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Improvement of the poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) production by dual feeding with levulinic acid and sodium propionate in *Cupriavidus necator*

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In the context of increasing volatility of oil prices, replacement of petroleum based plastics by bioplastics is a topic of increasing interest. Poly(hydroxyalkanoate)s (PHAs) are among the most promising families in this field. Controlling composition of the polymer on the monomeric level remains a pivotal issue. This control is even more difficult to achieve when the polymer is not synthesized by chemists, but produced by nature, in this case, bacteria. In this study mechanism and role of two 3-hydroxyvalerate (3-HV) inducing substrates on the production of PHBV with high, 80%, 3-HV content were evaluated. It was found that levulinic acid contributes to biomass and bio-polymer content enhancement, whereas sodium propionate mainly contributes to 3-HV enhancement. Optimized proportions of feeding substrates at 1 g/L and 2.5 g/L, respectively for levulinic acid and sodium propionate allowed a 100% productivity enhancement, at 3.9 mg/L/hour, for the production of PHBV with 80% 3-HV.

Introduction

Necessity of more sustainable and responsible behaviour rose these last years all around the world. Different aspects were concerned so far: energy, fuel, plastics, waste management, recycling, etc. [1]. Plastics are ubiquitous in today's society, unfortunately the mainly used are from fossil origin and non-biodegradable. Substitution of traditional petroleum-based plastic materials, by bio-plastics, is not an easy task, new materials have to reach economic and technical constraints, or present specific characteristics to meet not yet addressed issues [2,3].

Poly(hydroxyalkanoate) (PHA) family represents a very promising group of bio-polymers [4]. This family presents a wide range of polymers with very diverse characteristics, depending on the length of the alkanoate substitute and of the co-polymers composition [5–7]. The most studied part of the PHA family concerns the short-chain length PHA (PHA_{SCL}), which main representatives are poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) [8]. The production of PHB can be achieved by many strains and at relevant yields and production rates [9–11]. These polymers can be used either for technical [12], medical [13] or bioremediation [14,15] purposes. However, certain applications require less brittle, more elastic polymer with lower glass transition temperature (T_g). This can be achieved by modulation of PHB composition and the incorporation of the 3-hydroxyvalerate (3-HV) monomeric units [16,17].

Some, rather rare, strains, such as *Haloferax mediterranei* [18,19] or *Halomans campisalis* [20], are able to produce PHBV on unrelated carbon sources. However, the main PHBV producers, such as *Cupriavidus* genus (previously known as *Alcaligenes*, among others) were shown to produce the biopolymers with noticeable 3-HV content when cultured on the odd-carbon numbered inducing substrates [21,22].

Recently, we have shown [23] that in the case of *Cupriavidus necator* DSM 545 the combination of levulinic acid with sodium propionate gave the best results for the enhancement of 3-HV content in PHBV. In the present study the interactions between those compounds, as well as their optimal concentrations and proportions were studied in order to enhance the PHBV production with high 3-HV content.

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Materials and methods

General

All chemicals were from Sigma Aldrich, *C. necator* DSM 545 was from the DSMZ collection. Sartorius Certomat IS incubators were used for the fermentations.

Fermentations

Mineral medium: 6.7 g of $Na_2HPO_4{\cdot}2H_2O$, 1.5 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.01 g of $CaCl_2{\cdot}2H_2O$, 0.06 g of $Fe(NH_4)_2(SO_4)_2$ and 1 mL of trace element solution for 1 L. Trace element solution: 0.3 g of H_3BO_3, 0.2 g of $CoCl_2$, 0.1 g of $ZnSO_4{\cdot}7H_2O$, 0.03 g of $MnCl_2{\cdot}4H_2O$, 0.02 g of $NaMoO_4{\cdot}2H_2O$, 0.02 g of $NiCl_2{\cdot}6H_2O$ and 0.01 g of $CuSO_4{\cdot}5H_2O$ for 1 L. The pH value was adjusted at 7.

100 mL inoculates were grown for 24 hours on mineral medium in 300 mL shaking flasks, at 30°C, 150 rpm. Further inoculations were performed with 5% of 24 hours starter culture and the only C source was a mix of levulinic acid and sodium propionate at different combinations with the concentrations ranging from 1 to 5 g/L. The screening experiments were performed in 300 mL shaking flasks filled with 100 mL medium and the scale up was made in the 3 L shaking flasks filled with 1 L medium.

Analysis

The 3-hydroxyvalerate content was determined from ^1H [24] and ^{13}C [25] NMR spectra, performed on a Bruker 500 MHz instrument, and is given in mol %.

The PHA content in cells was determined by the thermogravimetric analysis (TGA) [26] performed with a Q500 TA instrument.

The concentrations of levulinic acid and sodium propionate were evaluated by HPLC, performed with an Alliance Waters instrument, equipped with a Photodiode Array (PDA) detector (Waters 2996) operating at 220 nm, using a Metacarb 67H column from Varian, and deionized water containing 1% H_2SO_4 as mobile phase.

All experiments were performed at least twice. All values presented here are averaged, and the error bars represent standard deviation.

Results and discussion

Screening of different proportions of levulinic acid and sodium propionate

To evaluate the most suitable proportions of the two, previously shown to be the most promising [23], 3-HV inducing substrates, the experiments with different initial concentrations ranging from 1 to 5 g/L of each, were performed (Fig. 1).

The time-course of biomass (Fig. 1a) shows that a rather long period, 24 hours, is needed in any case for the acclimation of cells to those substrates. Also, as soon as the concentration of sodium propionate reaches 5 g/L, the biomass enhancement is no longer observed. The experiments with low amount of sodium propionate (1 g/L) concomitant with levulinic acid concentration below 5 g/L, have reached the maximum cell dry mass (CDM), after 48 hours of experiment. The highest biomass was observed with the highest concentration of levulinic acid (5 g/L) when the sodium propionate concentration was kept at a minimum (1 g/L).

These observations are consistent with the analysis of PHA content in cells (Fig. 1b), no PHA were produced at 24 hours of fermentation. The amount of PHA was more relevant at higher

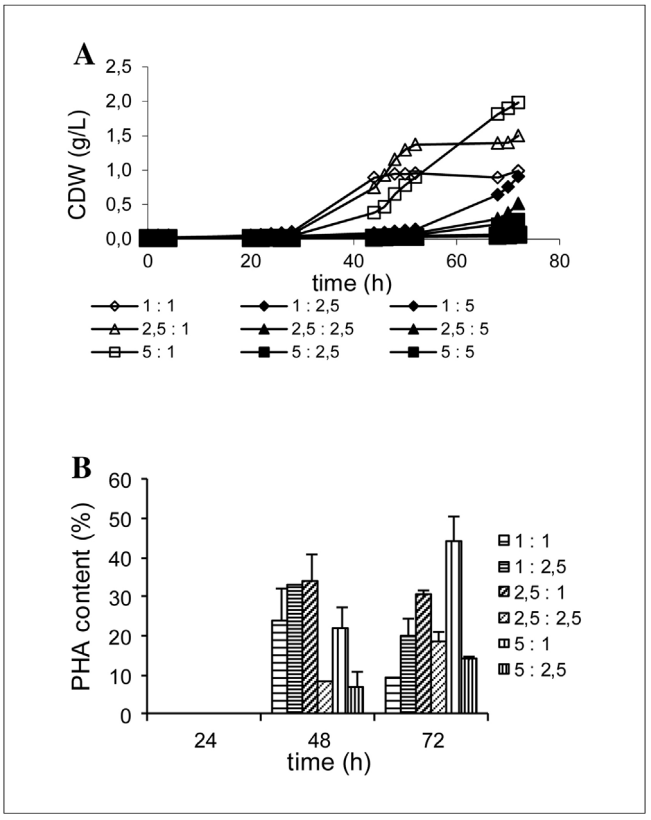

FIGURE 1

Time courses for CDM **(a)** and PHA content in cells **(b)**, during the screening experiments. The different initial concentrations of levulinic acid and sodium propionate are indicated in g/L, following this order (1:2.5 corresponds to the experiment with the initial concentrations of levulinic acid and sodium propionate of 1 and 2.5 g/L, respectively).

concentration of levulinic acid. It decreased between 48 and 72 hours when levulinic acid was fed at 1 g/L and increased when it was at 5 g/L. At 2.5 g/L of initial levulinic acid, the evolution of PHA content was dependant on the concentration of sodium propionate: it slightly decreased (merely remained stable) at 1 g/L and increased at 2.5 g/L of the latter substrate.

For a better understanding of the phenomena occurring during these fermentations we have also monitored the consumption of substrates (Fig. 2). With an initial sodium propionate concentration of 5 g/L no consumption of either sodium propionate or levulinic acid was observed (Fig. 2c,f,i), consistent with the above observations (Fig. 1a). Also, at small concentration of sodium propionate, 1 g/L, it has been almost linearly consumed in 48 hours. Levulinic acid at small concentration, 1 g/L, was concomitantly consumed (Fig. 2a), its consumption being delayed at higher concentrations, 2.5 g/L (Fig. 2d) and 5 g/L (Fig. 2g). This can explain the limited to 48 hours growth of cells at small, 1 g/L, sodium propionate concentrations concomitant with small to moderate, 1 to 2.5 g/L, levulinic acid concentrations; as well as the decrease of PHA content in cells between 48 and 72 hours of cells' cultivation in those experiments, since, after the exhaustion of sodium propionate and levulinic acid, the PHA become the only source of carbon for cells. Initial sodium propionate at 2.5 g/L requires 72 hours for being totally consumed, and levulinic acid concentration is hardly modified during the whole fermentation process in these experiments (Fig. 2b,e,h). These observations