

Copolymerization of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) produced by *Azotobacter vinelandii* OPNA is determined by the oxygen transfer rate of the culture

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Abstract

The aim of this study was to evaluate the changes in the composition of poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) by an OPNA mutant strain of *Azotobacter vinelandii* cultured under different conditions. In shaken flasks, the oxygen transfer rate (OTR) significantly affected the PHBV composition, the highest content of 3-hydroxyvalerate (3HV) (28%) was achieved from the cultures developed at high OTR max (5.87 mmol L⁻¹ h⁻¹). In the bioreactor, the cultures were grown under oxygen limited conditions and different OTRs, varying the agitation rate. The highest PHBV accumulation (85%) was achieved at low OTR max (4.96 mmol L⁻¹ h⁻¹), whereas, the maximum content of 3HV (8.5 mol%) was obtained at higher OTR max (20.3 mmol L⁻¹ h⁻¹). Our results reveal that increasing the OTR rises the molar fraction of 3HV in the PHBV copolymer. Therefore, the manipulation of OTR could be a feasible strategy to produce PHBV copolymers with different monomeric compositions at industrial level.

1. Introduction

Polyhydroxyalkanoates (PHAs) are linear intracellular polyesters, which accumulate as insoluble granules in the cytoplasm of bacteria, which serve as carbon and energy storage material [3, 22]. They are biodegradable, biocompatible, and with thermomechanical properties similar to those of conventional petroleum-based plastics, such as polypropylene and polyethylene, which make them suitable for the production of biodegradable plastics [3, 14, 17].

According to the carbon chain lengths of their monomers PHAs can be classified, in short-chain length (scl; three to five carbon atoms) and medium-chain length (mcl; six or more carbon atoms) [29]. In addition, they can be found as homopolymers (only have one type of monomer) or as copolymers (two or more type of monomers) [17, 37].

The homopolymer poly-3-hydroxybutyrate (PHB) is the most common member of the PHAs family. It has high hydrophobicity, brittle mechanics and low rate of biodegradation that limit its processing and therefore, its biomedical and pharmaceutical applications [3, 25]. However, the incorporation of monomers with different chain length in the polymer chain improves the physical properties of PHB; for example, the incorporation of 3-hydroxyvalerate (3HV) result in a copolymer of poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) in which thermomechanical properties are improved, making it a material less brittle, more flexible and with a lower melting temperature than PHB [22, 34, 38]. Moreover, these properties can vary depending on the mol percentage of 3HV in the polymer chain [20]. Previous studies have shown that, increasing the fraction of 3HV from 0 to 20 % in the PHBV, there is a significant reduction in the melting temperature, crystallinity, and stiffness in the polymer produced [24, 36]. Also, the processing capacity of the material improves, expanding potential applications of these polymers, especially in the pharmaceutical, medical and industrial fields [3]. For example, PHBV is a biomedical material used in drug release and transport systems, in the fabrication of implants, biosensors, tissue patches, and surgical sutures [34].

Several bacterial strains including wild-type and recombinant bacteria such as *Ralstonia eutropha*, *Cupriavidus necator*, *Pseudomonas sp*, *A. vinelandii* and recombinant *Escherichia coli* have been studied for PHBV copolymers production [3, 4, 14, 27, 35]. PHBV copolymer is obtained by adding different carbon sources; frequently organic acids and alcohols are used. The choice of carbon sources depends on the type of microorganisms [4, 16, 24, 33, 42]. *C. necator* produces the PHBV, when organic acids (propionic acid or levulinic acid) or alcohols (ethanol or pentanol) are used as a precursor [4, 30]. It has been observed that in *R. eutropha*, propionic acid can serve as the precursor of HV monomers by the condensation of propionyl-CoA and acetyl-CoA to generate hydroxyvaleryl-CoA. Subsequently, hydroxyvaleryl-CoA is incorporated into the polymer by the PHA synthase [25, 42]. In contrast, species of *Azotobacter* are able to produce PHBV, when valeric or heptanoic acid is added as a precursor. The use of propionic, hexanoic, octanoic acid or alcohol precursors did not result in the formation of PHBV [24, 27, 33, 40]. Previous studies by Page et al. [28] have shown that in *A. vinelandii*, HV production involves intermediate products of β -oxidation pathway. In that sense, valeric acid is converted to (R)-3-hydroxyvaleryl-CoA, that can be condensed by a PHA synthase to form PHBV [19, 28]. Similarly, recombinant strains of *E. coli* can use organic acids as a precursor to PHBV production [4].

It has been reported that, the monomeric composition of the copolymers can be modulated by culture parameters such as the carbon source, the timing of carbon source addition into the culture medium, and the oxygen transfer rate (OTR) [27, 40]. There are some studies regarding the effect of culture parameters on the monomeric composition of copolymers [4, 16, 27, 33, 40]. In this context, the influence of carbon source concentration on PHBV production has been studied. For example, Page et al. [27] reported that increasing the valerate concentration (from 10 to 40 mM) had a positive effect on the molar fraction of 3HV (from 17 to 24 %) in the PHBV copolymer produced in cultures in bioreactor by *A. vinelandii* UWD [27]. Likewise, Pozo et al. [33] observed that in shaken flasks cultures, when the valerate concentration was increased from 10 to 40 mM, the 3HV molar ratio increased 2-fold in the PHBV copolymer produced by *A. chroococcum* H23.

On the other hand, it was previously reported that oxygen influences the monomeric composition of PHA copolymers [4]. In that sense, Lee et al. [16] found that in cultures conducted in shaken flasks with *Comamonas acidovorans*, the oxygen transfer rate (OTR) had a negative effect on the incorporation of 4HB monomers. In that study the aeration conditions were manipulated using different filling volumes (50 to 300 mL) in 500 mL shaken flasks. In cultures grown at high aeration (50 mL) the molar fraction of 4HB was 18 %; whereas, in the culture grown at low aeration (300 mL) the content of 4HB increased to 50 %. More recently, Ryu et al. [35] reported that in shaken flasks cultures of *A. vinelandii* UWD at filling volumes of 50 and 100 mL (in 250 mL flasks) the molar fraction of 3HV monomers were similar at 48 h of cultivation. In contrast, Urtuvia et al. [40] reported that in cultures of *A. vinelandii* strain OP in bioreactor, the OTR positively affected the production of 3HV monomers. In that study two OTR_{max} conditions were tested and were varied, adjusting the agitation rate at 300 rpm and 700 rpm. In the cultures at low OTR_{max} ($4.3 \text{ mmol L}^{-1} \text{ h}^{-1}$, 300 rpm) the maximum 3HV fraction was 18 %, whereas, in

cultures at high OTR_{max} ($17.2 \text{ mmol L}^{-1} \text{ h}^{-1}$, 600 rpm) the content of 3HV in the copolymer produced was 35 %.

It is important to point out that the reports by Ryu et al. [35] and Urtuvia et al. [40] are the only studies regarding to the influence of OTR on the monomeric composition of PHBV produced by *A. vinelandii* strains. However, in the case of Ryu et al. [35], the authors did not find an influence of the OTR on the monomeric composition of PHBV copolymers. This could be due to genetic differences between the strains used, the UWD strain has mutations affecting the respiratory chain [28]. Also in the study of Urtuvia et al. [40] the cultures were conducted under nitrogen-fixation conditions, and it is known that under those conditions changes in metabolic pattern are found (metabolite biosynthesis, carbohydrate consumption and growth rate), compared to what happens in cultures where a fixed nitrogen source is added to the medium [12, 40]. Therefore, the aim of the present study was to analyze the effect of culture conditions on the composition of the PHBV produced by the OPNA mutant strain of *A. vinelandii*.

2. Materials And Methods

Microorganism

The *A. vinelandii* strain used was OPNA, which is a mutant derived from strain OP. This strain carries mutations that inactivate the genes coding for the IIA-PTS^{Ntr} and RsmA regulatory proteins, which act negatively on the PHB synthesis [11, 26]. The cells of strain OPNA were grown in Burk' agar supplemented with spectinomycin and kanamycin and were maintained by monthly subculture and cryopreserved at $-70 \text{ }^{\circ}\text{C}$ in 40% glycerol solution [9].

Culture medium

A. vinelandii OPNA was grown in Burk' medium with the following composition (in g L^{-1}): sucrose 20, yeast extract 6.5, MOPS 31, K_2HPO_4 0.66, KH_2PO_4 0.16, CaSO_4 0.05, NaCl 0.2, MgSO_4 0.2, $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 0.0029, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.027. The pH was adjusted to 7.2 with a concentrated 2 N NaOH solution. In shaken flask cultures, the organic nitrogen source (yeast extract) was replaced by an inorganic source (ammonium sulfate) using 1.4 g L^{-1} . In order to promote the biosynthesis of PHBV, valeric acid were added to the medium without neutralize during the exponential growth phase (on the most-active biosynthesis of PHB), at the final concentration of 10 and 40 mM.

Shaken flask cultures

The strain was cultured in 100 mL Burk' medium in 500 mL shaken flasks at 200 rpm and $29 \text{ }^{\circ}\text{C}$ during 20- 24 hours. The inoculum was used when it reached an optical density between 0.16 - 0.18 determined at 540 nm (corresponding to a biomass in dry weight between 0.08 to 0.1 g L^{-1}) [9, 21]. In order to evaluate the influence of aeration conditions, the strain was grown during 60 h under different filling volumes at high aeration (100 mL) and low aeration (200 mL) in 500 ml shaken flask. Every 12 h one shaken flask was sacrificed for analytical measurements. All experiments were conducted in triplicate.

Batch cultures in bioreactor

A. vinelandii cells were grown at 29 °C in 500 mL shaken flasks, containing 100 mL of Burk' medium (supplemented with yeast extract) at 200 rpm for 24 hours. Subsequently, this inoculum was used when it reached an optical density between 0.16 - 0.18 determined at 540 nm. The biomass obtained was collected by centrifugation at $15,500 \times g$, for 10 minutes. The supernatant was discarded, and the cells were suspended in 150 mL of fresh Burk' medium. This suspension was inoculated into the bioreactor containing 1350 mL of fresh culture medium. Batch cultures were carried out in a 3L Applikon bioreactor, with a 1.5 L working volume. The pH was controlled to 7.2 by the automatic addition of NaOH solution (5N). Foam was controlled by manual addition of 10 % w/v antifoam (silicone). Dissolved oxygen tension (DOT) was measured by an Ingold polarographic probe and was not controlled. Cultures were performed in duplicate at 29 °C. In order to evaluate the influence of OTR, two agitation rates (300 and 700 rpm) were tested.

OTR was determined by online measurement of O₂ in the exit gas and compared with measurements from the O₂ from in the inlet gas flow rate [8]. Two gas analyzers (BCP-O2, BCP-CO2, BlueSens) were used for gas (O₂ – CO₂) measurement. The equation (Eq. 1) used for determination of oxygen transfer rate (OTR) was described by Zeng et al. [43] as follows:

$$OTR = \frac{V_G}{V_L V_N} (X_{in} - X_{out}) \quad \text{Eq. [1]}$$

In which V_G is the gas inlet flow (L h⁻¹), V_L is the working volume in the fermentation (L), V_N is the molar volume (L mol⁻¹), X_{in} and X_{out} are the mole fractions of oxygen at the inlet and outlet, respectively.

Factorial design 2³

A factorial design 2³ was used to evaluate the main effects of culture conditions on the biosynthesis of PHBV produced by *A. vinelandii* in 500 mL shaken flasks cultures. The three factors: oxygen transfer rate (OTR), concentration, and addition time of valeric acid, were tested at a low and a high value represented by the levels (+1) and (-1), respectively (Table 1). The OTR was modified through changes in the filling volume (100 and 200 mL), the valeric acid was not neutralized before to be added, in two final concentrations (10 and 40 mM), at 12 and 24 h (during and after the most-active biosynthesis of PHB, respectively). The response variable was the percentage of 3HB and 3HV present in the copolymer at 48 h of cultivation. A total of eight experiments were conducted, all of them were done in triplicate. The statistical analysis (analysis of variance (ANOVA), coefficients) was carried out in Statgraphics software version 18.1.12. The p-value for statistical significance was set to 0.05.

Analytical determinations

The cell dry weight was determined by the gravimetric method using 5 mL of culture broth, it was centrifuged at 12,000 rpm for 5 min at 25 °C. Subsequently, the supernatant was discarded, recovering the cell pellet in previously weighted Eppendorf tubes (1.5 mL). The tubes were dried for 24 h at 70 °C. [9]. Protein concentration was determined by the Lowry method, using bovine serum albumin as standard [18]. The specific growth rate (μ) was calculated based on protein using the logistic model reported previously [15]. The sucrose determination was assayed by quantifying the hydrolysis products (glucose and fructose) using β -fructofuranosidase enzyme [5]. Sucrose and valeric acid were measured using a high-performance liquid chromatography (HPLC) (Waters 2695, USA), with an Aminex HPX-87H ion-exclusion organic acid column (Biorad), and the refractive index IR / UV detectors (Waters 2414 / 2996 USA). Elution was performed with 5mM of H_2SO_4 as the mobile phase, at a flow rate of 0.65 mL min^{-1} at 50 °C. The quantification of PHB as crotonic acid was assayed using a HPLC system, with an Aminex HPX-87H ion-exclusion organic acid column and a UV detector (Waters 2996, USA). H_2SO_4 (5mM) was used as the mobile phase at a flow rate of 0.65 mL min^{-1} and 50 °C [9].

Molecular Mass of PHBV copolymers

The extraction of PHBV was performed as described by Millán et al. [21]. The molecular mass was estimated by gel permeation chromatography (GPC), using a Shodex GPC K-807 L column, in an HPLC system (Waters 2695, USA) and a refractive index detector (Waters 2414, USA). The mobile phase was chloroform at 30° C at a flow rate of 1 mL min^{-1} . The calibration curve was constructed using polystyrene standards (2.9×10^3 to 1.0×10^7 Da). The samples were dissolved in chloroform and were filtered using membranes of $0.45 \mu\text{m}$ pore size [10].

Determination of PHBV and its monomeric composition

In order to convert the PHAs monomers into methyl esters, the samples were derivatized following the protocol proposed by Juengert et al. [13]. Lyophilized biomass (10 mg) was subjected to acid methanolysis, the propyl-ester extracts were injected into an HP-5MS capillary column (Agilent Technologies 30m x 0.25mm x 0.25 μm) and coupled to masses analyzer (Agilent Technologies, U.S.A), with a Split ratio of 10: 1. The carries gas was helium with a glow rate at 0.7 mL min^{-1} . The injector and detector temperature were 50 °C and 270 °C, respectively. Methyl 3-hydroxybutyrate and methyl valerate were used as an external standard, and benzoic acid as internal standard.

3. Results And Discussion

Influence of culture factors on the monomer composition of PHBV.

To study the influence of OTR, valeric acid concentration, and valeric acid addition time on PHBV copolymer production, a factorial design 2^3 was used (Table 1). Gas chromatography-mass spectrometry (GC-MS) analysis was performed to characterize the monomeric composition of copolymers produced by *A. vinelandii* OPNA. As shown in Fig. 1a peaks obtained at retention time 4.52, 6.18, and 8.64 were

identified as 3HB, 3HV and benzoic acid (internal standard), respectively. For each compound, the analysis of the fragmentation pattern of the peaks, matches with methyl esters of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) showed in Fig. 1b.

Table 2 shows the results obtained under the different conditions evaluated. The highest molar fraction of 3HV (28 ± 2.3 %) was obtained by adding 40 mM of valeric acid (A +1), after 12 h of cultivation, during the onset of the most-active PHA biosynthesis (B -1) and cultures at 100 mL (C -1) of filling volume. On the other hand, a mathematical model was generated and showed in Eq. (2), where the filling volume (C) and therefore the OTR_{max} , is the variable that had the highest effect on monomeric composition of PHBV.

$$\% \text{ 3HV} = 12.50 + 0.775A - 0.508 B - 7.26 C \text{ Eq. (2).}$$

It is known that changes in the filling volume on shaken flasks has an influence on the oxygen transfer rate (OTR), and therefore in the availability of oxygen in the liquid [30]. Previously, Lee et al. [16] observed that a decrease in the OTR_{max} (increasing the filling volume from 50 to 300 mL in 500 mL shaken flasks) increased the molar fraction of 4HB monomers (from 18 to 50 %) in P(3HB)-co-4HB copolymer produced by *Comamonas acidovorans*. In the case of *A. vinelandii*, the influence of OTR in monomeric composition seems to be dependent on the strain and culture condition employed. Ryu et al. [35] did not find an influence of OTR_{max} on monomeric composition of PHBV produce by UWD strain in shaken flasks culture. This study was carried out in two filling volume (50 and 100 mL in 250 shaken flasks), where in both conditions the content of 3HV was 4.1 ± 0.4 and 4.3 ± 0.8 %, respectively. In contrast, Urtuvia et al. [40] reported that the OTR has a positive influence on the production of 3HV monomers in PHBV copolymer produced by *A. vinelandii* OP in bioreactor cultures. These authors observed that increasing agitation rate (300 to 600 rpm), increased the OTR_{max} (4.3 to $17.2 \text{ mmol L}^{-1} \text{ h}^{-1}$), and therefore the molar fraction of 3HV monomers (18 to 35 %).

Characterization of PHBV produced in shaken flasks cultures.

Considering that the higher molar fraction of 3HV was achieved with 40 mM of valeric acid added at 12 h and at high OTR ($5.87 \text{ mmol L}^{-1} \text{ h}^{-1}$, 100 mL) (Run 3, Table 2), the effect of that concentration of valeric acid added at 12 h was evaluated on cell growth, production, and accumulation of PHBV. Figure 2 shows the growth kinetics, PHBV production, sucrose, and valeric acid consumptions by the OPNA mutant, with respect to those of in the control condition (strain without valeric acid addition). Valeric acid (40 mM) was added at 12 h of cultivation.

A specific growth rate (μ) of $0.047 \pm 0.001 \text{ h}^{-1}$ was achieved in the cultures conducted without adding valeric acid and the maximal protein concentration was about $0.9 \pm 0.012 \text{ g L}^{-1}$. A feeding strategy in fed-batch culture, it has been suggested to avoid the inhibition of cell growth and PHA accumulation, without affecting the 3HV content in the copolymer. Page et al. [27] reported that in fed-batch cultures of *A. vinelandii* UWD, the addition of valerate in a final concentration of 20 mM (6 mM in the vessel), did not affect the PHA content (65 %) when it is compared with the control (without addition of precursor);

whereas, the maximum content of 3HV was 23 %. (Fig. 2a). In the case of PHBV production, it was affected by the addition of valeric acid. A maximum accumulation of 38 ± 1.6 % was achieved in the control condition (where only PHB was produced); whereas, only 22 ± 2.4 % were obtained from the cultures conducted with the addition of valeric acid (Fig. 2b).

Our results are in agreement with those previously reported by Myshkina [24] and Page et al. [27], who found that the growth and the polymer production were inhibited 21 and 10 %, respectively, by the addition of valerate (10 mM) in the medium. Myshkina et al. [24] reported, that in shaken flasks cultures of *A. chroococcum* strain 7B, the addition of valerate (20 mM), inhibits the growth (43 %) and the polymer accumulation (7 %) at 48 h of cultivation. However, these authors evaluated the production of copolymers in species of *Azotobacter*, adding sodium salts as precursors (valerate), instead of carboxylic acids (valeric acid). In our study, the valeric acid was not neutralized before to be added, and to keep the pH constant during cultivation a high concentration of MOPS was employed. However, the pH was 5.2 ± 0.8 at the end of the culture. It is possible that inhibition of cell growth and polymer accumulation could be related to an effect caused by pH. In this line, Myshkina et al. [23] evaluated the effect of pH on the cell growth and PHB produced by *A. chroococcum* 7B in shaken flasks cultures. These authors observed that the optimum pH was at 7.0, whereas, alkaline (8.0) or acid pH (6.0) conditions negatively influence the growth and accumulation.

On the other hand, Chung et al. [7] reported that pH plays a relevant role in dissociation of acids and their transport. In that sense, it was previously shown that acids as acetic or propionic migrates by anti-carrier transport (dissociated form) or by simple diffusion through the cell membrane (undissociated form). In the last, the acid works as a proton uncoupler, which generates a dissipation of the proton-pumping force affecting ATP synthesis and therefore an inhibition of the cell growth [41]. In the case of propionic acid, is thought to involve an uncoupling role, because the fraction of undissociated forms increases when pH decreases [7]. Therefore, it is possible that a similar behavior occurs during the valeric acid transport; however, further studies will be needed to elucidate how valeric acid inhibits bacterial growth. It is important to point out that as described in bioreactor cultures, there was an inhibition on the cell growth and the polymer accumulation when valeric acid was added, this effect cannot be attributed to pH, because it was controlled during the cultivation.

As it was expected, 3HV monomers were synthesized only when valeric acid was added (Fig. 2c). The highest content of 3HV (39 ± 3 %) was obtained at 36 h of cultivation. It was previously reported that *Azotobacter* species are able to produce PHBV copolymers, when precursors are added to growth medium [24, 27, 40]. Table 3 shows the highest content of 3HV obtained from *Azotobacter* species cultured in shaken flasks under different precursor concentrations. It is important to note that, HV monomers production depends on several factors including culture conditions such as type and concentration of precursor; and biochemical and genetic background of the strain. To our knowledge, this molar fraction of 3HV (39 ± 3 %) in the PHBV copolymer obtained, is the highest reported until now, in *Azotobacter* species cultured in shaken flasks cultures.

Effect of valeric acid concentration and OTR on growth and PHBV production in bioreactor cultures.

In order to investigate the influence of valeric acid concentration on production of PHBV, batch cultures were carried out in a 3.0 L Applikon bioreactor. This was operated at 700 rpm (OTR_{max} 20.3 mmol L⁻¹ h⁻¹) and 29 °C. The pH was controlled at 7.2 ± 0.5. PHBV production was evaluated under two concentrations of valeric acid (10 and 40 mM), the precursor (valeric acid) was not neutralized before to be added at 6 h (during the most-active PHA biosynthesis).

It has been reported that increasing precursor concentration, increases the negative effect on cell growth and polymer accumulation [27]. Figure 3 shows grown kinetics (measured as protein), PHBV accumulation and content of 3HV monomers by strain OPNA of *A. vinelandii* cultured under two concentrations of valeric acid. As expected, a high precursor concentration (from 10 to 40 mM), negatively influenced specific growth rate (μ), reaching 0.11 ± 0.002 h⁻¹ at 40 mM, compared to those cultures developed at 10 mM of valeric acid, where the μ was 0.14 ± 0.003 h⁻¹. However, this effect was not reflected in the maximal protein concentration, because in both conditions tested the concentration of protein was similar at the end of the culture (Fig. 3a, Table 4).

It is important to point out that the negative effect on cell growth could be related with valeric acid concentration. Previously, Page et al. [27] reported that in bioreactor cultures of the UWD strain of *A. vinelandii*, increasing the precursor concentration (from 10 to 40 mM), decreased the cell growth around 25 %. However, there is not an explanation about this effect.

The precursor concentration also affected the accumulation of PHBV. Under both conditions the percentage of accumulation was the same at the beginning of the cultivation; however, after the addition of valeric acid (6 h), accumulation of PHBV decreased in the cultures developed at 40 mM, reaching a maximal percentage of 51 ± 3.6 % at 21 h. In contrast, the maximal percentage of 80 ± 0.7 % was achieved in the cultures carried out at 10 mM (Fig. 3b). This was consistent with the highest yield of PHBV based on sucrose and with the highest volumetric uptake sucrose rate (Table 4).

As shown in figure 3c, the highest content of 3HV was obtained using 40 mM of valeric acid; under this condition, 21 ± 0.5 % of 3HV was obtained at 15 h of cultivation, whereas, in cultures using a lower concentration of precursor (10 mM), the highest production reached was 8.2 ± 0.03 % at 12 h of cultivation. These results reveal that by increasing the concentration of the precursor from 10 to 40 mM, it is possible to increase the content of 3HV about three-fold. It is important to note that the molar fraction of 3HV increased with the valeric acid concentration; however, under this condition the accumulation of PHBV decreased (Fig.3b). Page and Manchak, [28] reported that in *A. vinelandii* a diminished accumulation of PHBV could be associated with the effect of valerate (precursor) on the beta-oxidation pathway. It was proposed that catabolism of valerate would inhibit 3-cetoacyl-CoA and acetoacetyl-CoA reductase activities, and these inhibitions could be reflected in a decrease in PHA yield. In addition, inhibition of 3-ketothiolase would be favored by the production of 3HV monomers [27].

The molar fraction of 3HV decreased with respect to time. A possible explanation for this phenomenon could be attributed to the fact that the valeric acid was exhausted around 15-18 h of cultivation (Fig. 3d). This gradual exhaustion would affect the synthesis of 3HV monomers, and incorporation of PHB monomers would be prevalent, modifying the molar fraction of both monomers in the copolymer [27]. The decrease in molar percentage of 3HV could not be due to degradation, because the 3HV concentration remains at values between 0.48 – 0.54 mg L⁻¹ and between 0.53 - 0.69 mg L⁻¹ at low and high OTR_{max}, respectively, during the rest of the culture. This is online with that reported by Volova et al. [42], who found that in batch cultures of *Ralstonia eutropha* B-5786, the concentration of 3HV remained nearly unchanged, whereby, the changes on molar fraction could be due to the continuous synthesis of PHB and the termination of 3HV production, related with the exhaustion of valerate in the medium.

Considering that the OTR has an important effect on the PHBV composition, experiments changing the OTR by the manipulation of the agitation rate at 300 (4.96 mmol L⁻¹ h⁻¹, low OTR_{max}) and 700 rpm (20.3 mmol L⁻¹ h⁻¹, high OTR_{max}), were performed. Valeric acid was added in a final concentration of 10 mM, to decrease its negative influence on cell growth and copolymer production. This addition was made during the cell growth phase of cultivation and the most-active PHA biosynthesis at 6 and 9 h for the cultures in the condition at high and low OTR_{max}, respectively.

Figure 4a, shows the evolution of dissolved oxygen tension (DOT) and oxygen transfer rate (OTR) in the cultures carried out at different agitation rates. In the cultures developed at 300 rpm, the DOT decreased during the first 3 h of cultivation and remained close to 0 % during the rest of the culture, whereas the OTR_{max} reached was 4.96 mmol L⁻¹ h⁻¹. At 700 rpm, the DOT progressively decreased and remained close to 2 – 4 % until 18 h of cultivation, the OTR_{max} was 20.3 mmol L⁻¹ h⁻¹. As expected, under both conditions the OTR was affected by the agitation rate. The OTR profile showed a characteristic region (plateau) during cell growth, that was associated with an oxygen limitation, as described previously by Anderlei et al. [2] and Díaz-Barrera et al. [8].

The cell growth (measured as protein) is shown in Fig. 4b. In the cultures conducted at low OTR_{max} the maximal protein concentration was about 1.26 ± 0.01 g L⁻¹, with a specific growth rate (μ) of 0.03 ± 0.002 h⁻¹, whereas in the cultures grown at high OTR_{max}, the protein concentration was 2.0 ± 0.2 g L⁻¹ and the μ was 0.14 ± 0.003 h⁻¹. This agrees with previous works that showed a negative influence on the growth rate when the OTR was decreased [9-10, 31].

As shown in Fig. 4c and 4d, sucrose and valeric acid were consumed simultaneously; however, sucrose was not consumed completely, remaining 4 and 1 g L⁻¹ at the end of the cultures developed at 300 and 700 rpm, respectively. On the other hand, valeric acid was completely exhausted after 27 h and 12 h in cultures carried out at 300 and 700 rpm. It is important to point out that the consumption rate of both substrates was negatively affected when the OTR_{max} decreased (from 20.3 to 4.96 mmol L⁻¹ h⁻¹) (Table 5).

It is important to point out that under the conditions used in the present study, the production of PHBV was growth associated, reaching a maximal concentration of 6.1 ± 0.9 and $6.4 \pm 0.2 \text{ g L}^{-1}$ in the cultures at high and low OTR_{max} , respectively (Fig. 5a). The highest PHBV accumulation ($85 \pm 3 \%$) was achieved at low OTR_{max} after 48h of cultivation; under this condition, the volumetric production rate was of $0.47 \text{ g}_{\text{PHBV}} \text{ L}^{-1} \text{ h}^{-1}$, whereas, at high OTR, the accumulation was $80 \pm 0.7 \%$ at 21 h of cultivation, and the volumetric production rate was of $0.33 \text{ g}_{\text{PHBV}} \text{ L}^{-1} \text{ h}^{-1}$ (Fig. 5b, Table 5). These results suggest that OTR did not influence the concentration or percentage of accumulation of the polymer. This agrees with a previous work that showed that PHAs accumulation in strain OPNA is around 80 %, regardless of the oxygen condition [9].

Fig. 5c shows the evolution of 3HV content in the polymer produced by *A. vinelandii* OPNA in batch cultures. The highest molar fraction of 3HV ($8.2 \pm 0.03 \%$) was obtained at high OTR_{max} ($20.3 \text{ mmol L}^{-1} \text{ h}^{-1}$), 6 h after the addition of the precursor. At low OTR_{max} ($4.96 \text{ mmol L}^{-1} \text{ h}^{-1}$) the highest content ($6.5 \pm 0.6 \%$) was reached 27 h after the addition of valeric acid. In both conditions evaluated, the content of 3HV dropped toward the end of the culture, to 5.2 ± 0.07 and $4.6 \pm 0.06 \%$, at high and low OTR_{max} respectively. As mentioned above, this could be related with the valeric acid depletion that affected the synthesis of the 3HV monomer [27, 42]. In accordance with this hypothesis, when valeric acid was exhausted, the molar fraction of 3HV decreased (Fig. 4d, Fig. 5c).

It has been reported that the molecular weight determines different thermomechanical properties of PHA polymers [22, 32]. Besides, the molecular weight depends on the culture conditions, and in the case of copolymers it can depend on the monomer composition [1, 23]. In the case of *Azotobacter* spp, it has been reported that this bacterium synthesizes PHB with a high and ultra-high molecular mass [10, 23, 32].

Figure. 6 shows the PHBV molecular mass distribution under different OTR_{max} conditions. The mean molecular mass (MMM) was between 6600 – 6700 kDa, during the exponential growth phase (at 12 h). After this time, the MMM remained constant between 6500 and 6600 until the end of the culture (21 h and 54 h for cultures developed at high and low OTR_{max} , respectively). These results indicated that there are no significant changes in MMM under the conditions evaluated. In contrast, Gómez-Hernández et al. [10], reported that in batch cultures of OP strain of *A. vinelandii*, the MMM was affected by the oxygen condition. At low OTR_{max} ($5 \text{ mmol L}^{-1} \text{ h}^{-1}$), the production of ultra-high molecular weight polymers (8300 kDa) was promoted; whereas, at higher OTR_{max} ($8\text{-}11 \text{ mmol L}^{-1} \text{ h}^{-1}$) the MMM decreased to 3500 kDa.

It is important to point out that, OPNA is a mutant strain derivate from OP; however, it has been previously reported that OP and OPNA strains have different phenotypic responses under several culture conditions [9]. Previously, Castillo et al. [6] reported that in fed-batch cultures of *A. vinelandii* OPNA under two C/N ratios (14 and 18) and different DOT profiles, the molecular weight was not affected by the changes on the oxygen condition.

From a technological point of view, it is highlighted that the production of copolymers with different monomeric composition is possible by the manipulation of the OTR, without affecting the molecular weight. Could allow expanding the potential application of these copolymers in the biomedical, pharmaceutical, and industrial fields.

Conclusions

In summary, our study has shown that a high valeric acid concentration (from 10 to 40 mM) has a negative effect on cell growth, and PHBV accumulation in the OPNA strain in shaken flasks and bioreactor cultures. However, this precursor concentration has a positive influence on the 3HV content in the copolymer, in comparison to that obtained at lower concentration of this precursor. In order to avoid the inhibition of cell growth and PHA accumulation, without affecting the 3HV content in the copolymer; the use of feeding- pulses fed-batch or pH-stat feeding strategy could be established [39].

On the other hand, increasing OTR_{max} from 4.96 to 20.3 $mmol L^{-1} h^{-1}$ increases the molar fraction of 3HV monomers (6.5 to 8,2 %) in batch cultures, indicating that OTR has a positive influence on 3HV content. It could be interesting, from a technological point of view, to establish strategies, through the manipulation of operational conditions as the OTR, to produce PHBV copolymers with different monomeric compositions at industrial level.

Declarations

Compliance with Ethical Standards

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors Contributions

AJTP designed experiments, performed the most of analysis, experiments, wrote and revised the manuscript, HSL conducted the analysis and manuscript writing, DS review and help in manuscript writing, and CP designed the experiments and reviewed and wrote the manuscript.

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Availability of data and materials

The authors agree to publish all the data in this article.

References

1. Albuquerque MGE, Martino V, Pollet E, Avérous L, Reis MAM (2011) Mixed culture polyhydroxyalkanoate (PHA) production from volatile fatty acid (VFA)-rich streams: effect of substrate composition and feeding regime on PHA productivity, composition and properties. *J Biotechnol* 151(1): 66-76.
2. Anderlei T, Zang W, Papaspyrou M, Büchs J (2004) Online respiration activity measurement (OTR, CTR, RQ) in shaken flasks. *Biochem Eng J* 17:187–194.
3. Anjum A, Zuber M, Zia KM, Noreen A, Anjum MN, Tabasum S (2016). Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: a review of recent advancements. *Int J Biol Macromol* 89: 161-174.
4. Blunt W, Sparling R, Gapes DJ, Levin DB, Cicek N (2018) The role of dissolved oxygen content as a modulator of microbial polyhydroxyalkanoate synthesis. *World J Microb Biot* 34(8): 1–13.
5. Castillo T, Heinzle E, Peifer S, Schneider K, Peña, C (2013). Oxygen supply strongly influences metabolic fluxes, the production of poly (3-hydroxybutyrate) and alginate, and the degree of acetylation of alginate in *Azotobacter vinelandii*. *Process Biochemistry* 48(7): 995-1003.
6. Castillo T, Flores C, Segura D, Espín G, Sanguino J, Cabrera E, Peña C (2017). Production of polyhydroxybutyrate (PHB) of high and ultra-high molecular weight by *Azotobacter vinelandii* in batch and fed-batch cultures. *J Chem Technol Biotechnol* 92(7): 1809-1816.
7. Chung YJ, Cha HJ, Yeo JS, Yoo YJ (1997). Production of poly (3-hydroxybutyric-co-3-hydroxyvaleric) acid using propionic acid by pH regulation. *J Ferment Bioeng* 83(5): 492-495.
8. Díaz-Barrera A, Peña C, Galindo E (2007). The oxygen transfer rate influences the molecular mass of the alginate produced by *Azotobacter vinelandii*. *Appl Microbiol Biotechnol* 76(4): 903-910.
9. García A, Segura D, Espín G, Galindo E, Castillo T, Peña C (2014). High production of poly- β -hydroxybutyrate (PHB) by an *Azotobacter vinelandii* mutant altered in PHB regulation using a fed-batch fermentation. *Biochem Eng J* 82: 117–123.
10. Gómez-Hernández E, Salgado-Lugo H, Segura D, García A, Díaz-Barrera A, Peña C (2020). Production of poly-3-hydroxybutyrate (P3HB) with ultra-high molecular weight (UHMW) by mutant strains of *Azotobacter vinelandii* under microaerophilic conditions. *Appl Biochem Microbiol*. 1-17.
11. Hernández-Eligio A, Castellanos M, Moreno S, Espín G (2011). Transcriptional activation of the *Azotobacter vinelandii* polyhydroxybutyrate biosynthetic genes phbBAC by PhbR and RpoS. *Microbiology* 157(11): 3014-3023.
12. Inomura K, Bragg J, Follows MJ (2017). A quantitative analysis of the direct and indirect costs of nitrogen fixation: a model based on *Azotobacter vinelandii*. *ISME J* 11(1): 166-175.
13. Juengert J, Bresan S, Jendrossek D (2018). Determination of polyhydroxybutyrate (PHB) content in *Ralstonia eutropha* using gas chromatography and Nile red staining. *Bio Protoc* 8: 27-48.

14. Khanna S, and Srivastava AK (2005). Recent advances in microbial polyhydroxyalkanoates. *Process Biochem* 40(2): 607-619.
15. Klimek J, and Ollis DF (1980). Extracellular microbial polysaccharides: kinetics of *Pseudomonas*, *Azotobacter vinelandii*, and *Aureobasidium pullulans* batch fermentations. *Biotechnol Bioeng* 22(11): 2321-2342.
16. Lee WH, Azizan MN, Sudesh K (2004). Effects of culture conditions on the composition of poly (3-hydroxybutyrate-co-4-hydroxybutyrate) synthesized by *Comamonas acidovorans*. *Polym Degrad Stabil* 84(1): 129-134.
17. Loo CY, and Sudesh K (2007). Polyhydroxyalkanoates: bio-based microbial plastics and their properties. *Malay Polym J*, 2(2): 31-57.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem* 193(1): 265-275.
19. Manchak J, and Page W (1994). Control of polyhydroxyalkanoate synthesis in *Azotobacter vinelandii* strain UWD. *Microbiology*, 140(4): 953–963.
20. Matsusaki H, Abe H, Doi Y. (2000) Biosynthesis and properties of poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant strains of *Pseudomonas* 61-3. *Biomacromolecules* 1, 17-22.
21. Millán M, Segura D, Galindo E, Peña C. (2016). Molecular mass of poly-3-hydroxybutyrate (P3HB) produced by *Azotobacter vinelandii* is determined by the ratio of synthesis and degradation under fixed dissolved oxygen tension. *Process Biochem* 51(8): 950-958.
22. Muhammadi, Shabina, Afzal M, Hameed S (2015). Bacterial polyhydroxyalkanoates-eco-friendly next generation plastic: Production, biocompatibility, biodegradation, physical properties and applications. *Green Chem Lett Rev* 8(3-4): 56-77.
23. Myshkina VL, Nikolaeva DA, Makhina TK, Bonartsev AP, Bonartseva GA (2008). Effect of growth conditions on the molecular weight of poly-3-hydroxybutyrate produced by *Azotobacter chroococcum* *Appl Biochem Microbiol* 44(5): 482-486.
24. Myshkina VL, Ivanov EA, Nikolaeva DA, Makhina TK, Bonartsev AP, Filatova EV, Bonartseva GA (2010). Biosynthesis of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer by *Azotobacter chroococcum* strain 7B. *Appl Biochem Microbiol* 46(3): 289-296.
25. Noda I, Green PR, Satkowski MM, Schechtman LA (2005). Preparation and properties of a novel class of polyhydroxyalkanoate copolymers. *Biomacromolecules* 6(2): 580-586.
26. Noguez R, Segura D, Moreno S, Hernandez A, Juarez K, Espin G (2008) Enzyme INtr, NPr and IIA Ntr are involved in regulation of the poly- β -hydroxybutyrate biosynthetic genes in *Azotobacter vinelandii*. *J Mol Microb Biotech* 15: 244–254.
27. Page WJ, Manchak JT, Rudy B (1992). Formation of poly (hydroxybutyrate-co-hydroxyvalerate) by *Azotobacter vinelandii* *Appl Environ Microbiol* 58(9): 2866-2873.
28. Page WJ, and Manchak J (1995). The role of β -oxidation of short-chain alkanates in polyhydroxyalkanoate copolymer synthesis in *Azotobacter vinelandii* *Can J Microbiol* 41(13): 106-114.

29. Pan P, and Inoue Y (2009) Polymorphism and isomorphism in biodegradable polyesters. *Prog Polym Sci* 34: 605–640.
30. Park CH, and Damodaran, VK (1994). Biosynthesis of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) from ethanol and pentanol by *Alcaligenes eutrophus*. *Biotechnol Prog* 10(6): 615-620.
31. Peña C, Galindo E, Peter C, Büchs J (2007). Evolution of the specific power consumption and oxygen transfer rate in alginate-producing cultures of *Azotobacter vinelandii* conducted in shaken flasks. *Biochem Eng J.* 36:73–80.
32. Peña C, López S, García A, Espín G, Romo-Urbe A, Segura D (2014). Biosynthesis of poly- β -hydroxybutyrate (PHB) with a high molecular mass by a mutant strain of *Azotobacter vinelandii* (OPN). *Ann Microbiol* 64(1): 39-47.
33. Pozo C, Martinez-Toledo MV, Rodelas B, Gonzalez-Lopez J (2002). Effects of culture conditions on the production of polyhydroxyalkanoates by *Azotobacter chroococcum* H23 in media containing a high concentration of alpechin (wastewater from olive oil mills) as primary carbon source. *J Biotechnol* 97(2): 125-131.
34. Rivera-Briso AL and Serrano-Aroca Á (2018). Poly (3-Hydroxybutyrate-co-3-Hydroxyvalerate): Enhancement strategies for advanced applications. *Polymers* 10(7): 732- 760.
35. Ryu HW, Cho KS, Goodrich PR, Park CH (2008). Production of polyhydroxyalkanoates by *Azotobacter vinelandii* UWD using swine wastewater: Effect of supplementing glucose, yeast extract, and inorganic salts. *Biotechnol Bioprocess Eng* 13(6): 651-658.
36. Savenkova L, Gercberga Z, Bibers I, Kalnin M (2000). Effect of 3-hydroxy valerate content on some physical and mechanical properties of polyhydroxyalkanoates produced by *Azotobacter chroococcum*. *Process Biochem* 36(5): 445-450.
37. Steinbüchel A, Hustede E, Liebergesell M, Pieper U, Timm A, Valentin H (1992). Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. *FEMS Microbiol Lett* 103(2-4): 217-230.
38. Sudesh K, Doi Y (2005). Polyhydroxyalkanoates. *Handbook of Biodegradable Polymers*, pp 219-256.
39. Mumtaz T, Abd-Aiz S, Rahman NAA, Yee PL, Shirai Y, Hassan MA (2009). Fed-batch production of P (3HB-co-3HV) copolymer by *Comamonas* sp EB 172 using mixed organic acids under dual nutrient limitation. *Eur J Sci Res.* 33(3), 374-384.
40. Urtuvia V, Maturana N, Peña C, Díaz-Barrera A (2020). Accumulation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by *vinelandii* with different 3HV fraction in shaken flasks and bioreactor. *Bioproc Biosys Eng* 43: 1469–1478.
41. Visser AS, and Postma PW (1973). Permeability of *Azotobacter vinelandii* to cations and anions. *BBA Biomembranes* 298(2): 333-340.
42. Volova TG, and Kalacheva GS (2005). The synthesis of hydroxybutyrate and hydroxyvalerate copolymers by the bacterium *Ralstonia eutropha*. *Microbiology* 74(1): 54-59.

Zeng A-P, Byun T, Posten C, Deckwer WD (1994) Use of the respiratory quotient as a control parameter for optimum oxygen supply and scale-up of 2,3-butanediol production under micro- aerobic conditions. *Biotechnol Bioeng.* 19: 1107–1114.

Tables

Table 1. Factors (variables) and levels (low (-1) and high (+1)). The OTR was calculated for 100 mL (5.8 mmol L⁻¹ h⁻¹), and for 200 mL (2.9 mmol L⁻¹ h⁻¹).

Factors	Levels	
	-1	+1
Valeric acid concentration (mM)	10	40
Filling volume (mL)	100	200
Timing valeric acid addition	12	24

Table 2 Values of response variables. Where capital letters are A: valeric acid concentration, B: Filling volume, C: Timing valeric acid addition, while the signs (-) and (+) refer to the low and high level evaluated for each factor, respectively

Run n°	Variable factors			Responses	
	A	B	C	% PHB ^a	% 3HV ^a
1	-1	-1	-1	88.3 ± 0.6	11.7 ± 0.6
2	-1	+1	-1	83.2 ± 1.2	16.8 ± 1.2
3	+1	-1	-1	72.0 ± 2.3	28.0 ± 2.3
4	+1	+1	-1	79.0 ± 2.0	22.6 ± 2.1
5	-1	-1	+1	88.7 ± 1.1	11.3 ± 1.1
6	-1	+1	+1	92.9 ± 4.3	7.1 ± 4.3
7	+1	-1	+1	95.5 ± 1.0	1.1 ± 0.4
8	+1	+1	+1	98.6 ± 0.2	1.4 ± 0.2

^aExperiments in triplicate.

Table 3 Synthesis of the PHBV copolymer by *Azotobacter* species, in shaken flaks adding valerate or valeric acid.

*(Without neutralize)

Precursor	Precursor concentration (mM)	HV content (mol%)	<i>Azotobacter</i> species	Reference
Valerate	20	21.6	<i>A. chroococcum</i> 7B	[24]
Valerate	40	24.0	<i>A. vinelandii</i> UWD	[27]
Valerate	10	27.4	<i>A. vinelandii</i> OP	[40]
Valeric acid*	40	39.0	<i>A. vinelandii</i> OPNA	This work

Table 4 Kinetic parameters of OPNA strain under different valeric acid concentrations in bioreactor cultures.

Parameters	Valeric acid concentration (mM)	
	10	40
Specific growth rate (μ)	0.14 ± 0.003	0.11 ± 0.002
Protein (g L^{-1})	2.0 ± 0.2	1.89 ± 0.1
Volumetric uptake sucrose rate ($\text{g}_{\text{sucrose}} \text{L}^{-1} \text{h}^{-1}$)	0.09 ± 0.01	0.07 ± 0.05
$Y_{\text{Biomass/Sucrose}} (\text{g}_x / \text{g}_{\text{sucrose}})$	0.32 ± 0.003	0.30 ± 0.004
$Y_{\text{PHBV/Sucrose}} (\text{g}_{\text{PHBV}} / \text{g}_{\text{sucrose}})$	0.33 ± 0.009	0.15 ± 0.007

Table 5 Kinetic parameters of OPNA strain under different oxygen transfer rate in bioreactor cultures.

Parameters	Oxygen transfer rate (mmol L ⁻¹ h ⁻¹)	
	4.96	20.3
Specific growth rate (μ)	0.03 ± 0.0002	0.14 ± 0.003
Volumetric uptake sucrose rate (g _{sucrose} L ⁻¹ h ⁻¹)	0.3 ± 0.03	0.9 ± 0.001
Volumetric uptake valeric acid rate (g _{valeric acid} L ⁻¹ h ⁻¹)	0.042 ± 0.001	0.11 ± 0.004
Y _{Biomass/Sucrose} (g _x / g _{sucrose})	0.42 ± 0.02	0.32 ± 0.003
Y _{PHBV/Sucrose} (g _{PHBV} / g _{sucrose})	0.47 ± 0.01	0.33 ± 0.05

Figures

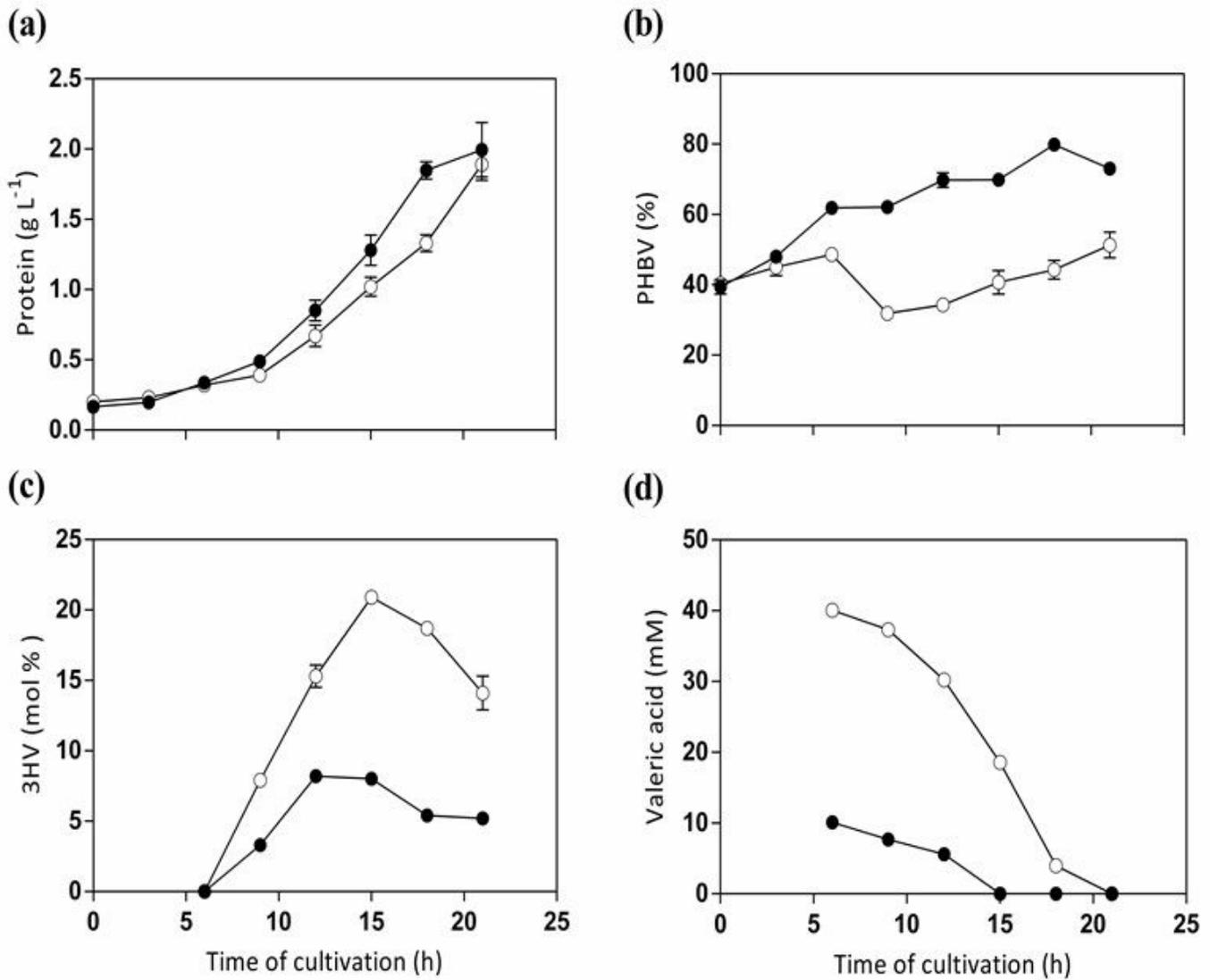


Figure 1

Gas chromatographic analysis of PHBV produced by *A. vinelandii* OPNA. 3HB; 3-hydroxybutyrate; 3HV: 3-hydroxyvalerate; Internal standard: Benzoic acid (a). Fragmentation patterns in the mass spectra of methyl-esters and chemical structure of 3HB and 3HV, respectively (b).

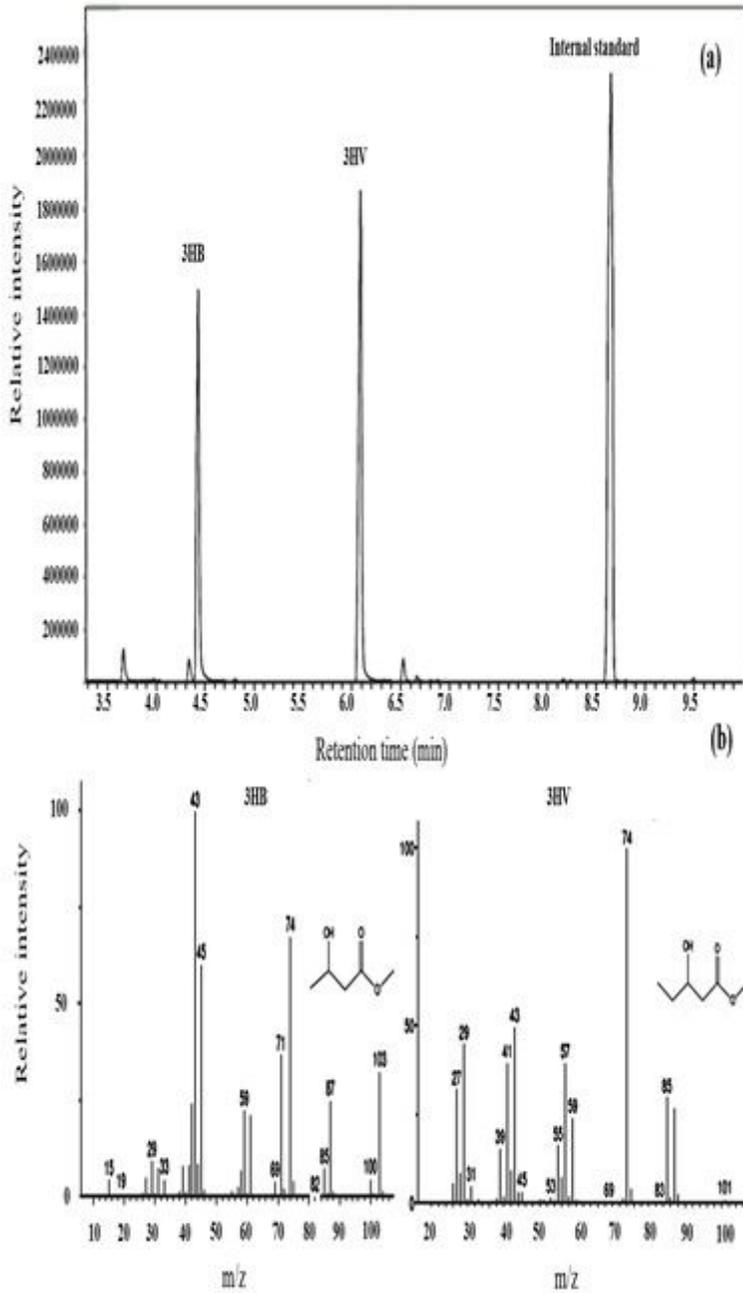


Figure 2

Protein concentration (a), percentage of accumulation of PHBV (b), molar fraction of 3HV (c) in 500 mL shaken flasks cultures of *A. vinelandii* OPNA. Culture with valeric acid was added at 12 h in a final concentration of 40 mM (Δ) and control condition (with no valeric acid) (\bullet).

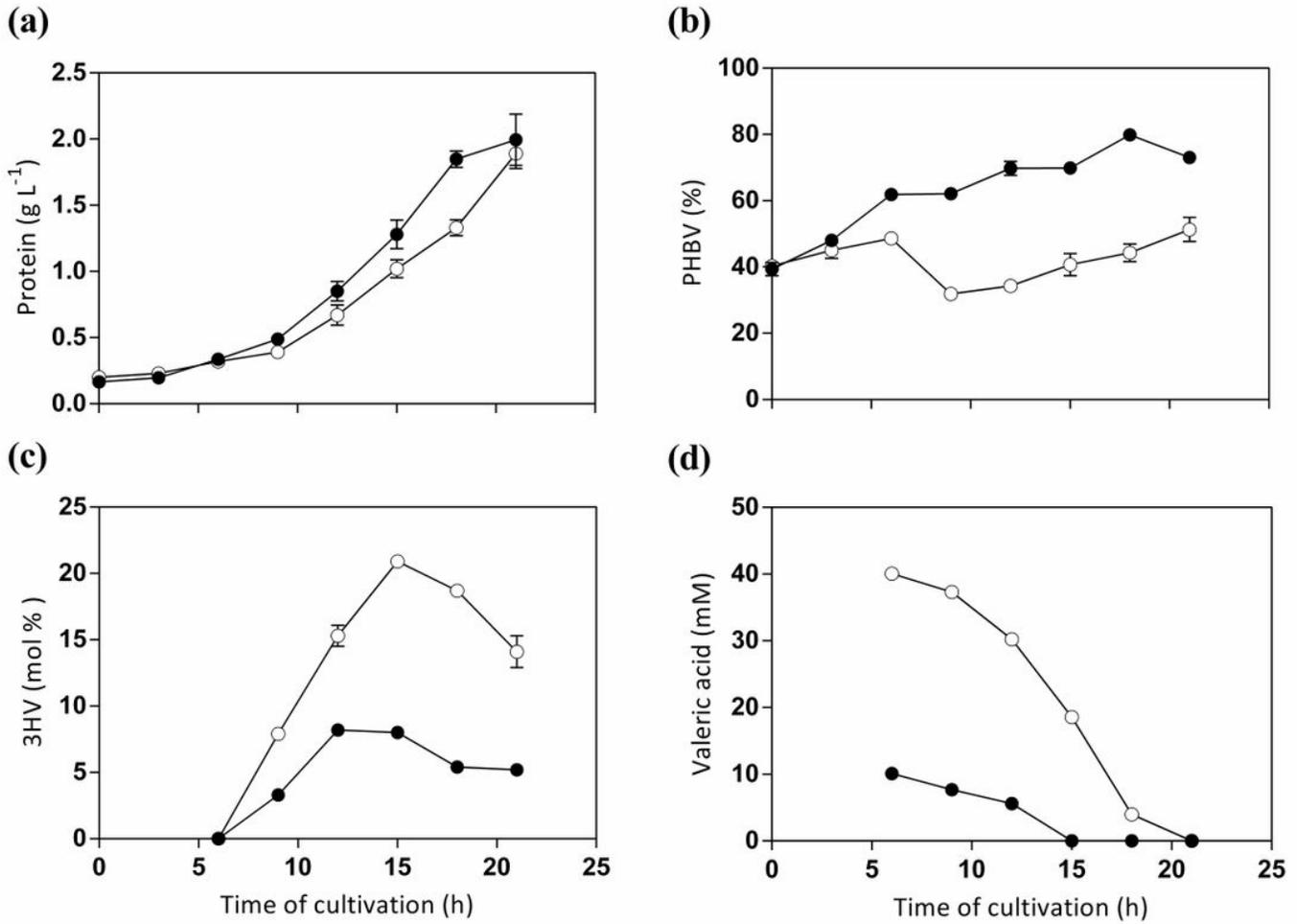


Figure 3

Growth kinetics estimated as protein concentration (a), PHBV accumulation (b), 3HV molar fraction (c) and valeric acid concentration (d) of *A. vinelandii* OPNA in batch cultures developed at constant agitation rate of 700 rpm with a concentration of valeric acid of 10 mM (●) and 40 mM (○). Valeric acid was added at 6 h of cultivation.

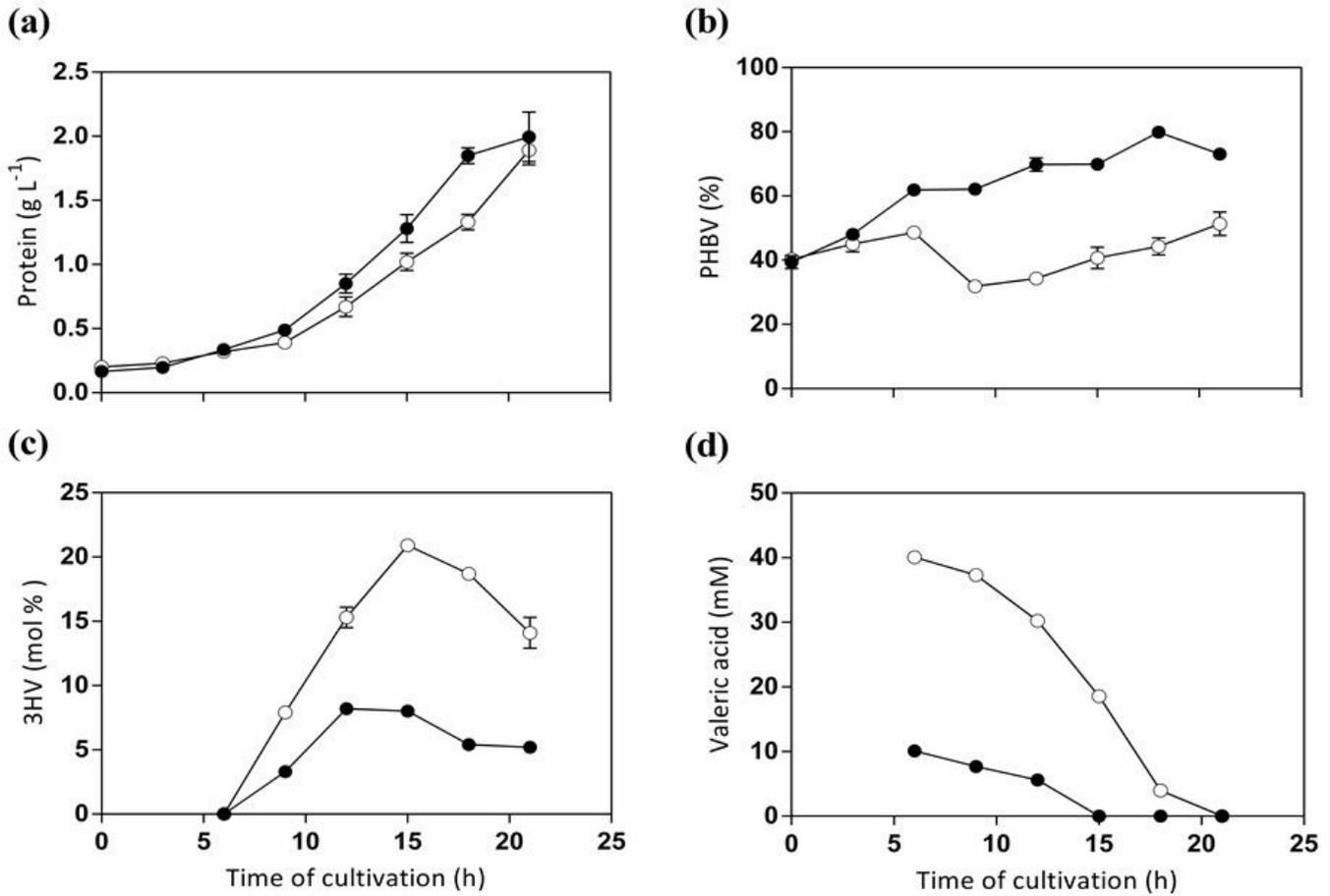


Figure 4

DOT and OTR profile in dashed and solid line, respectively (a), protein growth kinetics (b), sucrose concentration (c) and valeric acid concentration (d) of *A. vinelandii* OPNA in batch cultures developed at constant agitation rate of 300 rpm (\square) and 700 rpm (\bullet). DOT and OTR profile for 300 rpm (thick lines) and for 700 rpm (thin lines). Valeric acid was added at 9 h and 6 h of cultivation, for 300 (dashed arrow) and 700 rpm (solid arrow), respectively.

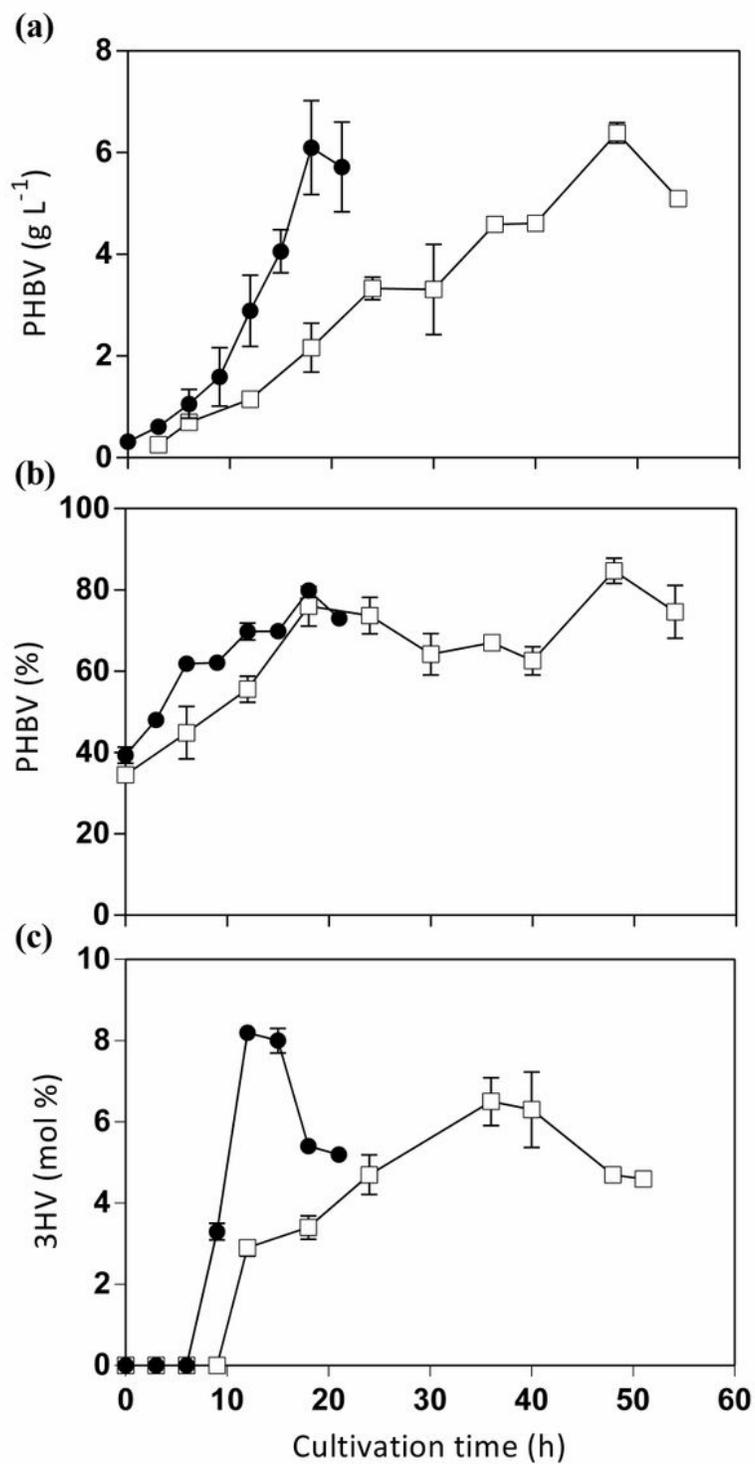


Figure 5

PHBV accumulation (a), PHBV concentration (b), 3HV molar fraction (c) of *A. vinelandii* OPNA in cultures developed at constant agitation rate of 300 rpm (□) and 700 rpm (●). A dashed and solid arrow indicates valeric acid addition for 300 and 700 rpm.

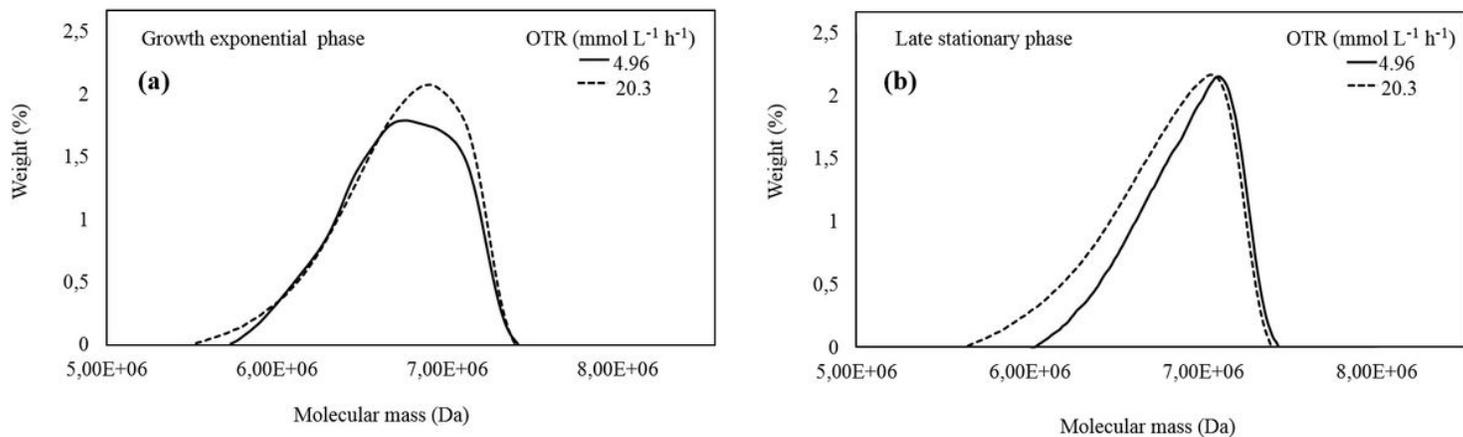


Figure 6

Molecular mass distributions of PHBV isolated from *A. vinelandii* OPNA in cultures developed at constant agitation rate of 300 rpm (—) and 700 rpm (---).