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Growth-associated production of poly(hydroxybutyric acid) by *Azotobacter beijerinckii* from organic nitrogen substrates

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Abstract Poly(hydroxybutyric acid) (PHB) was produced by a selectant of *Azotobacter beijerinckii* in media containing only organic nitrogen sources such as N substrates. The chosen compounds were casein peptone, yeast extract, casamino acids and urea, each combined with carbon substrates glucose or sucrose. The PHB was synthesized under growth-associated conditions. The concentrations amounted to more than 50% of cell dry mass on casein peptone/glucose as well as urea/glucose medium within 45 h fermentation time. Corresponding to these yields, productivities of about 0.8 g PHB l^{-1} h^{-1} were discovered. The highest values increased to 1.06 g PHB l^{-1} h^{-1} on casein peptone/glucose medium and 1.1 g PHB l^{-1} h^{-1} on yeast extract/glucose medium after a period of 20 h. It was found that oxygen limitation was essential for successful product formation, as demonstrated earlier. These data from basic research may support further investigations into the use of technical proteins from renewable sources as substrates for PHB production by a strain of *A. beijerinckii*.

Introduction

A wide variety of bacteria synthesize polyhydroxyalkanoates (PHA) as intracellular storage materials (Anderson and Dawes 1990) as well as components of the PHA/polyphosphate/calcium complex integrated into the plasma membrane (Huang and Reusch 1995). Since the discovery of thermoplastic and biodegradable qualities of bioplasts, many efforts have been made to develop industrial processes for their production. The reasons for obtaining such natural products include their applications in packaging (Chiellini and Solaro 1996) or as biocompatible materials in medicine (Yamamoto et al. 1996). However, besides the marketing of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate), e.g. Biopol by Zeneca Bio Products (Lee 1996), no other PHA have been used commercially. Until now the main obstacle for the replacement of petrochemical plastics by ecologically advantageous biopolymers is the extraordinary economic difference. Whereas, for instance, Biopol is sold at U.S. \$ 16 kg^{-1}, the price for polypropylene amounts to less than U.S. \$ 1 kg^{-1} (Hänggi 1995). Considerable scientific efforts are therefore constantly being directed towards the improvement of fermentation productivity. On the one hand, the biosynthetic capacity of relevant organisms has been investigated. This means screening new PHA-producing organisms (Remer et al. 1996) as well as increasing PHA yields by homologous or heterologous gene expression for PHA-synthesizing enzymes in strains of *Alcaligenes eutrophus* (Park et al. 1995), *Escherichia coli* (Lee et al. 1995) and plants (Blohm 1996). On the other hand, because of the enhanced efficiency of bioprocesses, metabolic design has been investigated using special precursors (Steinbüchel and Valentin 1995), cheaper raw materials (Lee and Chang 1995) or particular regimes for substrate feeding (Kim et al. 1996; Eggink et al. 1996).

Since the costs of production depend, first of all, on prices for feedstock of fermentations, it is necessary to find inexpensive suitable carbon and nitrogen sources. In this case, however, one has to take into account the fact that high-yield organisms have to tolerate relatively non-standardized raw or waste materials. From this point of view, the fermentation of *Pseudomonas* sp. on lipids (Huijberts et al. 1996) or octanoates (Samain et al. 1996), as well as *A. eutrophus* on waste water from alcoholic distilleries (Sou et al. 1996) or *Methylobacterium* sp. on methanol (Kim et al. 1996), demonstrates biotechnically relevant possibilities. Other useful carbon sources, for instance, are several carbohydrates, hydrocarbons, oils, alcohols and organic acids (Lee 1996). Complex nitrogen substrates has been applied successfully for recombinant strains of *Escherichia* sp. in media containing peptones (Kalousek and Lubitz 1995) or protein hydrolysates (Lee et al. 1995) as supplements. Very complex carbon

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substrates were utilized by a mutant of *Azotobacter vinelandii* because the strain UWD produced PHA on types of several molasses (Page 1989).

We were interested in *Azotobacter beijerinckii*, whose synthesis of poly(hydroxybutyric acid) (PHB) had been analyzed earlier (Senior and Dawes 1973). With previous experiments we had found that organic nitrogen substrates were able to substitute totally for ammonia in the fermentation medium. We compared any natural nitrogen sources in the production of PHB. The choice was made with the intention of checking biotechnologically important model components.

Materials and methods

Bacterial strain and growth conditions

A strain of *Azotobacter beijerinckii* DSM 1041 was selected from continuous culture by Dr. Christner (personal communication). Stock cultures were grown on sucrose/yeast extract/agar composed as follows (g/l): 5 yeast extract, 20 sucrose, 2.45 KH_2PO_4, 3.13 K_2HPO_4, 0.005 $CaCl_2$; 1 ml trace elements, 20 g/l agar, and distilled water to 1 l, pH 7, were added. The media were generally supplemented by stock solutions of trace elements (g/l): 71.2 $MgSO_4 \cdot 7H_2O$, 0.44 $ZnSO_4 \cdot 7H_2O$, 0.812 $MnSO_4 \cdot 4H_2O$, 0.785 $CuSO_4 \cdot 5H_2O$, 0.252 $Na_2MO_4 \cdot 2H_2O$, 4.98 $FeSO_4 \cdot 7H_2O$, 1.02 H_3BO_3, with 0.05 M H_2SO_4 to 1 l. All components were sterilized for 20 min at 120 °C separately. A 1-ml cell suspension (0.9% NaCl) of agar slants cultivated for 48 h at 30 °C was taken as seed material. The inocula for fermentations were grown in 500-ml flasks with 100 ml medium for 24 h at 30 °C under aerobic conditions. These media as the well as fermentation media included the same mineral salt components as described above but different nitrogen (5 g/l) and sugar sources (35 g/l). The following five media were chosen in accordance with fermentation variations: pancreatic digested casein peptone/glucose (PG), casamino acids/glucose (CG), yeast extract/glucose (YG), yeast extract/sucrose (YS), urea/glucose (UG). The growth and kinetics of substrates and PHA production were studied in 2.5-l fermentors filled with 2 l medium and equipped with two six-bladed disc impellers and the usual systems for regulation of pH, temperature, speed, aeration and prevention of foam. For the experiment with PO_2-coupled stirrer speed, a 2-l fermentor from Braun Bioinstruments was used. In fermentation media, the contents of casein peptone, casamino acids

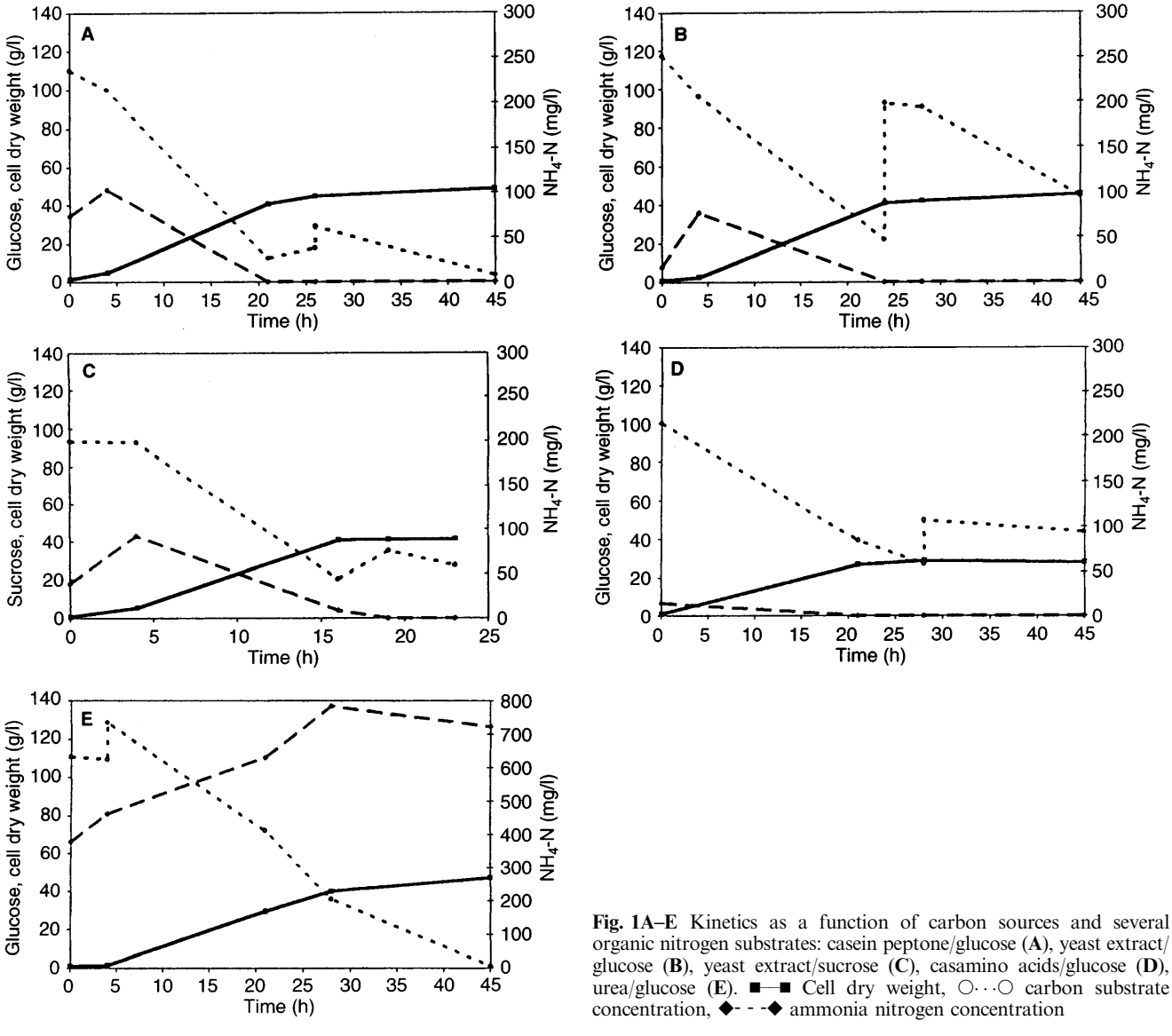

Fig. 1A–E Kinetics as a function of carbon sources and several organic nitrogen substrates: casein peptone/glucose (**A**), yeast extract/glucose (**B**), yeast extract/sucrose (**C**), casamino acids/glucose (**D**), urea/glucose (**E**). ■—■ Cell dry weight, ○···○ carbon substrate concentration, ◆- - -◆ ammonia nitrogen concentration

and yeast extract amounted to 20 g/l and urea was added up to 5 g/l or 10 g/l at the beginning. Glucose or sucrose, initially at 100 g/l, were supplemented in the course of fermentation by feeding dry sugars to exclude limitation. The pH was regulated with 6 M NaOH and 2 M H_2SO_4 at 6.8, the aeration rate amounted to 0.5:1 (vvm). If not otherwise specified, the stirrers were adjusted to 850 rpm. The fermentation media were inoculated with 200 ml cell suspension from preculture flasks; 20-ml samples of each culture were collected periodically. The supernatants obtained by centrifugation at 10 000 rpm for 10 min at 4 °C were used for substrate analysis. The contents of cell dry mass and PHA were estimated on the basis of sediments.

Analysis of culture broth

The determination of glucose concentration in the medium was carried out with on ESAT 6660-2 glucose analyzer from Prüfgerätewerk Medingen GmbH.

The ammonia nitrogen content was measured by the Kjeldahl method. The soluble phosphorus of the medium was examined by a commercial test (Merck) with a SQ 118 photometer. Dry-weight determinations of the growing cultures were made at 420 nm by using a spectrophotometer (Specol 11 of Zeiss Jena) gravimetry of cell dry mass.

Isolation and estimation of PHB

Cells were collected by centrifugation from 1 to 5-ml samples of the culture suspended in 10 ml of alkaline hypochlorite reagent (pH 10.0–10.5, NaOCl content 5.25%–5.5%) according to Williamson and Wilkinson (1958). After 2 h at 37 °C, the reaction mixture was centrifuged at 5000 rpm for 10 min and the solid pellet was washed successively with water, alcohol and acetone. Finally, the polymer was dried for 2 h at 105 °C and then weighed.

A gas-chromatographic method was used to determine both the content and the composition of PHA copolymers in cells.

Results

The bioprocesses on media with organic nitrogen substrates are demonstrated by typical diagrams of fermentation. They were generally characterized first by considerable growth for up to 30 h of cultivation. Figure 1A–E shows that, during that time, about 40 g/l cell dry mass was grown on casein peptone (Fig. 1A), yeast extract (Fig. 1B, C) and urea (Fig. 1E) whereas about 30 g/l cell dry mass was obtained on casamino acids (Fig. 1D). This was obviously caused by different amounts and qualities of nitrogen within the media. Whereas the first three nitrogen sources mentioned have a content of 10%–12% nitrogen, the casamino acids contain only 8% nitrogen besides a considerable quantity of ash. Therefore, the media differed in nitrogen content from 2–2.4 g N/l to 1.6 g N/l respectively.

The sugar compounds were taken up intensively during this time, while ammonium increased at the beginning but decreased later in the course of cultivation. This was initially caused by deamination of nitrogen substrates and subsequently by consumption of ammonia ions. But, whereas in the case of complex nitrogen sources, inorganic nitrogen was completely exhausted after about 25 h, there was no ammonia limitation when urea was used.

In order to estimate the production of PHB, several experiments with different media were arranged simultaneously. The gravimetric assay was used routinely to determine the product because the polymeric material was confirmed to be PHB by gas chromatography. It was found that the concentration of poly(hydroxyvaleric acid) amounted to less than 0.2%. Tables 1–3 show the average values and variations of product concentration with respect to the nitrogen and carbohydrate substrates used. It can be seen that about 27 g PHB/l or 20 g PHB/l was produced in experiments with casein peptone (Table 1) or urea (Table 3), whereas with media containing yeast extract (Table 2) or casaminoacids (Table 3) about 11–14 g PHB/l was produced. Productivities were calculated on the basis of fermentation times. Their average values obviously corresponded with PHB concentration, but a maximum level of more than 1 g PHB l^{-1} h^{-1} was determined after 20 h of special fermentations on casein peptone and yeast extract.

The PHB contents of different biomasses are shown in Fig. 2. Their average values ranged from 35% to 57%, showing the lowest cellular concentrations associated with increased variations for yeast extract. First it was of interest that, in experiments with urea, the PHB

Table 1 Concentration of poly(hydroxybutyric acid) (*PHB*) and productivities during fermentation on casein peptone/glucose medium (*PG*)

Experiment	Time (h)	PHB (g/l)	Productivity (gPHB l^{-1} h^{-1})
PG 1	22	11.9	0.54
PG 2	20	21.3	1.06
PG 3	26	22.4	0.86
PG 4	21	16.1	0.76
PG 5	21	20.2	0.96
Mean ± SD		18.37 ± 4.34	0.84 ± 0.20
PG 1	44	30.6	0.70
PG 3	44	28.6	0.65
PG 4	45	18.0	0.40
PG 5	45	31.0	0.69
Mean ± SD		27.05 ± 6.13	0.61 ± 0.14

Table 2 Concentration of PHB and productivities during fermentation on yeast extract/glucose medium (*YG*) and yeast extract/sucrose medium (*YS*)

Experiment	Time (h)	PHB (g/l)	Productivity (gPHB l^{-1} h^{-1})
YG 1	20	21.9	1.00
YG 2	25	8.7	0.35
YG 3	45	5.7	0.13
YG 4	24	20.4	0.85
YG 5	45	5.7	0.13
YG 6	45	7.5	0.17
Mean ± SD		11.66 ± 7.47	0.45 ± 0.42
YS 1	23	22.5	0.98
YS 2	24	16.2	0.67
YS 3	44	6.3	0.14
YS 4	45	8.2	0.18
Mean ± SD		13.29 ± 7.48	0.49 ± 0.40

Table 3 Concentration of PHB and productivities on casamino acids/glucose medium (*CG*) and urea/glucose medium (*UG*)

Experiment	Time (h)	PHB (g/l)	Productivity ($gPHB\ l^{-1}\ h^{-1}$)
CG 1	45	13.5	0.30
CG 2	26	16.4	0.63
CG 3	45	13.3	0.30
CG 4	45	13.2	0.29
Mean ± SD		14.10 ± 1.55	0.38 ± 0.17
UG 1	28	23.3	0.83
UG 2	23	18.0	0.78

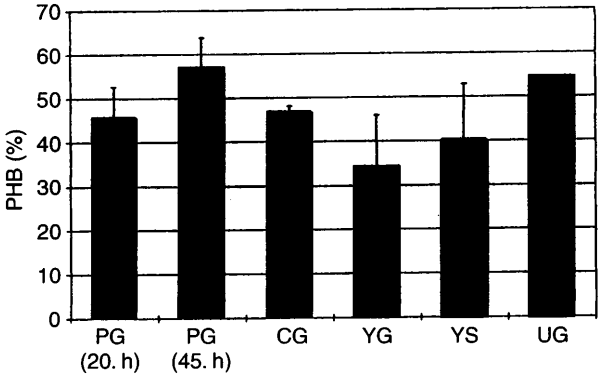

Fig. 2 Poly(hydroxybutyric acid) (*PHB*) accumulation as a function of carbon/organic nitrogen sources. *PG* casein peptone/glucose, *CG* casamino acids/glucose, *YG* yeast extract/glucose, *YS* yeast extract/sucrose, *UG* urea/glucose

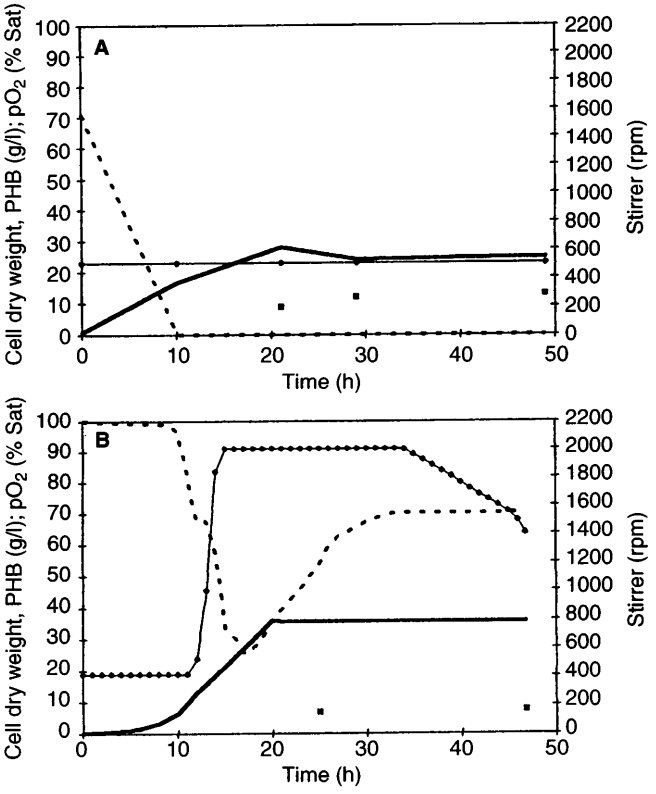

Fig. 3A, B Growth and PHB accumulation without control (**A**) and with control (**B**) (cascade control PO_2: stirrer) of the PO_2. ■ PHB, ◆ stirrer rotation, - - - PO_2, — cell dry weight

content had reached about 55% before 30 h of fermentation time. With casein peptone, however, this level was not reached for 44 h. From these results the question arose whether oxygen, besides other substrate components, influences the accumulation of PHB. Therefore, two fermentations were carried out with different oxygen concentrations. Figure 3A shows a run with oxygen limitation after 10% of the time course, achieved by constant stirrer speed at 500 rpm. The production of PHB was completed after 29 h and was clearly growth-associated. By contrast, as Fig. 3B demonstrates, fermentation without oxygen limitation showed other kinetics. The oxy-STAT regime used meant that the concentration of oxygen only decreased to 35% of saturation. The stirrer speed increased up to 2000 rpm. In contrast to the parallel experiment, the cell dry mass increased more but the concentration of PHB remained low.

Discussion

Bacteria that are used for the production of PHA can be divided into two groups (Lee 1996). The first group requires the limitation of an essential nutrient e.g. N, P, Mg etc. for efficient biosynthesis of PHA on the basis of excess carbon. The second group does not require such limitation but accumulates PHA during growth. This group includes *Alcaligenes latus*, a mutant strain of *A. vinelandii* and recombinant *E. coli* harboring the *A. eutrophus* PHA biosynthesis operon. These physiological characteristics of the strains are important for bioprocesses to produce PHA.

The data of our experiments show that PHB accumulation was achieved independently from ammonia limitation or excess in the early periods of cultivation. The microorganisms produced a little more than 20 g PHB/l within 24 h of fermentation time. Therefore, *A. beijeinckii* belongs to the second group of bacteria if it is cultivated on organic nitrogen substrates. But the accumulation of PHB could also be continued during a stationary growth phase if casein peptone was applied. This is a surprising result because we had used organic nitrogen sources alone instead of supplements at low concentrations (Page and Cornish 1993; Lee and Chang 1995). But the efficiency of PHB synthesis obviously depends on the type of nitrogen source and dissolved oxygen concentration simultaneously. Comparing the different substrates it is evident, on the one hand, that the average PHB accumulation was highest and showed least variation when casein petone was used. On the other hand, oxygen limitation seems to be essential for efficiency of product formation, as the experiment with stirrer speed of 2000 rpm showed, in contrast to all other fermentations, which were moved constantly at 850 rpm. From these results it can be concluded that the

components of the protein hydrolysate are convenient precursors for a successful metabolic balance of growth and PHB biosynthesis (Lee 1996). The variations in ammonia over time indicate a growth-coupled metabolic feedback involving the degradation and consumption of complex substrates including urea. In addition, the advantage of insufficient oxygen supply indicates an internal bottleneck within the tricarboxylic acid cycle, inducing an overflow of C_2 metabolites for biosynthesis of PHB (Ackermann and Babel 1997). This may be caused by decreased oxidation of NADH (Anderson and Dawes 1990; Kim et al. 1996).

The concentrations of PHB within the biomass as well as productivities demonstrate biotechnologically interesting aspects. First, one can aim to develop an optimized one-step fed-batch process because of the cellular level of about 50% PHB. The metabolic conditions for growth-associated PHB synthesis are comparable for active growth and sufficient product biosynthesis over a long time. Secondly, average productivities of 0.84 g PHB l^{-1} h^{-1} on protein hydrolysate and 0.8 g PHB l^{-1} h^{-1} on urea have never before been described for *Azotobacter* sp.. Lee (1996) published a productivity of 0.68 g PHB l^{-1} h^{-1} obtained by *A. vinelandii* on glucose medium supplemented with complex nitrogen sources.

These values may justify attempts to use technical proteins for the production of PHB by *A. beijerinckii* or other producing microorganisms. There are a lot of industrial nitrogen compounds, originating from renewable sources (Hauthal 1996), that can be used as cheap substrates. But such efforts must investigate the efficient hydrolysis of raw materials and, moreover, determine the homo- or heteropolymeric structure of the polymeric materials. Nakamura et al. (1992) discovered several relationships between PHB and poly(hydroxyvaleric acid) synthesized from individual amino acids in resting cells of *A. eutrophus*. Last but not least, the release of PHB from cells grown on complex media has to be taken into account (Page et al. 1995).

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