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Accumulation of polyhydroxyalkanoates by *Microlunatus phosphovor* under various growth conditions

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Abstract *Microlunatus phosphovor* is an activated-sludge bacterium with high levels of phosphorus-accumulating activity and phosphate uptake and release activities. Thus, it is an interesting model organism to study biological phosphorus removal. However, there are no studies demonstrating the polyhydroxyalkanoate (PHA) storage capability of *M. phosphovor*, which is surprising for a polyphosphate-accumulating organism. This study investigates in detail the PHA storage behavior of *M. phosphovor* under different growth conditions and using different carbon sources. Pure culture studies in batch-growth systems were conducted in shake-flasks and in a bioreactor, using chemically defined growth media with glucose as the sole carbon source. A batch-growth system with anaerobic–aerobic cycles and varying concentrations of glucose or acetate as the sole carbon source, similar to enhanced biological phosphorus removal processes, was also employed. The results of this study demonstrate for the first time that *M. phosphovor* produces significant amounts of PHAs under various growth conditions and with different carbon sources. When the PHA productions of all cultivations were compared, poly(3-hydroxybutyrate) (PHB), the major PHA polymer, was produced at about 20–30% of the cellular dry weight. The highest PHB production was observed as 1,421 mg/l in batch-growth systems with anaerobic–aerobic cycles and at 4 g/l initial glucose concentration. In light of these key results

regarding the growth physiology and PHA-production capability of *M. phosphovor*, it can be concluded that this organism could be a good candidate for microbial PHA production because of its advantages of easy growth, high biomass and PHB yield on substrate and no significant production of fermentative byproducts.

Keywords Activated-sludge systems · *Microlunatus phosphovor* · Polyhydroxyalkanoate · Poly(3-hydroxybutyrate) · Bacterial storage · PHA · PHB

Introduction

Microlunatus phosphovor is an activated-sludge bacterium which shows high levels of phosphorus-accumulating activity and phosphate uptake and release activities similar to those at activated-sludge conditions [1]. Therefore, it is an interesting model organism to study biological phosphorus removal.

Under anaerobic conditions, polyphosphate-accumulating organisms (PAOs) use the energy obtained from the hydrolysis of polyphosphate for substrate uptake and conversion to internal carbon reserves [2]. It is accepted that PAOs usually take up short-chain fatty acids, accumulate them in the form of polyhydroxyalkanoates (PHAs), and release phosphorus. Under aerobic conditions, those stored PHAs are used for growth, glycogen synthesis, and as an energy source to restore the polyphosphate pool [3].

Since its first isolation from activated sludge in 1995 [1], there have been some studies on the physiology and polyphosphate metabolism of *M. phosphovor*. However, there are almost no studies about its PHA storage capability, which is surprising for a PAO. In only one study, the phosphorus and carbon metabolism of *M. phosphovor* was investigated using repetitive

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anaerobic–aerobic cycles in a sequential batch reactor with ^{13}C -labeled glucose. However, the organism showed typical phosphorus uptake and release which was not coupled to PHA turnover [3]. Anaerobically assimilated glucose was not used by *M. phosphovorius* for growth, but was fermented to acetate. Acetate was not converted to PHA, but utilized aerobically for growth [3]. The fact that *M. phosphovorius* lacks key metabolic characteristics so that it neither takes up acetate nor accumulates PHA under anaerobic conditions has also been stated elsewhere [4]. Thus, the metabolism of *M. phosphovorius* is not typical for a PAO [5].

The aim of this study was to characterize the PHA storage behavior of *M. phosphovorius* in detail, under different growth conditions and using different carbon sources. For this purpose, pure culture studies in batch-growth systems were conducted in shake-flasks and in a bioreactor, using chemically defined growth media with glucose as the sole carbon source. A batch-growth system with anaerobic–aerobic cycles and varying concentrations of glucose or acetate as the sole carbon source, similar to enhanced biological phosphorus removal (EBPR) processes, was also employed. PHA analyses of culture samples were made both qualitatively (microscopic staining) and quantitatively (gas chromatography analysis). The obtained results for different cultivations were compared with each other and discussed.

Materials and methods

Microorganism and growth medium

M. phosphovorius (DSM 10555) was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). For all cultivations, M9 minimal medium was used with varying concentrations of glucose (Sigma, USA) or acetate (Sigma) as the sole carbon source. The other ingredients (per 1 l of this medium) were 12.8 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 3.0 g K_2HPO_4 , 0.5 g NaCl, 1.0 g NH_4Cl , 1 ml of 1 M MgSO_4 stock solution, and 1 ml of 0.1 M CaCl_2 stock solution. The pH of the medium was adjusted to 7.4.

Culture conditions

Batch cultivations in three parallel shake-flask sets were performed using 1-l baffled Erlenmeyer flasks (Sigma) with 250 ml culture volume. The inoculum size was 4%. The cultivations were performed at 30°C and 250 rpm in an orbital shaker (model S-2; Certomat, Germany).

Aerobic batch cultivations in a 2 l-bioreactor (Biostat B; Braun, Germany) were performed at 30°C and 250 rpm with 1.5 l working volume and 20% inoculum size. The air flow rate was set to 3 l/min. The preculture was grown overnight at 30°C and 250 rpm in an orbital shaker (Certomat S-2) to a final optical density at 600 nm (OD_{600}) of 1.04, and centrifuged at 5,320 g for 10 min

(Allegra 25R; Beckman Coulter, USA). The resulting cell pellet was then resuspended in an equal volume of fresh medium and used to inoculate the bioreactor medium.

Anaerobic–aerobic cycles in a batch growth system were performed according to Nikata et al. [6], with some modifications. Briefly, 50 ml of culture were grown in 250-ml Erlenmeyer flasks at 30°C and 250 rpm for 9 h during aerobic cultivation. Subsequently, anaerobic cultivation was performed by transferring the centrifuged cell pellets of aerobic cultivation into 50-ml centrifuge tubes containing 50 ml of fresh medium sparged with N_2 gas prior to cultivation and incubating the culture anaerobically at 30°C and 100 rpm for 15 h. This was followed by transfer and aerobic incubation of cells in Erlenmeyer flasks as described above, but without changing the medium. Centrifugation to obtain cell pellets prior to transfers was done at 5,320 g for 10 min (Allegra 25R). The anaerobic–aerobic cycles were repeated eight times by supplying fresh medium only once at the beginning of each cycle, which was the beginning of the anaerobic phase.

Analytical methods

Cell growth was monitored spectrophotometrically (UV-1601; Shimadzu, Japan) by OD_{600} measurements and by cellular dry weight (CDW) determinations using 0.2- μm filters (Schleicher & Schuell, Germany). Briefly, 10-ml culture samples were filtered on preweighed filters using a vacuum filtration system. The filters were heated at 105°C for 1 h and cooled in a desiccator for 30 min prior to weighing. Biomass concentrations were calculated using a CDW versus OD_{600} calibration line.

Residual glucose concentrations in filtered culture supernatants were determined using a HPLC system (Shimadzu) with a refractive index detector (RID-10A; Shimadzu) and Class-VP software (Shimadzu). A HPX87H column (BIORAD, USA) was used for the analysis. The mobile phase was 2.5 mM H_2SO_4 and the column temperature was 60°C.

The concentrations of acetate and other volatile fatty acids (propionate, butyrate, isobutyrate, valerate, isovalerate) in filtered culture supernatants were determined using a HP gas chromatography (GC) system (Agilent Technologies, USA) equipped with a DB-FFAP capillary column (J&W Scientific, USA) and ChemStation Software (Agilent Technologies). The injection (splitless) temperature was 260°C and the flame ionization detector (FID) temperature was 300°C. The carrier gas was helium. The column pressure was set to 145.3 kPa and the flow rate was 4.5 ml/min. In the detector, the hydrogen flow was set to 40 ml/min, airflow to 400 ml/min, and the make-up flow (helium) to 30 ml/min. The oven temperature had the following profile: initially at 100°C for 5 min, rising at 10°C/min to 160°C and holding for 5 min, then rising at 80°C/min to 230°C and holding for 3 min. A volatile fatty acids (VFA) standard solution (0.1% in water) was used for calibrations (FA-MIX-02; Alltech, USA).

For PHA sample preparation and analysis, the following procedure was used. Culture samples (10 ml) were taken into glass tubes containing formaldehyde and centrifuged at 5,320 g for 7 min (Allegra 25R). The resulting cell pellets were collected in a tube and frozen at -20°C and then freeze-dried at -50°C for 48 h. They were then crushed, weighed, and put in glass tubes with PTFE-lined screw caps (GL18; Schott, Germany). Then, 50 μl of benzoic acid (internal standard) was added and the tubes were boiled at 100°C for 2 h, with shaking every 15 min. After they cooled, 3 ml of distilled water were added and the tubes were shaken for 10 min, centrifuged at 1,330 g for 5 min at room temperature, and left for phase separation. An amount of 1 ml was taken from the lower (organic) phase and filtered by passing through a sodium sulfate column. The PHA concentrations were then determined using a GC system (Agilent Technologies) equipped with an Innowax capillary column (J&W Scientific). The data were analyzed using Chemstation software (Agilent Technologies). The injection split (10:1 ratio) temperature was 200°C and the FID detector temperature was 250°C . The carrier gas was helium. The column pressure was set to 101 kPa and the flow rate was 3 ml/min. In the detector, the hydrogen flow was set to 30 ml/min, airflow to 400 ml/min, and the make-up flow (helium) to 25 ml/min. The oven temperature had the following profile: initially at 80°C , rising at $25^{\circ}\text{C}/\text{min}$ to 130°C , then rising at $15^{\circ}\text{C}/\text{min}$ to 210°C and holding for 12 min. Known concentrations (1, 2, 3 mg/l) of a poly(3-hydroxybutyrate) (PHB) standard (Sigma-Aldrich, USA), a PHB/PHV copolymer powder, and beads (Sigma-Aldrich) were used for calibrations.

Microscopy

PHB staining was done using Sudan black B (0.3% in 60% ethanol) and Safranin O (0.5% aqueous solution) solutions [7]. The microscopy samples were examined under 1,000 \times magnification (oil immersion) using a light microscope (BX60; Olympus, Japan) equipped with a camera and imaging software (Spot Advanced Software, Japan).

Results

Aerobic batch cultivations in shake-flasks

In the first part of this study, the growth behavior of *M. phosphovorus* was investigated in a defined medium (M9) under aerobic batch-growth conditions in three parallel shake-flask experiments. Glucose (4 g/l) was used as the sole carbon source. Cell densities, volatile fatty acids, and residual glucose concentrations in the medium were determined at various time-points during cultivation. The changes in cell density (OD_{600}) and residual glucose concentration with respect to cultivation time are shown in Fig. 1. Under shake-flask growth

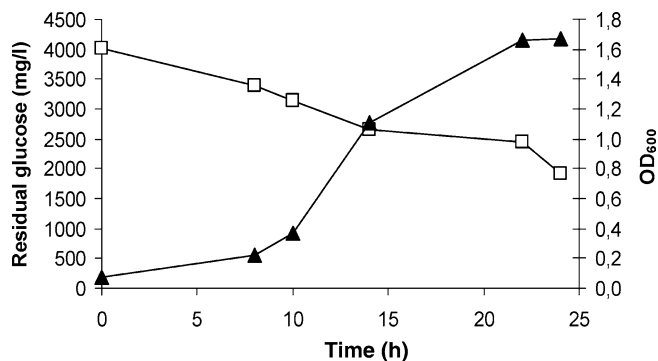


Fig. 1 The OD_{600} values (filled triangles) and residual glucose concentrations (open squares) of *M. phosphovorus* grown in baffled shake-flasks using a defined medium containing 4 g/l glucose as the sole carbon source

conditions, *M. phosphovorus* could utilize only up to about 50% of the initial glucose provided at 4 g/l in the medium and the cells entered the stationary phase at about 25 h of cultivation, by attaining a constant OD_{600} level. Additionally, only low levels of acetate were detected in the culture medium, starting with 157 mg/l and decreasing to 62 mg/l at the end of the cultivation. No other volatile fatty acids were detected in the culture medium. The average final cellular dry weight concentration was 1,103 mg/l and the biomass yield on glucose ($Y_{x/s}$) was calculated as 0.53 g CDW/g glucose.

Aerobic batch cultivations in a bioreactor

In order to monitor the growth and storage behavior of *M. phosphovorus* under controlled conditions, aerobic batch cultivations were performed in a 2-l bioreactor with 1.5 l working volume. As in the shake-flask experiments, the same defined medium was used, with 4 g/l glucose as the sole carbon source. Cell densities, volatile fatty acids, and residual glucose concentrations in the medium were determined at various time-points during cultivation. Additionally, culture samples were taken for

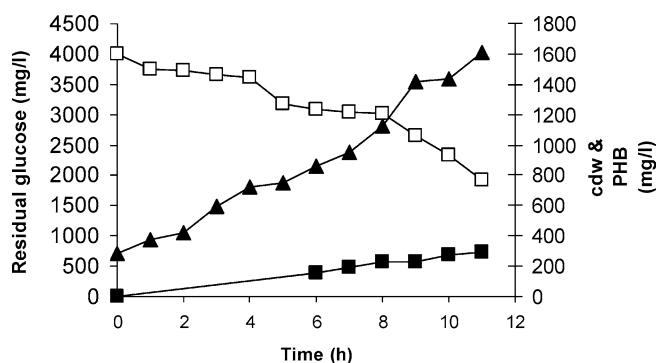


Fig. 2 Residual glucose (open squares), CDW (filled triangles), and PHB (filled squares) concentrations of *M. phosphovorus* batch-grown aerobically in a bioreactor using a defined medium containing 4 g/l glucose as the sole carbon source

Table 1 CDW, PHB, and PHV concentrations obtained during various cultivations in this study

Sample	CDW (mg/l)	PHB (% of CDW)	PHB (mg/l)	PHV (% of CDW)	PHV (mg/l)
Bioreactor, $t = 6$ h	860.3	19.78	170.2	0	0
Bioreactor, $t = 7$ h	954.0	21.79	207.9	0	0
Bioreactor, $t = 8$ h	1,127.7	23.34	263.2	0	0
Bioreactor, $t = 9$ h	1,408.3	20.50	288.7	0	0
Bioreactor, $t = 10$ h	1,433.7	27.01	387.3	0	0
Bioreactor, $t = 11$ h	1,610.1	22.43	361.2	0.16	2.6
Bioreactor, $t = 24$ h	1,626.6	23.97	390.0	0.33	5.3
Batch, anaerobic/aerobic cycles (1 g/l glucose)	2,016.2	15.80	316.8	0.18	3.7
Batch, anaerobic/aerobic cycles (2 g/l glucose)	2,668.6	33.15	882.3	0.22	5.9
Batch, anaerobic/aerobic cycles (4 g/l glucose)	5,485.7	25.91	1,421.4	0.20	11.2
Batch, anaerobic/aerobic cycles (2 g/l acetate)	2,938.7	23.23	682.6	0.16	4.6

both qualitative (microscopic staining) and quantitative (GC analysis) estimation of possible PHA formation at different time-points in cultivation. Figure 2 shows the changes in CDWs, PHB, and residual glucose concentrations of the batch cultures in bioreactor with respect to cultivation time. As in the case of the shake-flask cultivations, the cells could only utilize up to 50% of the initial glucose provided at 4 g/l concentration. However, compared with the shake-flask cultivations, they had up to 50% higher final cell densities and attained a stationary phase at a shorter cultivation time. Significant amounts of PHB accumulation was observed, up to about 25% of CDW (Table 1). Additionally, *M. phosphovor* was found to contain PHV up to less than 0.5% of CDW; and thus the cells were producing PHB as the major polymeric form under these experimental conditions (Table 1). As in the case of the shake-flask experiments, the only detectable VFA in the culture medium was acetate, starting with 85 mg/l and decreasing to 18 mg/l at the end of the cultivation. The average final CDW concentration was 1,627 mg/l and the biomass yield on glucose ($Y_{x/s}$) was calculated as 0.78 g CDW/g glucose. The PHB yield on glucose ($Y_{p/s}$) was calculated as 0.19 g PHB/g glucose.

Repeated anaerobic–aerobic cycles in a batch-growth system

In this part of the study, the effects of repeated anaerobic–aerobic cycles on the batch growth and storage metabolism of *M. phosphovor* were investigated. Glucose (at 4.0, 2.0, 1.0, 0.5 g/l) or acetate (2 g/l) was used as the sole carbon source in M9 medium. Cell densities and residual glucose concentrations in the medium were determined at the end of both anaerobic and aerobic phases of each cycle. To monitor storage behavior, culture samples were taken for both qualitative (microscopic staining) and quantitative (GC analysis) estimation of possible PHA formation at the end of each cultivation. Table 2 summarizes the residual glucose concentrations of the cultures at the end of different growth phases for each cultivation with anaerobic–aerobic cycles. The glucose-utilization behavior of the cultivation at 4 g/l glucose was similar to those in shake-flasks and

bioreactor, utilizing here even less than about 50% of the glucose provided. When 2,000 mg/l glucose was provided, the amount of glucose was again higher than the cells could consume aerobically. With 1,000 mg/l and 500 mg/l initial glucose concentrations, however, the cells could consume all the glucose at the end of the aerobic growth phase in a cycle. Also, with these initial glucose concentrations, during anaerobic growth phases, they could consume almost all of the glucose (Table 2). A comparison of final CDW and PHA concentrations of each culture with anaerobic–aerobic cycles is given in Table 1. The highest PHB concentrations (1,421 mg/l) were obtained with anaerobic–aerobic cycles at 4 g/l initial glucose concentration, being about 26% of CDW. When acetate was used as the sole carbon source at 2 g/l initial concentration, PHB accumulation was still observed, though less than at 2 g/l initial glucose concentration. At 0.5 g/l initial glucose concentration, however, no PHB accumulation was observed (data not shown).

Figures 3, 4 show PHB(–) and PHB(+) cells, respectively, after microscopic staining. In cultures grown with 4, 2, 1 g/l glucose and 2 g/l acetate, staining of the culture samples gave PHB(+) results only from the beginning of the fifth cycle on. For these four cultivations, except from the culture grown at 1 g/l glucose, PHB accumulation was observed during both aerobic and anaerobic phases of these cycles, the intensity of staining being higher at the end of the anaerobic phases.

Table 2 Residual glucose concentrations at the end of the aerobic and anaerobic phases of batch cultivations with anaerobic–aerobic cycles at different initial glucose concentrations. The results are average values of three measurements

Initial glucose (mg/l)	Cycle phase	Residual glucose (mg/l)
4,000	Aerobic (end)	2,532 ± 87
	Anaerobic (end)	3,528 ± 73
2,000	Aerobic (end)	1,102 ± 73
	Anaerobic (end)	1,588 ± 66
1,000	Aerobic (end)	0 ± 0
	Anaerobic (end)	525 ± 70
500	Aerobic (end)	0 ± 0
	Anaerobic (end)	64 ± 13

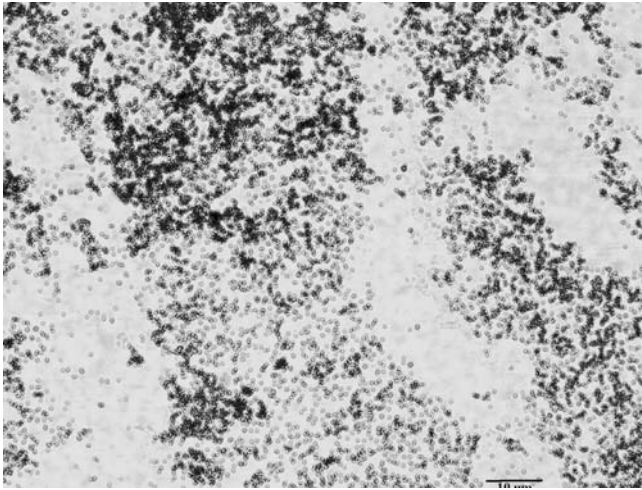


Fig. 3 PHB(-) cells observed under 1,000 \times magnification after PHB staining. The image was obtained at the end of the anaerobic phase of the second cycle during batch cultivation with anaerobic-aerobic cycles at 4 g/l glucose concentration

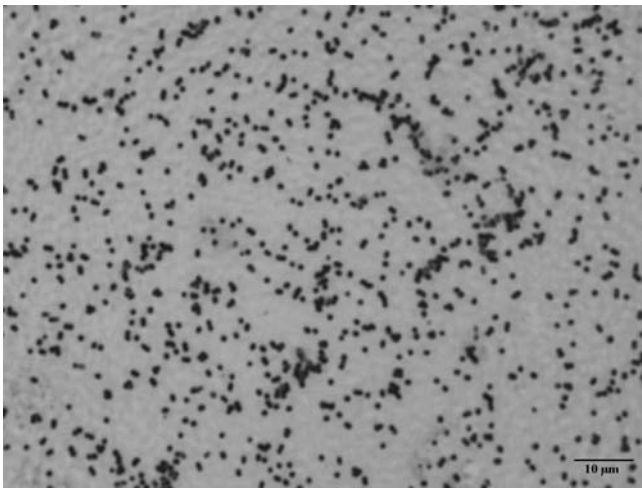


Fig. 4 PHB(+) cells observed under 1,000 \times magnification after PHB staining. The image was obtained at the end of the anaerobic phase of the sixth cycle during batch cultivation with anaerobic-aerobic cycles at 4 g/l glucose concentration

However, in the case of the 1 g/l glucose culture, PHB(+) cells were observed only in the anaerobic phases of the cycles. In the culture grown with 0.5 g/l glucose, however, no PHB(+) cells were observed throughout the cultivation. In parallel with the quantitative results obtained for PHB accumulation, the intensity of the PHB staining increased as the initial glucose concentration increased, with the highest accumulation at 4 g/l initial glucose concentration.

Discussion

This study investigated in detail the PHA storage behavior of *M. phosphovorus* under different growth conditions and using different carbon sources. Pure

culture studies in batch-growth systems were conducted in shake-flasks and in a bioreactor, using chemically defined growth media with glucose as the sole carbon source. A batch-growth system with anaerobic-aerobic cycles and varying concentrations of glucose or acetate as the sole carbon source, similar to EBPR processes, was also employed.

Despite the limited literature information that *M. phosphovorus* shows typical phosphorus uptake and release which is not coupled to PHA turnover [3], the results of our study demonstrates for the first time that *M. phosphovorus* produces significant amounts of PHAs under various growth conditions and with different carbon sources. In contrast to the general assumption that, under anaerobic conditions, PAOs accumulate PHAs and then use them for growth and as an energy source under aerobic conditions, we also observed PHA production under aerobic batch-growth conditions with glucose as the sole carbon source. In our batch-growth systems with anaerobic-aerobic cycles, however, the highest intensity of PHB(+) staining was observed in those cells at the end of the anaerobic phase of the cycle. This may imply that, in batch systems with anaerobic-aerobic cycles, the PHA production of *M. phosphovorus* occurs also during the anaerobic phase. The concentration and degree of utilization of the carbon source certainly becomes important here: we observed PHB(+) stained cells during both aerobic and anaerobic phases of the cycles when the initial glucose concentration was 4 g/l and 2 g/l. At the end of the aerobic phase, the cells still could not consume all of the glucose supplied during the anaerobic phase. Thus, they did not need to utilize the PHA produced during the anaerobic phase. This may explain the PHB(+) cells observed during the aerobic phases of growth in anaerobic-aerobic batch cycle systems. Indeed, we observed PHB(+) cells only during anaerobic phases of the batch cycles when 1 g/l glucose was provided as the sole carbon source. Our residual glucose data showed that, at 1 g/l glucose concentrations, *M. phosphovorus* consumes the majority of the glucose at the end of the aerobic cycle and thus it may be utilizing its PHA during the aerobic phase of growth. At 0.5 g/l glucose in an anaerobic-aerobic batch system, no PHB(+) cells were observed in any of the phases. This is probably due to a rapid consumption of glucose, followed by a rapid production and consumption of PHA.

Another interesting finding is that, in a batch-growth system with anaerobic-aerobic cycles at 2 g/l acetate as the sole carbon source, PHA accumulation was observed. PHB(+) cells were obtained during both aerobic and anaerobic phases of growth. This result clearly demonstrates the utilization of acetate and its conversion to PHA, which seemingly disagrees with the previous findings that *M. phosphovorus* respire acetate during the aerobic stage, without the formation of carbon reserves [3]. However, the growth medium used in that study contained only 0.25 g/l glucose and there was also yeast extract in the medium. In our experiments, we

used a chemically defined growth medium with glucose or acetate concentrations at 0.5 g/l and higher. Thus, the importance of medium composition becomes clear: at 0.5 g/l glucose concentration, we did not observe any PHA accumulation, either, which does not disagree with the literature [3]. Additionally, we observed PHA production on acetate at 2 g/l acetate concentration, a reasonably high value.

In a previous study with a newly isolated bacterium that morphologically and physiologically resembles *M. phosphovorius*, it was demonstrated that the bacterium showed anaerobic utilization and aerobic accumulation of polyP only after alternating anaerobic and aerobic conditions were applied a few times [8]. It was thus concluded that the enzyme system for the polyP metabolism is not constitutive but inducible. A similar behavior was also observed in the case of the PHB(+) staining of the cell samples taken from each anaerobic–aerobic cycle in our batch-growth system. In all cultivations, PHB(+) cells appeared only starting from the fifth anaerobic–aerobic cycle, indicating also a possibly inducible mechanism for PHA production by *M. phosphovorius*.

When PHA productions of all cultivations were compared, it was observed that, in all cultivations, almost exclusively one type of PHA polymer, PHB, was formed. Additionally, the PHB produced was about 20–30% of the CDW. The highest PHB production was observed in batch-growth systems with anaerobic–aerobic cycles and at 4 g/l initial glucose concentration. The final PHB concentration in this system was 1,421 mg/l. As the initial glucose concentrations decreased, there was a parallel decrease in the PHB produced. At 2 g/l acetate as the initial substrate, significant PHB production was also observed. Compared with a simple batch-growth system, feeding the system at each cycle during an anaerobic–aerobic batch system certainly improved biomass and PHB production significantly.

In the light of our key results regarding the growth physiology and PHA-production capability of *M. phosphovorius*, it can be concluded that *M. phosphovorius* could be a good candidate for microbial PHA production. The major advantage is easy growth and production of biomass as the major product upon a low-level glucose utilization. Most of the carbon taken up by the cells seems to be used for biomass and hence PHA production. Unlike *Escherichia coli*, there is no significant production of fermentative byproducts like acetate, etc., even in the presence of high glucose concentrations. This may significantly increase the PHA product yield (up to 80% biomass, about 20% PHB yield on glucose in our batch cultivation in a bioreactor). In previous studies on metabolic flux distributions in two yeasts, *Saccharomyces cerevisiae* and *Pichia stipitis*, it was found that *S. cerevisiae* produced significant amounts of the fermentative product ethanol along with biomass formation under aerobic conditions. The other yeast, *P. stipitis*, however, consumed less glucose but produced

more biomass, with no significant fermentation products [9, 10]. The major physiological difference between the two yeasts is that the latter is an efficient pentose utilizer, whereas *S. cerevisiae* is unable to do so. As these differences could be explained by a difference in pentose phosphate pathway utilization by these yeasts, according to flux analysis studies, it is also likely that *M. phosphovorius* may be an efficient pentose utilizer. Thus, it is possible that it may grow on cheaper substrates like hemicellulose hydrolysates and produce PHA. This could be an industrially more feasible process for PHA production, rather than using expensive substrates like glucose. It remains thus to be investigated under which other growth conditions and with which other interesting substrates PHB accumulation occurs and to what extent.

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