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I CONGRESSO BRASILEIRO DE BIOTECNOLOGIA INDUSTRIAL
XXIV SINAFERM ·· XV SHEB ·· XV ENZITEC FLORIANÓPOLIS ·· 2024
Creating connections between biotechnology and industrial sustainability
August 25 to 28, 2024
Costão do Santinho Resort, Florianópolis, SC, Brazil
INDUSTRIAL MICROBIOLOGY: PROSPECTING AND APPLIED MOLECULAR BIOLOGY

EVALUATION OF A NEW GENETIC TARGET FOR IMPROVING PHENAZINE PRODUCTION IN *Escherichia coli*

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ABSTRACT

Phenazines represent a class of microbial secondary metabolites that have been employed in various applications, including the production of biopesticides, the construction of biosensors, and pharmaceutical applications. The biosynthesis of phenazines occurs from chorismate, the final product of the shikimate metabolic pathway. Over the years, metabolic engineering strategies have been developed to optimize the production of aromatic compounds from this pathway. In this project, we overexpressed a genetic target to evaluate its impact on the heterologous production of phenazine-1-carboxylic acid (PCA) by recombinant *E. coli* cells. To achieve this objective, the gene was cloned into a plasmid and expressed in a phenazine-producing strain of *Escherichia coli*. The experiments demonstrated the assessed genetic target was capable of enhancing PCA production.

Keywords: Phenazines. Metabolic engineering. Synthetic biology. Secondary metabolite.

1 INTRODUCTION

Phenazines represent a class of natural products with potential applications in biopesticide production and drug development. This is due to their broad spectrum of antimicrobial, antifungal, and antitumor properties[1 2].

Despite their potential for biotechnological application, the production of these compounds has until recently been the subject of study only in naturally producing microorganisms. This fact constrained the feasibility of their application, as some of these organisms are pathogenic and others have very limited production. Recently, the heterologous production of phenazines was investigated in *E. coli* cells [3], thus enabling their production in a system free from the metabolic regulations found in the original organisms.

After the creation of the first recombinant strain of *E. coli* capable of producing phenazine-1-carboxylic acid (PCA), the optimization of the aromatic pathway in *E. coli* with the objective of enhancing the production of the phenazines was initiated. In our previous work (unpublished results), the conversion reactions of chorismate to PCA were incorporated into the genome-scale metabolic model iJO1366 [4] with the aim of identifying potential target reactions for modification in order to enhance phenazine production. The Optflux software [5] was employed to run the optimizations with the modified model using evolutionary algorithms. The *in silico* results identified three main reactions in the shikimate pathway as potential targets for improving phenazine production. One of the target reactions on this pathway is 3-dehydroquinate dehydratase, expressed by the *aroD* gene. From this result, the objective of the present study was to overexpress the *aroD* gene in a recombinant strain of *E. coli* in order to assess its impact on the heterologous production of PCA.

2 MATERIAL & METHODS

The pRaD plasmid was constructed in a monocistronic configuration by ligating the pRSM4 [6] vector and the *aroD* insert. The amplicon containing the *aroD* gene was amplified from the genomic DNA of *E. coli* K12 MG1655 using the Platinum SuperFi II 2x master mix (Thermo Scientific). The amplicon was then digested with the restriction enzymes NdeI and XhoI (FastDigest, Thermo Scientific). Similarly, the pRSM4 plasmid was digested with the same enzymes. Once the digestion reactions had been purified, the pRSM4 vector was ligated to the *aroD* insert and the ligation reaction was used to transform *E. coli* DH5α by heat shock. The transformation was plated on LB agar medium + kanamycin (50 µg/µL). The colonies that grew were evaluated by polymerase chain reaction (PCR) screening and had their plasmids extracted for further confirmation by a digestion reaction using XhoI and NdeI enzymes. Once the plasmid construction was confirmed, the colonies were inoculated into Luria-Bertani (LB) medium with kanamycin (50 µg/µL) at 37 °C overnight and stored in cryotubes with 20% glycerol.

Finally, the pRaD plasmid was transformed by electroporation into the *E. coli* QH4 Δ*tyrR* pE-PCA strain, which contained the pETM7_phzABGFCDE plasmid [3], responsible for the synthesis of phenazine-1-carboxylic acid. Cultures of the *E. coli* QH4 Δ*tyrR* pE-PCA pRaD (named QTPAD) strain, and *E. coli* QH4 Δ*tyrR* pE-PCA strain (named QTPA0) used as a comparative standard, were carried out for 33 hours in 250 mL flasks at 30 °C and 225 rpm in 50 mL of Terrific Broth (TB) medium supplemented with 2% glycerol, kanamycin (50 µg/µL) and ampicillin (80 µg/µL). The optical densities of the cultures were monitored at 600 nm in a spectrophotometer until they reached a range of 0.8 up to 1. At this point, they were induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). Then 1 mL samples were collected for subsequent characterization using High Performance Liquid Chromatography (HPLC). The characterization was carried out by HPLC (Shimadzu Pumps HPLC 510 system, W717 injector, W410 refractometer and PDA W996 UV reader). A C18 column (Sunfire Waters, Milford, USA) was used at 25 °C connected to the 274 nm UV/VIS detector, with 1 L of solution A characterized as Milli-Q® water containing 0.1% (v/v) trifluoroacetic acid; and 1 L of solution B composed of acetonitrile containing 0.1% (v/v) trifluoroacetic acid, both at a flow rate of 0.6 mL/min.

3 RESULTS & DISCUSSION

The cloning of the *aroD* gene into the pRSM4 plasmid, which map is shown in Figure 1-A, was successfully confirmed by colony PCR as illustrated in Figure 1-B, and by the digestion of the plasmid, as represented in Figure 1-C.

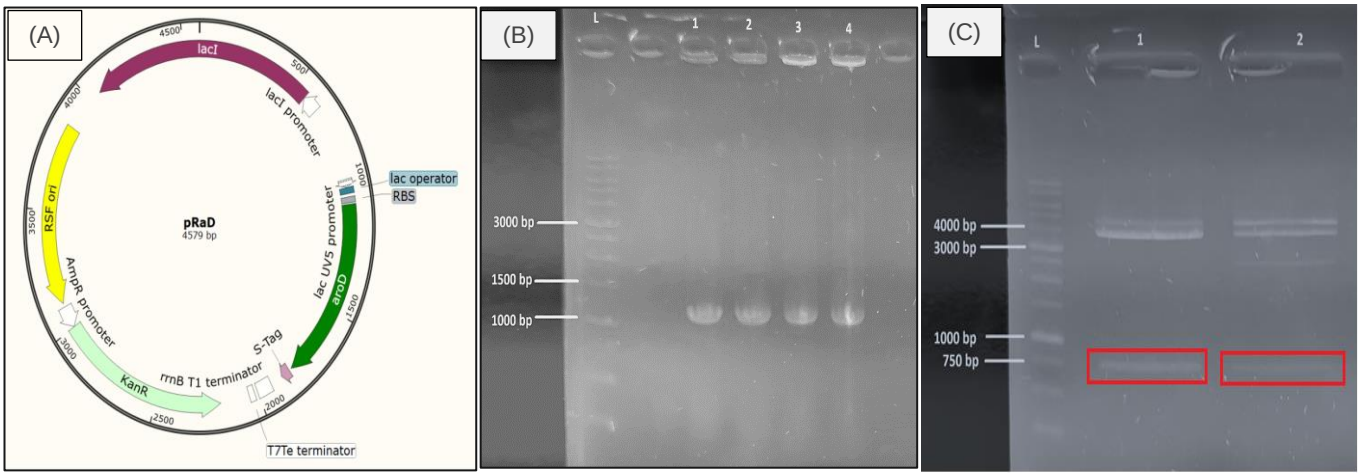

Figure 1 Confirmation of the pRSM4 plasmid construct containing the *aroD* gene. (A) Illustrative map of the pRaD plasmid visualized in SnapGene software. (B) Agarose gel electrophoresis of the products of the PCR reactions of colonies to confirm the pRaD construct. The molecular weight marker (ladder) is visible on the left in L. The numbers 1, 2, 3, and 4 indicate the colonies that were screened, where the positive reaction would amplify a band of 1161 bp. (C) Agarose gel electrophoresis of the products of the digestion reactions of the pRaD plasmid. The molecular weight marker (Ladder) is located on the left side of the gel, indicated by the letter L. The numbers 1 and 2 represent the digestion reactions conducted in duplicate, which would yield two distinct bands: one of 3818 bp and the other of 761 bp.

The characterization of PCA by HPLC enabled the quantification of the concentration of PCA at the end of cultivation for both strains. Figure 2 illustrates the production of phenazine by the QTPAD and QTPA0 strains.

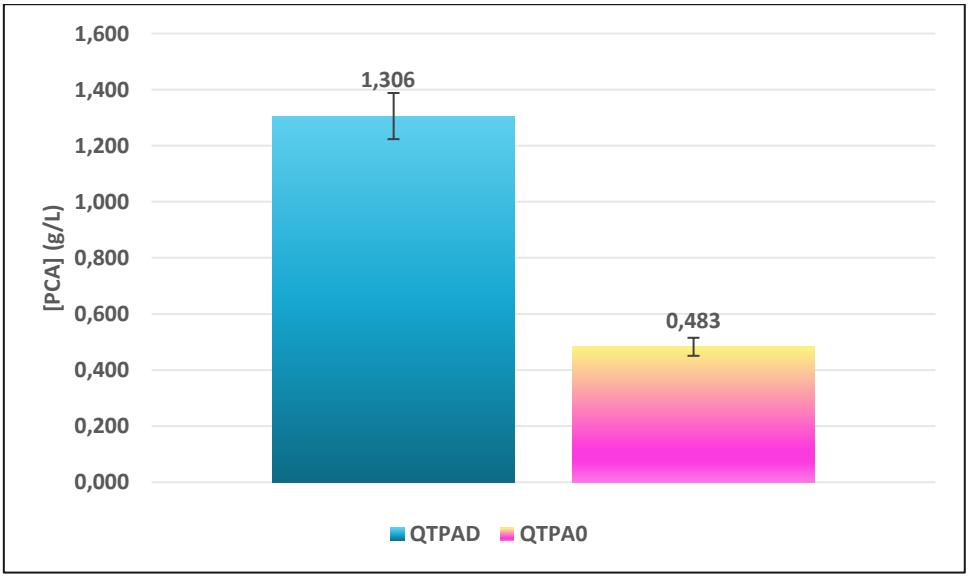

Figure 2 Comparison of the PCA production by the strains expressing (QTPAD) or not (QTPA0) an extra copy of the *aroD* gene.

The results demonstrate that the QTPAD strain achieved a PCA production of 1.306 g/L after 33 hours of cultivation, which is 170% higher than that of the QTPA0 strain that does not overexpress the *aroD* gene. Consequently, the *aroD* gene was confirmed as a genetic target whose overexpression can enhance phenazine production from the shikimate metabolic pathway. In addition to *aroD* expression, future work will investigate other genetic targets and fermentation conditions towards to increase phenazine production in *E. coli*.

4 CONCLUSION

Based on *in silico* results and experimental validation, the *aroD* gene was found to be a successful target for increasing phenazine production by more than 2.5-fold in the QTPAD strain. These results also reinforce the usefulness of stoichiometric models as powerful tools for metabolic engineering applications.

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ACKNOWLEDGEMENTS

This research was sponsored by Fundação de Amparo à Pesquisa do Estado de São Paulo (Processo Fapesp 2022/07833-7).