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Production of poly-D(-)-3-hydroxybutyrate and poly-D(-)-3-hydroxyvalerate by strains of *Alcaligenes latus*

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Abstract

Alcaligenes latus strains can accumulate poly-D(-)-3-hydroxybutyrate (PHB) up to about 85% of cell dry weight. The abilities to store poly-D(-)-3-hydroxyvalerate (PHV) of three strains of *A. latus* were investigated. With Na-propionate as PHV precursor, strain *A. latus*DSM 1122 had better PHV accumulation ability than strains *A. latus* DSM 1123 and 1124. Strain *A. latus* DSM 1123 could store PHV when Na-valerate but not Na-propionate served as the PHV precursor. PHB and PHV accumulation by *A. latus* DSM 1124 rapidly increased when propionic acid and acetic acid were together added to the fermentor. This increase was not obtained in the culture shaker flask and fermentor growing the same strain when Na-propionate alone served as a PHV precursor.

Introduction

Poly-D(-)-3-hydroxybutyrate (PHB) is a biodegradable, biocompatible thermoplastic made by microorganisms. The material can be formed into films, fibres and sheets and moulded into shapes and bottles. Copolymers with hydroxyvalerate units, poly-D(-)-3-hydroxyvalerate (PHV) can be made by some organisms such as *Alcaligenes eutrophus* when propionic acid or pentanoic acid was added into the culture (Holmes 1981). These are usually random arrangements of hydroxybutyric and hydroxyvaleric acid residues. The copolymers have superior mechanical properties to the homopolymer, they are more flexible and tougher which extends their versatility in end use to applications such as the manufacture of bottles and films which are not possible with PHB (Bauer & Owen 1985; Hartley 1987; Lafferty et al. 1988). PHB, and its copolymer with valerate units is under development in a wide range of applications by a number of companies using product supplied by Marlborough Biopolymers, a subsidiary established by Imperial Chemical Industries (ICI) Plc to exploit the material.

Since strains of *Alcaligenes latus* can use some cheap carbon sources, such as sucrose, glucose, fructose or corn syrup, cane molasses and beet molasses in a mineral medium, for the production of PHB, they become very attractive for the large-scale production of PHB. Furthermore, with a carbon source like sucrose *A. latus* DSM 1123 and 1124 strains are capable of rapidly accumulating the polymer during the growth phase and up to high concentration (80%) in the biomass (Palleroni & Palleroni 1978; Bucnik 1984; Küng 1982). *A. latus* is possibly of more interest than *A. eutrophus* for the production of PHB in a cheaper and easier way, as compared with other organisms discussed for other biotechnological processes in the past (Chen 1989).

As there is not any published research on the

production of PHB and PHV copolymer in *A. latus* strains, it would be very interesting to know if the strains can produce copolymer with HB and HV units in the presence of some precursors like propionate, valerate and a mixture of propionate and acetate.

Our present work describes the production of PHB and PHV with these three strains under different growth and accumulation conditions.

Materials and methods

Microorganisms and cultivation conditions

The following bacterial strains were used in this study: *Alcaligenes latus* DSM 1122, 1123 and 1124. These three strains were isolated (Palleroni & Palleroni 1978) from earth probes in California and Australia. Strains *A. latus* DSM 1122 and 1124 are unable to utilize propionate and valerate as sources of carbon for growth. The strains were grown heterotrophically at 35° C in a mineral medium supplemented with (g/l) sucrose 10–20, $Na_2HPO_4 \cdot 2H_2O$ 4.5, KH_2PO_4 1.5, $(NH_4)_2SO_4$ 1.0, $MgSO_4 \cdot 7H_2O$ 0.2, Fe(III)-NH_3-Citrat 0.05, $CaCl_2 \cdot 2H_2O$ 0.02 and 1 ml/l trace element solution containing (mg/l) $ZnSO_4 \cdot 7H_2O$ 100, $MnCl_2 \cdot 4H_2O$ 30, H_3BO_3 300, $CoCl_2 \cdot 6H_2O$ 200, $CuSO_4 \cdot 5H_2O$ 70, $NiCl_2 \cdot 6H_2O$ 20, $NaMoO_4 \cdot 2H_2O$ 30. The accumulation of PHB and PHV was studied at first in shake flasks with vigorous orbital shaking at 110 rpm, then in a glass fermentor (Biostat V, Braun Melsungen, Germany) equipped with a draught tube. In time-course sampling, 10 ml culture was collected from every shake flask or fermentor.

Fermentation conditions

All fermentations were carried out in Biostat V 5-Liter fermentors, the initial batch fermentation volume was 3 l, 300 ml inoculum was added with aseptic precautions into the fermentor. The temperature and pH of all fermentations were 35° C and 7.0, respectively, the oxygen concentration in culture was kept between 40–50% of air saturation, and stirring agitation was at 640 rpm.

Chemical analysis of cells and culture fluids

The cells were separated from culture fluids by centrifuging for 10 minutes in a centrifuge (Heraeus Christ, Germany) at 3000 g force. Cells from 10 ml culture were washed once with about 10 ml water and separated again. These cells were kept for about 3 h at − 70° C and then dried for 12 h in vacuum. The dry weight of cell materials was obtained from this dried biomass. The sucrose and propionate remaining in the culture supernatant fluid were estimated by HPLC and gas chromatography.

Table 1. Accumulation of PHV in strains of *A. latus* and *A. eutrophus* H16 in shake flasks at different concentrations of Na-propionate in the absence of sucrose and nitrogen source.

Strain	Na-propionate conc. (g/l)	DSM 1122	DSM 1123	DSM 1124	*A. eutrophus* H16
Before accumulation, polymer content (%)		61 PHB	57 PHB	52 PHB	56 PHB
After 24 hours of accumulation, polymer content (%)	1	35 PHB 10 PHV	37 PHB 4 PHV	29 PHB 8 PHV	47 PHB 2.5 PHV
	2	40 PHB 14 PHV	34 PHB 3 PHV	25 PHB 9 PHV	48 PHB 13 PHV
	3	53 PHB 13 PHV	32 PHB 0 PHV	57 PHB 3 PHV	51 PHB 11 PHV

Biomass was separated from the solid free medium, washed with water, centrifuged and kept at − 70° C for 2 hours and finally in vacuum for 12 hours. This dried biomass is cell dry weight (CDW) and the PHB and PHV in the biomass could be determined by GC (Braunegg 1978).

PHB/PHV analysis

PHB/PHV samples were prepared according to Braunegg (Braunegg 1978), the resulting methyl esters of the PHB/PHV monomers were quantified by using a model HP 5840 A (Hewlett-Packard, USA) gas chromatograph with a integrator. The nitrogen flow rate through the 4 m long column (5% Reoplex 400 on chromosorb G/AW 60/80, Germany) was 30 ml/min. The flame ionization detector was supplied with hydrogen and oxygen. 2 µl injections were made. The internal standard was benzoic acid, and the temperature-time profile was the same as that used by Braunegg. The external standard was poly-HB-co-HV (80% HB and 20% HV) copolymer (Biopol) obtained from ICI.

Medium component analysis

Sucrose, glucose and fructose concentrations were determined by high-pressure liquid chromatography with a Biorad HPX 87-H column with 0.01 N H_2SO_4 as the mobile phase. A differential refractometer (Knauer) detector was used.

Propionic, valeric, acetic and formic acids were also measured by gas chromatography. The nitrogen flow rate through the 2-m column (Chromosorb 101, 80–100 mesh) was 30 ml/min. The flame ionization detector was at 250° C, the oven temperature 180° C. The injections were 1 µl and temperature at 200° C.

Ammonium ion concentration in the culture broth supernatant was quantified by using a modified Berthelot reaction (Klinisches Labor, Merck, Germany 1974).

Results and discussion

Accumulation of PHV in the presence of Na-propionate in shake flasks

Three strains of *A. latus*, together with *A. eutrophus* H16 as a comparator were grown in the sucrose medium with nitrogen source. After incubation for 24 h, the biomass in each shake flask was

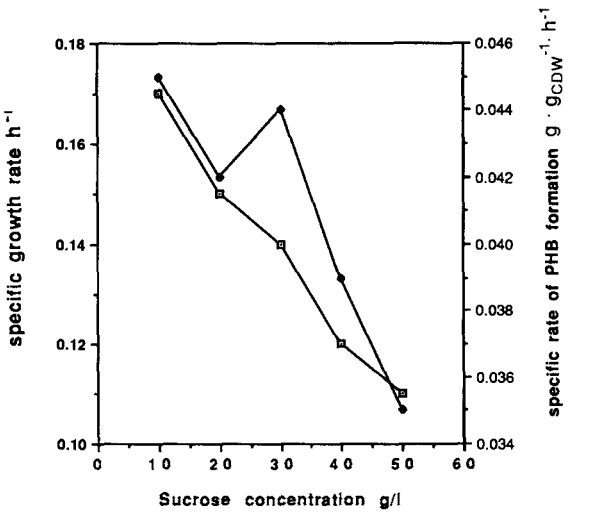

Fig. 1. Influence of sucrose concentration on growth and PHB formation by *A. latus* DSM 1122. The experiment was carried out in 1 l shake flasks contained 300 ml culture with vigorous orbital shaking at 110 rpm. The analysis conditions were the same as in Table 1. —□— specific growth rate μ; —●— specific rate of PHB formation $q_{p(PHB)}$.

centrifuged and then transferred into nitrogen-free media with three different concentrations of Na-propionate as the sole carbon source. After 24 h the polymer contents of the cells were measured (Table 1).

The results (Table 1) demonstrate that strain *A. latus* DSM 1122 possesses better ability than the other strains to accumulate PHV under the same conditions at all the concentrations of Na-propionate tested. Strain DSM 1124 produced a significant concentration of PHV (9%) in the cell. Under identical conditions, in the presence of 10 g sucrose/l and 1.5 g propionate/l the strain *A. latus* DSM 1122, which had been grown for many generations in media containing 1 g propionate/l showed a better accumulation of PHV than DSM 1124, e.g. 17% PHV, 29% PHB compared with 3% PHV, 40% PHB.

The data suggested that *A. latus* DSM 1122 could accumulate more PHV than strains *A. latus* DSM 1123 and 1124 under the conditions of N-limitation at Na-propionate concentrations up to 3 g/l. A study of the effect of growth conditions in shake flasks on PHV accumulation by *A. latus* DSM 1122 was therefore carried out. The specific growth rate

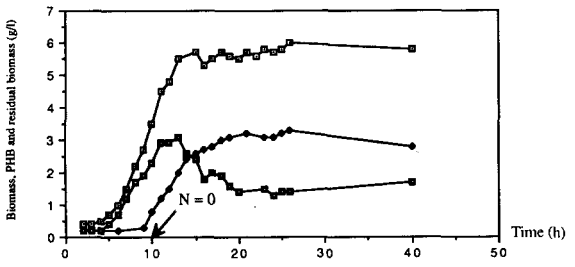

Fig. 2. Growth, PHB and residual biomass formation of *A. latus* DSM 1122 in a Biostat V 5 fermentor, 3 L culture, temperature 35° C, pH 7. Oxygen concentration 40–50% of air saturation. At the beginning of the fermentation there was 20 g/l Sucrose present. After 26 h 3 g/l sucrose still left in the fermentor. The arrow indicates the exhaustion of nitrogen source. —□— biomass; —◆— PHB; —■— residual biomass.

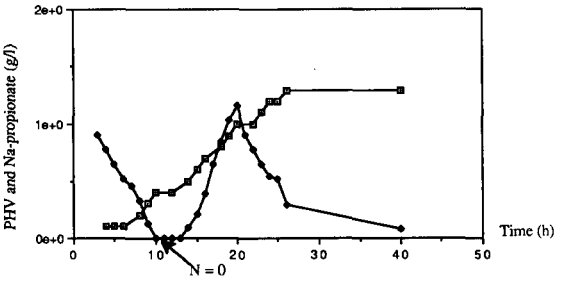

Fig. 3. PHV production and propionate consumption during the fermentation by strain *A. latus* DSM 1122. Experimental conditions were the same as those in figure 2. —□— PHV; —◆— Na-propionate.

μ and the specific PHB formation rate $q_{p(B)}$ tended to decrease with increasing sucrose concentrations (Fig. 1). The sucrose concentration had little effect on the PHV accumulation which remained at 3–4% over the range of sucrose concentrations tested in Fig. 1.

The Na-propionate was toxic for the cells when its concentration exceeded 2.0 g/l (Chen 1989). Up to that concentration of propionate the cells exhibited slow growth and a long lag phase. Because of this growth inhibition the Na-propionate concentration should be maintained lower than 2 g/l in the fermentor during culture growth with the 'fed-batch' process.

Growth experiments

The kinetics of growth and PHB/PHV formation by *A. latus* DSM 1122 in the fermentor were studied (Fig. 2). At the beginning of the fermentation there was 1.2 g Na-propionate/l present as the PHV precursor; after about 11 h no propionate could be detected by GC analysis. At this time 5.4 g Na-propionate (in 60 ml water solution) was added to the fermentor at a rate of 0.09 g/min (Fig. 3). The PHV concentration rapidly increased during the addition. A PHV concentration of 1.3 g/l, about 22% of the cell dry weight together with 3.4 g PHB/l, about 52% of cell dry weight, was achieved after 26 h. Table 2 shows the results of this growth experiment. It could be suggested that further addition of Na-propionate might result in a further increase of PHV accumulation.

Because strain DSM 1122 can not use propionate

Table 2. Important data of fed-batch fermentation with strains of *A. latus* DSM.

Strain	μ_{max}	$q_{p(B)}$	$q_{p(V)}$	P_B	P_V	$Y_{B+V/S}$	$Y_{V/prop}$	$Y_{X/S}$
1122	0.30	0.17	0.011	0.10	0.04	0.22	0.46	0.31
1123	0.45	0.13	0.036	0.21	0.14	0.43	0.43	0.77
1124	0.42	0.13	0.070	0.15	0.16	0.37	0.36	0.64

Conditions see text and figures for details.

μ_{max} = maximal specific rate of growth h^{-1}.
$q_{p(B)}$ = maximal specific rate of PHB formation $g \cdot g_{CDW-1} \cdot h^{-1}$.
$q_{p(V)}$ = maximal specific rate of PHV formation $g \cdot g_{CDW-1} \cdot h^{-1}$.
$Y_{B+V/S}$ = yield coefficient for products (PHB + PHV) with respect to Na-propionate consumption (g/g).
$Y_{V/prop}$ = Yield coefficient for PHV with respect to Na-propionate consumption (g/g).
$Y_{X/S}$ = Yield coefficient for biomass formation with respect to substrat consumption (g/g).

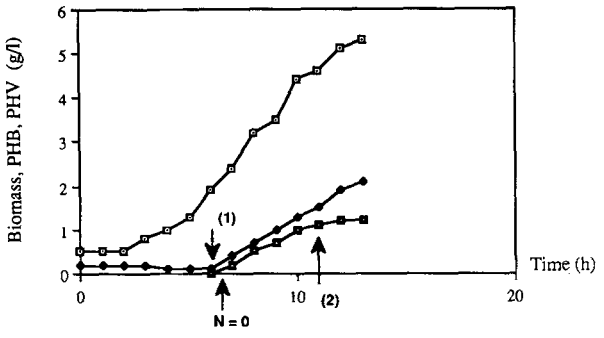

Fig. 4. Biomass, PHB and PHV formation, and ammonium consumption by *A. latus* DSM 1124 in a medium with 10 g sucrose/l and 1 g ammonimsulfate/l at the beginning. Experimental conditions: During the accumulation phase 10.5 g propionic acid and 3.5 g acetic acid (in 300 ml solution) were added to the culture with an addition rate of 1 ml/min. Arrow 1 indicates the addition of precursors (propionate and acetate) and arrow 2 the exhaustion of presursors. —□— biomass; —◆— PHB; —□— PHV.

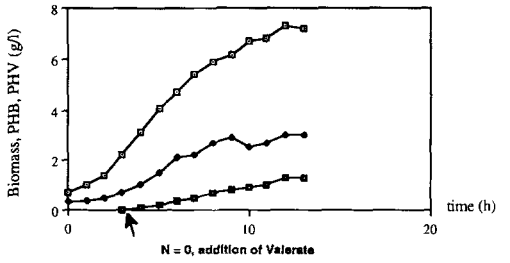

Fig. 5. Biomass, PHB, PHV formation and ammonium consumption by *A. latus* DSM 1123 in a medium with 10 g sucrose/l and 1 g ammonimsulfate/l at the beginning. Conditions: During the accumulation phase (N-limitation) 300 ml solution containing 9 g Na-valerate was added to the fermentor at an addition rate of 1 ml/min. Fermentation temperature: 35°C, pH 7.0. —□— biomass; —◆— PHB; —□— PHV.

as carbon source for growth, it might be expected that all the propionate would be turned into propionyl-CoA and then converted into PHV. However, the yield coefficient for PHV with respect to Na-propionate consumption, $Y_{V/prop}$ is only 0.46 (maximal $Y_{V/prop}$ is 1). That means, some propionate could be changed into other substrates which resulted in a decrease of $Y_{V/prop}$. We did find some acetate and formate in the culture liquid by the GC analysis and it is possible herefore that propionate was degraded to acetate and formate leading to the observed decrease of $Y_{V/prop}$.

In the presence of Na-propionate as the PHV precursor, strain *A. latus* DSM 1124 could not produce a significant amount of PHV (less than 3% of CDW) (Chen 1989). However, when propionic acid and acetic acid were added to the culture during the accumulation phase, a rapid increase of PHV content was observed (Fig. 4), with a maximal specific rate of PHV formation of 0.07 $g \cdot g_{CDW-1} \cdot h^{-1}$. Simultaneously PHB was rapidly accumulated to 42% of CDW after 12 h, at a maximal specific rate, $q_{p(B)}$ of 0.13 $g \cdot g_{CDW-1} \cdot h^{-1}$.

The results of this fed-batch fermentation with strain DSM 1124 were summarized in Table 2. Compared the PHV productivity of DSM 1122 with that of DSM 1124, we note that the addition of propionic acid and acetic acid to the culture containing DSM 1124 is important for PHV production because of the increases of specific PHV formation rates and PHV productivities (Table 2).

As *A. latus*DSM 1124 is unable to use propionate as carbon source for growth (Palleroni & Palleroni 1978), all propionate would be incorporated in PHV (1 g Na-propionate leads to about 1 g PHV). Nevertheless, as the coefficient $Y_{V/prop}$ of this experiment is 0.36, only some propionate was incorporated in PHV, other propionate might be expected to have been used by the cells to generate additional energy for the biosynthesis of polymer, this additional energy resulting in the increases of PHV and PHB formation.

A similar result had been obtained with strain *A. latus* DSM 1123 which has the best ability of the three *A. latus* strains to use propionate as the sole carbon source. The PHV accumulation ability of strain *A. latus* DSM 1123 in the presence of propionate, however, was worse than the other two. We found that propionate was degraded to acetate and formate during the fermentation of strain DSM 1123 (Chen 1989). Nevertheless, the addition of valerate to the fermentor containing strain DSM 1123 resulted in a rapid increase of PHB and PHV formation (Fig. 5).

In this fermentation of DSM 1123 we did not find any acetate and formate in the culture, although PHV was synthesized rapidly during the addition of valerate. The majority of the valerate was turned into PHV and a high yield coefficient for biomass

formation with respect to substrate consumption can be observed (Table 2).

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