Appl Microbiol Biotechnol (1998) 50: 604–607

SHORT CONTRIBUTION

E. J. Bormann · M. Leißner · M. Roth
B. Beer · K. Metzner

Production of polyhydroxybutyrate by *Ralstonia eutropha* from protein hydrolysates

Received: 8 July 1998 / Accepted: 26 August 1998

Abstract Polyhydroxybutyrate (PHB) was produced by *Ralstonia eutropha* DSM 11348 (formerly *Alicaligenes eutrophus*) in media containing 20–30 g l^{-1} casein peptone or casamino acids as sole sources of nitrogen. In fermentations using media based on casein peptone, permanent growth up to a cell dry mass of 65 g l^{-1} was observed. PHB accumulated in cells up to 60%–80% of dry weight. The lowest yields were found in media without any trace elements or with casamino acids added only. The residual cell dry masses were limited to 10–15 g l^{-1} and did not contain PHB. The highest productivity amounted to 1.2 g PHB l^{-1} h^{-1}. The mean molecular mass of the biopolymer was determined as 750 kDa. The proportion of polyhydroxyvalerate was less than 0.2% in PHB. The bioprocess was scaled up to a 300-l plant. During a fermentation time of 39 h the cells accumulated PHB to 78% w/w. The productivity was 0.98 g PHB l^{-1} h^{1}.

Introduction

In line with the ecological principle of sustainable production (Adam 1993), very many attempts have been made to avoid harmful environmental effects of petrochemical plastics by use of available natural products. Several bacteria are able to accumulate biopolymers as granules consisting of polyhydroxyalkanoates accompanied by phasines (Steinbüchel et al. 1995). Such homo- and/or heteropolymeric biomaterials could be produced and used for biodegradable packaging (Chiellini and Solaro 1996) or as biocompatible materials in medicine (Yamamoto et al. 1996). However, general substitution of conventional plastics has been limited by high production costs of the relevant bioproducts (Hänggi 1995). Different lines of experiment have been pursued to improve bioprocesses for the production of polyhydroxybutyrate (PHB). In addition to the homologous or heterologous expression of *phbCAB* genes in *Ralstonia eutropha* (formerly Alicaligenes eutrophus; Yabuuchi et al. 1995), (Park et al. 1995), *Methylobacterium extorquens* (Valentin and Steinbüchel 1993) or *Arabidiopsis thaliana* (Blohm 1996) the regulation of β-ketothiolase by CoA has also been studied biochemically (Mansfield et al. 1995). Furthermore, cheap technical materials (Kim et al. 1994) have been used as renewable nitrogen and carbon sources for growth and product formation. PHB was efficiently produced by cultivation of recombinant *Escherichia coli* strains in modified Luria-Bertani media (Kalousek and Lubitz 1996; Lee and Chang 1995; Lee 1996). Moreover, some amino acids stimulated PHB formation in resting cells of *R. eutropha* (Nakamura et al. 1992; Fujita et al. 1993). Productivities of more than 1 g PHB l^{-1} h^{-1} were achieved by cultivation of *Azotobacter beijerinckii* in media containing 20 g casein peptone 1^{-1} (Bormann et al. 1998).

We studied the production of PHB by *R. eutropha* DSM 11348, a mutant of *R. eutropha* DSM 531 (Metzner et al. 1997), cultivated in media with glucose and casein hydrolysate as the sole source of nitrogen.

E. J. Bormann (✉) · B. Beer
Innovent e.V./Biomaterialien, Felsbachstr. 5,
D-07747 Jena, Germany
Tel.: +49-36-41-28-25-22
Fax: +49-36-41-28-25-30

M. Leißner
Fachhochschule Jena, Bereich Wirtschaftsingenieurwesen,
D-07743 Jena, Germany

M. Roth
Hans-Knöll-Institut für Naturstoff-Forschung e.V,
Bereich Bioverfahrensentwicklung, D-07708 Jena, Germany

K. Metzner
BSL Olefinverbund GmbH Werk Schkopau,
D-06258 Merseburg, Germany

Materials and methods

Bacterial strain and growth conditions

Ralstonia eutropha DSM 11348 is a derivative of *R. eutropha* DSM 531 selected from a continuous culture after mutagenic

treatment (Dr. A. Christner, Hans-Knöll-Institut für Naturstoff-Forschung, Jena). The mutant showed increased growth rates on succinate, 2-oxoglutarate or glucose compared with the parental strain. The pattern of chromosomal DNA fragments after digestion by *Ssp*I and pulsed-field gel electrophoresis was also different (Metzner et al. 1997). A series of stock cultures stored at −18 °C were used to inoculate seed cultures. Each tube contained 5 ml identical cell suspension cryoprotected by 80% aqueous glycerol. Casein peptones (Fluka Chemie AG, Switzerland) and casamino acids (Berlin Chemie Germany) were used. The medium of the first seed cultures contained (g l^{-1}) peptone 10, glucose 20, KH_2PO_4 1, $CaCl_2$ 0.005 and 2.5 ml trace elements solution, reported elsewhere (Bormann et al. 1998), tap water was added to 1 l, pH 6.8. All constituents were sterilized separately and mixed thereafter. Seed cultures were grown at 30 °C in 500-ml flasks containing 100 ml medium inoculated with 1 ml stock culture. The cultivation was finished after 24 h or 41 h of shaking at 200 rpm. The media used for the seed cultures of the second stage and the fermentation cultures are described in the legends of the figures and the table. The fermentations were performed in home-made 2.5-l fermenters (working volume: 2 l). The vessels were equipped with two six-bladed disc impellers and the usual systems for the regulation of pH, temperature, stirrer speed, and aeration and for preventing foaming. The scaling-up was performed in a 300-l plant (Chemap, Switzerland). The fermentation culture was inoculated with 10% of its volume. The pH was regulated at 6.8 by 6 M NaOH and 2 M H_2SO_4. The stirrer speed was adjusted at 800–1000 rpm. The aeration rate was 0.5:1 (vvm).

Analysis of the culture broth

Samples of the fermentation cultures (20 ml) were collected periodically. The supernatants obtained by centrifugation at 10 000 rpm for 10 min at 4 °C were used for substrate analysis. The glucose concentration was determined using the glucose analyser ESAT 6660-2 (Prüfgerätewerk Medingen GmbH, Germany). The content of ammonia nitrogen was determined according to Kjeldahl. Organic nitrogen was estimated following its mineralization by hot sulphuric acid. Inorganic phosphorus was determined by means of commercial test kits using the photometer SQ 118 (Merck, Germany). The cell dry mass was calculated from the absorbance at 420 nm measured on a Specol 11 (Carl Zeiss, Jena, Germany) spectrophotometer. Determinations of cell dry masses were made after drying at 105 °C.

Analysis of PHB

The cells were collected by centrifugation and resuspended in 10 ml alkaline hypochlorite reagent (pH 10.0–10.5, NaOCl 5.25%–5.5%) according to the method of Williamson and Wilkinson (1958). Following a 2-h incubation at 37 °C, the mixture was centrifuged at 5000 rpm for 10 min. The solid pellets were successively washed with water, alcohol and acetone, and dried at 105 °C before weighing. The mean molecular mass was estimated by viscosimetry using an Ubbelohde apparatus adapted by BSL Olefinverbund GmbH, Merseburg, Germany. The limit viscosity, was calculated from the equations of Hagenbach and Huggins using data from three repeated measurements. The mean molecular mass was evaluated according to the Kuhn-Mark-Hauwink equation. The content of copolymers was determined by means of gas chromatography.

Results

Growth

The kinetics of growth and substrate utilization are demonstrated by a representative diagram of the fermentations (Fig. 1). The cells were growing during the whole of the cultivation time. The cell dry mass increased to at least 25 g l^{-1} during 24 h of fermentation, whereas 50–65 g l^{-1} was achieved after 40 h. The highest cell concentration was detected in medium containing 30 g casein peptone l^{-1}. The residual cell dry masses, e.g. real biomass without PHB, remained at 10–15 g l^{-1}. A logarithmic plot of the mean values of the cell dry masses of all fermentations shows an exponential growth phase between 5 h and 16 h of the fermentation period. The calculated growth rate, μ, was 0.18 h^{-1}.

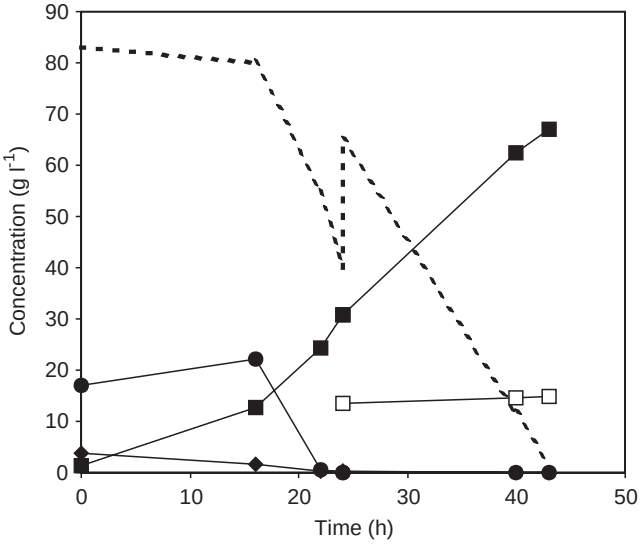

Fig. 1 Kinetics of a fermentation performed in a 2.5-l fermenter using a second-stage seed culture. Medium composition (g l^{-1}): peptone 20, glucose 100, KH_2PO_4 0.5, $CaCl_2$ 0.005, and 2.5 ml trace elements solution. The medium of the second seed culture contained 50 g glucose l^{-1}. ■ Cell dry mass, □ residual cell dry mass, --- glucose, ● $10^2 \times$ [ammonia], ◆ phosphorus

Substrates

Glucose was intensively metabolized during the fermentations and was fed, if necessary, in distinct doses. The ammonia concentration temporarily increased between 10 h and 15 h of the cultivation time and decreased to zero thereafter. The content of inorganic nitrogen at the beginning of the fermentations was caused by ammonia brought in by sterilization or seed cultures. The temporary increase of ammonia was probably caused by deamination of protein components as the only sources of nitrogen in the media. No phosphorus limitation was detectable because its level decreased finally to about 50 mg P l^{-1}. There was no limitation by organic nitrogen or by oxygen (data not shown). The concentrations of organic nitrogen amounted finally to about 1 g N l^{-1}. Dissolved oxygen was adjusted permanently to at least 30% saturation by regulation of the stirrer speed.

PHB concentration

Table 1 summarizes the mean values and deviations of the PHB accumulation and productivities in repeated experiments of fermentations differing in media composition and cultivation time. The biopolymers synthesized were almost pure PHB, the content of polyhydroxyvalerate amounting to less than 0.2%.

The cultures inoculated with first-stage seed cultures (SF) produced less PHB during 24 h of fermentation time than those inoculated with second-stage seed cultures. Different initial concentrations of glucose in the seed cultures ($SS_{50}F$: 50 g l^{-1}; $SS_{25}F$: 25 g l^{-1}) had no evident effects. The highest yields of PHB were achieved in media supplemented with 30 g peptone l^{-1} (SSP). However, the absence of trace elements (NT) or the use of casamino acids (CAS) instead of peptone resulted in only moderate PHB yields. During the scaling up of the fermentation to the pilot plant (300) PHB concentrations achieved the level reached in the laboratory fermenter.

Obviously the mean values of productivities correlated with the PHB concentrations, so the highest productivity being calculated as 1.2 g PHB l^{-1} h^{-1} when medium with 30 g peptone l^{-1} was used. The lowest productivities occurred either when the fermentation time was short (SF) or when trace elements were lacking (NT) and casamino acids (CAS) were used as the sole sources of nitrogen. The PHB contents of the biomasses are presented in Fig. 2. The accumulation of PHB achieved 84% of the cell dry mass in media containing 30 g peptone l^{-1}.

As the practical use of PHB is dependent on the degree of polymerization, the mean molecular masses of PHB isolated from seven fermentations were estimated. Notwithstanding the various culture conditions, the mean molecular mass differed only slightly and was calculated as 750 ± 70 kDa.

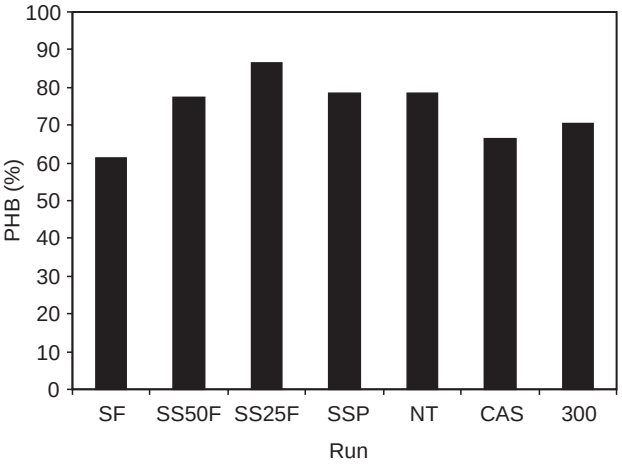

Fig. 2 Cellular accumulation of polyhydroxybutyrate [PHB]. *SF* First-stage seed culture inoculum, *$SS_{50}F$* second-stage seed culture inoculum and 50 g glucose l^{-1}, *$SS_{25}F$* similar inoculum with 25 g glucose l^{-1}, *SSP* medium supplemented with peptone, *NT* no trace elements, *CAS* casamino acids replaced peptone, *300* pilot plant scale

Discussion

Bacteria able to synthesize PHA can be divided into two groups (Lee 1996). The first group, accumulating PHA during the stationary growth phase, requires limitation of N, P, Mg, for example, and an excess of the carbon sources. The most important microorganism for industrial PHA production, *R. eutropha*, belongs to this group. The second group, accumulating PHA during the growth phase, includes *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii*, *Azotobacter beijerickii* (Bormann et al. 1998) or recombinant strains of *E. coli* bearing the PHA operon of *R. eutropha*. Our strain, *R. eutropha* DSM 11348, belongs to the second group, because PHB accumulates during growth on protein hydrolysates. However, the relative constancy of the residual cell dry mass indicated that, in the final fermentation phase, the increase of cell dry weights was actually caused by synthesis of PHB.

We think that this result derives from the phenomenon of "loose nitrogen limitation" (Fujita et al. 1993). Obviously the cultures were limited by balanced rates of formation (deamination) and utilization of ammonia. It is worth mentioning that our data do not match the results obtained from resting-cell experiments. According to Nakamura et al. (1992) and Fujita et al. (1993), PHB-deficient cells of *R. eutropha*, precultivated in complex medium and transferred to mineral salt medium supplemented with threonine or isoleucine, thereafter contained very low levels of PHB, including a high portion of polyhydroxyvalerate. By contrast we found high yields of cellular accumulation and productivity of PHB containing less than 0.2% polyhydroxyvalerate. Growth and product formation were probably metabolically balanced by use of a complete set of amino acids.

It can be concluded that protein hydrolysates consisting of amino acids and/or oligopeptides might be used as sources of nitrogen at concentrations of about 30 g l^{-1} by *R. eutropha* DSM 11348 instead of the lower concentrations previously used (Lee and Chang 1995). Such simplified fermentation procedures without special

Table 1 Polyhydroxybutyrate (PHB) concentrations and productivities of studied fermentations. Described in the text and in legend to Fig. 2

Series	Runs	Time (h)	PHB (g l^{-1})	Productivity (g l^{-1} h^{-1})
SF	3	24	16.2 ± 1.8	0.67 ± 0.08
$SS_{50}F$	3	41	42.7 ± 4.3	0.97 ± 0.04
$SS_{25}F$	3	43	43.0 ± 4.8	1.04 ± 0.10
SSP	3	43	52.2 ± 1.5	1.21 ± 0.02
NT	2	41	28.3 ± 0.1	0.68 ± 0.02
CAS	1	47	25.3	0.53
300	1	39	38.4	0.98

feeding strategies for inorganic nitrogen sources might be useful for an efficient production of PHB. The basic regimes studied have to be improved in order to achieve higher cell densities and utilize cheap industrial proteins. Several renewable materials (Hauthal 1996), e.g. casein hydrolysates or glutene, are known to be available from agriculture as nitrogen sources. However, a method for cleaving raw materials by careful hydrolysis ought to be developed for gram-negative bacteria deficient in extracellular proteases. Finally, special problems in the isolation of PHB have to be considered (Page et al. 1995) if complex media are used for cultivation.

Acknowledgements We wish to thank J. Gemeinhardt, I. Krüger, C. Kunze, G. Müller and J. Reinhard for technical assistance, and Dr. A. Christner and K. Menzel for cooperation. We thank B. Fahnert for critical reading of the manuscript. This work was supported by BSL Olefinverbund GmbH, Merseburg, Germany.

References

Adam D (1993) Ökologische Anforderungen an die Produktion. In: Gabler Th, Schriften zur Unternehmensführung. Betriebswirtsch. Wiesbaden, pp 5–31

Blohm D (1996) Trends: Biochemie und Molekulargenetik – Biotechnologie. Nachr Chem Tech Lab 44: 180–182

Bormann EJ, Leißner M, Beer B (1998) Growth-associated production of polyhydroxybutyric acid by *Azotobacter beijerinckii* from organic nitrogen substrates. Appl Microbiol Biotechnol 49: 84–88

Chiellini E, Solaro R (1996) Biodegradable polymeric materials. Adv Mater 8: 305–312

Fujita M, Nakamura K, Kuroki H, Yoshie N, Inoue Y (1993) Biosynthesis of polyesters from various amino acids by *Alcaligenes eutrophus*. Int Biol Macromol 15: 253–255

Hänggi UJ (1995) Requirements of bacterial polyesters as future substitute for conventional plastics for consumers goods. FEMS Microbiol Rev 16: 213–220

Hauthal HG (1996) Nachwachsende Rohstoffe – Perspektiven für die Chemie. Nachr Chem Tech Lab 44: 32–35

Kalousek S, Lubitz W (1996) High-level poly(ß-hydroxybutyrate) production in recombinant *E. coli* in sugar free complex medium. Can J Microbiol 41 [Suppl 1]: 216–221

Kim BS, Lee SC, Lee SY, Chang NH, Chang YK, Woo SJ (1994) High cell density culture technique for *Alcaligenes eutrophus*. Biotechnol Bioeng 43: 892–898

Lee SY (1996) Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnol 14: 431–438

Lee SY, Chang HN (1995) Production of poly(3-hydroxybutyric acid) by recombinant *Escherichia coli* strains: genetic and fermentation studies. J Can Microbiol 41 [Suppl 1]: 207–215

Mansfield DA, Anderson AJ, Naylor LA (1995) Regulation of PHB metabolism in *Alcaligenes eutrophus*. Can J Microbiol 41: 44–49

Metzner K, Berndt C, Christner A, Hilliger M, Geuther R, Perlet K, Roth M, Bormann EJ, Leißner M (1997) Mikrobielles Verfahren zur Herstellung von Polyhydroxyalkanoaten. German patent 19704045.4

Nakamura K, Goto Y, Yoshie N, Inoue Y (1992) Biosynthesis of poly(3-hydroxyalkanoate) from amino acids. Int J Macromol 14: 321–325

Page WJ, Sherburne R, D'Elia L, Graham LL (1995) Poly(β-hydroxybutyrate) extrusion from pleomorphic cells of *Azotobacter vinelandii* UWD. Can J Microbiol 41 [Suppl 1]: 22–31

Park JS, Park HC, Huh TL, Lee YH (1995) Production of poly-β-hydroxybutyrate by *Alcaligenes eutrophus* transformants harboring cloned *phb*CAB genes. Biotechnol Lett 17: 735–740

Steinbüchel A, Aerts K, Babel W, Föllner C, Liebergesell MN, Madkour R, Mayer F, Peter-Fürst U, Pries A, Valentin HE, Wiezcorek R (1995) Considerations on the structure and biochemistry of bacterial polyhydroxyalkanoic acid inclusions. Can J Microbiol 41 [Suppl 1]: 94–105

Valentin HE, Steinbüchel A (1993) Cloning and characterization of the *Methylobacterium extorquens* polyhydroxyalkanoic-acid-synthase structural gene. Appl Microbiol Biotechnol 39: 309–317

Williamson DH, Wilkinson JF (1958) The isolation and estimation of the poly-β-hydroxyburytate inclusions of *Bacillus* species. J Gen Microbiol 19: 198–209

Yabuuchi E, et al. (1995) Microbiol Immunol 39: 897–904

Yamamoto Y, Abe H, Doi Y (1996) Developments in the area of medical or implantable polymers. In: Withold B, (Eds) Abstracts of the International Symposium on Bacterial Polyhydroxyalkanoates. Davos