International Journal of Biological Macromolecules 98 (2017) 654–663

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Combining molecular and bioprocess techniques to produce poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) with controlled monomer composition by *Burkholderia sacchari*

Thatiane T. Mendonça[a], Rafaela R. Tavares[a,1], Lucas G. Cespedes[a], Ruben J. Sánchez-Rodriguez[b], Jan Schripsema[b], Marilda K. Taciro[a], José G.C. Gomez[a], Luiziana F. Silva[a,*]

[a] *Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brazil*
[b] *Centro de Ciências e Tecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brazil*

ARTICLE INFO

Article history:
Received 6 November 2016
Accepted 2 February 2017
Available online 4 February 2017

Keywords:
Burkholderia sacchari
Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
Modulated PHA composition
pha *PCJ* operon
Aeromonas sp.

ABSTRACT

Biopolymers as polyhydroxyalkanoates (PHA) composed by different *co*-monomers 3-hydroxybutyrate and 3-hydroxyhexanoate [P(3HB-*co*-3HHx)] has attracted interest since its properties are similar to low density polyethylene. *Burkholderia sacchari* produces this copolymer with a very low 3HHx molar fraction, about 2 mol%. *B. sacchari* mutant (unable to produce polymer) was engineered to host PHA biosynthesis genes (*phaPCJ*) from *Aeromonas* sp. In addition, a two-step bioprocess to increase biopolymer production was developed. The combination of these techniques resulted in the production of P(3HB-*co*-3HHx) with 3HHx content up to 20 mol%. The PHA content was about 78% of dry biomass, resulting in PHA volumetric productivities around 0.45 g l^{-1} h^{-1}. The P(3HB-*co*-3HHx) containing 20 mol% of 3HHx presented an elongation at brake of 945%, higher than reported before for this PHA composition. Here we have described an approach to increase 3HHx content into the copolymer, allowing the precise control of the 3HHx molar fractions.

1. Introduction

Polyhydroxyalkanoates (PHA) are accumulated as intracellular granules by several bacteria, more expressively under limitation of an essential nutrient and excess of carbon source [1]. Huge efforts have been undertaken to achieve good producers of PHA able to synthesize polymers presenting monomer compositions covering the market demands, to make these polyesters competitive substitutes to the petrochemical polymers. PHA as a biodegradable plastic could be an alternative to solve environmental problems inherent to the quick disposal and accumulation of conventional plastics, since it is completely broken into water and CO_2 much faster than the first ones [2,3].

Among PHA containing short chain-length (SCL – C3–C5) monomers poly-3-hydroxybutyrate (P3HB) has been the most studied. Although having characteristics similar to polypropylene, its brittleness and stiffness limit its range of applications. To overcome this issue, the incorporation of medium chain-length (MCL – C6–C14) monomers has been studied for the production of copolymers with more flexibility [2,4].

The monomer composition of PHA determines polymer mechanical properties and enables its use in several applications. This diversity, among other factors, is controlled by the specificity of the enzyme PHA synthase present in the polymer-producing microorganism [5].

Fig. 1 depicts some pathways supplying monomers to the PHA biosynthesis from carbohydrates and fatty acids [6]. Extensive work has established *Ralstonia eutropha* as a platform for PHA production [7] and a number of bioprocesses were developed using this bacterium.

Abbreviations: PHA, polyhydroxyalkanoates; 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HHp, 3-hydroxyheptanoate; 3HHx, 3-hydroxyhexanoate; GC, gas chromatography; HPLC, high pressure liquid chromatography; GPC, gel permeation chromatography; DSC, differential scanning calorimetry; TGA, thermogravimetric analysis; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; Mw, weight average molecular weights; Mn, number-average molecular weight; Mw/Mn, polydispersity index; T_g, glass transition temperature; T_c, crystallization temperature; T_m, melting temperature.

* Corresponding author at: Departamento de Microbiologia, Instituto de Ciências Biomédicas II, Universidade de São Paulo. Av. Prof. Lineu Prestes, 1374, Cidade Universitária, CEP 05508-000, São Paulo, SP, Brasil.
E-mail addresses: thatianemendonca@usp.br (T.T. Mendonça), lukneif@usp.br (L.F. Silva).
[1] Present address: Biotechnology Department, PDEng Programm on Bioprocess Engineering Designer, TU Delft, Delft, Netherlands.

http://dx.doi.org/10.1016/j.ijbiomac.2017.02.013
0141-8130/

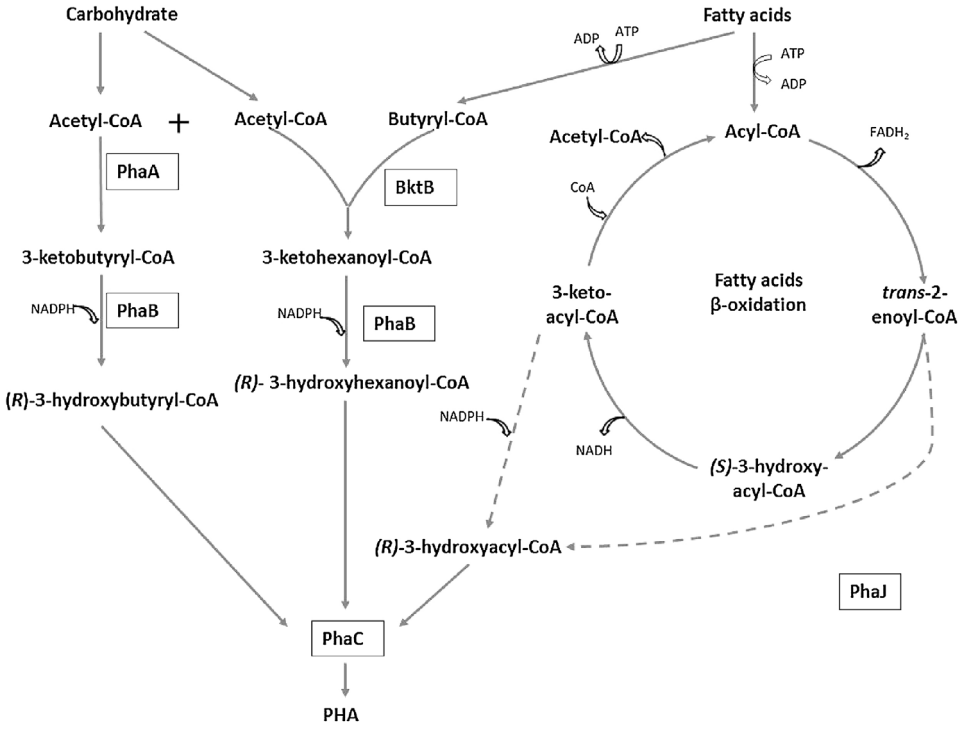

Fig. 1. Reactions and genes involved on PHA (3HB and $3HA_{MCL}$) biosynthesis from carbohydrates and organic acids. Enzymes: PhaA and BktB: β-ketotiolase; PhaB: 3-ketoacyl-CoA redutase/acetoacetyl-CoA reductase); PhaC: PHA synthase; PhaJ: enoyl-CoA hydratase (*R*)-specific.

Burkholderia sacchari is a bacterium isolated from soil in Brazil and described as a new species after polyphasic taxonomic studies [8], and recently its genome has been sequenced [9]. This strain proved to be able to use sucrose to grow to high cell densities and to produce up to 75% of cell dry weight (CDW) as poly-3-hydroxybutyrate (P3HB), presenting specific growth rates (up to 0.39 h^{-1}) higher than *Ralstonia eutropha* (up to 0.30 h^{-1}) [10–12]. Differently from *Raltonia eutropha*, *B. sacchari*, besides the ability to grow in sucrose, is an attractive strain to industrial processes since it can also use xylose as a sole carbon source and even hemicellulosic hydrolysates [13,14]. Due its ability in metabolizing these substrates, *B. sacchari* could be used to produce PHA, linked to the sugar cane production mill expanding the concept of biorefineries in these production units [15,16], thus *B. sacchari* constituting a Brazilian platform for PHA production.

This strain has been successfully improved to produce P(3HB-*co*-3HV) with a high efficiency in the conversion of propionate into 3HV units [17–19]. Different organic acids were also evaluated as co-substrates to generate monomers different of 3HB [20]. Besides 3HV monomers produced from odd-chain fatty acids (propionic, valeric, heptanoic, nonanoic and undecanoic acids), 4HB and 3HHx were detected, respectively, from 4-hydroxybutyric and hexanoic acids, though representing 9.1 and 1.6 mol%, respectively in the PHA [20]. Micro-reactor experiments were performed using glucose and hexanoic acid as carbon sources and the maximum 3HHx content reached was 2.4 mol% [21]. Results suggest a high capability to oxidize hexanoic acid through β-oxidation combined with a low specificity of PHA synthase to medium-chain-length (C6–C14) monomers in *B. sacchari*.

PHA synthases presenting higher specificity to 3HHx monomer have been describing in *Aeromonas* spp. in reports of PHA production by these bacteria from plant oils and fatty acids and also by recombinant strains expressing *Aeromonas phaC* genes [22–26].

With the propose of improving the efficiency of *B. sacchari* to produce P(3HB-*co*-3HHx), in the present work, a recombinant strain, harboring *Aeromonas* sp. PHA biosynthesis genes, was developed. A bioprocess was also established allowing the precise control in a wide range of 3HHx molar fraction in the PHA.

2. Material and methods

2.1. Bacterial strains and plasmid

Burkholderia sacchari LFM 101 (LMG 19450^T, CCT 6971^T) and its PHA negative UV mutant (LFM344), obtained in our laboratory, belonging to our bacteria collection, were used in this study. Engineering construction in LFM 344 was done amplifying PHA biosynthesis genes from *Aeromonas* sp. (LFM897), isolated by our group and characterized by ARDRA (Amplified Ribosomal DNA Restriction Analysis) [27]. The plasmid pBBR1MCS-2 [28] was used as a cloning and expressing vector.

2.2. Recombinant strain construction

B. sacchari LFM344 was used in this study as a host for *phaPCJ* from *Aeromonas* sp. genes involved on PHA accumulation. To amplify the PHA biosynthesis operon from *Aeromonas* sp. (LFM897), primers from Lu et al. [24] were used: 5′ TTTGGTACCTGGAGACCGATGATGAATATGG 3′ (underlined sequence shows *Kpn*I restriction site) 5′ACGAAGCTTTTAAGGCAGCTTGACCACGG 3′ (underlined sequence shows *Hind*III restriction site. Restriction sites for appropriate cloning in the plasmid were designed into the 5′ ends. PCR reaction was set up using Phusion High Fidelity DNA polymerase (Thermo Scientific, Waltham, Massachusetts, USA), following the manufacturer's instructions, running the reaction in a thermal cycler (Eppendorf) and using an appropriate program. The amplicon (2.7-kb DNA fragment) obtained was inserted into pBBR1MCS-2, under control of the *lac* promoter, by ligation reaction using DNA T4 ligase (Fermentas Inc./Thermo-Fisher, Waltham, Massachusetts, USA), according to the protocol suggested by manufacturer. The plasmid thus obtained pBBR1MCS-2:$phaPCJ_{Asp}$ was introduced

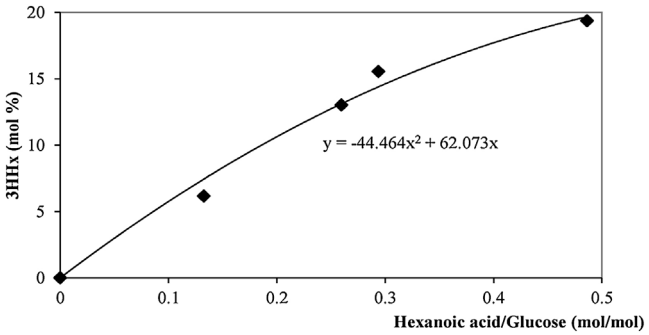

Fig. 2. Relation between hexanoic acid: glucose molar ratio supplied to the bacteria and 3HHx molar fraction in the copolymer synthesized by *B. sacchari* pBBR2*phaPCJ*$_{Asp}$ using the two-step bioprocess.

into *E. coli* XL1- Blue (Stratagene, Agilent, Santa Clara, California, USA) using chemical transformation. Transformants were selected by antibiotic resistance, blue/white colonies detection using IPTG/XGal and PCR screening were employed. Positive clones were confirmed by plasmid extraction (kit Qiagen) followed by digestion (Fermentas *Fast Digestion* Restriction Enzymes) and sequencing the DNA inserted. Plasmid pBBR2*phaPCJ*$_{Asp}$ (Fig. 2) was introduced in *E. coli* S17-1 [29] using chemical transformation, and then transferred to *B. sacchari* by conjugation. Positive clones were recovered on solid mineral medium containing glucose (1 g l^{-1}) and kanamycin. All procedures for DNA manipulation were performed according to the literature [30]. As a control condition to the accumulation experiments, a strain harboring only pBBR1MCS-2 was constructed.

2.3. PHA accumulation assays

Bacterial recombinant strains were cultivated during 24 h (30 °C, 150 rpm) in Luria Bertani broth (LBK: 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl, and 50 μg l^{-1} kanamycin. This previous culture was used as inoculum (10% v/v) to mineral salts medium (MM) [18] under nitrogen limitation to PHA accumulation: 3.5 g l^{-1} Na_2HPO_4; 1.5 g l^{-1} KH_2PO_4; 1.0 g l^{-1} $(NH_4)_2SO_4$; 0.2 g l^{-1} $MgSO_4{\cdot}7H_2O$; 0.01 g l^{-1} $CaCl_2{\cdot}2H_2O$; 0.06 g l^{-1} $C_6H_8O_{7.}{}^{*}{}_{x}Fe^{*}{}_{x}NH_3$; g l^{-1} $C_6H_8O_{7.}{}^{*}{}_{x}Fe^{*}{}_{x}NH_3$; 1.0 ml l^{-1} Trace elements solution. Trace elements solution composition: 0.30 g l^{-1} H_3BO_3; 0.20 g l^{-1} $CoCl_2{\cdot}6H_2O$; 0.10 g l^{-1} $ZnSO_4{\cdot}7H_2O$; 0.03 g l^{-1} $MnCl_2{\cdot}4H_2O$; 0.03 g l^{-1} $NaMoO_4{\cdot}2H_2O$; 0.02 g l^{-1} $NiCl_2{\cdot}6H_2O$; 0.01 g l^{-1} $CuSO_4.5H_2O$. Glucose (10 g l^{-1}) was added at the beginning of the experiment, and fatty acids (0–1.5 g l^{-1}) after 24 h of cultivation, as carbon sources, to promote the synthesis of copolymers. The cultures were incubated at 150 rpm under 30 °C for 72 h. Kanamycin (50 μg ml^{-1}) was added to the medium. Cell dry weight, cellular PHA content and composition were determined at 24 and 72 h of cultivation.

2.4. Bioreactor experiments

A set of fed-batch experiments was performed in a Biostat® B (BBraun – Biotech, Hamburg, Germany) bioreactor 2-liters working volume at 30 °C. The pH was controlled at 7.0 by adding NaOH (1 mol l^{-1}) or H_2SO_4 (1 mol l^{-1}). Dissolved oxygen (DO) was monitored with a polarographic electrode (Ingold, Mettler-Toledo, Greifensee, Switzerland) and maintained above 40% of saturation by varying stirring. Cultures were performed under nitrogen limitation in the accumulation phase. Recombinant strain was pre-cultured in 200-ml MM [18], containing 3 g l^{-1} nitrogen and 6 g l^{-1} glucose (incubation conditions 24 h, 30 °C, 150 rpm). This culture was inoculated in a bioreactor with a reformulated mineral salts medium, presenting the following composition (in g l^{-1}): KH_2PO_4 (0.388); $(NH_4)_2SO_4$ (2.907); $MgSO_4.\ 7H_2O$ (0.310); $CaCl_2{\cdot}2H_2O$ (0.010); $C_6H_8O_{7.}{}^{*}{}_{x}Fe{\cdot}{}^{*}{}_{x}NH_3$ (0.060); NaCl (1.000); Glucose (15.000); trace elements solution (2 ml l^{-1}), which was prepared with H_3BO_3 (0.30 g l^{-1}); $CoCl_2{\cdot}6H_2O$ (0.20 g l^{-1}); $ZnSO_4{\cdot}7H_2O$ (0.10 g l^{-1}); $MnCl_2{\cdot}4H_2O$ (0.03 g l^{-1}); $NaMoO_4{\cdot}2H_2O$ (0.03 g l^{-1}); $NiCl_2{\cdot}6H_2O$ (0.02 g l^{-1}); $CuSO_4{\cdot}5H_2O$ (0.01 g l^{-1}). Immediately after the end of growth phase, indicated by decreasing of O_2 consumption and CO_2 production, a solution containing glucose and hexanoic acid with a glucose:hexanoic acid ratio equal to 140:0–45 g l^{-1} was added as carbon sources. Two feeding rates were tested: 4.95 and 9.90 ml l^{-1} h^{-1}. The following parameters were periodically determined: CDW, carbohydrates, hexanoic acid and ammonium concentrations, PHA content and composition, partial pressures of CO_2 and O_2 in the outlet gas.

2.5. Analytical methods

2.5.1. Cell dry weight (CDW)

Gravimetric method was used to determined CDW.10 ml of culture were harvested by centrifugation at 10,600*g* and lyophilized in microtubes. Dry biomass was weighed using analytical balance (Ohaus, Adventurer, Parsippany, New Jersey, USA).

2.5.2. Carbohydrate and organic acid determination

Glucose and hexanoic acid concentrations were determined by liquid chromatography (HPLC) and gas chromatography (GC), respectively, using methods described previously [10,20].

2.5.3. Ammonium concentration

Ammonium was determined as described before [20]. Briefly, after alkalinization of the sample, an ion-selective electrode (Orion 9512HPBNWP) in a potenciometer (Thermo Scientific, Orion 4 Star, pH-ISE Benchtop, Singapore) was used to measure the ammonium gas formed. From a standard curve of a $(NH_4)_2SO_4$ solution, the amount of nitrogen was calculated.

2.5.4. PHA extraction

PHA was extracted by chloroform from freeze-dried cells in shaken flasks incubated at 30 °C, 150 rpm for 24 h. The PHA-chloroform solution was concentrated in a Rotavapor (Büchi, Rotavapor R-114, Water Bath B-480, Switzerland) and precipitated in ethanol. The polymer extracted was purified after at least three successive chloroform solubilizations and ethanol precipitations.

2.5.5. PHA amount and composition

Freeze-dried cells or purified PHA were subjected to propanolysis [31]. PHA content and composition were determined using a GC method as described previously [10,20]. Briefly, the polyesters were analyzed in an Agilent 7890A GC System (Agilent Technology, Santa Clara, California, USA) equipped with a HP5 capillary column after sample split (1:25). Helium (0.6 ml min^{-1}) was used as carrier gas. Injector and FID temperature were 250 °C and 300 °C, respectively. The oven was programmed to keep temperature at 100 °C for 3 min, increasing temperature at a rate of 6 °C min^{-1} up to 240 °C, which was then maintained for 6 min. Benzoic acid was used as the internal standard. External standards were P3HB (Sigma-Aldrich, Saint Louis, Missouri, USA), P3HB-*co*-3HV (Sigma-Aldrich, Saint Louis, Missouri, USA) and, PHA_{MCL} produced by *P. putida* ATCC29347 from different fatty acids or by *Pseudomonas* sp LFM046 from glucose.

2.6. Molecular weight

PHA samples were analyzed by gel permeation chromatography (GPC). A Waters 1515 pump (Waters Corporation, Nilford,

Massachusetts, USA), a degasser (Viscotek VE7510, Malvern Instruments, Malvern, Worcestershire, UK), an auto-sampler injector 717, a refractive index detector (2414, Waters, USA), associated to a duo detector (Viscotek TDA302, Malvern Instruments, Malvern, Worcestershire, UK) were employed. GPC columns (Phenomenex, Torrance, California, USA) with diameters ranging from 500 a 10^4 Å were used. A calibration curve was prepared with low dispersity polystyrene solubilized in chloroform.

2.7. Thermal properties analysis

2.7.1. Differential scanning calorimetry (DSC)

DSC data of copolymers were recorded on a TA Instruments Q-DSC 2000 instrument calibrated with indium. Samples weighing between 4 and 8 mg were encapsulated in hermetic aluminum pans and heated from −120 to 190 °C at a heating rate of 10 °C min^{-1}, for the first run. After, the samples were cooled to −120 °C and a second heating was carried out at the same heating rate to 190 °C. From this analysis *Tg*, *Tc*, *Tm* and ΔH were calculated.

2.7.2. Thermogravimetric analysis (TGA)

TGA was performed on a TA Instruments TGA 5000. About 10 mg of sample were applied to analyses into platinum pans with dynamic nitrogen atmosphere (25 ml min^{-1}) and 10 °C min^{-1} heating rate from room temperature to 700 °C.

2.8. Nuclear magnetic resonance (NMR) analysis

Samples of copolymers were dissolved in deuterated chloroform and the NMR spectra were recorded on a JEOL ECLIPSE+ 400 (Jeol LTD, Akishima, Tokyo, Japan) spectrometer operating at 400 MHz for ^{1}H. Chemical shifts are given in ppm relative to the signal of tetramethylsilane (TMS), used as an internal chemical shift standard.

3. Results and discussion

In previous studies wild type *B. sacchari* has been shown to accumulate different copolymers when supplied with various carbon sources [20]. From hexanoic acid as co-substrate wt *B. sacchari* accumulated P(3HB-*co*-3HHx) containing up to 1.6 mol% of 3HHx. A cultivation strategy in micro-bioreactor, supplying combinations of glucose/hexanoic acid as feeding solutions, was applied in order to raise the amount of 3HHx in the copolymer produced by wt *B. sacchari*. Applying this cultivation method the strain had reached the accumulation of a PHA containing the maximum molar fraction of 3HHx of 2.4% [21].

From the results obtained with wt *B. sacchari* using those strategies, we concluded that *B. sacchari* PHA synthase specificity to the MCL monomers was the bottleneck to produce P(3HB-*co*-3HHx) containing higher molar fractions of 3HHx. Then, we engineered a *pha* negative mutant of *B. sacchari* inserting the *Aeromonas* sp. PHA biosynthesis genes, which has a higher specificity to 3HHx [22].

The PHA biosynthesis operon *phaPCJ* from *Aeromonas* sp. (LFM897) was selected to construct the recombinant. The DNA fragment of 2716-pb corresponding to $phaPCJ_{Asp}$ was amplified by PCR and successfully cloned into the vector, resulting in pBBR2$phaPCJ_{Asp}$. After that, recombinant *B. sacchari* harboring pBBR$phaPCJ_{Asp}$ was constructed and its ability to produce PHA tested under some conditions.

3.1. PHA production by recombinant B. sacchari

Two sets of experiments were performed in shaken flasks to evaluate polymer accumulation by recombinant *B. sacchari* harboring pBBR2$phaPCJ_{Asp}$. In the first set of experiments, 10 g l^{-1} glucose and 0–1.5 g l^{-1} hexanoic acid were supplied. This condition should promote the accumulation of P(3HB-*co*-3HHx) containing different molar fraction of 3HHx [20]. Previous experiments indicated that hexanoic acid does not inhibit the growth of *B. sacchari* up to 1.5 g l^{-1} (data not shown). In these experiments, the glucose supplied would promote the cellular growth and 3HB accumulation, while hexanoic acid would result on 3HHx monomers formation.

Results obtained are shown on Table 1. The recombinant strain was able to accumulate copolymers with 3HHx molar fraction ranging from 4 to 18 mol%. The molar fraction of 3HHx in the PHA produced by the wt strain was below 0.5 mol%.

Copolymer modulation was obtained by modification of the *co*-substrates concentration offered. From the results obtained, it was possible to establish a correlation between the concentration of hexanoic acid supplied and 3HHx content accumulated by the recombinant strain (Fig. S1, Supplementary material). In general, the PHA contents calculated to the control (2.6 g l^{-1}) and engineered (2.5 g l^{-1}) strains were quite similar, indicating that the recombinant *B. sacchari* constructed maintained the ability to accumulate polymer, with the advantage of accumulating a PHA with a highly modified composition, as expected. Clearly the results showed a better performance in the recombinant strain when compared to the wild type regarding the conversion of hexanoic acid into 3HHx.

In the second set of shaken flasks experiments, PHA accumulation cultures were performed supplying glucose plus butyric, valeric, heptanoic, octanoic, lauric, oleic or linoleic acids as co-substrates (Table 2). From butyric, octanoic, lauric and oleic acids 6C-monomers were expected. Butyril-CoA would condense with acetyl-CoA from glucose catabolism or octanoic or oleic acids would also give rise to 6C-monomers when metabolized through β-oxidation (Fig. 1). However, when butyric, octanoic, and oleic acids were supplied only the production of 3HB monomers was detected (Table S1 Supplementary material).

When valeric acid was supplied, 3HV monomers were inserted in the PHA (Table 2) The fatty acids β-oxidation could simply lead to the formation 3-hydroxyvaleryl-CoA or give rise to three-carbon intermediates, which would condense with acetyl-CoA to form 3HV [17].

From glucose and butyric acid, a PHA containing only 3HB monomers was produced. From glucose and valeric acid, only 3HB and 3HV monomers were detected. Therefore, there is no evidence of Claisen condensation occurring to synthesize 3HHx or 3HHp.

The use of heptanoic acid as a co-substrate by recombinant *B. sacchari* resulted in the accumulation of a terpolymer containing 61.84 mol% of 3HB, 34.17 mol% of 3HV and 3.99 mol% of 3HHp (Table 2), demonstrating the ability of the PHA synthase from *Aeromonas* sp. to incorporate 3HHp monomers. Similar results were reported when other bacteria hosting PHA biosynthesis genes from *Aeromonas* were tested. Recombinant *R. eutropha*, harboring *phaPCJ* from *Aeromonas caviae* produced a copolymer containing 95 mol% of 3HV from valeric acid and also a terpolymer with 33 mol% of 3HB, 61 mol% of 3HV and 6 mol% of 3HHp from heptanoic acid [32]. *Burkholderia* sp. JCM15050 containing $phaC_{Ac}$ accumulated a copolymer composed of 91 mol% 3HV when 0.5% (w/v) sodium valerate was offered [33].

Lauric and linoleic acids are present in plant oils being cheaper carbon sources to promote the insertion of $3HA_{MCL}$ into the PHA. Using lauric and linoleic acids as co-substrates, copolymers P(3HB-*co*-3HHx) were obtained, containing 4.82 and 3.11 mol% 3HHx, respectively. Here, the total amount of PHA accumulated was around 46–50% of cell dry weight (Table 2).

There are many studies reporting the use of these carbon sources to produce PHA. Recombinant *Burkholderia* sp. USM (JCM15050) containing $phaC_{Ac}$ when grown on 0.5% lauric acid (w/v) yielded a

Table 1
P(3HB-*co*-3HHx) production from glucose (10 g l^{-1}) and hexanoic acid (0–1.5 g l^{-1}) by wild type and recombinant strain of *B. sacchari* harboring the *phaPCJ* operon from *Aeromonas* sp.

Recombinant strain	Hexanoic acid	CDW	PHA (mol%)		PHA
	(g l^{-1})	(g l^{-1})	3HB	3HHx	(%CDW)
B. sacchari	0	5.76 ± 0.8	100.00 ± 0.0	0	53.59 ± 1.0
pBBR1MCS-2 (LFM936)	0.25	5.70 ± 0.0	99.91 ± 0.1	0.14 ± 0.0	48.54 ± 1.8
	0.50	5.70 ± 0.0	99.70 ± 0.1	0.30 ± 0.1	37.25 ± 6.0
	1.00	5.43 ± 0.0	99.66 ± 0.0	0.34 ± 0.0	43.07 ± 4.0
	1.50	5.11 ± 0.2	99.54 ± 0.0	0.46 ± 0.0	42.39 ± 3.4
B. sacchari	0	5.43 ± 0.7	100.00 ± 0.0	0	49.25 ± 1.0
pBBR2*phaPCJ*$_{Asp}$(LFM1034)	0.25	5.99 ± 0.7	95.62 ± 0.3	4.38 ± 0.3	44.58 ± 3.9
	0.50	6.49 ± 0.3	88.58 ± 1.2	11.42 ± 1.2	32.46 ± 1.9
	1.00	6.78 ± 0.3	84.84 ± 0.3	15.16 ± 0.3	36.69 ± 4.4
	1.50	6.85 ± 0.4	81.73 ± 4.7	18.27 ± 4.7	38.74 ± 6.8

The cells were inoculated in mineral medium containing glucose (10 g/L) as carbon source and kanamycin (50 μg ml^{-1}). Different concentration of hexanoic acid were added after 24 h of culture, when the limiting nutrient (nitrogen) was exhausted: CDW: Cell dry weight; 3HB: 3-hydroxybutyrate (4C); 3HHx: 3- hydroxyhexanoate (6C). Average and standard deviation of triplicate cultivation.

Table 2
PHA production from glucose and different fatty acids by *B. sacchari* recombinant harboring *phaPCJ*$_{Asp.}$(LFM1034).

Carbon source	CDW	PHA (mol%)				PHA
(g l^{-1})	(g l^{-1})	3HB	3HV	3HHx	3HHp	(CDW%)
Glucose	5.59 ± 0.7	100.00 ± 0.0	0	0	0	38.34 ± 1.5
Valeric acid[a]	5.39 ± 0.2	80.39 ± 1.9	19.61 ± 1.9	0	0	35.31 ± 6.7
Heptanoic acid[a]	5.35 ± 0.5	61.84 ± 3.9	34.17 ± 3.9	0	3.99 ± 0.3	50.53 ± 1.4
Lauric acid[a]	5.29 ± 1.6	95.18 ± 0.4	0	4.82 ± 0.4	0	46.00 ± 1.1
Linoleic acid[a]	5.35 ± 0.4	96.89 ± 0.1	0	3.11 ± 0.1	0	50.05 ± 4.0

CDW: Cell dry weight; 3HB: 3-hydroxybutyrate (4C); 3HV: 3-hydroxyvalerate (5C); 3HHx: 3- hydroxyhexanoate (6C); 3HHp: 3- hydroxyheptanoate. Average and standard deviation of triplicate cultivation.

[a] The cells were inoculated in mineral medium containing glucose (10 g l^{-1}) as carbon source and kanamycin (50 μg ml^{-1}). Fatty acids (1 g l^{-1}) were added after 24 h of culture, when the limiting nutrient (nitrogen) was exhausted.

copolymer P(3HB-*co*-3HHx) containing 1 mol% 3HHx [33]. *E. coli* LS5218 harboring *phaJ*4 gene from *P. putida* KT2440, *phaC*1 from *Pseudomonas* sp 61-3 and *phaAB* from *R. eutropha* cultivated in glucose and lauric acid as carbon sources produced a copolymer containing up to 8 mol% 3HHx and another 3HA$_{MCL}$ monomer [34]. *R. eutropha* H16-PHB^{-4} hosting *phaC*$_{Ac}$ when grown for 96 h in soybean oil accumulated a P(3HB-*co*-3HHx) containing 5 mol% 3HHx [35].

Performing this set of shaken flasks experiments, we have shown the ability of recombinant *B. sacchari* to produce P(3HB-*co*-3HHx) from hexanoic, lauric or linoleic acid as co-substrates. The feasibility of modulating the content of 3HHx into the copolymer was demonstrated. Valeric and heptanoic acids as co-substrates were metabolized by the recombinant to produce co-monomers 3HV and/or 3HHp, respectively.

From a metabolic point of view, we showed that the PHA synthase (PhaC) from *Aeromonas* sp. (LFM897) has specificity to short and medium chain-length monomers (3HB, 3HV, 3HHx and 3HHp), as described before in the literature [22,32,33]. However, the PhaC specificity observed to 3HHp in this study was lower than to the other monomers inserted into the PHA produced by recombinant *B. sacchari*.

Aiming at developing a bioprocess allowing the synthesis of P(3HB-*co*-3HHx) and controlling the 3HHx molar fraction, bioreactor experiments were designed and performed.

3.2. Bioprocess strategy to control 3HHx content

The previous experiments performed in shaken flasks using wild type *B. sacchari* [20] or recombinant *B. sacchari* (see experiments above) showed that the supply of increasing concentrations of hexanoic acid results on a PHA containing increasing 3HHx molar fractions.

A two-step bioreactor cultivations for the production of P(3HB-*co*-3HHx) that allows to modulate the monomer composition of the PHA was established. During the first step, all nutrients were supplied to promote the exponential cell multiplication. In the second step, the cellular multiplication was prevented by nutrient limitation (nitrogen) and the PHA was produced from the carbon sources supplied. During the second step, hexanoic acid and glucose were supplied at different ratios in the feeding solution promoting the production of P(3HB-*co*-3HHx) with variable 3HHx molar fraction. The fed-batch experiment approach was designed to maintain the feeding solution rate lower than the consumption rate, which means there was no hexanoic acid detected during the cultivation, because the total amount of hexanoic acid supplied was promptly consumed by the culture. This strategy was made to avoid the influence of the toxicity of this acid to the cells.

Each bioreactor culture started with 15 g l^{-1} of glucose. In the accumulation phase (second step), a feeding solution was supplied, containing glucose 140 (±2.6) g l^{-1} and hexanoic acid at the following concentrations: 44.5 g l^{-1} (B1), 28.0 g l^{-1} (B2), 23.7 g l^{-1} (B3), 12.1 g l^{-1} (B4) and 0 g l^{-1} (B5) (Fig. S2 and S3, Supplementary material). The growth phase in all experiments lasted 13–18 h and the specific growth rates were around 0.22 h^{-1}.

Table 3 summarizes data from B1 to B5 bioreactor experiments. PHA production by engineered *B. sacchari* was around 70–78% of CDW, which means that the efficiency in accumulating is quite similar to the wild type strain [20]. 3HHx yields from hexanoic acid were between 0.36 and 0.50 g g^{-1}, these values correspond, respectively, to 36.76 and 51% of the maximum theoretical yield (0.98 g g^{-1}). Wild type *B. sacchari* converted only 2% of the hexanoic acid effectively in 3HHx [20]. Therefore, the expression of PHA biosynthesis genes from *Aeromonas* sp. increased by 25 times the capability of *B. sacchari* to insert 3HHx monomers into the PHA. On the other hand, even in the recombinant strain the achieved

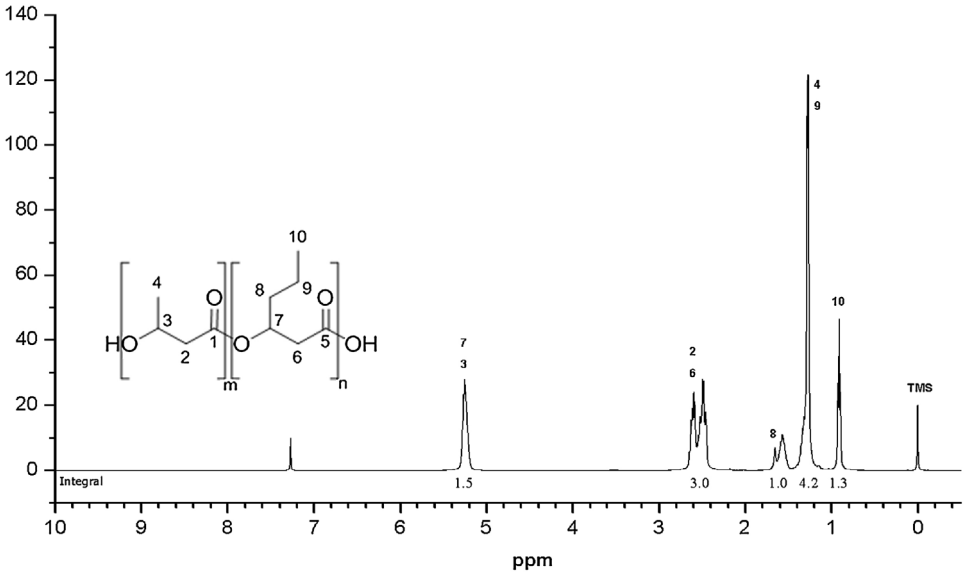

Fig. 3. ^{1}H NMR spectrum (400 MHz) of copolymer P(3HB-*co*-20mol%3HHx) produced by recombinant *B. sacchari* from glucose and hexanoic acid as a co-substrate. 3HB: 3-hydroxybutyrate; 3HHx: 3-hydroxyhexanoate. *m and n represent 76% of 3HB and 24% of 3HHx, respectively, in the PHA molecule.

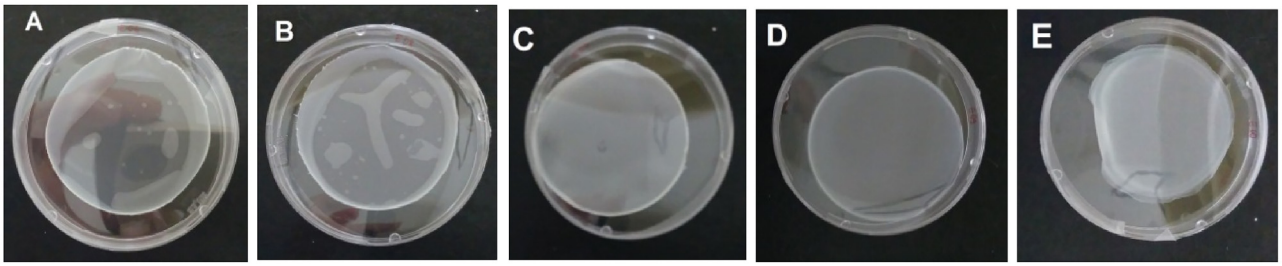

Fig. 4. Films of copolymers P(3HB-*co*-3HHx) with composition ranging from 5 to 20 mol% of 3HHx produced by recombinant *B. sacchari*. A – P(3HB-*co*-20mol%3HHx); B – P(3HB-*co*-15mol%3HHx); C – P(3HB-*co*-10mol%3HHx); D – P(3HB-*co*-5mol%3HHx); E – P(3HB).

Table 3
Summary of bioreactor experiments to modulate the composition of P(3HB-*co*-3HHx) produced by *B. sacchari* pBBR2*phaPCJ*$_{Asp}$. supplied with feeding solutions containing different concetrations of hexanoic acid.

Bioreactor cultivation	Time	Xt	%PHA	PHA	3HHx	μXr	μPHA	$Y_{Xr/Gli}$	$Y_{3HB/Gli}$	$Y_{3HHx/AHex}$	P
	(h)	($g l^{-1}$)	(CDW)	($g l^{-1}$)	(mol%)	(h^{-1})	($mg g^{-1} h^{-1}$)	($g g^{-1}$)	($g g^{-1}$)	($g g^{-1}$)	($g l^{-1} h^{-1}$)
B1	27	17.84	71.76	12.80	19.69	0.23	161.3	0.156	0.320	0.503	0.47
B2	32	17.63	69.55	12.26	13.57	0.20	118.6	0.138	0.267	0.408	0.38
B3	34	20.13	75.85	15.27	10.25	0.21	173.6	0.113	0.311	0.366	0.45
B4	32	18.17	73.46	13.35	5.85	0.22	131.0	0.128	0.323	0.493	0.42
B5	32	16.24	76.92	12.49	–	0.20	117.9	0.097	0.313	–	0.39

B1–bioreactor cultivation fed by glucose ($140 g l^{-1}$) and hexanoic acid ($45 g l^{-1}$).
B2–bioreactor cultivation fed by glucose ($140 g l^{-1}$) and hexanoic acid ($33 g l^{-1}$).
B3–bioreactor cultivation fed by glucose ($140 g l^{-1}$) and hexanoic acid ($22 g l^{-1}$).
B4–bioreactor cultivation fed by glucose ($140 g l^{-1}$) and hexanoic acid ($11 g l^{-1}$).
B5–bioreactor cultivation fed by glucose ($140 g l^{-1}$).
Xt-cell dry biomass.
CDW – cell dry weight.
μXr – maximum growth rate in the exponential phase.
YXr/Gli – Xr yield from glucose (residual biomass).
Y3HB/Gli – 3HB yield from glucose.
Y3HHx/AHex – 3HHx yield from hexanoic acid.
P = Volumetric productivity.

is still only a half of the maximum theoretical value. A partial β-oxidation of hexanoic acid should be important to reach the maximum theoretical yield. The interruption of β-oxidation pathway enhances the supply of medium-chain-length 2-enoyl-CoA and has been reported to increase and to control the PHA composition in *E. coli*, *P. putida* and *R. eutropha* [36–38].

The specific rate of P(3HB-*co*-3HHx) production varied between 118 and $173 mg g^{-1} h^{-1}$, the average value being $140 \pm 26 mg g^{-1} h^{-1}$ (Table 3). The PHA volumetric productivities were around $0.4 g l^{-1} h^{-1}$, which is a good value since these experiments were not performed to reach high cell densities. Comparing to the literature, yields reported using high cell density cultures

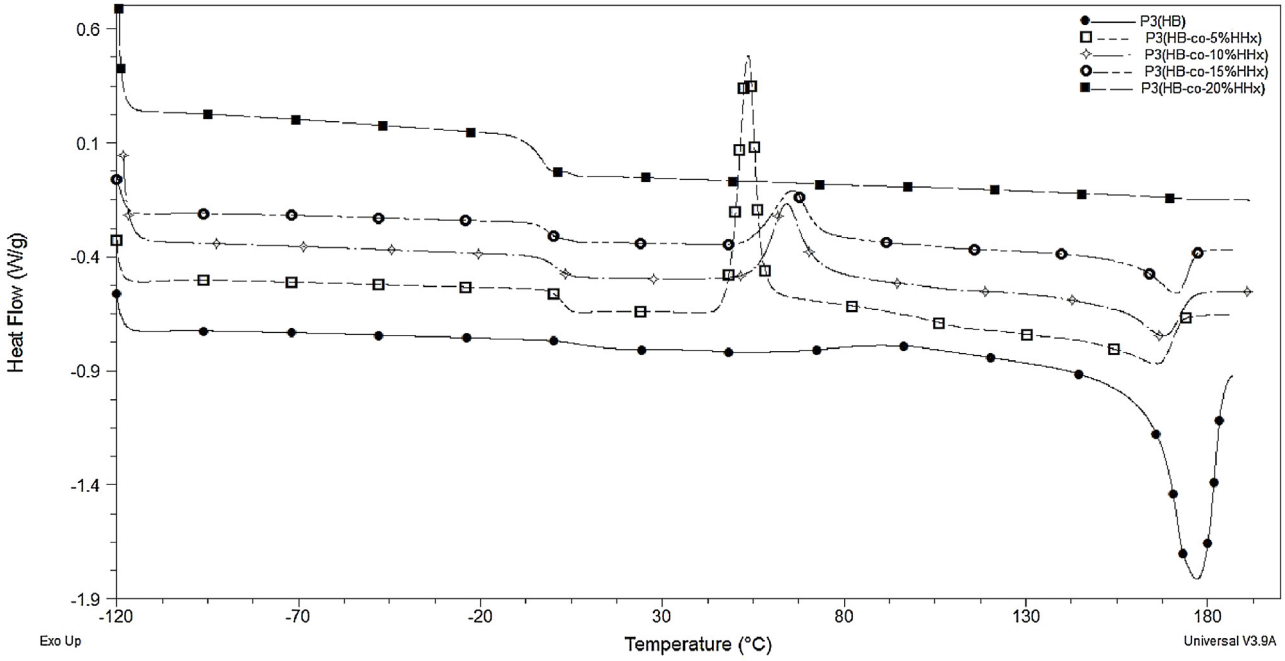

Fig. 5. DSC thermograms of second heating runs of P(3HB-*co*-x%3HHx) with different 3HHx contents.

of *R. eutropha*, *Alcaligenes latus*, *Methylobacterium organophilum* or recombinant *E. coli* reached volumetric productivities between 1.9–2.5 g l^{-1} h^{-1} [39]. *R. eutropha* cultivation reached 1.1 g l^{-1} h^{-1} of PHA from urea as nitrogen source and vegetable oils as carbon source [40]. *B. sacchari* in an airlift bioreactor cultivation supplied with sucrose as the carbon source has reached a productivity of 1.7 g l^{-1} h^{-1} P(3HB) [12]. The same strain has reached a PHA productivity of 0.7 g l^{-1} h^{-1} when grown in a batch, fed with glucose and gamma-butyrolactone [14]. Considering a two-step bioprocess starting with 0.1 g l^{-1} of residual biomass growing exponentially at a specific growth rate of 0.22 h^{-1}, a residual biomass of 30.5 g l^{-1} would be reached after 26 h of culture. If, in the second step, P(3HB-*co*-3HHx) is accumulated at a specific production rate of 140 mgg^{-1} h^{-1}, after more 19 h of culture (accumulation phase), a total biomass of 111.6 g l^{-1} would be reached, containing about 72.7% of PHA and corresponding to a volumetric productivity of 1.8 g l^{-1} h^{-1}. Thus, the volumetric productivities achieved by *B. sacchari* pBBR2*phaPCJ*$_{Asp}$ in the experiments presented here suggests that it is possible to develop an industrially interesting process to attain high cell density cultures.

Data from the set of bioreactor experiments allowed to draw an equation that correlates the ratio of hexanoic acid/glucose supplied (mol.mol^{-1}) in the feeding solution and the 3HHx content in the copolymer produced by *B. sacchari* pBBR2*phaPCJ*$_{Asp}$ (Fig. 2). Using this equation, it is possible to calculate the composition of the feeding solution to be supplied to biosynthesize tailored polymers, and therefore, to modulate the 3HB and 3HHx contents.

Production of copolymers with flexible contents of 3HHx was described in *Aeromonas hydrophila* overexpressing acyl-CoA dehydrogenase (*yafH*) from *E. coli* β-oxidation pathway, when supplied with lauric acid and glucose. According to this report, the product of the *yafH* gene enhances the production of 3HHx, promoting the increase of enoyl-CoA intermediates. Furthermore, acetyl-CoA from glucose metabolism would inactivate 3-ketocyl-CoA thiolase from β-oxidation pathway (Fig. 1) and thus 3HHx accumulation could control by offering different glucose concentrations [24]. Here we described a more precise method to modulate the 3HHx content, since it is based only the supply of different concentrations of hexanoic acid as precursor. Moreover, a direct relation between co-substrate supply and monomer production allow us to quantify and modulate de 3HHx content in the PHA (Fig. 2).

3.3. Nuclear magnetic resonance (NMR) analysis

The ^{1}H NMR spectrum (Fig. 3) of P(3HB-*co*-20mol%3HHx) allowed the exact calculation of the percentage of 3HHx in the copolymer through the integration of specific signals. The multiplet at 5.25 ppm is associated with H-3 of 3HB and 3HHx, while the multiplets between 2.4 and 2.7 ppm are associated with the H-2 hydrogens in both. The triplet at 0.91 ppm is associated with the hydrogens of the terminal methyl group of the side chains of 3HHx [41,42,20]. The relative integral of this latter signal, referred to the first mentioned signals provides the 24.2% 3HHx molar contents in the copolymer.

3.4. Molecular weight and thermal properties analysis of P(3HB-co-3HHx) with different 3HHx contents

Polymers produced by recombinant *B. sacchari*, containing different compositions from bioreactor experiments, were extracted and purified. Films of P(3HB-*co*-3HHx) with different amounts of 3HHx were prepared (Fig. 4), from A (higher amount of 3HHx) to E (only 3HB monomers) and their thermal properties and molecular weights were analyzed (Table 4). The picture E shows clearly a brittle material when compared with that more flexible in A (Fig. 4).

The weight average molecular weights (Mw) obtained from these copolymers were at the same order (10^5 Da), regardless to the 3HHx content, although P(3HB-*co*-20mol%3HHx) presented a Mw (2.3×10^5 Da) slightly lower than the others (Table 4). These biopolymers with relative low Mw have a potential and practical application as arrays in controlled drugs release, due to their higher degradation rate, and consequently easier bio-absorption of the polymeric material [43]. It is known that molecular weight depends on the PHA synthase and the cultivation time [44]. P(3HB-*co*-3HHx) molecular weights reported by other strains were around 9.8×10^5 Da from *E. coli* LS5218 containing *phaC*$_{Ac}$ [32], and 6.5×10^5 Da from recombinant *R. eutropha*, harboring *phaC*$_{Ac}$ [45].

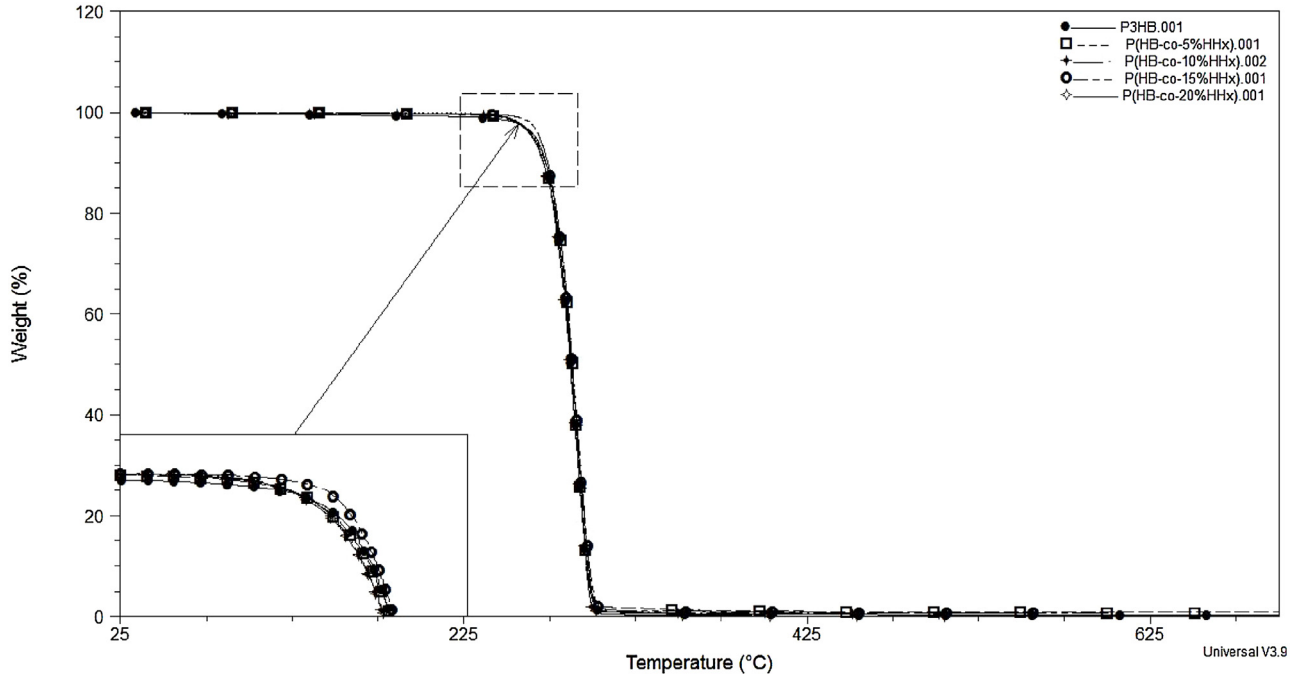

Fig. 6. Thermogravimetric measurements of P(3HB-*co*-x%3HHx) with different 3HHx contents.

Table 4
Composition, molecular weight and thermal properties of P(3HB-*co*-3HHx) films with different amounts of 3HHx produced by recombinant *B. sacchari*.

Films composition	Molecular Weight		Thermal properties				
(mol%)	Mw (Da)	Mw/Mn	T_g (°C)	T_c (°C)	ΔHc	T_m (°C)	ΔHm
P(3HB-*co*-19.69mol%3HHx)	232833	1.96	−3.22	NO	NO	NO	NO
P(3HB-*co*-13.57mol%3HHx	302592	1.42	−1.21	65.9	19.7	171.4	19.9
P(3HB-*co*-10.25mol%3HHx	285562	1.49	0.71	64.3	24.0	167.9	24.2
P(3HB-*co*-5.85mol%3HHx	303685	1.43	2.87	53.6	39.0	166.3	41.6
P(3HB)	270633	1.56	4.20	102	72.1	177.3	73.5

3HB: 3-hydroxybutyrate (4C); 3HHx: 3-hydroxyhexanoate (6C); Mw: weight average molecular weights; Mw/Mn: polydispersity index; T_g: glass transition temperature; T_c: crystallization temperature; T_m: melting temperature; NO: not observed at experimental conditions.

Fig. 5 shows the DSC second heating thermograms and cristalization (T_c), melting (T_m) and glass transition (T_g) temperatures. Glass transition observed in these analysis indicates that increasing 3HHx contents (0–20 mol%) decrease the T_g between 4.2 to −3,2 °C (Table 4). This behavior obtained without a significant influence of molecular weight or molecular distribution (Mw/Mn) is due to the inclusion of 3HHx co-monomer in polymer backbone, which introduce side chains, decreasing the conformational energies and increasing the segment mobility of molecules, and consequently the T_g is lower in the relation to P(3HB). Similar T_g results were observed by other authors [22,46], which reported T_g values between 0 to − 4 °C for copolymers P(3HB-*co*-3HHx) with 3HHx ranging from 5 to 25 mol%.

Temperatures associated to crystalline phase were also affected by 3HHx contents. The T_c varied between 54 °C to 66 °C as much as the 3HHx fraction (5–15 mol%) in the copolymers, however, a mild impact was detected in the T_m, in which the transition temperature was 10 °C lower to P3(HB-*co*-5%HHx) than to P(3HB) (Table 4). The same behavior was reported to copolymers containing 3HHx ranging from 1.5–25 mol% [22,34,47]. These changes here observed were because of the 3HHx amount in the copolymers, which could introduce disturbance in the crystalline lattice of P(3HB). The non-regular change observed to the T_c and T_m could be associated to the 3HHx distribution in copolymer backbones.

The ΔHc associated to crystalline phase formation (Table 4) and ΔHm due to melting process of the crystalline phase decreased as much as the 3HHx contents increased, and these measures were lower than that observed to P(3HB). The side chains associated to the 3HHx fractions (0–20 mol%) allowed a regular impact on the crystalline degree of these copolymers.

P(3HB-*co*-3HHx) copolymers analyzed showed a similar thermodegradation temperature to the P(3HB) (Fig. 6). These results were associated to the lower melting points of these copolymers, which are around 171–166 °C, similarly to P(3HB) (177 °C). The ideal situation would be to obtain a larger difference between degradation and melting temperatures. For example, decreasing the T_m, by adding 3HHx co-monomers to the P(3HB) chain, would enhance the industrial processability of these copolymers [42,48].

Mechanical properties analysis showed that the content of 3HHx in the copolymers tested have improved the mechanical properties, as expected. Copolymer films were softer and more flexible. The elongation to break presented by P(3HB-*co*-20%3HHx) was 945%, which is associated to high segment mobility and low or no crystallinity degree (Fig. S4, Supplementary material). Other reports registered elongation to break of 400 and 850% to the copolymers containing 12 or 17 mol% of 3HHx, respectively [22,49].

4. Conclusions

We successfully established the P(3HB-*co*-3HHx) production controlling 3HHx fraction up to 20 mol% by the combination of recombinant *B. sacchari* pBBR2*phaPCJ*$_{Asp}$ construction and the

development of a two-step bioprocess. The 3HHx yield reached up to 50% of the maximum theoretical value ($0.50\,gg^{-1}$), increasing 25-fold the ability to accumulate this monomer compared to the wild type. Recombinant *B. sacchari* is also able to produce 3HHx, 3HV and 3HHp from lauric/linoleic or valeric or heptanoic acids respectively. T_g and T_m of copolymers analyzed varied according to the 3HHx content, showing that the copolymers obtained here can cover specific requirements of applications.

Funding source

The research of the manuscript was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil (fund numbers 10/51935-1 and 12/51533-6).

Acknowledgment

The authors thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2017.02.013.

References

[1] A. Steinbüchel, T. Lütke-Eversloh, Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms, Biochem. Eng. J. 16 (2003) 81–96.
[2] S.J. Park, T.W. Kim, M.K. Kim, S.Y. Lee, S.C. Lim, Advanced bacterial polyhydroxyalkanoates: towards a versatile and sustainable platform for unnatural tailor-made polyesters, Biotechnol. Adv. 30 (2012) 1196–1206.
[3] M. Koller, G. Braunegg, Potential and Prospects of continuous polyhydroxyalkanoate (PHA) production, Bioengineering 2 (2015) 94–121.
[4] J.M. Jeon, C.J. Brigham, Y.h. Kim, Y.H. Kim, D.H. Yi, H. Kim, C.K. Rha, A.J. Sinskey, Y.H. Yang, Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(HB-co-HHx)) from butyrate using engineered Ralstonia eutropha, Appl. Microbiol. Biotechnol. 98 (2014) 5461–5469.
[5] B.H.A. Rehm, Bacterial polymers: biosynthesis: modifications and applications, Nat. Rev. Microbiol. 8 (2010) 578–592.
[6] K. Sudesh, H. Abe, Y. Doi, Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters, Prog. Polym. Sci. 25 (2000) 1503–1555.
[7] F. Reinecke, A. Steinbüchel, *Ralstonia eutropha* strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers, J. Mol. Microbiol. Biotechnol. 16 (2009) 91–108.
[8] C.O. Brämer, L.F. Silva, J.G.C. Gomez, P. Vandamme, A. Steinbüchel, *Burkholderia sacchari* sp. nov. a polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar cane plantation in Brazil, Int. J. Syst. Evol. Microbiol. 51 (2001) 1709–1713.
[9] P.M.R. Alexandrino, T.T. Mendonça, L.P. Guamán-Bautista, J. Cherix, G.C. Lozano-Sakalauskas, A. Fujita, E. Ramos-Filho, P. Long, G. Padilla, M.K. Taciro, J.G.C. Gomez, L.F. Silva, Draft genome sequence of the polyhydroxyalkanoate-producing bacterium Burkholderia sacchari LMG 19450 isolated from Brazilian sugarcane plantation soil, Genome Announcement 3 (2015) 1–2.
[10] J.G.C. Gomez, M.F.A. Rodrigues, R.C.P. Alli, B.B. Torres, C.L. Bueno-Netto, M.S. Oliveira, L.F. Silva, Evaluation of soil gram-negative bacteria yielding polyhydroxyalkanoic acids from carbohydrates and propionic acid, Appl. Microbiol. Biotechnol. 45 (1996) 785–791.
[11] J.G.C. Gomez, V. Fontolan, R.C.P. Alli, M.F.A. Rodrigues, C.L. BuenoNetto, L.F. Silva, D.A. Simões, Production of P3HB-co-3HV by soil isolated bacteria able to use sucrose, Rev. Microbiol. 28 (1997) 43–48.
[12] J.G. Pradella, M.K. Taciro, A.Y. Mateus, High-cell-density poly(3-hydroxybutyrate) production from sucrose using Burkholderia sacchari culture in airlift bioreactor, Bioresour. Technol. 101 (2010) 8355–8360.
[13] L.F. Silva, M.K. Taciro, M.E.M. Ramos, J.M. Carter, J.G.C. Pradella, J.G.C. Gomez, Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugar cane bagasse hydrolysate, J. Ind. Microbiol. Biotechnol. 31 (2004) 245–254.
[14] M.T. Cesário, R.S. Raposo, M.C.M.D. Almeida, F. Van Keulen, B.S. Ferreira, J.P. Telo, M.M.R. Fonseca, Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) by Burkholderia sacchari using wheat straw hydrolysates and gamma-butyrolactone, Int. J. Biol. Macromol. 71 (2014) 59–67.
[15] R.V. Nonato, P.E. Mantelatto, C.E.V. Rossell, Integrated production of biodegradable plastic: sugar and ethanol, Appl. Microbiol. Biotechnol. 57 (2001) 1–5.
[16] L.F. Silva, M.K. Taciro, G. Raicher, R.A.M. Piccoli, T.T. Mendonca, M.S.G. Lopes, J.G.C. Gomez, Perspectives on biopolymer production from biorefineries associated with sugar and ethanol production, Int. J. Biol. Macromol. 71 (2014) 2–7.
[17] L.F. Silva, J.G.C. Gomez, M.S. Oliveira, B.B. Torres, Propionic acid metabolism and poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P3HB-co-3HV) production by Burkholderia sp, J. Biotechnol. 76 (2000) 165–174.
[18] R.C.S. Rocha, L.F. Silva, M.K. Taciroa, J.G.C. Pradella, Production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV) with a broad range of 3HV content at high yields by Burkholderia sacchari IPT 189, World J. Microbiol. Biotechnol. 24 (2008) 427–431.
[19] E.M. Pereira, S.R. Silva-Queiroz, J.G.C. Gomez, L.F. Silva, Disruption of the 2-methylcitric acid cycle and evaluation of poly-3-hydroxybutyrate-co-3-hydroxyvalerate biosynthesis suggest alternate catabolic pathways of propionate in Burkholderia sacchari, Can. J. Microbiol. 55 (2009) 688–697.
[20] T.T. Mendonça, J.G.C. Gomez, E. Buffoni, R.J. Sánchez-Rodriguez, J. Schripsema, M.S.G. Lopes, L.F. Silva, Exploring the potential of *Burkholderia sacchari* to produce polyhydroxyalkanoates, J. App. Microbiol. 116 (2013) 815–829.
[21] T.T. Mendonça, J.G.C. Gomez, M. Tortajada, D.R. Vidal, A. Rojas, D. Bustamante, L.F. Silva. Effects of the supply of different glucose/hexanoic acid ratios in the composition of the copolymer poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) P(3HB-co-3HHx) produced by Burkholderia sacchari. In: M.A. Reis, B.S. Ménde (Eds.) Bioplásticos. PRIBOP–CYTED. 1, 2014, 9–21.
[22] Y. Doi, S. Kitamura, H. Abe, Microbial synthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), Macromolecules 28 (1995) 4822–4828.
[23] S.H. Lee, D.H. Oh, W.S. Ahn, Y. Lee, J. Choi, S.Y. Lee, Production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) by high-cell-density cultivation of *Aeromonas hydrophila*, Biotechnol. Bioeng. 67 (2000) 240–244.
[24] X.Y. Lu, Q. Wu, W.J. Zhang, G. Zhang, G.Q. Chen, Molecular cloning of polyhydroxyalkanoate synthesis operon from *Aeromonas hydrophila* and its expression in *Escherichia coli*, Biotechnol. Prog. 20 (2004) 1332–1336.
[25] Y.Z. Qiu, J. Han, G.Q. Chen, Metabolic engineering of *Aeromonas hydrophila* for the enhanced production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), Appl. Microbiol. Biotechnol. 69 (2006) 537–542.
[26] J. Jian, Z.J. Li, H.M. Ye, M.Q. Yuan, G.Q. Chen, Metabolic engineering for microbial production of polyhydroxyalkanoates consisting of high 3-hydroxyhexanoate content by recombinant *Aeromonas hydrophila*, Bioresour. Technol. 101 (2010) 6096–6102.
[27] T.S. Matsuda, Isolation of Bacteria Producing Polyhydroxyalkanoates Containing Short-chain-length and Medium-chain-length Monomers from Plant Oil Master of Sciences Tesis, University of Sao Paulo, 2009, 84 p.
[28] M.E. Kovach, P.H. Elzer, D.S. Hill, G.T. Robertson, M.A. Farris, R.M. Roop, K.M. Peterson, Four new derivatives of the broad-host-range cloning vector pBBR1MCS: carrying different antibiotic-resistance cassettes, Gene 166 (1995) 175–176.
[29] R. Simon, U. Priefer, A. Puhler, A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria, Nat. Biotechnol. 1 (1983) 784–791.
[30] J. Sambrook, D.W. Russell, Molecular Cloning: a Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York, NY, 2001.
[31] V. Riis, W. Mai, Gas chromatography determination of poly-β-hydroxybutyric acid in microbial biomass-esther hydrochloric acid propanolisis, J. Chromatogr. 445 (1988) 285–289.
[32] T. Kichise, T. Fukui, Y. Yoshida, Y. Doi, Biosynthesis of polyhydroxyalkanoates (PHA) by recombinant Ralstonia eutropha and effects of PHA synthase activity on in vivo PHA biosynthesis, Int. J. Biol. Macromol. 25 (1999) 69–77.
[33] J.Y. Chee, N.S. Lau, M.R. Samian, T. Tsuge, K. Sudesh, Expression of *Aeromonas caviae* polyhydroxyalkanoate synthase gene in *Burkholderia* sp. USM (JCM15050) enables the biosynthesis of SCL-MCL PHA from palm oil products, J. Appl. Microbiol. 112 (2011) 45–54.
[34] Q. Wang, C. Zhu, T.J. Yancone, C.T. Nomura, The effect of *co*-substrate feeding on polyhydroxyalkanoate (PHA) homopolymers and copolymer production in recombinant *Escherichia coli* LS5218, J. Bioprocess Eng. Biorefin. 1 (No.1) (2012) 86–92.
[35] P. Kahar, T. Tsuge, K. Taguchi, Y. Doi, High yield of polyhydroxyalkanoates from soybean oil by *Ralstonia eutropha* and its recombinant strain, Pol. Deg. Stab. 83 (2004) 79–86.
[36] R.C. Tappel, Q. Wang, C.T. Nomura, Precise control of repeating unit composition in biodegradable poly(3-hydroxyalkanoate) polymers synthesized by *Escherichia coli*, J. Biosc. Bioeng. 113 (2012) 480–486.
[37] L. Tripathi, L. Wu, M. Dechuan, J. Chen, Q. Wu, G. Chen, Pseudomomas putida KT2442 as a platform for the biosynthesis of polyhydroxyalkanoates with adjustable monomer contents and compositions, Bioresour. Technol. 142 (2013) 225–231.
[38] C. Insomphun, J. Mifune, I. Orita, K. Numata, S. Nakamura, T. Fukui, Modification of β-oxidation pathway in *Ralstonia eutropha* for production of

poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from soybean oil, J. Biosci. Bioeng. 117 (2014) 184–190.
[39] J. Choi, S.Y. Lee, Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation, Bioprocess. Eng. 17 (1997) 335–342.
[40] S.L. Riedel, J. Bader, C.J. Brigham, C.F. Budde, Z.A.M. Yusof, C. Rha, A.J. Sinskey, Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) by Ralstonia eutropha in high cell density palm oil fermentation, Biotechnol. Bioeng. 109 (2012) 2847–2854.
[41] C.T. Nomura, T. Tanaka, Z. Gan, K. Kuwabara, H. Abe, K. Takase, K. Taguchi, Y. Doi, Effective enhancement of short-chain-length (SCL) medium-chain-length (MCL) polyhydroxyalkanoate copolymer production by co-expression of genetically engineered 3-ketoacyl-acyl-carrier protein synthase III (fabH) and polyhydroxyalkanoate synthesis genes, Biomacromolecules 5 (2004) 1457–1464.
[42] C.T. Nomura, T. Tanaka, T.E. Equen, A.S. Appah, K. Matsumoto, S. Taguchi, C.L. Ortiz, Y. Doi, FabG mediates polyhydroxyalkanoate production from both related and nonrelated carbon sources in recombinant Escherichia coli LS5218, Biotechnol. Prog. 24 (2008) 341–351.
[43] C.S. Reddy, R. Ghai, V.C. Rashmi Kalia, Polyhydroxyalkanoates: an overview, Bioresour. Technol. 87 (2003) 137–146.
[44] S. Tomizawa, M. Hyakutake, Y. Saito, J. Agus, K. Mizuno, H. Abe, T. Tsuge, Molecular weight (PHA) caused by the PhaC subunit of PHA synthase from Bacillus cereus YB-4 in recombinant Escherichia coli, Biomacromolecules 12 (2011) 2660–2666.
[45] T. Tsuge, S. Watanabe, S. Sato, T. Hiraishi, H. Abe, Y. Doi, S. Taguchi, Variation in copolymer composition and molecular weight of polyhydroxyalkanoate generated by saturation mutagenesis of *Aeromonas caviae* PHA synthase, Macromol. Biosci. 7 (2007) 846–854.
[46] M.K. Cheung, K.P.Y. Wan, P.H. Yu, Miscibility and morphology of chiral semicrystalline poly-(R)-(3-hydroxybutyrate)/chitosan and poly-(R)-(3-hydroxybutyrate-*co*-3-hydroxyvalerate)/chitosan blends studied with DSC, ^{1}H T_1 and $T_1\rho$ CRAMPS, J. Appl. Polym. Sci. 86 (2002) 1253–1258.
[47] C.Y. Loo, K. Sudesh, Polyhydroxyalkanoates: bio-based microbial plastics and their properties, Malays. Polym. J. 2 (2007) 31–57.
[48] S.R. Montoro, C.Y. Shigue, M.L.T. Sordi, A.M. Santos, M.I. Ré, Kinetic study of the molecular weight reduction of poly (3-hydroxybutirate-*co*-3-hydroxyvalerate) (PHBHV), Polímeros: Ciência e Tecnologia 20 (2010) 19–24.
[49] H.M. Chang, Z.H. Wang, H.N. Luo, M. Xu, X.Y. Ren, G.X. Zheng, B.J. Wu, X.H. Zhang, X.Y. Lu, F. Chen, X.H. Jing, L. Wang, Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)-based scaffolds for tissue engineering, Braz. J. Med. Biol. Res. 47 (2014) 533–539.