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Melhoramento da eficiência de conversão de xilose e co-substratos em copolímeros híbridos P(3HB-*co*-3HA$_{MCL}$) por *Burkholderia sacchari*.

Tese apresentada ao programa de Pós-Graduação Interunidades em Biotecnologia USP/Butantã/IPT, para obtenção de título de Doutor em Ciências.

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Improvement of the conversion efficiency of xylose and co-substrates to P(3HB-*co*-3HA$_{MCL}$) copolymers by *Burkholderia sacchari*

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RESUMO

OLIVEIRA-FILHO, E.R. **Melhoramento da eficiência de conversão de xilose e co-substratos em copolímeros híbridos P(3HB-*co*-3HA$_{MCL}$) por *Burkholderia sacchari***. 2021; 154 f. Tese (Doutorado em Biotecnologia) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2021.

Burkholderia sacchari LFM101, linhagem isolada de solo de canavial do interior do Estado de São Paulo, está sendo proposta como plataforma bacteriana para a produção biotecnológica de bioprodutos. LFM101 se destaca por sua capacidade de metabolizar ampla gama de substratos, como açúcares provenientes de hidrolisados hemicelulósicos e diversos ácidos graxos. Esta linhagem bacteriana vem sendo estudada por diversos grupos de pesquisa por produzir eficientemente poli(3-hidroxibutirato) [P(3HB)], biopolímero biocompostável e biocompatível da família dos polihidroxialcanoatos (PHA), além de outros bioprodutos como ácido xilônico e xilitol. A produção desses biopolímeros vem sendo explorada mundialmente como alternativa ao uso de plásticos derivados de petróleo, devido aos diversos problemas ambientais causados pelo seu uso. *B. sacchari* é também capaz de incorporar outros monômeros na cadeia polimérica nascente, como 3-hidroxivalerato (3HV), 4-hidroxibutirato (4HB) e 3-hidroxihexanoato (3HHx) quando suprida com substratos relacionados. Copolímeros de 3HB e 3HHx têm sido descritos como promissores para a área médica por suas propriedades termomecânicas. Porém, a produção industrial de PHA ainda não é completamente viável devido aos custos relacionados às matérias-primas utilizadas e etapas de purificação. Visando contribuir para a produção industrial de PHA a partir de xilose, matéria-prima barata e abundante, utilizando *B. sacchari*, neste trabalho foram abordados os seguintes tópicos: (1) o efeito de diferentes limitações nutricionais sob o crescimento e acúmulo de P(3HB) em biorreator suprido com xilose como única fonte de carbono; (2) a construção de linhagens recombinantes, seu teste em relação a velocidade específica de crescimento em xilose e eficiência de conversão de hexanoato em 3HHx, assim como a produção em biorreator de copolímeros contendo diferentes frações molares de 3HHx e a caracterização dos copolímeros produzidos; (3) o teste dos diferentes copolímeros produzidos por *B. sacchari* como materiais para fabricação de nanopartículas, testadas *in vitro* quanto a sua citotoxicidade e capacidade de *drug delivery*.

Palavras-chave: Xilose. *Burkholderia sacchari*. Bipolímeros. P(3HB-*co*-3HHx). Nanopartículas.

ABSTRACT

OLIVEIRA-FILHO, E.R. **Improvement of the conversion efficiency of xylose and co-substrates to P(3HB-*co*-3HA$_{MCL}$) copolymers by *Burkholderia sacchari***. 2021. 154 p. Doctoral Thesis (Biotechnology) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2021.

Burkholderia sacchari LFM101, an isolated strain of sugarcane soil in the interior of the State of São Paulo, is being proposed as a bacterial chassis for the biotechnological production of bioproducts. LFM101 stands out for its ability to metabolize a wide range of substrates, such as sugars from hemicellulosic hydrolysates and various fatty acids. This bacterial strain has been studied by several research groups for efficiently producing poly(3-hydroxybutyrate) [P(3HB)], a biocompostable and biocompatible biopolymer from the family of polyhydroxyalkanoates (PHA), in addition to other bioproducts such as xylonic acid and xylitol. The production of these biopolymers has been explored worldwide as an alternative to the use of oil-based plastics, due to the well-known environmental problems caused by their use. *B. sacchari* is also capable of incorporating other monomers into the nascent polymeric chain, such as 3-hydroxyvalerate (3HV), 4-hydroxybutyrate (4HB) and 3-hydroxyhexanoate (3HHx) when co-supplied with related substrates. Copolymers of 3HB and 3HHx have been described as promising for the medical field due to their thermomechanical properties. However, the industrial production of PHA is not yet completely viable due to the costs related to the raw materials used and the purification steps. In order to contribute to the industrial production of PHA from xylose, a cheap and abundant raw material, using *B. sacchari*, the following topics were addressed in this work: (1) the effect of different nutritional limitations on the growth and accumulation of P(3HB) in a fed-batch bioreactor supplied with xylose as the sole source of carbon; (2) the construction of recombinant strains, their evaluation regarding to the specific growth rate in xylose and to hexanoate conversion efficiency in 3HHx, as well as the production in fed-batch bioreactor of copolymers containing different 3HHx molar fractions, and the characterization of the produced copolymers; (3) the test of the different copolymers produced by *B. sacchari* as materials for the manufacture of nanoparticles, tested *in vitro* for their cytotoxicity and drug delivery capacity.

Keywords: Xylose; *Burkholderia sacchari*; Biopolymers; P(3HB-*co*-3HHx); Nanoparticles.

1 INTRODUCTION

Modern society is highly dependent on the use of plastic materials. Plastics are used from the manufacturing packages to noble materials, such as suture lines, prostheses, and surgical supplies, for example. Although conventional oil-based plastics are non-biodegradable, they are used in huge quantities and not recycled in most countries, therefore its residues are accumulating in the environment. Some eco-friendly materials have been proposed as alternatives to oil-based plastics, such as polyamines, polycaprolactones, polylactic acid, gutta-percha, and polyhydroxyalkanoates (PHA).

The PHA are natural biopolyesters naturally produced and accumulated by bacterial and archaeal strains in specific conditions. Intracellular PHA granules serve as an energy and reducing power source for the microorganism. PHA are truly green plastics, since they can be produced from different carbon sources, including waste biomass, and are fully biodegradable in the environment (under adequate pH, humidity, temperature) by depolymerases, being converted to CO_2 and H_2O or to CH_4.

PHA biosynthesis is mainly influenced by three factors: (a) the supplied carbon source; (b) the bacterial metabolic pathways responsible for converting substrates or co-substrates to 3-hydroxy acid monomers; (c) the PHA synthase, the key enzyme in PHA biosynthesis. Accordingly, short- (HA_{SCL}: 3 to 5 carbon atoms) or medium-chain-length (HA_{MCL}: 6 to 14 carbon atoms) monomers can be produced and incorporated into the nascent PHA polymeric chain. PHA copolymers are composed of short and medium-chain-length monomers, and can present improved properties, as being less brittle and more elastomeric, which favors their use to manufacture different products. In fact, the copolymer composed of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx) [P(3HB-*co*-3HHx)] was indicated as the most promising PHA for biomedical applications.

However, the PHA production costs are still too high for industrial applications, mainly affected by the used carbon source, which represents from 30 to 50% of the final costs. In this scenario, the use of byproducts, especially lignocellulosic hydrolysates, came out as an alternative production route. A few bacterial strains are able to produce these interesting copolymers from cheap substrates as the main carbon source. One of the most interesting of them is *Burkholderia sacchari* LFM101.

Burkholderia sacchari was isolated in the 1990s from sugarcane crop soil in Brazil, in a joint research project aiming to identify microorganisms capable of producing new products from sucrose. The isolate presented high yields to convert sucrose to poly(3-hydroxybutyrate), also assimilating 3HV with spectacular efficiency from propionate. Later, LFM101 potential to produce PHA with different monomeric compositions, and other value-added industrially relevant biomolecules, like xylitol and xylonic acid, was evidenced. Although a non-model bacterium, *B. sacchari* is currently studied by different research groups worldwide distributed (Brazil, Portugal, Austria, Czech Republic, Ireland, the United States of America, Mexico, Ecuador, Canada and Oman) and proposed as a bacterial chassis to produce PHA and other bioproducts. *B. sacchari* can catabolize different carbon sources, is resistant to some of the growth inhibitors present in lignocellulosic hydrolysates, does not present virulence-associated gene sequences, and is sensitive to clinically relevant antibiotics.

In this context, the aim of the research presented in this thesis was to study the production of PHA copolymers by *B. sacchari* and define its applications, through (1) investigate nutrient limitations role in growth and PHA production in fed-batch cultivations; (2) construct recombinants with improved xylose catabolism and P(3HB-*co*-3HHx) production, aiming to tailor the 3HHx molar fraction in fed-batch cultivations; (3) apply different P(3HB-*co*-3HHx), with various 3HHx molar fractions, produced by recombinant *B. sacchari* from xylose and hexanoate as materials to produce nanoparticles.

7 CONCLUSIONS AND FUTURE PERSPECTIVES

The main conclusions obtained in this work are the following:

a. The great potential of *B. sacchari* to produce PHA and other fine chemicals is presented, also as a source of genes and for the identification of new pathways. Its integration to biorefineries is a promising, but some aspects should be the subject to future studies, such as the use of alternative bioreactors and alternative substrates, obtaining clones resistant to toxic compounds present in hemicellulose hydrolysates, and extending the knowledge on the metabolic routes and cultivation conditions to produce new fine chemicals.
b. In fed-batch bioreactor using xylose as the sole source of carbon, phosphorus concentration is a defining factor to achieve higher growth rates on xylose. Phosphorus limitation (nitrogen excess) resulted in P(3HB) concentration, although polymer accumulation phase started later. PHO regulon expression studies would reveal new aspects regarding phosphate assimilation in *B. sacchari*. The accumulation of polyphosphate by *B. sacchari* is also a hot-topic to be studied in the next years.
c. Recombinant *B. sacchari* expressing genes encoding for xylose catabolism enzymes and heterologous PHA biosynthesis genes was described as a good strategy to produce of P(3HB-*co*-3HA$_{MCL}$) containing from different HA$_{MCL}$ molar fractions. The biopolymers produced by B. sacchari demonstrated interesting characteristics to be applied as biomaterials.
d. PHA copolymers containing different 3HA$_{MCL}$ molar fractions were successfully tested to produce monodisperse nanoparticles using a highly reproducible technique. The encapsulation of different hydrophobic drugs was successfully achieved. *In vivo* application of the PHA produced by *B. sacchari* is an ambitious perspective contribution for the application of PHA biopolyesters as nanomaterials.

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