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Study on the Effect of Levulinic Acid on Whey-Based Biosynthesis of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Hydrogenophaga pseudoflava*

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Abstract

Background and Objective: Production of polyhydroxyalkanoate copolyesters consisting of 3-hydroxybutyrate and 3-hydroxyvalerate units was for the first time studied using the production strain *Hydrogenophaga pseudoflava* based on sustainable raw materials. This strategy provides for increased cost efficiency in polyhydroxyalkanoate production and in enhanced material quality.

Materials and Methods: As a particularity, production of these poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolyesters was based on a novel substrate/co-substrate combination: whey permeate from dairy industry, acted as substrate for biomass and 3-hydroxy-butyrate biosynth-esis; on the other hand, levulinic acid, accessible from various renewable resources, was used as 3 hydroxyvalerate related precursor compound. The experiments were carried out on shaking flask scale using defined nutrient media.

Results and Conclusion: Applied during nutritionally balanced growth of *H. pseudoflava*, levulinicacid displays drastic growth inhibition at rather low concentrations of 0.2 g l^{-1} (growth inhibition constant K_i = 0.032), which suggests the careful supply of this compound in the first phase of cultivation. Under nitrogen-free cultivation conditions, inhibition of the strain´s metabolism by levulinic acid was less pronounced. Here, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) concentrations up to 4.2 g l^{-1} and volumetric poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) productivities up to 0.06 g l^{-1} h^{-1} were achieved in dependence on the precursor supply. Investigating poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) composition in setups supplied with differently composed whey/levulinic acid mixtures revealed 3-hydroxyvalerate fractions in the polymer between 0 and 0.6 mol mol^{-1}. This study successfully demonstrates the feasibility of combined utilization of different waste- and by-products from food industry and agriculture for generation of value-added 2nd generation biopolymers.

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1. Introduction

Current efforts to replace environmentally precarious end-of pipe products from petrochemistry by ecologically benign compounds based on renewable resources also encompass materials with plastic-like characteristics [1]. Here, numerous activities are reported to make production of "green" alternatives like polyhydroxyalkanoates (PHA) efficient in terms of both economics [2] and material performance [3]. PHA, from the chemical point of view prokaryotic polyoxoesters of hydroxyalkanoates, are acc-umulated as highly organized "carbonosomes" by numerous prokaryotic microbes, acting as carbon and energy storage and protectants against exogenous stress factors [4,5]. Typical conditions boosting microbial PHA biosynthesis are excessive availability of carbon substrates in parallel to limitation of other growth essential nutrients like nitrogen or phosphate [6]. Only in the recent years, it was demonstrated that also oxidative stress [7] or suboptimal addition of certain organic solvents [8] provoke increased PHA accumulation. Moreover, presence of PHA granules offers the enzymatic machinery in microbial cells a chaperon function against heat [9], freezing [10], or high osmolarity [11].

Techno-economic aspects are currently addressed by investigating inexpensive raw materials as next generation feedstocks for PHA production [12]. Such "2nd generation

feedstocks" are identified among various by-products from agriculture and food processing [13-18]. To an increasing extend, CO_2 from exhaust gas is investigated as feedstock for production of "3rd generation PHA" by hydrogen oxidizing microbes [19-21] or phototrophic cyanobacteria [22, 23], whereas applying recombinant organisms for CO_2-based PHA biosynthesis is referred to as production of "4th generation PHA" [24]. These next generation feedstocks constitute sustainable alternatives to "1st generation feed-stocks" like purified sugars or noble oils typically used for large scale biotechnology, which are in direct conflict with nutritional purposes [25]. In this context, whey, a side stream of dairy industry, constitutes a prime example of such novel feedstocks due to its high lactose content. While this high lactose content causes severe ecological problems when disposing surplus whey, it provides at the same time a precious carbon source for biotechnological purposes [26-29]. Beside the selection of adequate feedstocks, economic efficiency of PHA production is also influenced by the applied techniques for PHA recovery from microbial cells [30-33], by the selected process regime [34,35], or by switching to mixed culture approaches [36,37]. For monoseptic cultivations, powerful novel production strains are needed which convert such inexpensive feedstocks at high yields and high productivity [38,39].

Material quality of PHA is highly determined by the polyester´s monomeric composition. In this context, one has to emphasize that PHA copoly-esters such as poly(3-hydroxybutyrate-*co*-3-hydro-xyvalerate) (PHBHV) display advanced material properties in terms of lower crystallinity, melting temperature, or glass transition point if compared to the most frequently occurring and best described representative of PHA, namely the rather brittle homopolyester poly(3-hydroxybutyrate) (PHB) [27,40,41]. Hence, copolyesters are the materials of choice when selecting the adequate PHA for processing towards vendible items, e.g., via melt extrusion, injection molding, or 3D-printing [42,43]. Materials processed by these techniques find use in different market fields, such as food packaging or medical applications [1,44]. Unfortunately, copolyester production, in most cases, requires the supplem-entation of 3-hydroxyvalerate (3HV)-related precursor substr-ates. Such precursors, predominately odd-numbered alkanoates like propionate or valerate, significantly contribute to the entire PHA production costs and often severely hamper growth and PHA production kinetics [25,27]. Therefore, one firstly has to search for inexpensive 3HV-precursors, and, secondly, to carefully evaluate the maximum acceptable actual concentr-ation level of these precursors for a given substrate-precursor-strain combination in order to avoid severely affecting the strain´s kinetics.

In the field of generating new, simple 3HV precursors, advanced strategies were developed based on the sophisticated conversion of abundant raw materials. Here, the oxidative ozonolysis of unsaturated fatty acids in waste lipids results in a cocktail of odd-numbered carboxylic acids which were successfully used for 3HV formation by the strain *Cupriavidus nectar* [36]. Moreover, acidic transformation of wood residues in a wicrowave process ("liquefied wood") provides a mixture rich in compounds like the 3HV precursor levulinic acid (LA) [46].

The bacterium *Hydrogenophaga (H.) pseudoflava* forms yellow rods (Fig. 1), grows optimally at 35-37ºC [47], and produces PHA from a range of diverse carbonaceous substr-ates, *inter alia* from sugars like lactose [48,49]. The conversion of hydrolyzed or intact whey lactose towards PHA was shown previously [26,50]. Also PHA copolyesters consisting of 3HB, 3HV, or 4HB building blocks are accumulated by this strain [26,48,49,51]. Whereas the supplementation with propionic acid generates PHBHV [46], both PHBHV and poly(3-hydroxybutyrate-*co*-4-hydroxy-butyrate) are accessible when co-feeding glucose plus different lactones as 3HV or 4HB precursors [49,50]. Regarding PHBHV production, the beneficial role of sodium valerate (SV) in comparison to propionic acid was demonstrated with this strain [51]. In this context, LA constitutes a highly versatile compound accessible from carbohydrates [53,54] or by converting lignocellulosics [46]. Using LA, *Alcaligenes* sp. SH-69 has been reported to be capable of PHBHV biosynthesis [55]. Further, the production co- and terpolyesters containing mainly 3HV, 4-hydroxyvale-rate (4HV), 3-hydroxybutyrate (3HB) monomers has been reported co-feeding octanoic acid and LA; here, recombinant *Pseudomonas* (*P.*) *putida* and *Ralstonia* (*R.*) *eutropha* (today *Cupriavidus necator*) harboring the *Thiocapsa* (*T.*) *pfennigii* PHA-biosynthesis genes *encoding* for Class-III PHA synthase enzymes, were cultured to high cell densities and high PHA contents [56].

As demonstrated before, *H. pseudoflava* accumulates PHA already during the exponential growth phase [26]. This "growth associated PHA production" kinetic requires the feeding of 3HV-related precursors already during balanced growth in order to avoid the formation of blocks of PHB in growing PHA chains during the early stage of the process. Formation of such blocks might result in a heterogeneous, blocky structured product instead of the desired randomly distributed copoly-ester, which is known to make the polymer more attractive for processing. Hence, it was important to study the influence of this precursor on kinetics of the production strain not only during conditions boosting PHA accumulation, but also already during the growth phase. Currently, no reports at all are available for LA utilization by *H. pseudoflava*, neither during microbial growth, nor during PHA accumulation under unbalanced conditions. Therefore, the first part of the present study investigates the effect of LA on growth of *H. pseudoflava* if cultured on glucose as model main carbon source, whereas the second part focuses on PHBHV copolyester production by this organisms under nitrogen-limited cultivation conditions by co-feeding hydrolyzed whey as inexpensive main carbon source stemming from food production, and LA as 3HV-struc-turaly related precursor compound accessible from inexpensive surplus materials.

2. Materials and Methods

2.1 Strain and strain maintenance

H. pseudoflava DSM 1034 was obtained from the strain and culture collection DSMZ, Germany, as freeze-dried sample. Strain maintenance was done by culturing the cells on solid H3 medium containing 10 g l^{-1} glucose as substrate (composition reported previously by [26]); transfer of colonies to fresh solid medium was accomplished regularly in two-weeks intervals.

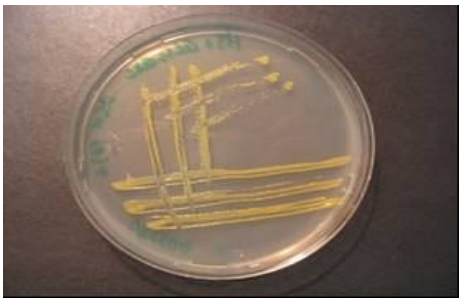

Figure 1. Typical yellow colonies of *H. pseudoflava* DSM 1034 cultivated on solid H3 medium containing hydrolyzed whey permeate as sole carbon source (own picture of the authors).

2.2 Impact of LA on growth of *H. pseudoflava*

For strain vitalization, fresh single colonies from H3 slants were transferred into 300 ml baffled shaking flasks containing 100 ml DSM 1 medium at 35°C, pH-value = 7.0. When the cultures had reached the late exponential phase (two days growth), 10 ml of culture broth was use to inoculate 250 ml of fresh DSM 1 medium in 1 l baffled shaking flasks. 10 ml each of this overnight pre-culture from the late exponential phase was used to inoculate a total of 12 shaking flasks containing 250 ml H3 medium (10 g l^{-1} of glucose, pH-value adjusted to 6.9) and the following concentrations of LA (g l^{-1}): 0; 0.2; 0.5; 1.0; 1.5; 2.0. The shaking flasks were cultivated under continuous shaking at 35ºC. Samples were taken about every 6 hours until the stop of the experiment after 48 h, and analyzed for optical density (OD), pH-value, LA-, SV-, glucose-, and ammonium con-centration. The last samples (after 48 h) were centrifuged (12,000 ×g, 20 min., Sorvall RC-5B Refrigerated Superspeed centrifuge), and the formed pellet was frozen and lyophilized for cell dry mass (CDM) and PHA analysis.

2.3 PHBHV copolyester production by *H. pseudoflava* on hydrolyzed whey permeate and LA

H. pseudoflava was inoculated from agar slants with H3 medium containing glucose (5 g l^{-1}) and galactose (5 g l^{-1}) as carbon source into four 300 ml baffled shaking flasks containing 100 ml DSM 1 medium. When the cells were at the end of the exponential growth phase, 20 × 1-l shaking flasks were inoculated with 10 ml of the pre-culture cultivated on DSM 1 medium. At the end of the exponential growth phase, the contents of all flasks were centrifuged and the resulting pellets from two of the setups were re-suspended in 250 ml of nitrogen-free medium. Medium pH-value: 7.0, composition (g l^{-1}): $NaHCO_3$, 0.47; Na_2HPO_4, 2.75; KH_2PO_4, 2.18; $MgSO_4$ $7H_2O$, 0.47; $CaCl_2$ $2H_2O$, 0.01; NH_4Fe (citrate) 0.047, SL6 4.7 (ml l^{-1}) and hydrolyzed whey permeate, 47 (ml l^{-1}). LA was added to the individual flasks at the following concentrations (g l^{-1}): 0, 0.2, 0.5 and 1.0. For comparison, SV was added as a second precursor in the concentration of 1 g l^{-1} in two flasks, together with 0.5 g l^{-1} LA. All flasks were inoculated at 35ºC and shaken continuously. Samples were taken in 6 h intervals and pH-value, OD, CDM, substrates (glucose, galactose, LA and SV) and PHA were analyzed. In order to avoid PHA degradation, additional precursor additions were carried out when concentrations were close to zero. At the end of the experiment, the polymer was extracted and analyzed with nuclear magnetic resonance (^{1}H-NMR; see 2.7).

2.4 Analytical procedures

- OD was measured at λ=420 nm against cell-free super-natant as zero-reference using a Spectronic genesys 2 PC spectrophotometer (purchased from Sigma Aldrich, Germany).
- For CDM determination, 5 ml of the liquid sample was centrifuged in pre-weighed glass tubes. The filtered supernatant was used for substrate (ammonium, glucose, galactose, LA) determination. The remaining pellet was frozen and lyophilized overnight to constant mass. After weighing, the difference between pellet containing and empty tube was used to calculate CDM.
- Ammonium ions were measured electrochemically with an ion-sensitive Orion electrode [57].
- The concentrations of glucose, galactose, and LA were measured using an HPLC equipment (purchased from Hewlett Packard, USA), consisting of a thermostated Aminex HPX 87H column (Bio-Rad, UK), an HP 7673 Contoller, a JASCO 880-PU intelligent HPLC pump, and a BISCHOFF RI-Detector 8110. The compounds to be analyzed were eluted with 0.005 M H_2SO_4 at a flow rate of 0.60 ml min^{-1}. Pure substrates (Sigma Aldrich) were used for external calibration.
- The frozen and lyophilized biomass pellets from CDM determination were used for GC-based PHA analysis via acidic methanolysis according to the standard process of Braunegg et al. [58]. As reference for external calibration, pure poly(3HB-*co*-19.1 mol% 3HV), Biopol™, Imperial Chemical Industries, UK, was used. Prior to injection, samples were neutralized after the acidic methanolysis with 10% aqueous $NaHCO_3$. The applied GC apparatus was an HP 6890 chromatograph (Hewlett Packard, USA) with an HP 6890 injector and a 15 m DB-WAX column. Flame ionization detection (FID) was used to monitor the methyl esters of the PHA monomers. He was used as carrier gas, H_2 and synthetic air as detector gases, and N_2 was used as auxiliary gas.

2.5 Substrate preparation

Hydrolysis of whey lactose, if used as substrate for *H. pseudoflava*, is beneficial for the strains´s kinetics, if compared to the utilization of non-hydrolyzed whey [24]. Enzymatic hydrolysis of lactose in whey permeate was performed by adding 2.5 ml of Maxilact 2000™, a commercially available *Klyveromyces lactis* β-galactosidase (EC 3.2.1.23) solution (DSM Food Specialties, UK), per liter whey permeate. The setup was continuously stirred for 25 hours at a pH-value of 6.5 and a temperature of 39°C. Completion of lactose hydrolysis was monitored via HPLC (*cf.* "Analytical procedures") [59].

2.6 Polymer recovery

PHA was isolated from microbial biomass as reported previously [45]. Shortly, cell suspension was in situ Pasteur-ized by heating (80°C, 30 min), centrifuged (12,000 ×g, 20 min., Sorvall RC-5B Refrigerated Superspeed centrifuge), frozen and lyophilized for 24 h. After degreasing the bioma-ss by overnight Soxleth-extraction with ethanol, PHA was Soxleth-extracted overnight with chloroform. Purity of the extracted PHA and completeness of extraction was determined by GC (*cf.* above). Exemplarily, pictures of cast films of PHBHV extracted from *H. pseudoflava* biomass cultivated on hydrolyzed whey are illustrated in Fig. 2.

Figure 2. Cast films of PHBHV extracted from *H. pseudoflava* biomass cultivated on hydrolyzed whey permeate (own pictures of the authors)

2.7 NMR characterization

^{1}H NMR spectra of PHA samples (5 g l^{-1} in $CDCl_3$) were recorded on a Varian Unity Inova 500 MHz Spectrometer (Varian Medical Systems, USA). Spectra were obtained at 20°C, 45° pulse, 6.0 s pulse repetition, relaxation delay 1.000 s, and 7000 Hz spectral width.

3. Results and Discussion

3.1 Impact of LA on growth of *H. pseudoflava*

H. pseudoflava was grown in H3 medium with different concentrations of LA as precursor substrate. During the experiment, OD and pH-value were monitored. In addition, substrate concentration and ammonium ion concentrations were measured during the whole experiment. CDM and PHA mass fraction in CDM were measured at the end of the experiment.

OD values of the different shaking flasks are illustrated in Fig. 3a-b. The cells grown on 10 g l^{-1} glucose as sole carbon substrate had a shorter lag phase as in setups containing LA. Already a LA concentration of 0.5 g l^{-1} caused a much longer lag phase and a slower growth in the exponential phase. After 40 h of growth, OD was highest for the cells grown with 0.5 g l^{-1} LA. When LA concentration exceeded 1 g l^{-1}, growth was significantly inhibited; at LA concentrations of 1.5 g l^{-1} and 2 g l^{-1}, practically no increase of OD was observed.

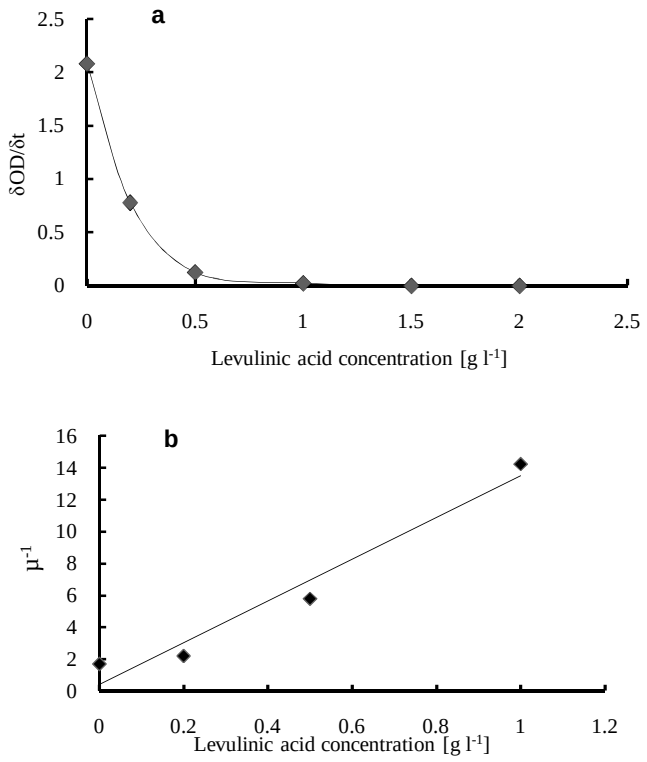

Figure 3. (a) Effect of levulinic acid (LA) concentration on the growth rate of *H. pseudoflava*, expressed as slope of optical density to time (δOD/δt); (b) Dixon plot for determination of growth inhibition constant K_i.

The growth rates of the cells, expressed as δOD/δt, were calculated for the individual LA between the h 15.15 and 19.45, hence from the end of the lag phase to the beginning of the exponential phase. As can be seen from Fig. 3a, the growth rate was significantly influenced by increasing LA concentrations. Based on these rates, specific growth rate μ was calculated as the quotient of δOD/δt and OD at t = 15.25 h, and used for drawing a Dixon plot by plotting the inverse value of μ *vs.* the LA concentration (Fig. 3b). By linear regression, the inhibition constant was calculated to be K_i = 0.032. This value is considerably lower than inhibition constants calculated for similar whey-based PHBHV production processes; e.g., in the case of *Burkholderia funghorum* (formerly known as *Pseudomonas hydrogenovora*), K_i for SV during growth phase was calculated with 1.84 g l^{-1} [27]. The low value of the inhibition constant signifies that the acid inhibits the growth of *H. pseudoflava* already in very low concentrations.

In addition to growth rates and OD, also the concentrations of the substrate glucose and the precursor LA were monitored. Figures 4 a-d illustrate the utilizations of the substrates and the corresponding OD values.

From Fig. 4 b-d, it can be seen that LA is used at faster rates than glucose. Both exponential growth phase and glucose conversion only starts after LA is completely depleted (Fig. 4 b,c). According to Fig. 3 d, OD stays at a low level as LA concentration is so high in the beginning that it cannot be utilized within 48 h; neither exponential growth nor glucose conversion was observed.

The ammonium sulfate concentrations in the media are consistent with the data from the OD measurements and the substrate analysis. Ammonium is used most rapidly when there is no LA present in the growth media. When LA concentration increases, less ammonium is consumed by the microbes (data not shown).

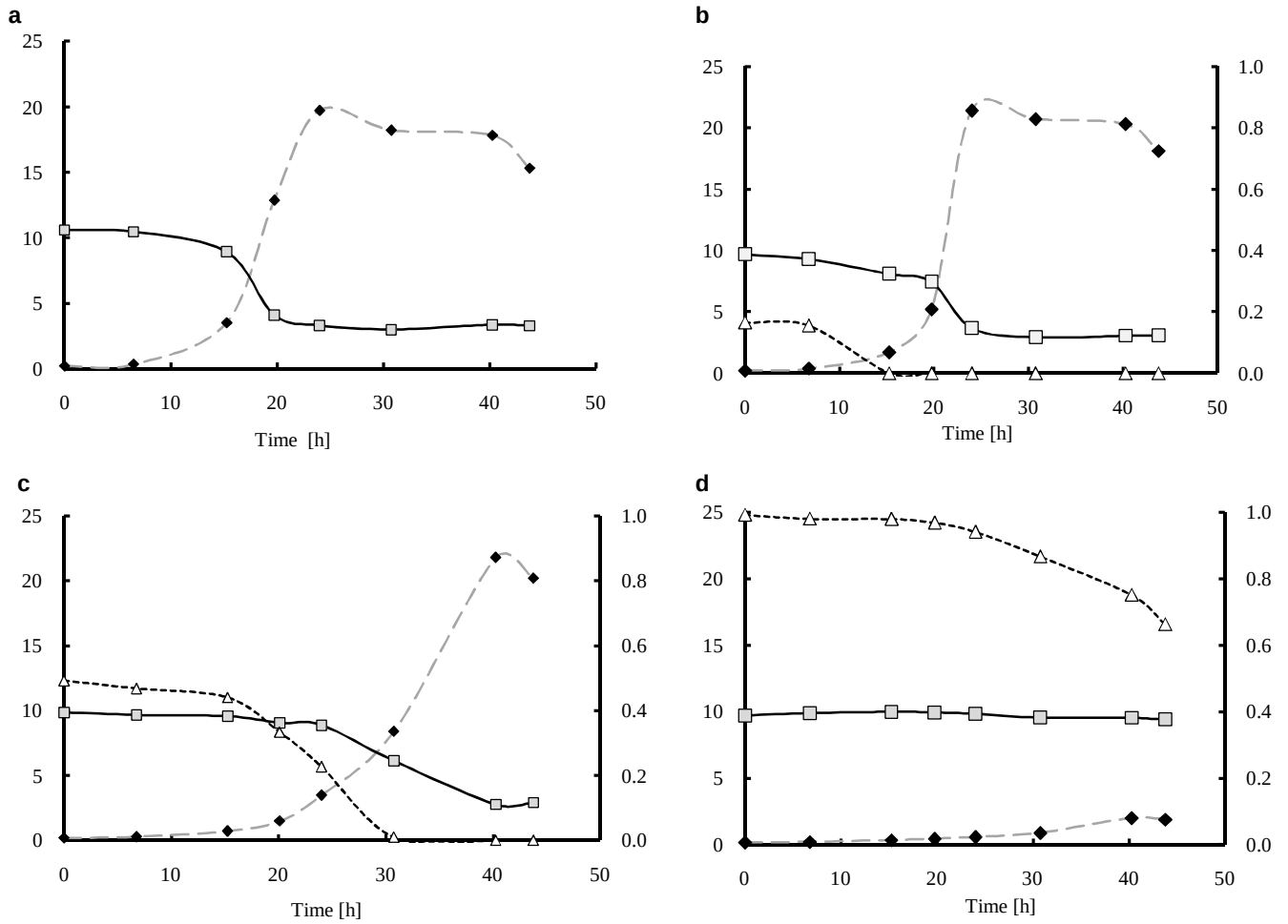

Figure 4. *H. pseudoflava* on glucose and different LA concentrations: (**a**) 0 g l^{-1}, (**b**) 0.2 g l^{-1}, (**c**) 0.5 g l^{-1} and (**d**) 1 g l^{-1}. Left axis: OD (rhombs), glucose concentration [g l^{-1}] (grey squares); right axis: LA concentration [g l^{-1}] (triangles).

GC-FID analysis revealed that the produced PHA constitutes a copolyester of 3HB and 3HV (PHBHV). After 48 h of growth, setups with 0.5 g l^{-1} LA displayed the highest CDM value, as shown in Fig. 5a. Also the total PHA concentration and the concentration of 3HB and 3HV were highest in setups with 0.5 g l^{-1} LA. At concentrations of 1.5 and 2.0 g l^{-1} LA, values for 3HB and 3HV were below the detection limit; the same was the case for 3HB concentration at 1 g l^{-1} LA.

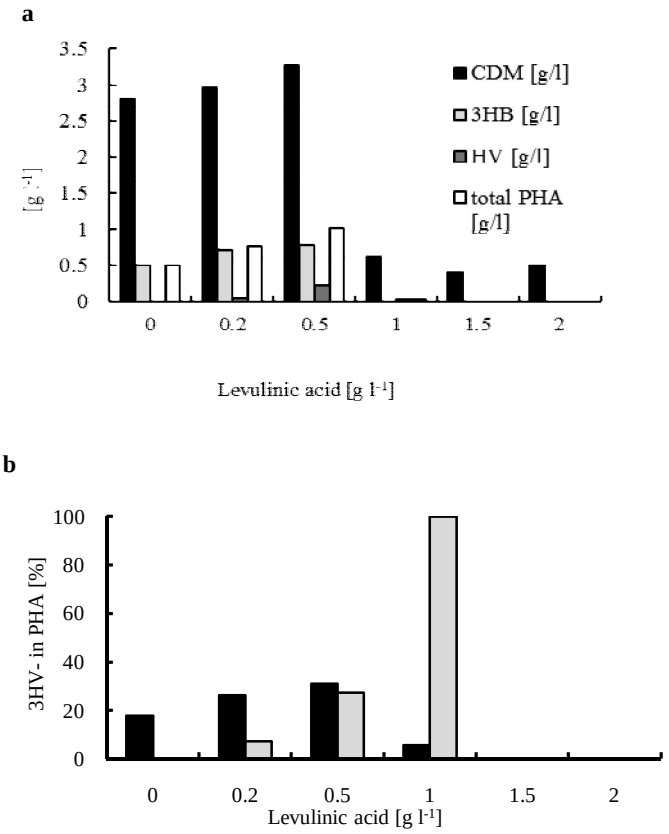

Figure 5. (**a**) Effect of different LA concentrations on final CDM, 3HB, 3HV and PHA concentration: *H. pseudoflava* under nutritionally balanced growth conditions. (**b**) Effect of LA on PHA content in cells (black bars) and on 3HV percentage on total polymer (grey bars).

Figure 5b illustrates the mass fractions of PHA in CDM and of 3HV in PHA for all investigated setups. Increasing LA concentration in the medium from 0.5 g l^{-1} to 1 g l^{-1} boosted the HV content from 27% to 100% (pure PHV homopolyester); here, the 3HB value was below the measuring accuracy. This is coherent to the finding displayed in Fig. 3d, where it is shown that LA levels as high as 1 g l^{-1} completely inhibit glucose catabolism, resulting in the inability of the strain to generate the required acetyl-CoA pool needed for biomass and 3HB biosynthesis. As expected, no 3HV was detected in setups without 3HV-related precursor compounds.

3.1 PHBHV copolyester production by *H. pseudoflava* on hydrolyzed whey permeate and LA

This growth experiment described above proofed that *H. pseudoflava* is able to use LA as substrate up to a concentration of 1 g l^{-1} and displays the desired capability to synthesize 3HV from LA. The subsequent experiment was designed to investigate how LA effects PHA synthesis under nutritionally unbalanced conditions, provoked by nitrogen deprivation. Concentrations exceeding 1 g l^{-1} were not investigated in this second series due to the high inhibition of *H. pseudoflava´*s metabolism manifested in the previous experiment (Fig. 2a). Further, significant amounts of the polyesters had to be produced in order to confirm their monomeric composition by means of nuclear magnetic resonance (^{1}H-NMR).

Hydrolyzed whey permeate was added to the medium to provide equimolar concentrations of glucose and galactose (5 g l^{-1} for each sugar). Depending on the precursor concentration, the sugars were utilized at different rates. Generally, glucose was used significantly faster than galactose. In the flasks containing 0.2 g l^{-1} LA, both sugars were utilized faster than in other setups. In those setups containing 1 g l^{-1} LA or mixtures of LA (0.5 g l^{-1}) plus SV (1 g l^{-1}), sugar utilization slowed down considerably. In the flasks containing 0 or 0.2 g l^{-1} LA, a total of 6.6 g l^{-1} sugars (glucose plus galactose) were utilized after 70.5 h of cultivation, whereas only 3.5 g l^{-1} of sugars where utilized at this time in setups containing 0.5 g l^{-1} or 1 g l^{-1} LA, and mixtures of LA plus SV (data not shown). The precursor concentrations (LA, SV) were followed during the experiment, and as the concentrations approached depletion, additional precursor feeds were accomplished according to Fig. 6.

In the used nitrogen-deficient minimal medium, the bacteria are not able to reproduce, but shift the intracellular carbon flux towards PHA biosynthesis. Therefore, the increase in CDM values illustrated in Fig. 8 reflects the amount of PHA synthesized by the cells. CDM values of *H. pseudoflava* grown on different precursor concentrations (Fig. 7a) illustrate that lowest PHA concentration was achieved without addition of 3HV precursors, hence, in cultivations on hydrolyzed whey lactose as only carbon source. Increasing LA concentrations resulted in increased PHA concentrations (Fig. 7b-d), with highest CDM values and 3HB concentrations monitored in setups containing 0.5 g l^{-1} LA plus 1 g l^{-1} SV (Fig. 7a and 7b, respectively); the amounts of 3HB were comparable in setups containing 0, 0.5, and 1 g l^{-1} LA, whereas in flasks containing 0.2 g l^{-1} LA and LA/SV mixtures, higher 3HB concentrations were obtained (Fig. 7b).

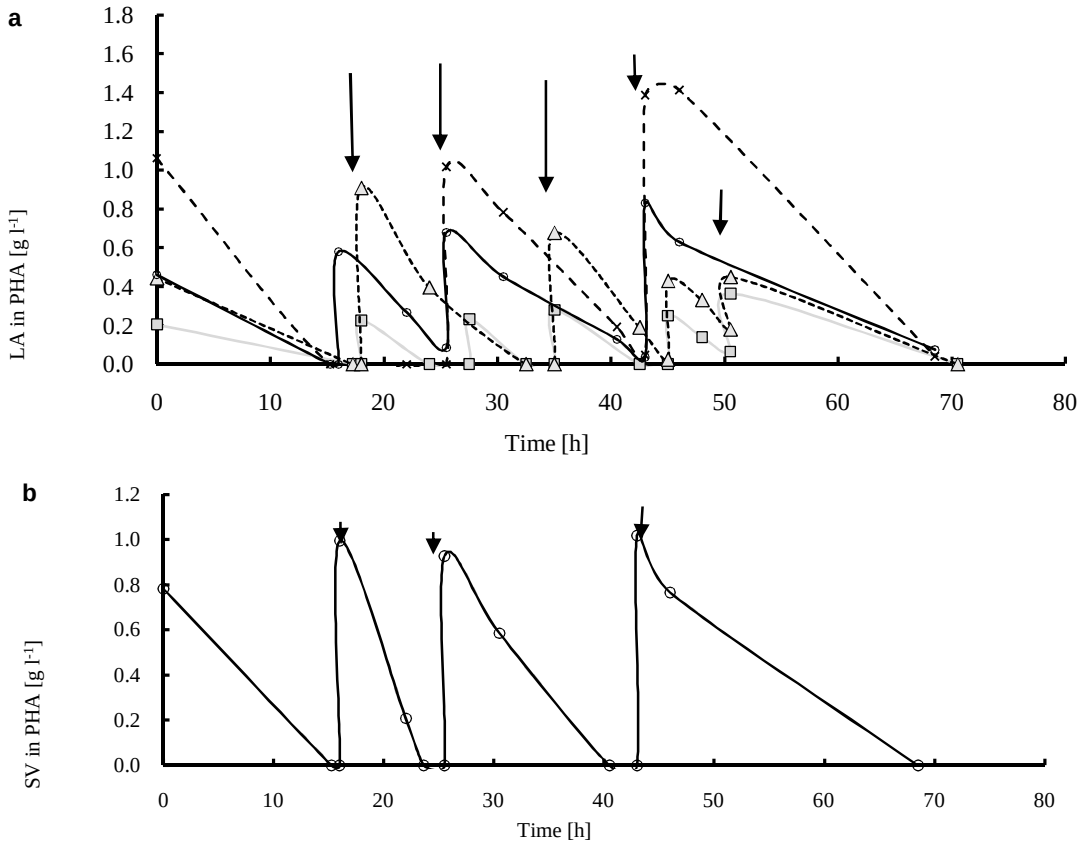

Figure 6. Utilization and refeeding of the 3HV precursors LA (**a**) and SV (**b**). Arrows indicate the time points of additions. Squares: 0.2 g l^{-1} LA; triangles: 0.5 g l^{-1} LA; asterisks: 1 g l^{-1} LA; spheres: 0.5 g l^{-1} LA plus 1 g l^{-1} SV

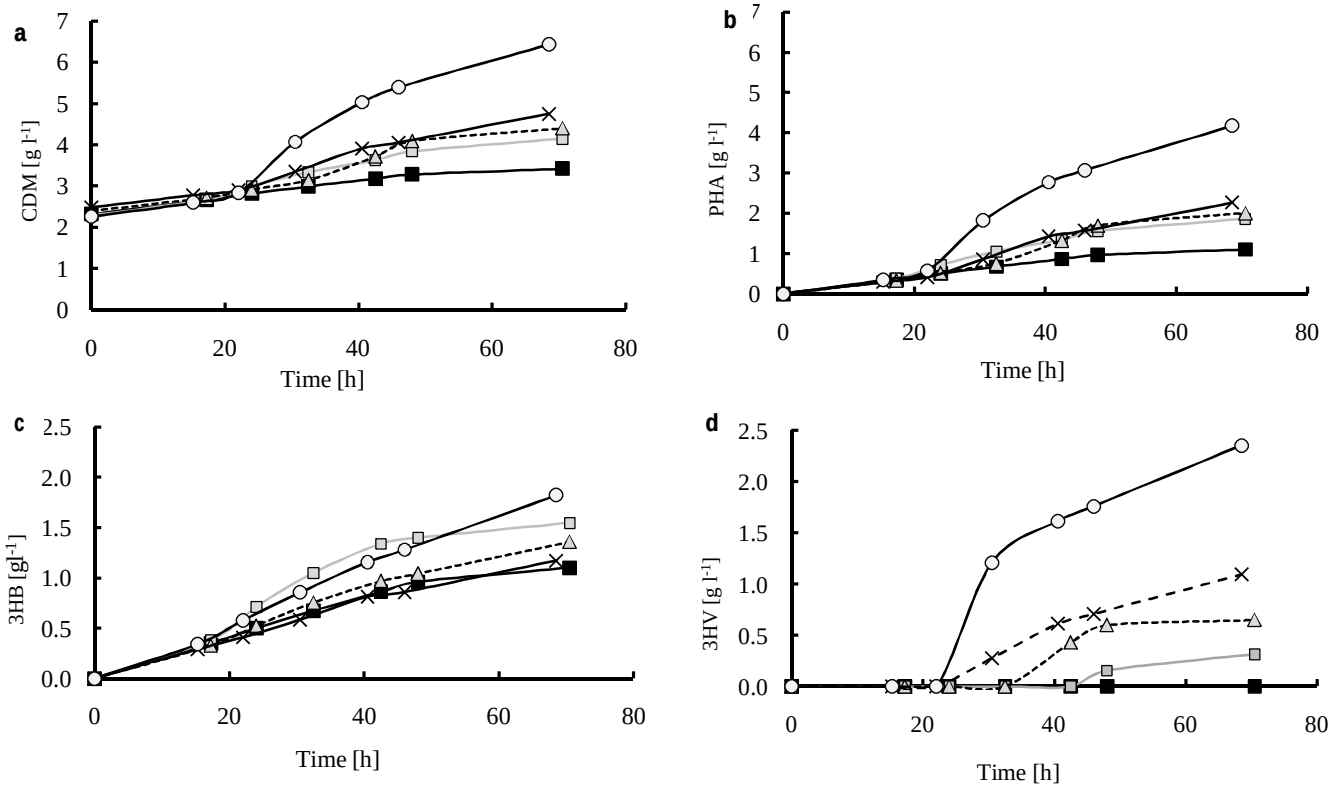

Figure 7. Time courses of (**a**) CDM, (**b**) PHA, (**c**) 3HB and (**d**) 3HV. *H. pseudoflava* on hydrolyzed whey permeate and 3HV-precursors under nitrogen-free cultivation conditions. Black squares: No precursor addition; grey squares: 0.2 g l^{-1} LA; triangles: 0.5 g l^{-1} LA; asterisks: 1 g l^{-1} LA; spheres: 0.5 g l^{-1} LA plus 1 g l^{-1} SV

3HV concentrations (Fig. 7c) and 3HV mass fractions in PHA (Fig. 8) directly correlated to the provided precursor concentration; the more precursor added, the higher the achieved 3HV concentration (Fig. 7c), and the higher the 3HV fraction in PHA (Fig. 8). Highest 3HV fractions of almost 0.6 g g^{-1} were achieved in setups containing LA/SV mixtures; as expected, the polyester produced without addition of 3HB-related precursors was identified as PHB homopolyester (Fig. 8).

The mass fraction of PHA in CDM at the end of the cultivation setups is illustrated in Fig. 9a. Lowest mass fractions were obtained in setups without precursor addition (hydrolyzed whey lactose as sole carbon source; setups A). In addition, volumetric PHA productivity was lowest in the precursor-free setups (32%; setups A), and highest in the flasks with 0.5 g l^{-1} LA plus 1 g l^{-1} SV (65%; setups E) (Fig. 9b). Volumetric productivity ranged from 0.0156 g^{-1} h^{-1} (setups A) to 0.061 g l^{-1} h^{-1} (setups E).

It is a well-known fact that conversion of 3HV-related compounds like propionic acid, SV, or LA not only initiates 3HV synthesis, but, by oxidative decarboxylation, also generates a pool of the C2-compound acetyl-CoA, which undergoes condensation towards the 3HB-intermediate acetoacetyl-CoA [58]. Therefore, 3HB yields exceeding the theoretical maximum value of 0.48 g g^{-1} were observed at the highest precursor concentrations (setups E; data not shown). The highest product yields obtained were monitored in setups containing 0.5 g l^{-1} LA plus 1 g l^{-1} SV, and amounted to 0.56 g 3HB per g consumed sugar (glucose and galactose), and 0.38 g 3HV per g 3HV precursor, respectively. The conversion yields of sugars towards 3HB are considerably higher than reported previously for bioreactor cultivations of this strain on non-hydrolyzed whey permeate (0.2 g g^{-1}) [26]. If further increase of the conversion yield towards 3HV is aspired, one could resort to techniques successfully demonstrated in the past by Lefebvre et al., who prevented the oxidative decarboxy-lation reaction by restricting the dissolved oxygen concentration (DOC) during the PHA biosynthesis in the case of PHBHV production by *C. necator* on glucose and the 3HV precursor propionic acid. This strategy, on the one hand, positively affected 3HV yields, hence, resulted in higher 3HV fractions in PHA, but on the other hand, negatively the entire PHA productivity by slowing down the sugar uptake rate [60].

When SV was fed at a concentration of 1 g l^{-1} in addition to 0.5 g l^{-1} LA and 5 g l^{-1} of glucose plus galactose (from hydrolyzed whey permeate), more polymer, higher 3HV fractions in PHA, higher yields and better PHBHV productivity were achieved than in any of the setups using LA as sole 3HV-related precursor. At a first glance, this makes SV the superior 3HV precursor if compared to SA; moreover, the price for LA as a pure compound is contemporarily higher if compared to SV. Nevertheless, as discussed above, it has to be emphasized that LA is accessible from various abundant raw materials by advanced technologies, which makes its future application in mixed substrates more feasible.

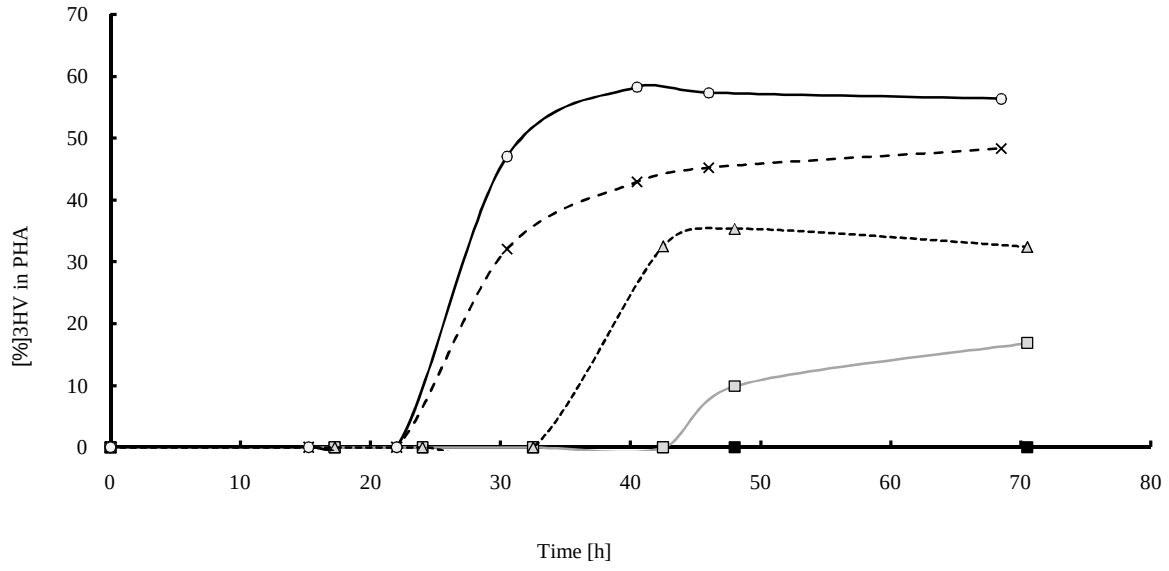

Figure 8. 3HV fraction in total PHA produced by *H. pseudoflava* on hydrolyzed whey permeate and 3HV precursors LA and SV. Black squares: No precursor addition; grey squares: 0.2 g l^{-1} LA; triangles: 0.5 g l^{-1} LA; asterisks: 1 g l^{-1} LA; spheres: 0.5 g l^{-1} LA plus 1 g l^{-1} SV

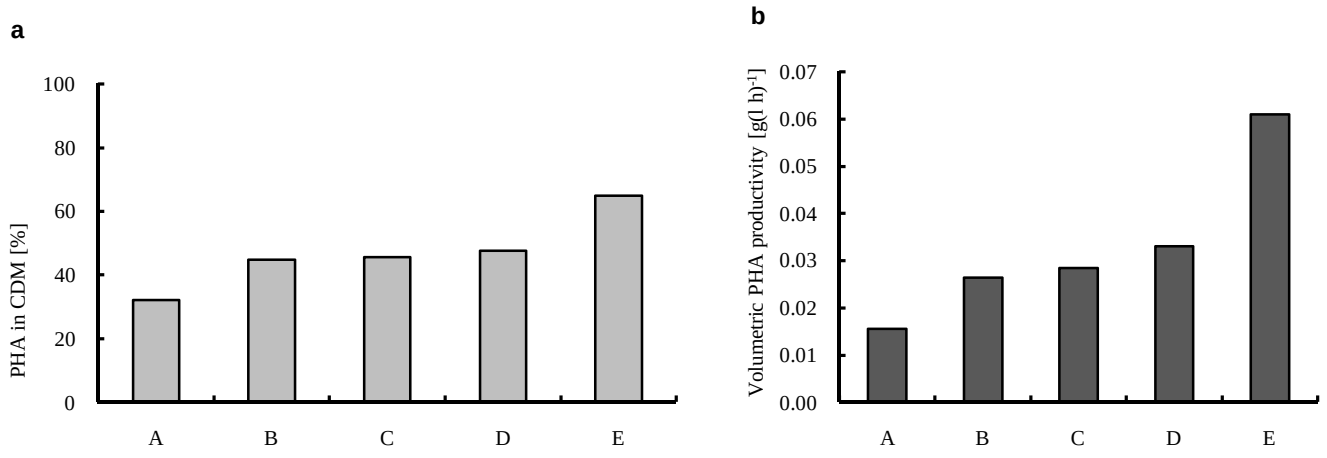

Figure 9. (**a**) Mass fraction of PHA in CDM; (**b**) volumetric productivity of PBHV. *H. pseudoflava* on hydrolyzed whey permeate plus 3HV precursors: A: 0 g l^{-1} LA, B: 0.2 g l^{-1} LA, C: 0.5 g l^{1} LA, D: 1 g l^{-1} LA, and E: 0.5 g l^{-1} LA plus 1 g l^{-1} SV.

^{1}H-NMR analysis of the polymer confirmed the GC results by showing that *H. pseudoflava* produces a PHBHV copolyester when cultivated on hydrolyzed whey permeate and LA under nitrogen limiting conditions. Exemplarily, Fig. 10a shows ^{1}H-NMR spectra of the polyester produced from the setups C (addition of 0.5 g l^{-1} LA) with a 3HV fraction in PHA of about 30%. As reference, the spectrum of a Biopol™ poly(3HB-*co*-19.1 mol-% 3HV) standard sample was also recorded (Fig. 10b). The described resonances of CH_3- (**2**), -CH_2- (**4**), and –HC- (**6**) protons in 3HB, and of CH_3- (**1**), -CH_2- (**3** and **5**), and –HC- (7) protons in 3HV, with **1** and **3** being the most significant peaks to trace 3HV units, as reported in previous studies [61,62], are well visible in the spectra, thus unambiguously identifying the products as PHBHV.

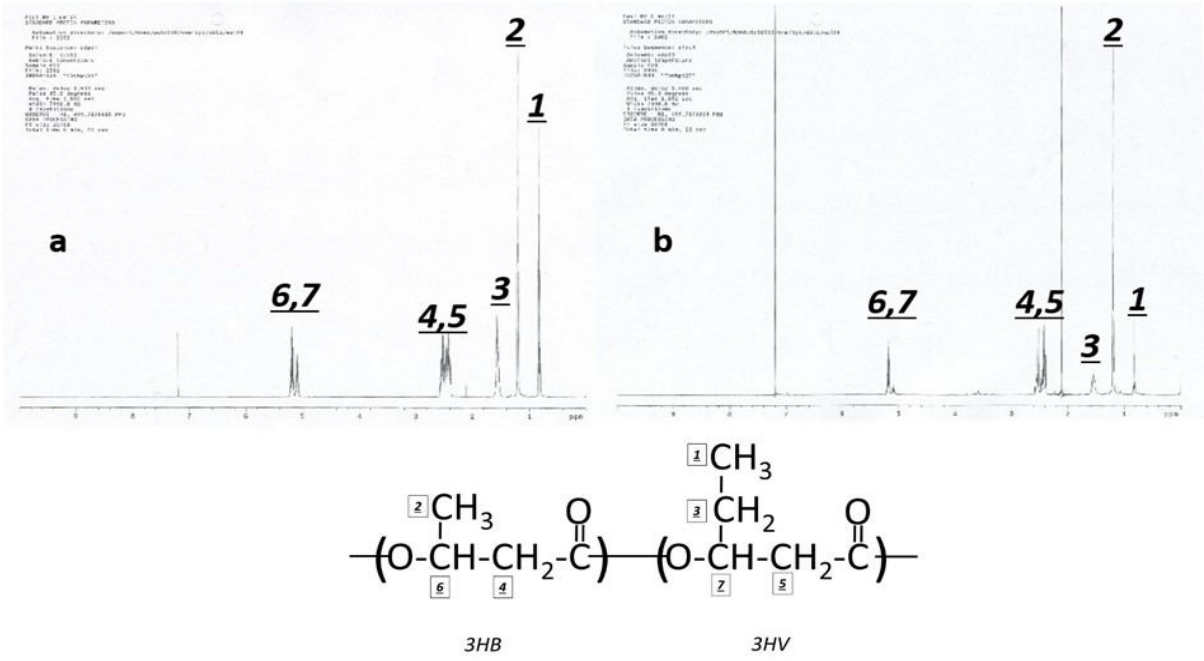

Figure 10. ^{1}H-NMR graphic of the polymer produced from hydrolyzed whey permeate and LA by *H. pseudoflava* (**a**) and of a Biopol ™ standard poly(3HB-*co*-3HV) with 19.1% 3HV (**b**). The structure below assigns the peaks to the characteristic 3HB and 3HV fragments.

4. Conclusion

The article provides a proof of concept for feasible PHBHV copolyester biosynthesis by the promising production strain *H. pseudoflava* based on abundant raw materials from food industry (whey permeate) and agriculture (LA). The results reveal that PHBHV production occurs at high rates and productivity, and results in high product fractions in microbial biomass. Based on these data, bioreactor experiments under controlled pH-value, DOC, and chemostat conditions should be carried out as the next steps in order to produce the PHBHV copolyesters at constant quality and tailored monomeric composition. Relevant product quantities allowing it´s in-depth material characterization (thermoanalysis, mechanical properties, etc.) are needed. Investigating the influence of DOC on the cultivation system: this will enable finding a compromise between optimized LA-to-3HV conversion yields, and acceptable productivity of the entire biopolyester. Importantly, the feeding regime has to be designed in a way addressing the different conversion rates of the main- and co-substrates in order to firstly prevent substrate limitation, and, secondly, to avoid inhibition of the strain´s metabolic apparatus by excessive precursor supply; here, it appears reasonable to resort to continuous chemostat feeding regimes, where substrates are fed according to their conversion by the cells. This will prevent the culture from toxic precursor concentrations, and will help to avoid the accumulation of unutilized substrate residues in the fermentation broth, which cause increased substrate costs and additional expenses for treatment of spent fermentation broth.

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6. Conflict of Interest

The authors declare that there exist no conflicts of any interest.

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مطالعه اثر لونیلیک اسید بر بیوسنتز پلی (3-هیدروکسی بوتیرات-کو-3-هیدروکسی والرات) با *هیدروژنوفاگا پسودوفلاوا*

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چکیده

سابقه و هدف: تولید پلی هیدروکسی آلکونوآت کوپلی استرها حاوی واحدهای 3-هیدروکسی بوتیرات و 3- هیدروکسی والرات برای اولین بار با استفاده از *هیدروژنوفاگا پسودوفلاوا* و بر اساس مواد اولیه پایدار مورد مطالعه قرار گرفت. این استراتژی تولید PHA با افزایش بهره‌وری هزینه و بهبود کیفیت مواد را موجب می‌شود.

مواد و روش‌ها: به عنوان یک ویژگی، تولید پلی استرهای پلی (3- هیدروکسی بوتیرات-کو-3- هیدروکسی والرات) براساس تلفیق بدیع سوبسترا- کمک سوبسترا بوده است؛ تراویده آب پنیر صنعت شیر، به عنوان سوبسترا برای توده زیستی و 3HB بیوسنتز عمل می‌کند و از سوی دیگر، از میان منابع گوناگون تجدیدپذیر لوولینیک اسید به عنوان پیش ساز مرتبط با 3HV آزمایش‌ها در ارلن آزمایشگاهی دوار با استفاده از محیط کشت حاوی مواد مغذی مشخص انجام شد.

یافته‌ها و نتیجه‌گیری: به‌کار بردن محیط کشت متوازن از نظر مواد مغذی برای رشد پسودوفلاو، لوولینیک اسید مهار رشد زیادی بمراتب بیشتر از غلظت‌های کمتر از $0/2\ g\ l^{-1}$ (ثابت مهار رشد K_i=0/032) را از خود نشان داد، که تامین دقیق این ترکیب در فاز اول کشت را پیشنهاد می‌کند. در شرایط کشت بدون ازت، مهار متابولیسم گونه با لوولینیک اسید کمتر مشخص بود اینجا، غلظت پلی (3-هیدروکسی بوتیرات-کو-3- هیدروکسی والرات) تا $4/2\ g\ l^{-1}$ و بهره وری حجمی پلی (3-هیدروکسی بوتیرات-کو-3- هیدروکسی والرات) $0/06\ g\ l^{-1}\ h^{-1}$، در صورت تامین پیش سازها، به‌دست آمد. با درنظر گرفتن ترکیب پلی (3-هیدروکسی بوتیرات-کو-3- هیدروکسی والرات) در تنظیم مخلوط‌های گوناگون آب پنیر/لوولینیک اسید، میزان 3-هیدروکسی والرات در پلیمر بین 0 و 0/6مول بر مول بود. این مطالعه به‌طور موفقیت آمیزی نشان داد که استفاده از مخلوط ضایعات و فراورده‌های جانبی گوناگون صنعت غذا و کشاورزی برای تولید دومین نسل زیست بسپارهایی با ارزش افزوده امکان‌پذیر می‌باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ تعارض منافعی وجود ندارد.

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واژگان کلیدی

- زیست بسپار
- لوولینیک اسید
- پلی هیدروکسی آلکانوآت (PHA)
- پلی (3-هیدروکسی بوتیرات-کو-3-هیدروکسی والرات)
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