CONSTRUCTION OF AN ARABINOSE-INDUCIBLE EXPRESSION SYSTEM FOR *Cupriavidus necator* TO PRODUCE A BLUE PIGMENT

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ABSTRACT

Gases are polluting agents that have a major influence on the global temperature rise. With the advent of climate change and global climate catastrophes, society has been pressuring industries for more sustainable production methods that could slow down or reverse these changes. The textile industry has been looking for good and sustainable alternatives to the materials that are currently used. One of these possible alternatives is the non-ribosomal peptide indigoidine, a blue molecule that can substitute the blue dye indigo. Current bioproduction methods still rely on sugars as the main carbon sources, as most used microbial chassis are heterotroph. Although more eco-friendly than traditional chemical processes, it cannot be considered the most sustainable alternative for long-term production. *Cupriavidus necator* is a gram-negative bacterium capable of growing on different carbon sources, including C1 gases. As it can grow on CO_2, it poses as a promising production alternative with low carbon emissions and climate impacts. Therefore, this work aims to establish a heterologous indigoidine production utilizing the facultative autotrophic organism *Cupriavidus necator*.

Keywords: Synthetic Biology, Sustainable production, Indigoidine, Gas fermentation.

1 INTRODUCTION

Due to the imminence of climate change and with it, the social pressure for more sustainable production methods, industrial sectors have been looking for alternatives that can meet these requirements, aiming for a transition to a sustainable bioeconomy. Major polluting agents are the gases produced by industries, mostly made up of one-carbon gases (CO_2, CO, CH_4). Their accumulation in the atmosphere has a direct impact on the increase in global temperature and the observed climate changes. A viable alternative for reducing these emissions is reusing these gases as a carbon source to produce new molecules by microorganisms capable of fixing these substrates.

Cupriavidus necator is a gram-negative bacterium capable of growing from different carbon sources such as sugars, fatty acids, amino acids, and CO_2, through the Calvin-Benson-Bassham cycle. The textile industry, in agreement with other industrial sectors, has been actively searching for new sustainable alternatives to its currently used raw materials. As is the case with indigo dye, synthesized from the toxic compound aniline. A sustainable alternative is the biomolecule indigoidine, a non-ribosomal peptide naturally produced by some bacteria from L-glutamine by indigoidine synthase. Hereby, this project aimed to construct a plasmid and introduce it in the bacterium *C. necator* for the future production of indigoidine in different conditions as a possible sustainable alternative for the currently used indigo.

2 MATERIAL & METHODS

The plasmid was constructed using the golden gate assembly[1] from parts that were amplified using PCR. Due to the high number of parts, we carried out a two-step golden gate cloning, connecting two blocks separately and then ligating them to complete the plasmid. In the first step, we connected the fragments pCAT204-*araC*-pBAD into one block and the *sfp*-pBAD-*bpsA* into another. After assembling the blocks, the fragments were selected by the correct size on agarose gel. The blocks were purified by band excision and another round of golden gate assembly was carried out to connect them and form the final plasmid. The reaction was used to transform chemically competent *E. coli* TOP10 for propagation and plasmid extraction. Successful transformants were selected by resistance to the antibiotic chloramphenicol and the induction of blue color by adding L-arabinose to the medium. The wild-type strain *C. necator H16* was conjugated by using the conjugation strain *E. coli* GM2163 (pUB307) that has been previously transformed with the plasmid. All *E. coli* strains were cultivated in test tubes filled with 5 mL LB liquid medium supplemented with 25 µg/mL chloramphenicol and 0.1% (m/v) L-arabinose. Cultures were incubated at 220 rpm and 37ºC. Cultivation of *C. necator* strains was carried out in test tubes filled with 5 mL LB liquid medium supplemented with 50 µg/mL Chloramphenicol, 10 µg/mL gentamycin, and 0,1% L-arabinose when confirmation was needed. Cultures were incubated at 200 rpm and 30ºC.

3 RESULTS & DISCUSSION

Aiming to produce indigoidine using *C. necator* in autotrophic conditions we constructed a plasmid with inducible production triggered by L-arabinose, the most common used inducer for *C. necator*. An inducible expression was chosen to ease the metabolic burden of production and mitigate possible toxic effects of indigoidine production. The final plasmid consists of: (1) the *bpsA* gene, which encodes the enzyme indigoidine synthetase; (2) the *sfp* gene, which encodes the enzyme 4-phosphopantetheinyl transferase that activates the indigoidine synthetase; (3) the arabinose regulator gene *araC;* and (4) the

inducible promoter pBAD that will drive the transcription of both *bpsA* and *sfp*. The backbone plasmid pCAT204[2] contains an origin of replication optimized for *C. necator* and a resistance gene for Chloramphenicol. The final plasmid contains 8593 bp all the necessary parts for the propagation and inducible production of indigoidine (Fig. 1A). The constructed plasmid was transformed in *E. coli* TOP10 and successfully transformants were selected on LB plates with 10 µg/mL chloramphenicol and 0.1% L-arabinose. The successful construction of the plasmid was confirmed by size in agarose gel containing *Xba*I digested plasmids (Fig. 1B).

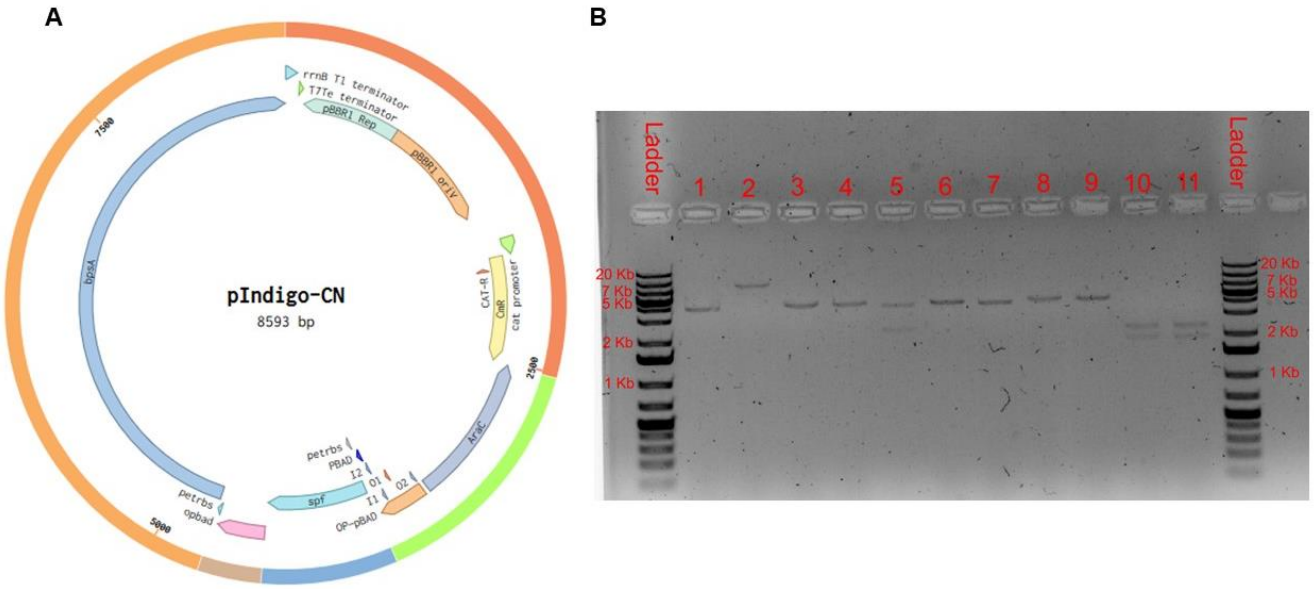

Figure 1 (A) Schematic of the plasmid constructed, the external circle shows the parts that were amplified and assembled by golden gate and the internal circle shows the individual components of the plasmid and **(B)** Agarose gel of plasmids digested with *Xba*I from 11 isolated colonies after transformation. Line number 2 represents the positive colony with the right plasmid size.

Plasmids were extracted from transformed *E. coli* Top10 cultures and used to transform chemically competent *E. coli* GM2163 (pUB307) cells. The transformed colonies were selected in LB plates with 25 µg/mL chloramphenicol and 0.1% L-arabinose. Transformed *E. coli* GM2163 was used for the conjugation of *C. necator* H16. After the conjugation process, colonies were selected on LB plates with 50 µg/mL chloramphenicol and 10 µg/mL gentamycin. Single colonies were isolated and striked out on LB plates with 50 µg/mL chloramphenicol, 10 µg/mL gentamycin, and 0.1% L-arabinose for confirmation of indigoidine production (Fig. 2).

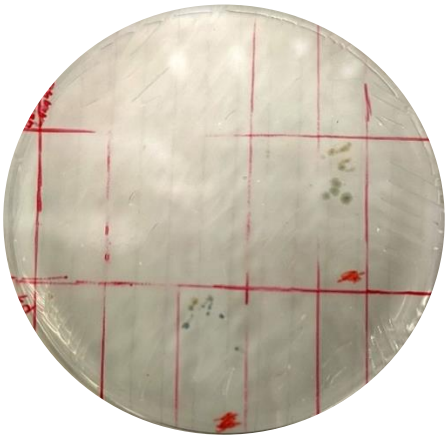

Figure 2 LB plate with *C. necator* blue colonies.

Successfully transformed colonies were cultivated in LB medium supplemented with 50 µg/mL chloramphenicol and 10 µg/mL gentamycin at 200 rpm, 30 ºC. After 48h, the culture was used to make 20% (m/m) glycerol stocks that were stored at -80 ºC for further cultivation and production evaluation.

4 CONCLUSION

We constructed a plasmid for the heterologous production of indigoidine induced by L-arabinose. The constructed plasmid was successfully used to transform *C. necator* H16. The transformants were able to produce indigoidine attested by the presence of blue color on liquid cultures and in plated colonies. In the future, this strain will be used to evaluate the production capacity of *C. necator* in heterotrophy, autotrophic, and mixotrophic conditions.

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