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Selective Enrichment of Bacteria Accumulating Polyhydroxyalkanoates in Multistage Continuous Culture

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Summary

The relationship between polyhydroxyalkanoate (PHA) fraction and cell survival under long-term carbon starvation was utilized for developing a method for the selective enrichment of PHA-forming bacteria from environmental samples. The first reactor of a five-stage continuous culture was fed with a carbon-rich and nitrogen poor medium at very low dilution rates. These conditions allowed not only growth but also PHA accumulation by competent organisms. In the following stages the culture was kept under carbon and nitrogen limiting conditions for a total of 100 to 200 hours. Fifteen different bacterial strains were isolated from various environmental samples. Four of them were able to accumulate high amounts of PHA (mass fraction > 10%), 3 accumulated minor amounts (mass fraction 1–10%), 2 strains accumulated trace amounts and 6 showed no PHA production at all. A control experiment was carried out with a mixed population containing one PHA-accumulating bacterium species (Ralstonia eutropha) and one unable to accumulate PHA (Escherichia coli). In this experiment significant enrichment of the slower growing but PHA-accumulating species, R. eutropha, was achieved.

Keywords: Ralstonia eutropha, polyhydroxyalkanoates, bacterial storage compounds

Introduction

Carbon and energy-reserve materials in plants and animals have been recognized for many years, leading to the discussion of a possible role of analogous compounds in bacteria. It was shown that in the absence of bacterial growth, energy is still required for the maintenance of the intracellular pH and osmotic pressure, for motility and the turnover of proteins and nucleic acids *(1–3)*. Besides polysaccharides, lipids, and polyphosphates, polyhydroxyalkanoates (PHAs) have been proposed as possible bacterial storage compounds *(4)*. PHAs are biologically formed polyesters of high molecular-mass which can serve as intracellular carbon and energy-reserve materials without affecting the internal osmotic pressure. When growth is restricted due to limitation in an essential medium component except carbon, competent bacteria start accumulating PHAs *(5,6)*. PHA accumulation is not an essential requisite for growth *(7)*, but a clear relationship between PHA content and survival under carbon/energy limiting conditions has been shown *(4,7–13)*. More recently PHAs have gained strong industrial interest due to their thermoplastic properties and their complete biodegradability, and they have been proposed as a possible alternative to many mineral oil derived plastics. Due to high production cost hitherto only one such polymer, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) has been commercially produced under the name BIOPOL™ (ZENECA, Billingham, England) with *Ralstonia eutropha* as production strain *(5,6)*. The finding of new bacterial strains with possibly improved PHA-production properties may help to make PHAs better competitive to traditional plastics. In this paper we describe the application of the above mentioned relationship between PHA content and cell survival for developing a method for the selective enrich-

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ment of PHA-forming bacteria from environmental samples.

Materials and Methods

Bacterial strains and culture maintenance

Ralstonia eutropha DSM 545 was originally obtained from DSM, Braunschweig, Germany. Ampicillin-resistent *Escherichia coli* K12 RR1 pBR322 was originally obtained from the Institute of Genetics, University of Köln, Germany. In these experiments no antibiotics were added to the media. For culture maintenance of all strains (including isolates), colonies that had grown on solid media for 3 to 4 days were kept at 4 °C and transferred to new plates every 4 to 6 weeks. For long-term maintenance the strains were stored in liquid nitrogen.

Media used

Each liter of the basic mineral-salts buffer consisted of: 4.5 g of $Na_2HPO_4 \cdot 2\ H_2O$, 1.5 g of KH_2PO_4, 0.20 g of $MgSO_4 \cdot 7\ H_2O$, 1 mL of trace-element solution SL-6: 0.02 g of $CaCl_2 \cdot 7\ H_2O$, and 0.05 g of NH_4Fe(III) citrate. Each liter of the trace-element solution SL-6 contained: 100 mg of $ZnSO_4 \cdot 7\ H_2O$, 300 mg of H_3BO_3, 200 mg of $CoCl_2 \cdot 6\ H_20$, 6 mg of $CuSO_4$, 20 mg of $NiCl_2 \cdot 6\ H_2O$, 30 mg of $Na_2MoO_4 \cdot 2\ H_2O$, and 25 mg of $MnCl_2 \cdot 2\ H_2O$. Mass of 10 g of sodium acetate together with 5 g of 1,4-butanediol and 1 g $(NH_4)_2SO_4$ were added to 1 L of the buffer as carbon and nitrogen source. Volume fraction 0.5 mL L^{-1} of antifoam agent polypropylene glycol was added to the feed medium for continuous culture.

Rolling-culture experiments for PHA production

Shaking flasks of 300 mL containing 70 mL of mineral-salt medium were inoculated with colonies from solid media. The cultures were incubated for 24 hours under reciprocal shaking at 130 r.p.m. After this growth phase, the biomass was harvested by a 20 min centrifugation at 4 °C and 7000 g. The pellets were resuspended in the mineral-salt buffer and transferred into test tubes containing different accumulation media (mineral-salt buffer plus carbon source and precursor) yielding a final volume of 10 mL. A 36 hour incubation phase on a rolling shaker was followed by the harvesting of the cells and determination of biomass and PHA.

Principle and description of the continuous enrichment plant

The selective enrichment was carried out in multistage continuous culture (five stages) to obtain a narrow residence time distribution. From a medium storage tank the first reactor was continuously fed with a carbon-rich and nitrogen-poor medium at very low dilution rates. These conditions allow not only growth, but also PHA accumulation by competent microorganisms. In the following stages the culture was kept under both nitrogen-limiting and carbon-limiting conditions for 100 to 200 hours (depending on the dilution rate). Cells having stored sufficiently large amounts of carbon reserve materials (PHA) could survive this starvation period, whereas non-accumulating bacteria died according to their death rate under carbon starvation. By recycling the culture from the last stage into the first stage, surviving cells could be used as a continuous inoculum, thus enhancing the enrichment effect.

The following reactor types were used for the setup of the plant: Braun Biostat glass fermenter (1.7 L) with 1L working volume, SGI glass fermenter (2 L) with 1L working volume, Plexiglas airlift reactor (2 L) with 1500 mL working volume, and Quickfit glass vessels (1 L) with magnetic stirring and 800 mL working volume. The Braun and SGI glass fermenters were equipped with pH measurement by Ingold combined glass electrodes and O_2 measurement by Ingold polarographic electrodes. The experiment was carried out under non-sterile conditions, as the main aim was the enrichment of as many different species as possible which are able to produce PHAs. To avoid contamination with *Alcaligenes latus*, which was worked with in the same laboratory, sodium acetate, which can not be utilized by *A. latus*, was used as the carbon source in the enrichment experiments.

Environmental samples (soil, sewage sludge and compost) were used to inoculate shaking flasks (1 L) containing 300 mL of sterile mineral-salts medium. After 24 hours of growth at 30 °C and 130 r.p.m., a well grown culture together with 1000 mL of fresh sterile medium was transferred to the first reactor of the enrichment plant as inoculum.

During the enrichment experiment, the temperature in all reactors was maintained at (30 ± 1) °C. The pH in the first reactor of the cascade was maintained at 7 by automatic addition of 10% (mass fraction) solutions of NaOH or H_3PO_4. Agitation and aeration in the first reactor were manually controlled to keep the dissolved-oxygen concentration above 50% of air saturation. Constant feeding rates of fresh medium to the reactor cascade were achieved by pressurizing the feed-medium reservoir to 1 bar gauge and connecting it to the reactor cascade via a Teflon capillary (0.2 mm internal diameter) of variable length. To obtain very small flows, a time-controlled electric valve was added. In the case of the control experiment with *Ralstonia eutropha* and *Escherichia coli*, the feed was supplied by a Watson-Marlow 101U peristaltic pump. Feeding rates were calculated from changes in the overflow reservoir over known period of time. Recycle flow was accomplished by a Watson-Marlow 101U peristaltic pump.

Determination of cell dry mass

A volume of 5 mL of the liquid culture were centrifuged for 15 min in a Hereaus Megafuge 1.0 R centrifuge at 3240 g and 4 °C, followed by a first washing of the pellet with 2 mL of 0.9% NaCl solution and a second washing with 2 mL of deionized H_2O. The pellet was freeze-dried for 12 h and weighed on an analytical balance.

PHA assay

PHAs were determined by gas chromatograph after methanolysis of freeze dried biomass samples with acidified methanol (5% volume fraction of H_2SO_4), containing 1 g L^{-1} of hexanoic acid as internal standard *(14,15)*. Biopol™ P(3-HB-co-3-HV) samples mixed with sodium 4-hydroxybutyrate were used as PHA standards.

Substrate analysis

The concentration of glucose was determined spectrophotometrically using a hexokinase test (DIPRO, Austria). The ammonium concentration was determined with an ion-sensitive electrode (model 95–12, Orion Research Inc., Boston). 1,4-butanediol and acetate were determined by gas chromatography as tetrahydrofurane and ethylacetate, respectively *(15)*.

Determination of death rate under carbon starvation conditions

Cultures (300 mL) of *Ralstonia eutropha* and *Escherichia coli* in mineral-salts buffer containing 10 g L^{-1} of glucose and 1 g L^{-1} of $(NH_4)_2SO_4$ were grown overnight at 30 °C and 130 r.p.m. in shaking flasks. The cultures were mixed and the concentration of residual glucose was followed. When glucose concentration had reached zero, a sample was taken and a 1:10 dilution series up to 10^{-9} was made. The volume of 0.1 mL of each dilution were plated out on solid media and incubated at 30 °C for three to four days. After that time cell counts of both strains were made. The two strains could easily be distinguished due to their characteristic colony colors. *R. eutropha* formed opaque yellowish colonies whereas *E. coli* formed translucent whitish colonies. For a total of 168 hours samples were taken every 24 hours.

Results

Enrichment experiments with environmental samples

In the first experiment a tropical soil sample from Jaco (Costa Rica) was used as inoculum. Quickfit reactors were used for the first, third, fourth and fifth stage, the air-lift reactor was used as the second stage of the reactor cascade. The feed rate to the first stage was 20 mL/h, and the recycle rate was set to 0.

After 16 days, samples were taken from all reactors. Subsequent analysis showed, that the desired enrichment conditions – nitrogen limitation and carbon excess in the first part of the enrichment plant and nitrogen plus carbon limitation in the following reactors – were accomplished. Ammonium sulfate concentrations were below detection level in all reactors. Concentrations of 0.17 g L^{-1} of sodium acetate and 4.28 g L^{-1} of 1,4-butanediol were found in the first reactor. Concentration of 0.59 g L^{-1} of the latter was also present in the second reactor. Neither acetate nor butanediol were found in the third to fifth reactor. A maximum biomass concentration of 2.33 g L^{-1} was found in the second reactor. PHA content of the biomass was 17% (mass fraction) in this reactor and decreased over the following reactors to reach 4% in the fifth reactor. After 25 days, samples were plated out and 4 different strains denoted 4V1, 4V2, 4V4, and 4V7 were isolated. PHA accumulation by these strains was tested in rolling-culture experiments and is summarized in Table 1. It should be noted that, despite the presence of 1,4-butanediol in the accumulation medium, only poly(3-hydroxybutyrate) homopolymers were found and neither strain produced a poly(3-hydroxybutyrate-co-4-hydroxybutyrate) copolymer.

In a second experiment a soil sample from the Sageney riverbanks (Canada) was used as inoculum. The SGI reactor was used as the first stage, the air-lift reactor was used as the second stage, and Quickfit reactors were used as the third, fourth and fifth stage, of the reactor cascade. The feed rate to the first stage was 35 mL h^{-1}, and the recycle rate was set to 0. Analysis of samples taken after 12 days showed an even higher degree of carbon limitation than in the first enrichment experiment. In all reactors the acetate concentration was below detection level and 1,4-butanediol was only found in a concentration of 1.03 g L^{-1} in the first reactor. Small amount of ammonium sulfate (0.14 g L^{-1}) was found in the third to fifth reactor and this could be due to protein degradation of starving cells *(16)*. A maximum biomass concentration of 1.19 g L^{-1} was found in the first reactor. PHA content of the biomass was 3% in this reactor and decreased over the following reactors to reach zero in the third reactor. Samples were plated out and 10 different strains, denoted 5I2, 5I3, 5III1, 5III2, 5III3, 5III4, 5IV5, 5V2 and 5V5, were isolated. PHA accumulation was tested in rolling-culture experiments and is summarized in Table 1.

In a third enrichment experiment sewage sludge from a pulp-and-paper waste water treatment plant was used as inoculum. The same setup as in the previous experiment was used. The feed rate was 31 mL h^{-1} and the recycle rate was 125 mL h^{-1}. Analysis of a biomass sample taken from the fifth reactor, after 12 and 18 days showed a PHA mass fractions of the biomass of 6 and 3%, respectively. After 39 days, samples were plated out and only 2 different strains, denoted 6V1 and 6V2, were isolated. PHA accumulation was tested in rolling-culture experiments and is summarized in Table 1.

Control experiment with Ralstonia eutropha and Escherichia coli

In order to show the efficiency of the enrichment method under defined conditions, a control experiment

Table 1. PHA accumulation in rolling-culture by isolated strains from the enrichment experiments. Nitrogen-free mineral-salts buffer containing 10 g L^{-1} of sodium acetate and 3 g L^{-1} of 1,4-butanediol was used as accumulation medium.

Strain	w^a (PHA) in biomass / %	x^b (4-HB in PHA) / %
4V1	0	—
4V2	4	0
4V4	64	0
4V7	5	0
5I2	0	—
5I3	traces	traces
5III1	1	traces
5III2	20	10
5III3	traces	0
5III4	0	—
5IV5	0	—
5V2	0	—
5V5	14	0
6V1	27	0
6V2	0	—

[a]w = mass fraction, [b]x = amount of substance (molar) fraction

with one fast-growing non-accumulating and one slow--growing, but PHA-accumulating bacterial strain was carried out. *E. coli* and *R. eutropha* were chosen and their specific growth rates were determined in batch fermentations as 0.43 and 0.28 h^{-1}, respectively (fermentation data not shown). The death rate of the two microorganisms under carbon-starvation conditions were determined as described in Materials and Methods. Over 7 days, *R. eutropha* showed no loss of viability, whereas *E. coli* cells died with a death rate of 0.011 to 0.015 h^{-1}. Shaking-flask (300 mL) cultures of both strains were mixed and used as inoculum for the enrichment plant. Braun reactors were used as the first and fifth stage, the SGI reactor was used as the third stage, and Quickfit reactors were used as the second and fourth stage. The feed rate and the recycle rate were both set to 20 mL h^{-1}. In this experiment all reactors were sterilized prior to sterily assembling the reactor cascade and no contamination occurred throughout the duration of experiment. The feed medium contained 10 g L^{-1} of glucose instead of acetate and 1,4-butanediol as the carbon source. For six days samples were taken from the first, third and fifth reactor every 24 hours and the viable-cell counts of both strains were determined in the same way as in the determination of the death rate. Results are shown in Table 2. As it is shown in Fig. 1, *R. eutropha* made up for

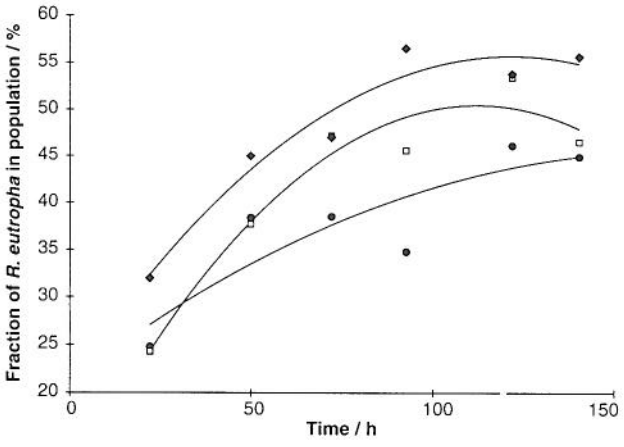

Fig. 1. Composition of the *Ralstonia eutropha* – *Escherichia coli* mixed culture during the enrichment experiment (● Reactor I, □ Reactor III, ◆ Reactor V).

Table 2. Viable-cell counts for *Ralstonia eutropha* and *Escherichia coli* during the enrichment experiment. Results are given in 10^{-7} CFU / mL.

Time/h	*Ralstonia eutropha*			*Escherichia coli*		
	R I	R III	R V	R I	R III	R V
22.0	74	27	23	224	84	49
50.3	66	68	27	106	113	33
72.3	116	67	30	185	75	34
93.0	106	87	35	199	104	27
121.8	115	78	36	135	68	31
140.5	101	92	34	124	97	27

25% of the total population at the beginning of the experiment, whereas after only 6 days it had enriched to more than 55%.

Discussion

Although the interest in new strains for the bacterial production of PHAs is high, hitherto no attempts to selectively isolation of PHA-accumulating bacteria from environmental samples have been described. The isolation of PHA-degrading microorganisms can simply be achieved by offering PHAs as the sole carbon source in the isolation process *(17)*, but such clear selection criteria are missing for the isolation of PHA-accumulating bacteria.

In the past, continuous culture methods have proved very useful for the selective enrichment of microorganisms from mixed cultures or the enrichment of mutants from axenic cultures. Usually the competition for the growth limiting substrate is used as the selection criterion. The organism with the highest growth rate under the prevailing conditions will completely replace all competitors *(18)*. Because PHA accumulation is not an essential requisite for growth *(7)*, the creation of a strong selective pressure is more difficult. The physiological role of PHA in bacteria has been studied extensively and it seems to be mainly one of osmotically neutral carbon and energy reserve material, enabling the survival under long term carbon- and energy-limiting conditions *(4, 7–13)*. In some organisms PHA production seems to be a possibility to generate $NAD(P)^+$ when the respiratory NADH oxidation is restricted *(19–21)*. More recently a low molecular mass form of the polymer has been found as a component of transmembrane ion transport systems in both prokaryotic and eukaryotic cells *(22)*.

Only the survival behavior under carbon starvation seemed to be suited to serve as selection criterion and was employed for our experiments. We were aware of difficulties in establishing such selective conditions, because interactions between bacteria in mixed cultures, such as antibiotic production, the possible excretion of organic substances or the production of other reserve materials are hardly known and can not be controlled. However, as it can be deduced from the PHA content of the mixed cultures, after enrichment PHA-accumulating bacteria made up for a significant fraction of the population.

A total of 15 different bacterial strains were isolated in the experiments. Four of them were able to accumulate high amounts of PHA (> 10%), 3 accumulated minor amounts (1–10%), 2 strains accumulated trace amounts and 6 showed no PHA production at all. More bacterial strains must have been present in the cultures, but due to the dilution of the samples taken before plating-out, strains, which were only present in low concentrations, were lost during the isolation process. Colony form and cell morphology under the phase-contrast light microscope were used for determining and excluding double isolates in one experiment.

1,4-butanediol was added during enrichment as a second carbon source, because we were interested in mainly isolating bacteria which are insensitive to this

substance and capable of producing poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3-HB-co-4-HB)] copolymers from it. Of all 15 strains isolated only 3 were able to produce such a copolymer on 1,4-butanediol. This is in accordance with the findings of Valentin *et al. (23)* studying the PHA metabolism of *Ralstonia eutropha*, and with observations we have made in other experiments, showing that the capability of PHA-accumulating bacteria to utilize 1,4-butanediol as carbon source is no indicator for possible P(3-HB-co-4-HB) production *(24)*.

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Selektivno obogaćivanje bakterija polihidroksialkanoatima višestupanjskim kontinuiranim uzgojem

Sadržaj

Odnos između udjela polihidroksialkanoata (PHA) u stanicama i njihova preživljavanja u uvjetima nedostatka ugljika iskorišten je za razradu postupka kojim se iz uzoraka bakterija iz okoliša mogu selektivno izdvojiti one koje akumuliraju PHA. U prvi reaktor petostupanjskog kontinuiranog uzgoja dodavala se podloga u velikom razrjeđenju, bogata izvorima ugljika, a siromašna dušikom. Pod tim su uvjetima određeni mikroorganizmi ne samo rasli već i nakupljali PHA. U sljedećim stupnjevima uzgoj se održavao pod ograničenim količinama izvora ugljika i dušika tijekom 100–200 sati. Iz raznih uzoraka bakterija iz okoliša izolirano je 15 različitih sojeva. Četiri su soja akumulirala PHA više od 10%, tri samo manju količinu (1–10%), dva soja samo u tragovima, a šest sojeva nije proizvelo PHA. Proveden je i kontrolni pokus s miješanom populacijom koja je sadržavala soj (Ralstonia eutropha) što akumulira PHA i soj (E. coli) koji ne proizvodi PHA. U tom je pokusu postignuto značajno obogaćivanje s PHA soja R. eutropha koji je polagano rastao.