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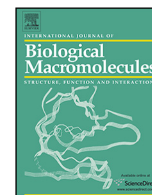

Combining molecular and bioprocess techniques to produce poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) with controlled monomer composition by *Burkholderia sacchari*

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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.

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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.

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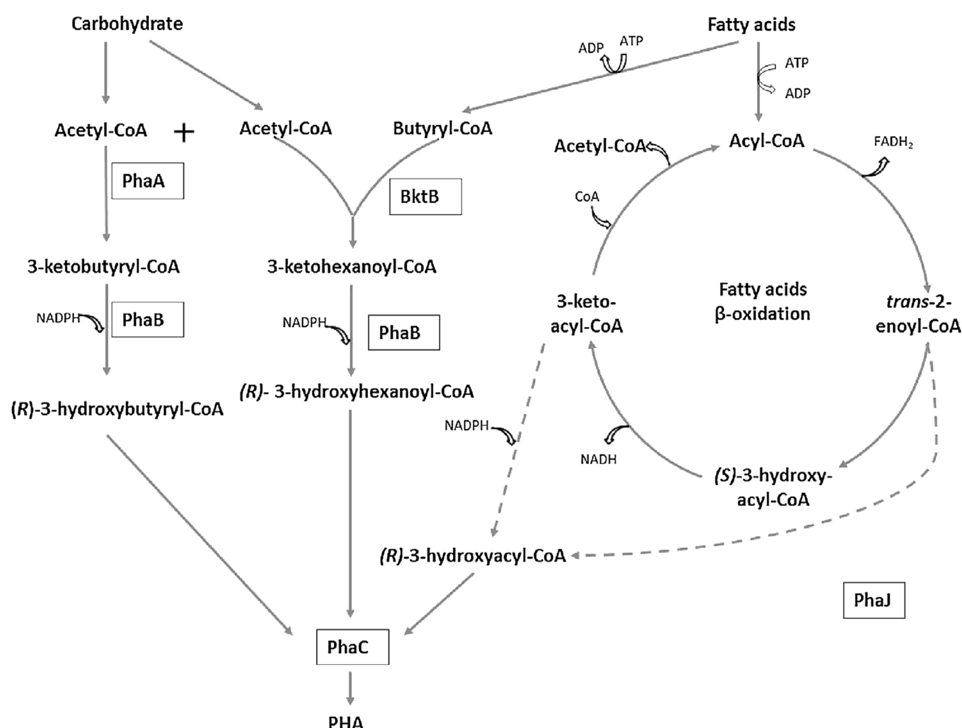

Fig. 1. Reactions and genes involved on PHA (3HB and $3HA_{MCL}$) biosynthesis from carbohydrates and organic acids. Enzymes: PhaA and BktB: β-ketotiolase; PhaB: 3-ketoacyl-CoA redutase/acetoacetyl-CoA reductase); PhaC: PHA synthase; PhaJ: enoyl-CoA hydratase (*R*)-specific.

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

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