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MAINTAINING A CONTROLLED RESIDUAL GROWTH CAPACITY INCREASES THE PRODUCTION OF POLYHYDROXYALKANOATE COPOLYMERS BY *ALCALIGENES EUTROPHUS.*

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Summary
The manner in which copolymer poly(HB-co-HV) production was influenced by different methods of limiting cell proliferation during the production phase was examined. Polymer production was significantly improved in fermentation strategies in which some growth was maintained, either by linear or exponential nitrogen source feeding as compared to cultures in which nitrogen supply was totally interrupted. Improved volumetric productivities were obtained in cultures fed with NH_4OH and the proportion of 3HV incorporated was approximately twofold higher in these cultures. These performance improvements were due to higher specific rates of glucose and propionate consumption in cultures in which true growth capacity was maintained.

Introduction

Polyhydroxyalkanoates (PHAs) are carbon and energy reserve compounds accumulating intracellulary as granules in certain bacteria during unbalanced growth (Anderson and Dawes, 1990; Doi, 1990). The polymer most studied is polyhydroxybutyrate (PHB), but copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate, poly(HB-co-HV), possess far more attractive thermoplastic properties (Byrom, 1987). Moreover, the relative proportions of each monomer determine such properties (Holmes, 1985). Industrial production of poly(HB-co-HV) uses two stage cultures of *Alcaligenes eutrophus*: an initial growth phase on glucose followed by a phosphate limited production phase fed with glucose + propionate mixtures. The proportion of 3HV monomers in this PHA is varied by the ratio of propionic acid/glucose during the substrate feeding phase to give polymers containing 0-20 mol% 3HV. Since propionic acid is toxic above 1g/l, fed-batch fermentation strategies are necessary to produce significant quantities copolymer (Byrom, 1987).

Production of PHA is most frequently studied under nitrogen starvation despite the fact that addition of a nitrogen source during the production phase has been shown to increase both the productivity and the substrate conversion yield for PHB (Suzuki et al. 1986a, 1986b; Bitar and Underhill 1990; Lee et al. 1993). Copolymer production by *Alcaligenes* SH-69 using glucose as the sole carbon source, has been shown by Rhee et al.

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(1993) to be perturbed by modifications of the C/N ratio. These authors observed that the proportion of 3HV in the copolymer increased when the C/N ratio was diminished. They suggested that the molar fraction of 3-HV in poly(HB-co-HV) can be controlled by regulating the concentration of ammonium salt in the feed. In general, however, constant C/N ratios are used in most fed-batch approaches (Suzuki et al. 1986a, 1986b; Lee et al. 1993). The influence of controlling growth of *A. eutrophus* by nitrogen feeding strategies has not been studied in respect to PHA production and furthermore, detailed kinetic analyses of PHA production are rarely reported.

In this study, the effect of various nitrogen feeding strategies on poly(HB-co-HV) production were compared for cultures of *A. eutrophus* grown on glucose and propionate, and feeding strategies enabling improved rates of production of copolymers enhanced in their 3HV composition are proposed.

Materials and methods

Micro-organism and culture medium

A glucose-utilising mutant of *Alcaligenes eutrophus*, DSM 545, was used throughout these investigations.

The seed medium composition (based on that of Ramsay et al. 1990) was (per litre of medium): 0.19g of nitrilotriacetic acid, 0.06g of ferrous ammonium citrate, 0.5g of $MgSO_4.7H_2O$, 0.01g of $CaCl_2.2H_2O$, 5.0g of $(NH_4)_2SO_4$ and 1ml of trace element solution. The trace element solution composition was (per litre of distilled water): 0.3g of H_3BO_3, 0.2g of $CoCl_2.6H_2O$, 0.1g of $ZnSO_4.7H_2O$, 0.03g of $MnCl_2.4H_2O$, 0.03g of $Na_2MoO_4.2H_2O$, 0.02g of $NiCl_2.6H_2O$, 0.01g of $CuSO_4.5H_2O$. After autoclaving the above medium, 40ml of a sterile phosphate solution containing 224g/l of $Na_2HPO_4.12H_2O$ and 37.5g/l of KH_2PO_4 was aseptically added to the fermentor. Likewise, carbon substrate was added as a sterile glucose solution at 700 g/l. The initial glucose concentration was 40 g/l.

Culture conditions

The reactor was a 20 liter Braun fermentor (Biostat E, Braun Diessel Biotech, Melsungen, Germany) with a pH controller, (Fermprobe Broadley James Corporation, USA) and a dissolved oxygen controller (Ingold, Urdorf, Switzerland). The pH was maintained at 7.0 by addition of a NaOH (5 M) solution or a H_3PO_4 (1M) solution. The dissolved oxygen concentration was maintained above 20% of air saturation by varying the agitation speed and/or the inlet air flow rate. Anti-foaming agent Strucktol Type J673 (Sanofi ELF BioRecherche, France) was used. The medium was inoculated with a 10% vol/vol inoculum prepared in seed medium.

The cultures were grown on the seed medium, except that the initial ammonium sulfate concentration was diminished to 2.3g/l. The initial growth phase ended after complete exhaustion of the nitrogen source and a poly(HB-co-HV) production phase was provoked in which different feeding strategies were used. At the onset of the production phase a propionate solution was added to obtain an initial concentration of 1g/l. The propionate concentration was maintained between 0 and 1g/l throughout the culture by further addition of concentrated propionate solution each time the on-line dissolved oxygen partial pressure increased indicating propionate depletion. Various conditions of nitrogen supply during the production phase were investigated. **Culture A** was fed with carbon substrate alone without further nitrogen supply. **Culture B** was fed with both carbon and nitrogen so as to maintain a constant rate of biomass production (r_X) of 0.11g of biomass/l.h by a linear feed profile (0.21 gNH_3/h) of ammonia solution which took into account the previously determined yield coefficient of biomass from nitrogen ($Y_{X/N}$) of 8.0 g/g. **Culture C** was also fed with both carbon and nitrogen substrates, but in this case, ammonia feeding was by an exponential profile (0.25-0.84 gNH_3/h) to maintain the specific growth rate constant at $0.02h^{-1}$.

Analytical procedures

Biomass: cells were harvested by filtering a given volume of culture broth comprised between 3 and 10 ml through preweighed polyamide membrane filters (0.2μm pore size), washed twice with distilled water and dried to constant weight at 60°C under vacuum. Cellular density was also determined by turbidimetric measurements at 600nm. *Glucose:* residual glucose concentration was measured using an YSI glucose analyser, (YSI Model 2000, Yellow Springs Instruments Co., Yellows Springs, Ohio, USA). *Proteins:* samples of culture broth (0.5-1.5ml) were centrifuged in Eppendorf tubes, the pellets were washed twice with distilled water and then frozen at -20°C for subsequent analysis by the Biuret reaction (Stickland, 1951) using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., USA) as a standard. *Poly(HB-co-HV) analysis:* cell samples used for poly(HB-co-HV) analysis was obtained as for protein analysis and these were prepared using the method of Braunegg et al. (1978) as modified by Brandl et al. (1988). Poly-(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) of natural origin with a defined 3HV content (14%) was used as the external standard. The resulting methyl esters were quantified by injection of 0.1μl in a gas chromatography (HP 5890) equipped with a semi-capillary column (Supelcowax 10, Supelco, Bellefonte, Pa., USA) 30m long and 0.1μ ID. *Propionate:* residual propionate concentration was measured by gas chromatography (HP 5890) with a Poraplot Q (Chrompack) 25m long and 0.53 ID column, with isobutyric acid as internal standard.

Kinetic Analysis

All specific rates were calculated from experimental data using a corrected residual biomass value in which the fraction of cell weight corresponding to PHA had been subtracted.

Results

Two distinct phases were observed in all cultures, corresponding to the period of unrestricted growth and the copolymer production phase. For the culture A, in which no further addition of nitrogen was made during the production phase (Fig 1), total biomass concentration (Xt) during the growth phase could be subdivided into PHB and true cell material: total biomass concentration when the nitrogen source had been depleted was 4.5g/l with a PHB content of 16.5% (w/w). In the ensuing production phase, protein production was halted while 3HB and 3HV (due to propionate addition) increased significantly. The overall behaviour of the cultures B and C, with nitrogen feeding (see above) was similar except that protein synthesis was maintained at feeding-dependent rates.

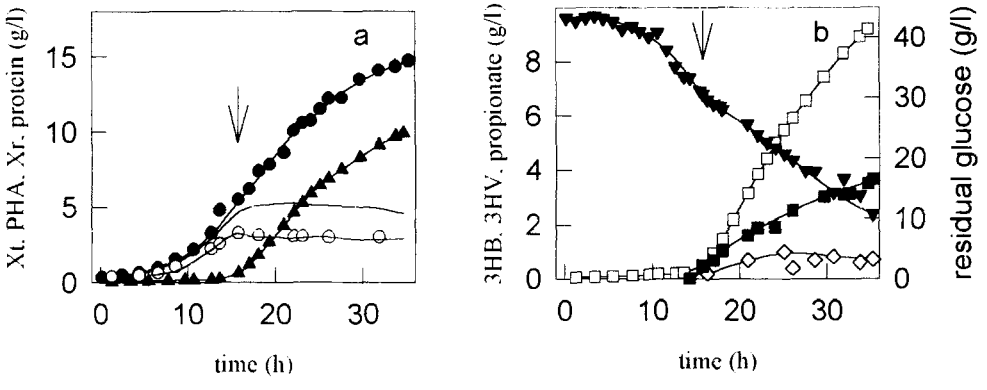

Fig.1. Time course of total biomass (Xt) (•), PHA (▲), Xr (Xt minus PHA) (—), protein (○) (1a) and 3HB (□), 3HV (◊), residual glucose concentration (▼) and total propionate consumed (■) (1b) concentrations for culture A. Arrow marks exhaustion of nitrogen source.

In all three cultures the specific growth rate (μ) observed during the nonlimited growth period was equal to $0.2h^{-1}$, value which fell rapidly as nitrogen source became limiting. For culture A, μ fell to zero, while cultures B and C maintained rates of biomass formation as would be expected from the nitrogen feeding rate once PHA accumulation had been allowed for. No trace of residual nitrogen was detected during any of the production phases. Specific rates of both glucose (q_{gluc}) and propionate (q_{prop}) consumption, calculated relative to cell material other than PHA (Xr) were significantly higher in cultures B and C than for culture A thus explaining the improved rates of PHA accumulation (see Fig 2). A similar effect was seen as regards the maximal specific rates of 3HB (q_{3HB}) and 3HV (q_{3HV}) production. In addition, these values were maintained for longer periods in nitrogen fed cultures than in the control culture (A) in which rates of production diminished rapidly. This fall off of the 3HB specific production rate from a maximum value of 0.125 g/g.h has also been observed by Braunegg et al. (1995) for cultures of *A. eutrophus* after nitrogen depletion. The maximum rates of 3HV accumulation (0.05 g/g.h) seen in cultures B and C are similar to those seen by Kim et al. (1992) for *A. eutrophus* with an optimised residual concentration of propionate of 0.5g/l.

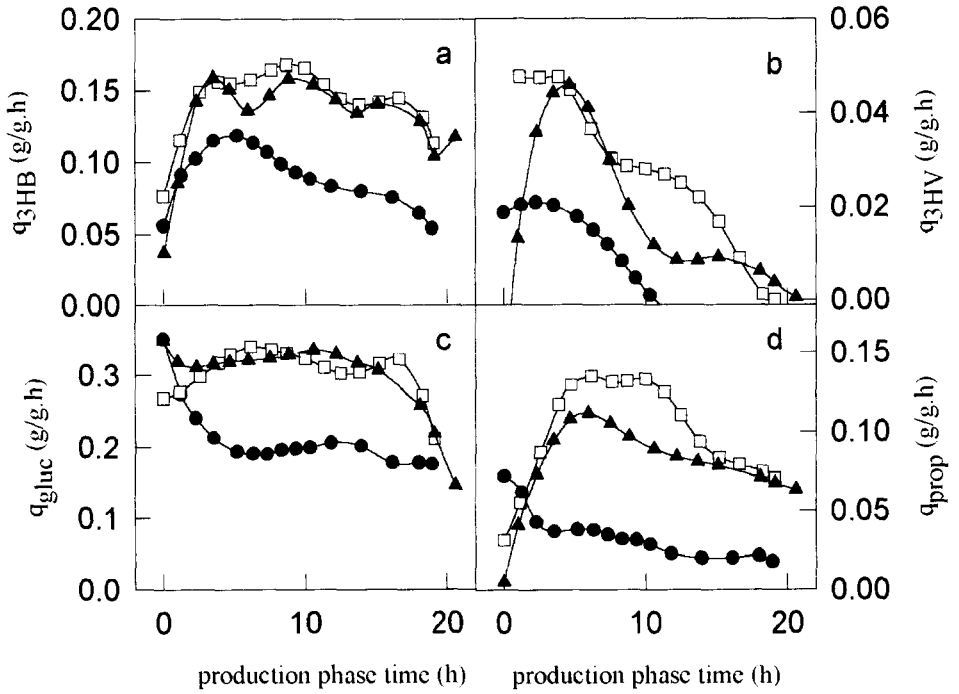

Fig. 2. Specific rates of 3HB production (q_{3HB}) (a), 3HV production (q_{3HV}) (b), glucose consumption (q_{gluc}) (c) and propionate consumption (q_{prop}) (d) for cultures A (•), B (□) and C (▲).

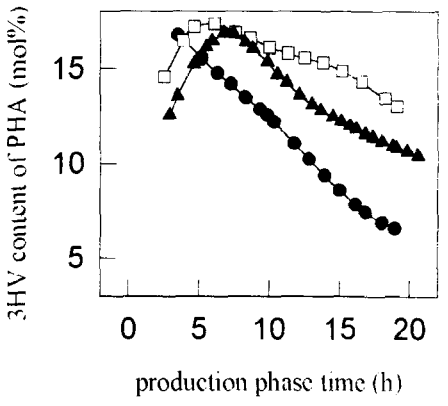

Fig.3.(above) Molar percentage of 3HV in poly(HB-co-HV) during production phase for cultures A (•), B(□) and C (▲).

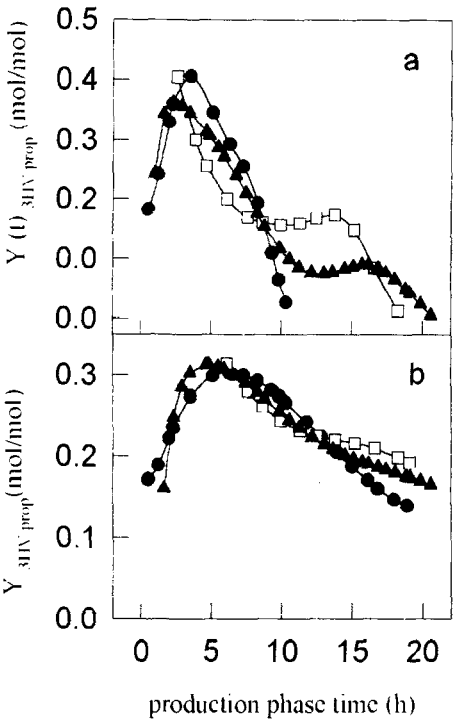

Fig.4. Instantaneous molar yield of propionate into 3HV ($Y(t)_{3HV\ prop}$) (a) and overall yield of propionate into 3HV ($Y_{3HV\ prop}$) (b) for cultures A(•), B (□) and C (▲).

The molar percentage of 3HV accumulated (%mol 3HV/PHA) was in all cultures close to 16% at the onset of the production phase but fell progressively to 6.5% in culture A (Fig 3) to give values similar to those obtained by Ramsay et al. (1990), while remaining significantly higher in the other cultures (13.1% and 10.4% for cultures B and C, respectively). Thus, not only was the overall bioconversion potential lower in nitrogen depleted cultures but the specific capacity to transform propionate to 3HV was particularly diminished. If molar conversion yields (Fig 4) are examined it may be seen that propionate is incorporated into 3HV at a maximum efficiency of only 0.4 mol/mol and in all cultures this value fell throughout the production period though less so in nitrogen fed cultures leading to somewhat better overall production yields in these cultures. When the experimental conversion yields for 3HB relative to glucose were examined, values of 0.37 g/g, 0.47 g/g and 0.50 g/g were obtained for cultures A, B and C respectively (results not shown). Previously, Yamane (1993) has estimated a theoretical PHB yield of 0.48 g/g from glucose using pathway stoichiometry which assumed that carbon was used exclusively for PHB production and hence that the only carbon loss was the CO_2 at the pyruvate dehydrogenase step. Similar values have been obtained here in cultures in which growth was also taking place which ought to have diminished this yield as would maintenance metabolism. Thus, it can be concluded that propionate contributed either to carbon flux towards biomass synthesis or/and PHB synthesis as has been proposed by Doi et al (1986,1987). In view of the results obtained in our study it would seem necessary to better establish the true carbon flux pattern associated with PHA production since further improvement will almost certainly necessitate manipulation of these carbon fluxes.

As has been shown for PHB production (Suzuki et al. 1986a, 1986b; Bitar and Underhill 1990; Lee et al. 1993), productivity of the copolymer can be greatly enhanced when nitrogen feeding at limiting rates is used: 0.72g poly(HB-co-HV)/l.h during the production phase for cultures B and C as opposed to 0.46g poly(HB-co-HV)/l.h in the nitrogen-depleted culture A.

Conclusion

Various characteristics of the production of poly(HB-co-HV) copolymers can be seen to be favoured by the use of a nitrogen feeding strategy in which true cell proliferation is maintained. Not only production rates but also the molar composition of the resulting polymer are improved with higher 3HV levels. Furthermore these improvements are not to the detriment of yields. Perhaps more important from a process engineering viewpoint, the results obtained here show for the first time that these high rates of production can be maintained for extended periods thus diminishing fermentation periods.

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