HETEROLOGOUS PRODUCTION OF A BLUE MICROBIAL DYE

Ana Paula M. L. Horta[1], Vitor L. Pantaleão[1], Adilson J. Silva[1*]

[1] *Department of Chemical Engineering, Federal University of São Carlos, São Carlos, Brazil.*
** Corresponding author's email address: adilsonjs@ufscar.br*

ABSTRACT

The demand for natural dyes to replace synthetic analogues is growing as society and the manufacturing sector become more interested in developing sustainable processes. Among the natural dyes being investigated for production by fermentative processes is indigoidine. This molecule is a microbial blue dye that has been studied as an indigo substitute for textile dyeing, but may have other applications, such as in the food and cosmetics industries. In this work, the genes required for indigoidine production were cloned and expressed in *E. coli*. The recombinant strain proved capable of producing the dye, paving the way for future studies to optimize its production.

Keywords: Indigoidine. *Escherichia coli*. Metabolic engineering. Synthetic biology.

1 INTRODUCTION

In recent decades, the public awareness of sustainability has increased. Among the concerns is the need to develop more environmentally friendly production methods to replace polluting industrial processes. In this context, indigoidine, which is a natural blue dye, has great potential to replace indigo, a chemical dye used in the textile industry, which is mostly produced through polluting chemical processes.

Therefore, obtaining microbial strains capable of efficiently producing this compound is highly desirable in order to make its production viable on a large scale. Some research groups have reported the heterologous production of indigoidine in different microorganisms[1,2,3,4,5,6]. The best results were observed in fed-batch cultivations of *Corynebacterium glutamicum* [2] and *Rhodosporidium toruloides* [4] strains. For *Escherichia coli*, only shaken flask assays were reported [6].

The biosynthesis of indigoidine occurs through the condensation of two glutamine molecules, and its production in *E. coli* requires the expression of two heterologous genes encoding an indigoidine synthetase and a phosphopantetheinyl transferase [6]. Therefore, the aim of this work was to clone and express these genes in *E. coli* to enable the heterologous production of indigoidine

2 MATERIAL & METHODS

The homologous recombination cloning method [7] was used to clone the *bpsA* gene from *Streptomyces lavendulae* genomic DNA and a functional version of the *sfp* gene from the genomic DNA of *Bacillus subtilis* strain 168 [8]. *Escherichia coli* DH5α was used for cloning and expression.

To construct the recombinant plasmids, an amplification PCR was initially carried out for the *sfp* and *bpsA* genes and the pETM7 plasmid [9,10], containing 1 µL of the specific primer mix for each reaction at a concentration of 10 pmol/µL, 1 µL of genomic DNA, 25 µL of 2X Platinum SuperFi II PCR Master Mix (Invitrogen) and water to complete 50 µL. The PCR was programmed with 1 cycle of 30 s at 98°C, 30 cycles of 10 s at 98°C, 10 s at 60°C and 2 min at 72°C, and the final extension for 5 min at 72°C. After completing the PCR, 1 µL of DpnI was added to the pETM7 amplification reaction, which was placed at 37°C for 1 hour to eliminate the template. The purified fragments (sfp 675 bp and bpsA 3852 bp) from the PCR were mixed in a reaction with 10 µL of water, combining the amplicons of the pETM7 plasmid and the *sfp* gene, and the pETM7 plasmid and the *bpsA* gene separately. After transformation by heat shock and obtaining colonies, the constructions were checked by colony PCR, digestion and Sanger sequencing.

To assemble the plasmid with both genes in pseudo-operon form, pETM7_sfp was used as the recipient plasmid and pETM7_bpsa as the donor, following the protocol described by Xu et al. (2012) [9]. The enzymes BcuI and BsPOI were used for pETM7_sfp, and XmaJI and BsPOI for pETM7_bpsa. The purified products were mixed in a 1:1 ratio (insert:vector) for heat shock transformation of *E. coli* DH5α cells. Some of the colonies obtained were analyzed by colony PCR and digestion.

The recombinant cells were cultivated in triplicate, using 50 mL of LB containing 20 g/L glucose and 50 µg/mL ampicillin at 37°C at 250 rpm. The OD was measured at 600 nm until it reached 0.5, when the cells were induced with 1mM IPTG. During induction, the temperature was shifted to 30°C, and samples were taken during 20 hours of induction. Indigoidine was analyzed by optical density readings at 612 nm. Samples were diluted 20x with DMSO + Tween 20 2% and sonicated for 2 min. The sonicated samples were centrifuged at 13,000g for 1 minute and the supernatant was diluted 5x with DMSO + Tween 20 prior to

OD reading. The calibration curve was prepared using indigoidine purified from a previous culture, following the protocol described by Banerjee et al. (2020)[1].

3 RESULTS & DISCUSSION

The individual cloning of indigoidine synthetase (*bpsA*) and phosphopantetheinyl transferase (*sfp*) genes was successful, as can be seen in Figure 1-A. Combination of both genes in the same plasmid was also achieved (Figure 1-B) in pseudo-operon configuration, favoring a higher transcription of the *bpsA* gene. Figure 1-C shows a scheme of the final recombinant plasmid.

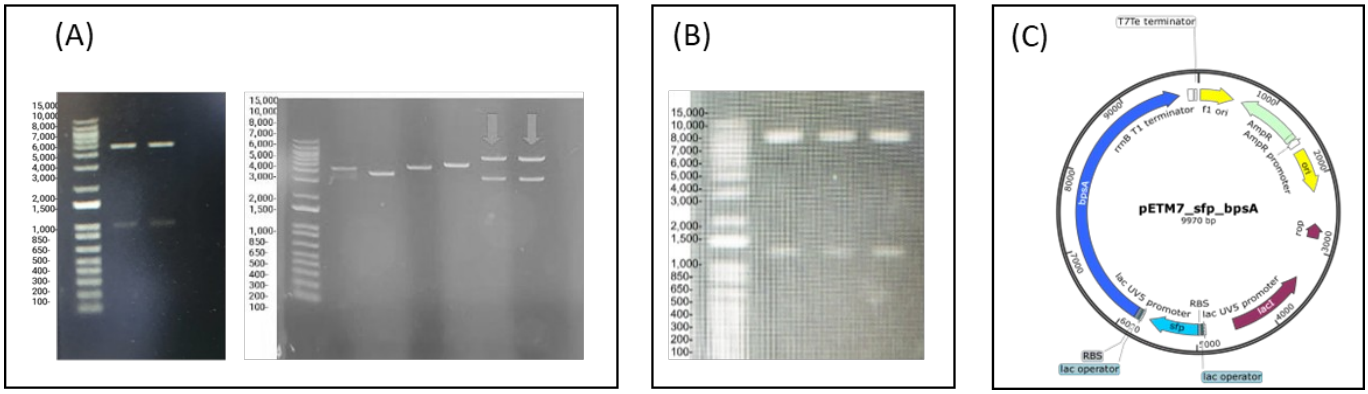

Figure 1 Confirmation of individual cloning of the *bpsA* and *sfp* genes into the pETM7 plasmid and the pETM7_sfp_bpsA construction containing both genes. (A) Agarose gel electrophoresis of the digested plasmids: pETM7_sfp (left), with expected bands at 4712 and 1005 bp; pETM7_bpsA (right), with expected bands at 5125 and 3925 bp. (B) Confirmatory digestion of pETM7_sfp_bpsA, with expected bands at 8705 and 1264 bp. (C) Schematic representation of the final construction of the pETM7_sfp_bpsA plasmid for indigoidine production in *E. coli*.

After transforming *E. coli* cells with the recombinant plasmid, the indigoidine production was observed even in the absence of IPTG, indicating a leaky expression of both genes. Dark blue colonies were visible on the transformation plates, indicating the production of the dye (Figure 2-A). Cultivation in shaken flasks showed dye formation only after induction with IPTG and lowering of temperature. Indigoidine was isolated and purified from a culture in a volume of 160 mL (Figure 2-B) for the preparation of a calibration curve.

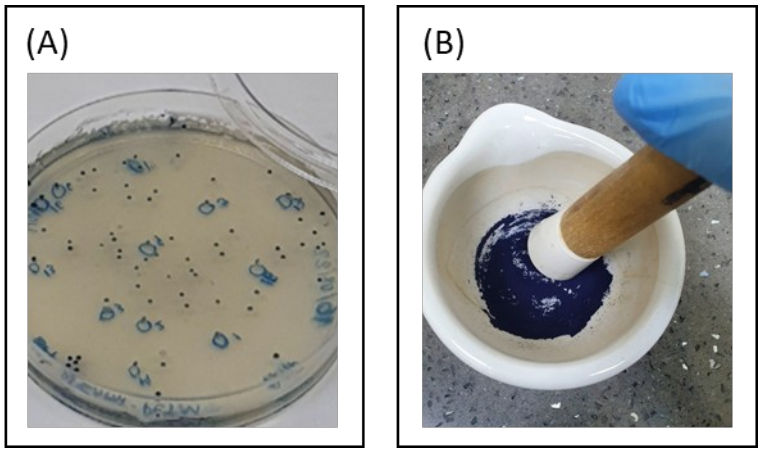

Figure 2 Indigoidine production and purification. (A) Petri dish with LB Agar, ampicillin and colonies carrying the pETM7_sfp_bpsA plasmid, with production of the indigoidine blue dye. (B) Purified indigoidine being pulverized using a mortar and pestle.

Once the calibration curve was obtained, a triplicate culture was performed to quantify production. The concentration of indigoidine reached its maximum value at about 20 h, reaching an average of 1,18 +/- 0.069 g/L. This result is higher than that reported for indigoidine production by *Sacharomyces cerevisiae* (0.98 g/L in fed-batch culture) [5] and close to *Aspergillus oryzae* production (1.4 g/L) [3]. After future studies to optimize cultivation conditions, we expect to obtain a strain that is competitive with the other microbial platforms under investigation.

4 CONCLUSION

Our results showed that the desired genes were successfully cloned and expressed in *E. coli cells*, and the indigoidine production was achieved, reaching a concentration of 1.18 g/L after 20h of cultivation. Process parameters will be optimized in future studies.

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