect is exploited for production of PHA in a bioreactor, the nitrogen, phosphorus, sulfur or magnesium sources are usually the ones chosen for exhaustion. Low dissolved oxygen concentrations lead too to enhanced PHA accumulation [5,6]. Most bacterial strains investigated to date require these unbalanced conditions for substantial PHB synthesis, and long accumulation phases must

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often be accepted if high concentrations of PHB are to be obtained [7-9]. PHB production processes, normally run in a discontinuous mode, are thus time consuming (> 100 hours of fermentation time). If a continuous production process is applied, a two-stage fermentation, where the growth phase in a first bioreactor is separated from the accumulation phase in a second vessel, is usually the recommended method [10,11]. But when certain strains, such as those of the bacterium *Alcaligenes latus*, are used, increased PHB biosynthesis is not dependent on growth limitation. In *A. latus* DSM 1122 for example, PHB is accumulated during growth in substantial amounts, its content being practically never below 50 % of the cell dry weight [12,13]. *Azotobacter vinelandii* also shows PHB hyperproduction during exponential growth [14]. A consequence of this behavior is that a discontinuous production process for PHB can be remarkably shortened (< 50 hours of fermentation time), or if a continuos process is considered, a simple single-stage system can suffice [15].

Production of PHAs

Homopolymer and Copolymers

The PHB homopolymer can be produced in high quantities from cheap raw materials, but there are limits to its industrial and commercial applications because of its less-than-perfect physical properties (namely its brittleness and high glass transition point). Therefore when hydroxyalkanoate monomers other than 3-hydroxybutyrate were found in PHA samples isolated from activated sludge [16], research for such copolymeric PHAs was intensified [17,18]. Since then it has been shown that besides PHB, a number of other PHAs are synthesized by various bacteria when suitable cosubstrates are fed to the fermentation medium during the product accumulation phase [19-26]. Varying molecular structures impart of course different physical properties to the copolymers, so PHAs with features ranging from crystalline thermoplasticity to complete elastomery can be produced.

But for the utilization of PHAs to become widespread, their production costs must be lowered and their quality must be increased. Many aspects must be considered to reach these goals, most of them having to do with the producing microorganism: it has to be perfectly suited to the task. It should grow fast on cheap carbon sources, preferably at relatively high temperatures (35 - 40 °C), and should not be sensitive to the precursors used for copolymer production. The inexpensiveness of the carbon source is very important, because the costs of a high-volume product are greatly influenced by the price of the raw

material, which might contribute to up to 50 % of the final price tag of a biotechnological product. Therefore agricultural waste materials should be very interesting renewable raw materials for PHA production. Processes other than simple batch or fed-batch fermentations should be considered; in particular, those which can take into account the fundamental differences between a microorganism in its growth phase and the same one in polymer accumulation conditions. In the case of a continuous production process, where cultures are commonly run over long periods of time, the strain should be genetically stable. And for any type of process, a microorganism capable of growth-associated polymer production is almost always preferable to one which is not.

Kinetics of PHA accumulation

The new findings reported above and those reviewed from the literature point out the existence of three distinct types of growth and PHA accumulation behavior, each one typified here by one or more microorganism-carbon source combinations:

1) PHA synthesis occurs in association with growth (example: *A. latus with sucrose or green syrup);*

2) PHA synthesis occurs in partial association with growth (example: *Ralstonia eutropha* G^{+3} *with glucose, A. latus with glucose or molasses*);

3) PHA is hyperproduced after a carbon starvation period (example: *Pseudomonas 2 F with glucose*).

In principle, the three behaviors can be exploited for PHA production in batch culture, but due to its higher productivity, a continuous production process is of higher commercial interest, especially for strains with a high maximum specific growth rate. To prove this point, the overall productivity of a batch system was compared to that of a continuous culture with *Ralstonia eutropha* G^{+3} and *Alcaligenes latus*. The use of values of μ_{max} for *A. latus* and *R. eutropha* G^{+3} and a maximum biomass concentration of 30 gL^{-1} gives a productivity ratio of 8.2 with *A. latus* and 5.25 with *R. eutropha* if t_0 (time between two production runs) is set to a low 10 h. This means that for a fixed desired amount of product per unit of time, the bioreactor volume can be substantially reduced if a continuous culture is chosen over a batch process. For continuous production of PHAs knowledge of as well growth as production kinetics is of importance in order to choose the optimum reaction

system, a system combining a continuous stirred tank reactor for microbial growth, and a tubular plugflow reactor for PHA storage [27]. In the case of *R. eutropha* G^{+3} just described a second-stage PFTR needs thus only have 11,4 % the volume of a CSTR to achieve the same results. In the case of *A. latus* a PFTR needs 19,8 % the size of a CSTR. The CSTR-PFTR arrangement not only guarantees maximum productivity, but also minimizes cosubstrate loss, and might be a tool for enhancing product quality, since very narrow residence time distributions (and therefore uniform cell population) are characteristic of plug-flow tubular reactors.

Production of P3HB with Alcaligenes latus Strain DSM 1123, and DSM 1124

Alcaligenes latus exhibits a behavior different from that of *R. eutropha* in both growth and P3HB accumulation. Its maximum specific growth rate μ_{max} is 0.45 to 0.48 h^{-1}, depending on the nature and the concentration of the carbon source (glucose or sucrose). If sucrose alone is used, P3HB accumulation is almost totally associated to growth [22], and recent research has showed that with glucose as substrate, up to 38 % of the dry biomass can consist of P3HB at the end of a growth phase [45] . The maximum specific P3HB production rate $q_{P3HB,\,max}$ can reach 0.50 h^{-1} during growth on sucrose, whereas a $q_{P3HB,\,max}$ of 0.29 h^{-1} is attainable with glucose alone. Therefore, cells of *A. latus* contain a high percentage of P3HB (about 60 % with sucrose, 38 % with glucose) even during non-limited multiplication.

In order to lower production costs for the polyester, maltose (starch hydrolysate), green syrup (an intermediate from sucrose production), and beet molasses have been chosen as cheap carbon sources for growth and polyester accumulation. All experiments were performed in identical 10L bioreactors under controlled conditions. As can be seen from Figure 1, the strains grew well on all substrates and accumulated PHB in concentrations between 28 and 43 % (w/w) of the dry biomass within only 15 hours. When green syrup or maltose were chosen as carbon sources, PHB was accumulated associated to the growth of both strains of *A. latus* used, whereas with beet molasses major PHB accumulation only occurred under growth-limiting conditions in a separate PHB accumulation phase. In Table 1 yields and specific rates for growth and PHA accumulation are shown for the same experiment.

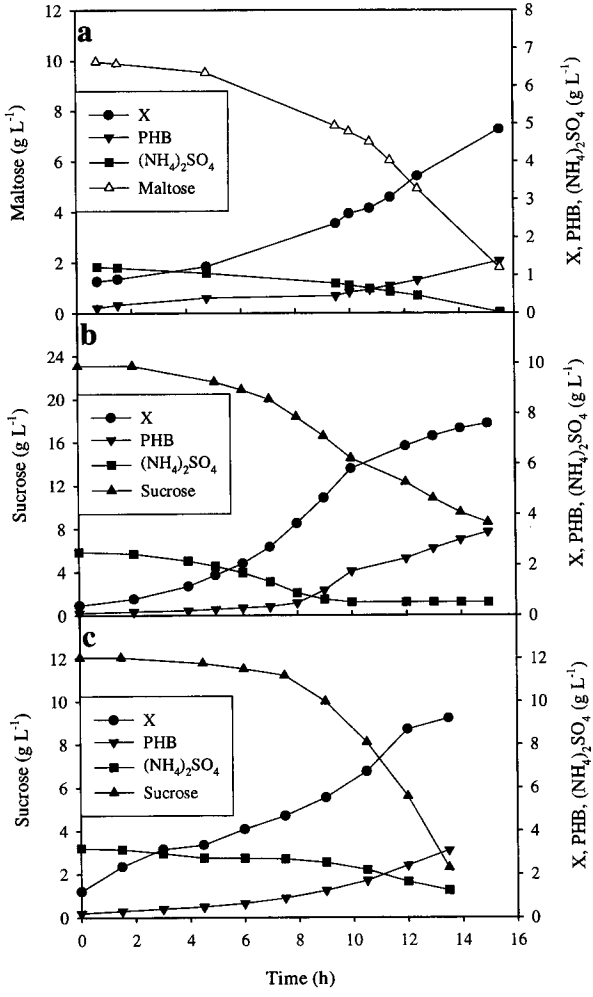

Figure 1: Concentrations of cell dry weight (X), Poly-3-hydroxybutyrate (PHB), growth-limiting substrate ($(NH_4)_2SO_4$) and carbon sources during discontinuous growth and PHB production experiments with: a: *A. latus* 1123 on starch hydrolysate (maltose) b: *A. latus* 1123 on green syrup (sucrose) c: *A. latus* 1124 on beet molasses (sucrose)

Table 1: *A. latus* strains DSM 1123, and 1124: yields and specific rates with maltose, green syrup, or beet molasses as carbon sources. Residual biomass X_R is defined as total dry biomass minus its PHA content, the specific growth rate μ (h^{-1}) is equal to $\mu = 1/X\ dX/dt$, and the specific production rate q_P (h^{-1}) is $q_P = 1/X\ dP/dt$. The yields are defined as $Y_{X,P/C}$=g biomass,PHA per g substrate used.

Carbon source: Maltose	**$Y_{X/C}$ (g/g)**	**$Y_{X/N}$ (g/g)**	**$Y_{P/C}$ (g/g)**	**μ (h^{-1})**	**q_P (h^{-1})**
A. latus DSM 1123	**0.49**	**3.33**	**0.151**	**0.15**	**0.16**
Carbon source: Green Syrup	**$Y_{X/C}$ (g/g)**	**$Y_{X/N}$ (g/g)**	**$Y_{P/C}$ (g/g)**	**μ (h^{-1})**	**q_P (h^{-1})**
A. latus DSM 1123	**0.497**	**3.80**	**0.22**	**0.286**	**0.14**
Carbon source: Molasses	**$Y_{X/C}$ (g/g)**	**$Y_{X/N}$ (g/g)**	**$Y_{P/C}$ (g/g)**	**μ (h^{-1})**	**q_P (h^{-1})**
A. latus DSM 1124	**0.82**	**4.10**	**0.297**	**0.123**	**0.11**

In the case of *A. latus* DSM 1123 on green syrup as carbon source specific growth rate μ and the yield coefficient $Y_{X/N}$ are nearby identical compared to the values measured in a defined sucrose medium, and $Y_{X/C}$ is even higher suggesting that an additional carbon source is available for the strain. On the other hand the yield coefficient for PHB is about 30 % lower, and specific production rate q_P is decreased as well. In total this means that the combination of the microbial strain and carbon source is very interesting for a production process, even though somewhat longer fermentation times are needed. If the same strain is used in combination with maltose as single carbon source, specific rates μ and q_P drop down dramatically, and also $Y_{P/C}$ is much lower. Fermentation times are longer here to come to the same PHA end-concentration, they are comparable now with those we have determined for *R. eutropha* on a glucose medium. The same is true for the combination of *A. latus* 1124 and molasses, but it has to be mentioned that the yield factors $Y_{X/C}$ and $Y_{P/C}$ are very high in this case.

Production of P3HB with Methylomonas extorquens

Another possibility to use agricultural waste materials for PHA production is based on the glycerol phase from rapeseed oil methylester (biodiesel) production. In the EU 550 x 10^3 tons of biodiesel have been produced 1997, and further plants with an additional capacity of 736 x 10^3 tons are planned. Therefore 57,3 x 10^3 tons of glycerol, respectively 134 x 10^3 tons adding the capacities planned, could be used for PHA production. *Methylomonas extorquens*, a facultative methylotrophic bacterium, was used in a batch process utilizing

raw glycerol phase containing 20 % methanol. The microorganism utilized both carbon sources, first methanol was metabolized, then glycerol was used for growth and PHB production. The polyester was accumulated in higher quantities only after the depletion of the nitrogen source in a separate accumulation phase. Unfortunately the strain grew quite slowly, and 90 hours were needed to reach the end concentration of 28 % (w/w) PHB in the dry biomass. Table 2 shows the yields and specific rates for growth and product formation for this strain.

Table 2: Yields and specific rates for growth and PHA production from glycerol phase containing 20 % methanol for *Methylomonas extorquens*. For definitions see Table 1.

Carbon source: glycerol phase	$Y_{X/C}$ (g/g)	$Y_{X/N}$ (g/g)	$Y_{P/C}$ (g/g)	μ (h^{-1})	q_P (h^{-1})
M. extorquens	**0,17**	**1,27**	**0,23**	**0,05**	**0,23**

Conclusion

It has been shown that a number of agricultural wastes can successfully be used for PHA production. Comparing yields and specific reaction rates for the strains of *A. latus* with those coming from experiments with a defined sucrose medium, velocities and yields are lower, and are in the typical range for the corresponding values for *Ralstonia eutropha*, a strain that is used for commercial production of PHAs. The results obtained therefore look very interesting, and suggest that PHB production costs can be lowered substantially compared to a production process with pure chemicals as raw materials.

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