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IN SILICO EVALUATION OF THE METABOLIC PATHWAY FOR 2-PYRONE-4,6-DICARBOXYLIC ACID PRODUCTION

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ABSTRACT

2-pyrone 4,6 dicarboxylate (PDC) is a promising monomer to produce biodegradable polyesters, but the lack of an efficient method for its large-scale production and the absence of a chemical synthesis method makes its industrial use difficult. In this work, metabolic flux analysis was conducted using the *Burkholderia sacchari* metabolic network to evaluate the ability of the recombinant strain to produce PDC from different pathways. The *in silico* analysis was able to predict a high conversion of up to 0.85 molPDC/molGlucose for the proposed metabolic network. The metabolic modeling and analysis presented in this study could potentially serve as valuable guidance for future metabolic engineering of *B. sacchari* for efficient PDC production.

Keywords: Elementary mode analysis. *Burkholderia sacchari* metabolism. PDC production. Microbial cell factory.

1 INTRODUCTION

2 pyrone 4,6 dicarboxylate (PDC) is a dicarboxylic acid with a (pseudo) aromatic ring and two carboxylic acids, identified as a possible successor to terephthalic acid due to its structural similarity. Still, it brings with its differences better characteristics for bioplastics produced from PDC, as they can present good mechanical and thermal resistance, as well as exhibit better biodegradability and high elasticity compared to PET (Michinobu et al., 2009; Lee, et al. al., 2022; Jin et al., 2024). PDC is an intermediate product of the protocatechuate acid (PCA) degradation pathway, generated from the degradation of aromatic compounds derived from lignin by bacteria, such as *Sphingomonas paucimobilis* (Masai et al., 1999) *and Comamonas testosteroni* using the 4,5 PCA route (Kamimura & Massai, 2014). Thus, the use of different microorganisms capable of producing PDC has been explored through the conversion of various substrates, such as lignin-derived aromatics and glucose degradation products, through the insertion of heterologous genes that lead to the insertion of the metabolic pathways necessary for their production (Nakajima et al., 2009; Otsuka et al., 2023; Zhou et al., 2023). However, large-scale PDC production faces challenges, such as low yield (production less than 100 g/L) and high market value of the co-substrate. An alternative to overcome this problem would be using a platform capable of using different carbon sources, enabling its application on an industrial scale. *Burkholderia sacchari*, recognized for its efficiency in PHA production and the use of many carbon sources, including xylose, the second most abundant sugar in lignocellulosic biomass, stands out as a promising candidate to face these challenges (Oliveira-Filho et al., 2021). One strategy for analyzing production performance is metabolic systems engineering, through metabolic modeling and pathway analysis. Metabolic flux analysis (MFA) allows the quantitative description of cellular fluxes in a metabolic network, allowing the analysis and proposal of metabolic modifications aimed at producing a specific product, and obtaining the conversion factors that define the metabolism of the cell in question (Falco et al., 2022). This allows analyzing the insertion of synthetic metabolic pathways and analyzing the possibility of constructing consistent metabolic pathways based on the selected parameters. Therefore, this *in silico* analysis will serve to guide the best way to use the metabolism of *B. sacchari* for the efficient production of PDC through the introduction of synthetic metabolic pathways.

2 MATERIAL & METHODS

IN SILICO SEARCH FOR GENES AND PATHWAYS

Based on a search in the Kyoto Encyclopedia of Genes and Genomes database (KYOTO ENCYCLOPEDIA OF GENES AND GENOMES, 2017) and in the literature, it was possible to identify two routes that could be used to produce PDC from the shikimic acid, the meta 4,5PCA pathway and the ortho 3,4PCA pathway (Kamimura & Masai, 2014; Kersten et al., 1982). To search for genes involved in the production and degradation of protocatechuate acid (PCA) in *B. sacchari*, the Rapid Annotation using System Technology (RAST) platform was used, and to provide information on the similarity and function of the enzymatic genes of the degradation pathway of protocatechuate present in *Comamonas* sp. using MUSCLE.

METABOLIC MODEL CONSTRUCTION OF *B. sacchari* LMF 101

The network was reconstructed based on genomic data on the pathways present in *Burkholderia* spp, including the Embden-Meyerhof-Parnas (EMP), Entner-Doudoroff (ED), Pentose phosphate pathway (VP), Krebs cycle (CK), glyoxylate shunt (GLX), anaplerotic reactions (AD), aerobic respiration of coenzymes (OXFAD and OXNAD), membrane (PNTAB) and soluble (SDH)

transhydrogenases, and with the exclusion of the PHB production pathway. The metabolic model constructed considered *B. sacchari* growing heterotrophically using glucose and oxygen as substrate, and the products formed carbon dioxide and PDC.

The premise was adopted that PDC production would occur after the growth phase, since the shikimate pathway leads to the production of essential amino acids, therefore the formation of biomass was not considered for PDC production. Thus, the network included the heterologous pathways of production and degradation of gallic acid and PCA. Therefore, to construct this metabolic network, *B. sacchari* Δ*phbC* was considered, containing a recombinant plasmid for expression of the 4,5 PCA pathway based on the work of Nakajima et al. (2009) and Zhou et al. (2023), which produces PDC through the rerouting of 3-dehydroshikimate (DHS) production in the shikimic acid pathway. The network was analyzed using metabolic flux analysis (MFA) based on the carbon balance, and stoichiometric and thermodynamic restrictions of the reactions, using the Metatool software (Peiffer et al., 1999) to determine the minimum possible paths that describe a stable network for PDC (elementary modes) production. These modes will guide the development of metabolic engineering strategies for efficient PDC production.

3 RESULTS & DISCUSSION

IN SILICO SEARCH FOR GENES AND PATHWAYS

Using RAST (Rapid Annotation using Subsystem Technology), a new sequence from a recent sequencing of *Burkholderia sacchari* LFM 101 (unpublished data) by the Laboratory of Bioproducts was used to search for genes, from which it was possible to identify the presence of genes similar to *pmdAB*, referring to the enzyme protocatechuate 4,5 dioxygenase (protocatechuate 4,5-dioxygenase alpha and beta subunit), noted in *B. sacchari* as *P45Dab* with 44% similarity, their low similarity may be due to the large phylogenetic distance between *Comamonas* sp. and *Burkholderia sp.* which justifies a greater difference between these proteins, however, the functional regions are well conserved, therefore, it is possible to affirm the identification of the existence of this protein in the genome, in addition, bench data corroborates the existence of this pathway since *B. sacchari* can grow in media with PCA (unpublished data). However, without the possibility of PDC formation, as the *pmdC* gene (4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase) was not found, demonstrating the need for the heterologous expression of this gene to enable the production of the target molecule. Furthermore, there is the possibility of accumulation due to the absence of genes similar to *pmdD* responsible for producing 2-pyrone-4,6-dicarboxylate hydrolase that would degrade the target molecule.

During the search for genes in the meta pathway (4,5PCA), annotations of genes in the protocatechuate 3,4 PCA pathway were found, such as *pcaHG*, which in *Pseudomonas* species is used in the degradation of gallic acid with the production of PDC (Kersten et al., 1982), with the presence of this pathway in *Burkholderia* there is the possibility of accumulation of the focus product since *B. sacchari* can grow in a medium with gallic acid, suggesting that this could be another synthetic pathway to be tested in the metabolic network of *B. sacchari*, but that this pathway could also mean an escape of PCA that would be used in pathway 4,5 by *Burkholderia*.

METABOLIC FLUX ANALISYS (MFA)

From the processing of the constructed network, a set of 83 elementary modes was generated together with their respective global reactions and enzymatic pathways. The networks were analyzed taking into account the highest conversion factor (molPDC/molGlucose) to indicate the best strategy for genetic manipulation to produce PDC. Thus, it was possible to identify that PDC could be produced through two pathways considering the conversion of DHS into PCA, from the conversion of gallate or CHMS into PDC, in which the two expressed heterologous pathways present the modes with greater conversion efficiency, resulting in 0.85 molPDC/molGlucose. However, there may be a PCA deviation for the production of gallic acid with the use of the 3,4pcaHG pathway, which may indicate that the exclusion of this pathway may result in better production without the drainage of carbons for gallic acid, as there is no a production flow using both routes at the same time. Thus, maintaining the main pathway with the insertion of heterologous genes for PDC production from the 4,5PCA pathway may be viable for its accumulation, maintaining production efficiency at the theoretical maximum value, but it is interesting to note that to achieve this performance, it is necessary to maintain a gluconeogenic cycle with the use of anapleurotic pathways, since its deactivation leads to lower efficiency in the conversion of PDC (0.5molPDC/molGlucose), which indicates that a genetic modification to maintain the intense flow of anapleurotic pathways would be necessary to reach the maximum theoretical efficiency, since with a zero flow on these pathways we have a decrease in conversion.

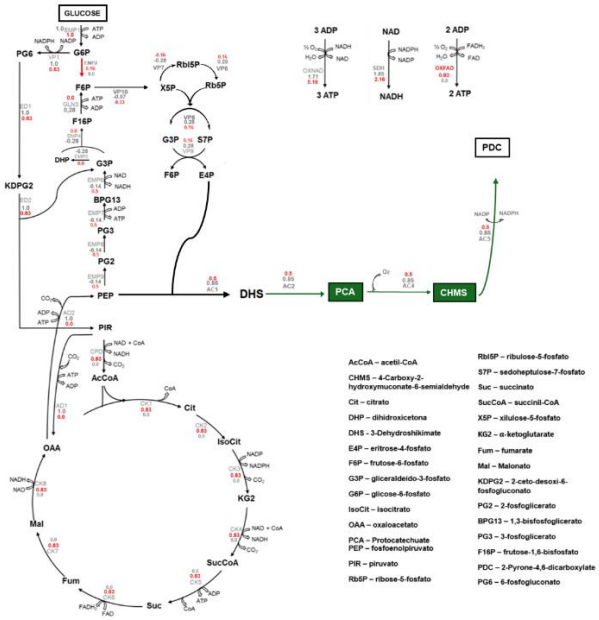

Image 1. Distribution of flows representative of PDC production generated from MFA in Metatool, where flows in gray lead to the theoretical maximum and flows in red decrease conversation efficiency with deactivating the anapleurotic pathways.

4 CONCLUSION

Based on the analysis of the metabolic flux within the proposed network for *Burkholderia sacchari* targeting PDC production, the *in silico* study underscores the potential of the synthetic pathways. Both pathways demonstrated viability for PDC synthesis. However, to achieve the theoretical maximum metabolic capacity, it is advisable to prioritize the anapleurotic pathways and the 4,5PCA pathway. This approach is recommended because the use of the 3,4PCA pathway may introduce a bottleneck, redirecting PCA towards gallic acid production and thereby limiting the efficiency of PDC synthesis.

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