

Engineering Probiotics for Serotonin Production as a Potential Adjunctive Therapy for Depression

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Abstract. Depression remains the main cause affecting global mental health, but current drug treatments are often limited by slow onset and significant side effects. As a potential alternative, we explored the possibility of programming bacterial hosts to directly synthesize serotonin (5-HT) in the gut. To this end, we constructed a synthetic operon containing tryptophan hydroxylase (TPH) and tryptophan decarboxylase (TDC) genes, which were fused by overlapping PCR and inserted under the control of the *trc* promoter in the pTrc99k plasmid. After transforming the plasmid into *E. coli* BL21 (DE3), we drove gene expression by gradient regulating IPTG concentration within the range of 0 to 10 mM. The SDS-PAGE electrophoresis results showed that both protein bands significantly increased with the increase of IPTG concentration and reached a plateau at approximately 5 mM. These results confirm that the dual enzyme 5-HT production module can operate normally in laboratory strains. However, applying this design to the field of probiotics is still a necessary next step, and we must further verify its actual production in the intestine.

Keywords: Serotonin; 5-hydroxytryptamine; Tryptophan decarboxylase; Tryptophan hydroxylase; Probiotics; Synthetic biology; Metabolic engineering; *Escherichia coli*; Depression.

1. Introduction

Depression is one of the most common neurological and psychiatric disorders worldwide, and the imbalance in the regulation of the serotonergic system, especially serotonin (5-HT), has been considered closely related to its underlying pathological mechanisms. At present, most prescription antidepressants act on 5-HT reuptake transporters, but there are still a considerable number of patients who experience inadequate efficacy, prolonged clinical benefit latency, or intolerable adverse reactions. This unmet need has prompted people to explore alternative solutions to improve the availability of central 5-HT, including strategies that can circumvent pharmacokinetic limitations of oral administration [1].

Microbial biosynthesis, as a feasible method for engineering complex bioactive molecules through heterologous metabolic pathways, has received widespread attention [2]. For therapeutic applications, careful consideration is needed when selecting suitable host bacteria. Some non pathogenic strains of *Escherichia coli*, especially laboratory variants such as Nissle 1917 and BL21 (DE3) that have been extensively studied, provide a practical starting point. These microorganisms have good genetic operability, combined with the potential for oral administration and intestinal survival, making them ideal candidates for local and sustained production of target compounds as cell carriers.

The synthesis of serotonin from tryptophan requires two consecutive enzymatic conversions: an initial hydroxylation to 5-hydroxytryptophan catalyzed by tryptophan hydroxylase (TPH), followed by decarboxylation to 5-hydroxytryptamine mediated by tryptophan decarboxylase (TDC) [3,4]. Nallasamy et al. [3] elucidated the catalytic mechanism of plant-derived TDC, including its pyridoxal phosphate (PLP) dependence, while Windahl et al. [4] determined the crystal structure of TPH, revealing the coordination chemistry of Fe^{2+} with tetrahydrobiopterin (BH_4) at the active site. This structural and functional knowledge provides the foundation for attempts to reconstitute this pathway in microbial hosts. However, the simultaneous expression of two heterologous enzymes within a single cell presents practical difficulties, as the relative abundance of each protein must be fine-tuned to minimize the metabolic burden while maintaining pathway throughput.

The previous research on expressing multiple genes in *Escherichia coli* provided useful guidance for this study. Huang et al. [5] reported that the yield of xanthine oxidase inhibitory peptides was improved by optimizing the sequence and using tandem gene organization; We hereby apply this method to our dual enzyme construct. In another study, Lee et al. [6] demonstrated that the natural RimI acetyltransferase in *Escherichia coli* can metabolize 5-HT to melatonin, revealing the pre-existing 5-HT metabolism ability in the strain. The extent to which this endogenous metabolic pathway will promote or hinder our engineering production is still an unresolved issue.

In this study, the coding sequences of TDC and TPH were concatenated by overlapping PCR and connected to the IPTG responsive vector pTrc99k. The obtained recombinant plasmid was transferred to *Escherichia coli* BL21 (DE3) and protein expression was induced by different concentrations of IPTG. Subsequently, the cell lysate was analyzed by SDS-PAGE to evaluate whether both enzymes accumulated in the soluble components. These data provide a preliminary framework for microbial 5-HT generation, which may be of great significance for the development of adjuvant interventions for depression.

2. Materials and Methods

2.1. Bacterial Strains, Vectors, and Reagents

Escherichia coli DH5 α and BL21(DE3) competent cells were used for cloning and protein expression, respectively. The prokaryotic expression vector was pTrc99k (Amann et al., 1985). Restriction endonucleases, T4 DNA ligase, plasmid miniprep kits, and DNA gel extraction kits were purchased from commercial suppliers. Isopropyl- β -D-thiogalactopyranoside (IPTG) was used for protein induction. Kanamycin was used for antibiotic selection at a final concentration of 50 μ g/mL.

2.2. Construction of Recombinant Plasmid pTrc99k-TDC-TPH

The coding sequences of TDC and TPH were amplified by gene-specific primers containing overlapping regions to achieve seamless fusion. The tdc-tph double gene cassette was constructed by connecting the two fragments by overlapping PCR. The fusion product was separated by agarose gel electrophoresis, and the target band was cut out and purified using a gel extraction kit. The purified fragment and pTrc99k vector were digested with the corresponding restriction enzymes, and the digested products were recovered. T4 DNA ligase was used for an overnight ligation reaction at 16 °C. The connecting mixture was transformed into *Escherichia coli* DH5 α competent cells, and the cells were coated on LB agar plate containing kanamycin, and then cultured overnight at 37 °C. A single colony with clear boundary was screened for colony PCR screening. The positive clone was amplified in LB liquid medium containing kanamycin, and the plasmid was extracted for restriction enzyme verification. Subsequently, the confirmed recombinant plasmid pTrc99k TDC TPH was transformed into *E. coli* BL21 (DE3) for protein expression.

2.3. Protein Expression, Extraction, and SDS-PAGE Analysis

The transformed BL21 (DE3) cells were cultured in LB medium containing kanamycin at 37 °C and 220 rpm. The overnight seed culture solution was diluted into fresh LB medium at the ratio of 1:100 and cultured to the mid-logarithmic phase (OD₆₀₀ $\approx$ 0.6). IPTG was added for induction, and the final concentrations were 0, 0.1, 0.5, 1, 5 and 10 mmol/L, respectively. The culture continued to oscillate at 37 °C for 4 hours. The cells were collected by centrifugation, resuspended with PBS buffer, and ultrasonically broken on ice (3-second pulse, 5-second interval, total 15 minutes). The cracking solution was centrifuged at 12000 $\times$ g for 10 minutes at 4 °C. The supernatant was collected as crude intracellular protein extract. The equal volume of protein sample was mixed with SDS loading buffer, boiled for 10 minutes, and then separated by SDS-PAGE (12% separation gel). The gel was dyed with Coomassie brilliant blue R-250 and decolorized to develop protein bands.

3. Results

3.1. Construction and Verification of the Recombinant Plasmid pTrc99k-TDC-TPH

The coding sequences of TDC and TPH have been successfully assembled into a double gene box and cloned into the ptrc99k vector. To confirm the presence of recombinant plasmids in host cells, the ligation mixture was transformed into *E. coli* competent cells and coated on LB agar plate containing kanamycin. As shown in Figure 1, a large number of well-defined colonies appeared on the selective plate, indicating that host cells carrying ptrc99k TDC TPH plasmid have successfully completed transformation and acquired antibiotic resistance.



Figure 1. *E. coli* transformation plate carrying recombinant plasmid ptrc99k TDC TPH was cultured on LB agar and kanamycin was used as screening agent. The appearance of colonies confirmed that the transformation was successful.

Figure 2 shows the physical map of the constructed plasmid ptrc99k TDC TPH. The coding sequences of TDC and TPH are arranged in tandem downstream of the TRC promoter, and the intergenic region between them contains a putative ribosomal binding site to promote the independent translation of the two enzymes from a single transcript. The plasmid carrying pmb1 replication starting point and kanamycin resistance marker could be stably maintained in *E. coli*.

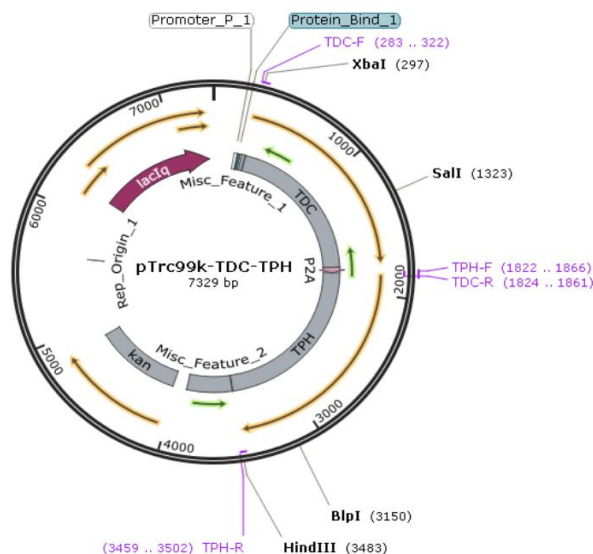


Figure 2. schematic diagram of recombinant plasmid ptrc99k TDC TPH (7329 BP). The coding sequences of TDC and TPH were spliced by overlapping PCR and cloned into the polyclonal site of ptrc99k under the control of TRC promoter. KanR, Kanamycin resistance gene; ori, Copy start point.

3.2. Restriction Enzyme Digestion Verification of the Recombinant Plasmid

To verify whether TDC and TPH were correctly inserted into the ptrc99k vector, plasmids extracted from positive clones were subjected to restriction enzyme digestion and analyzed by agarose gel electrophoresis (Figure 3).

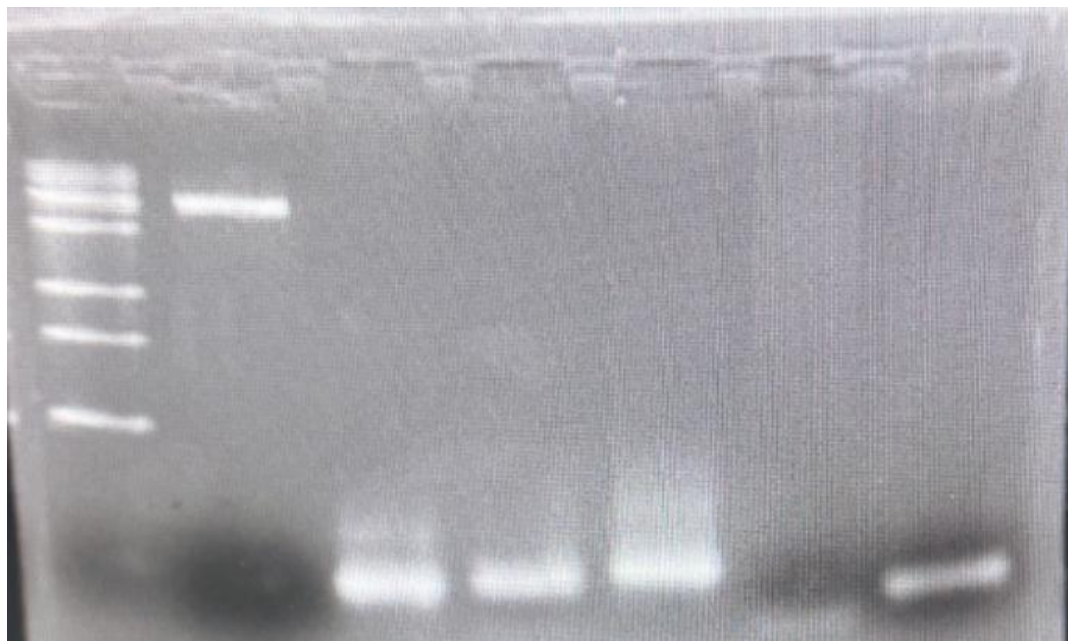


Figure 3. agarose gel electrophoresis of plasmid and PCR product after restriction enzyme digestion. Lane 1, linearized ptrc99k empty vector; Lane 2, TDC fragment; Lane 3, TPH fragment; Lane 4, ptrc99k TDC TPH digested with 25 μ L of enzyme; Lane 5, ptrc99k TDC TPH digested with 43 μ L of enzyme; Lane 6, TDC fragment (positive control). All lanes showed clear bands of the expected size.

The results confirmed the composition of the recombinant plasmid. Lane 1 shows the linearized empty ptrc99k vector. Lanes 2 and 3 show separate segments of TDC and TPH, respectively. The 4th and 5th lanes contained ptrc99k TDC TPH plasmids digested with 25 μ L and 43 μ L restriction enzymes, respectively. The two swimlanes showed clear bands corresponding to the carrier skeleton and tdc-tph insertion fragments, respectively. It is worth noting that compared with 25 μ L enzyme reaction (Lane 4), 43 μ L enzyme reaction (Lane 5) produced sharper and more complete bands, indicating that the smaller enzyme reaction volume is not enough to achieve complete linearization of plasmids. Lane 6 serves as a positive control for TDC fragments. The expected bands appeared in all swimming lanes, which jointly confirmed the successful construction of ptrc99k TDC TPH and the correct insertion of the two target genes.

3.3. IPTG-Induced Expression of TDC and TPH in *E. coli* BL21(DE3)

The recombinant plasmid ptrc99k TDC TPH was transformed into *E. coli* BL21 (DE3) for protein expression. To optimize the induction conditions, different concentrations of IPTG gradients (0, 0.1, 0.5, 1, 5 and 10 mm) were used, and the intracellular protein mass spectra were analyzed by SDS-PAGE (Figure 4).

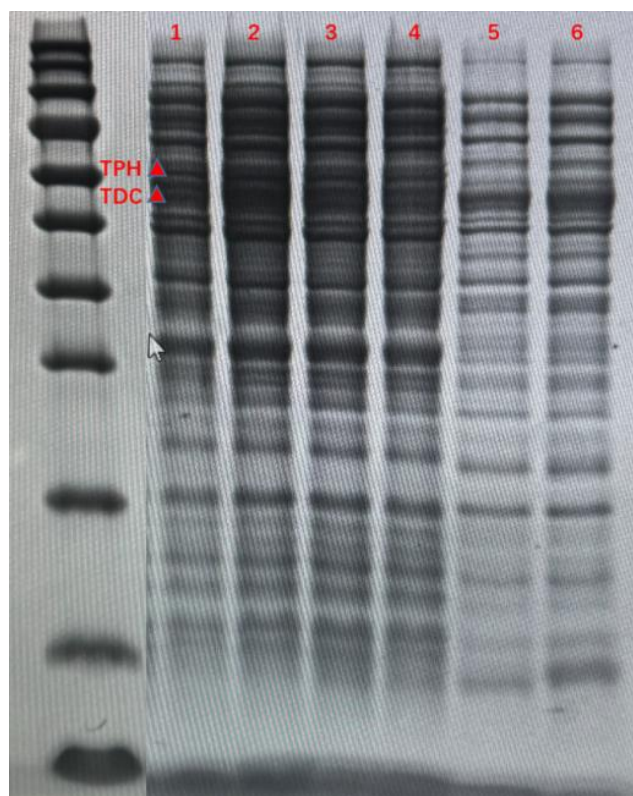


Figure 4. SDS-PAGE analysis of intracellular proteins of *Escherichia coli* BL21 (DE3) carrying ptrc99k TDC TPH plasmid induced by different IPTG concentrations. M channel, protein molecular weight labeling; 1 channel, 0 mM IPTG (uninduced control); 2 channels, 0.1 mM IPTG; 3 channels, 0.5 mM IPTG; 4 channels, 1 mM IPTG; 5 channels, 5 mM IPTG; 6 channels, 10 mM IPTG. Arrows indicate the expected position of the TDC and TPH.

In the absence of IPTG (Lane 1), no obvious target protein band was observed, indicating that the TRC promoter was strictly inhibited and the leakage expression was minimal. After adding IPTG, two obvious protein bands gradually appeared at about 55 kDa, corresponding to the expected molecular weights of TDC and TPH, respectively. Under the condition of 0.1 mM IPTG (the second lane), a weak but detectable target band appeared, indicating that it can be successfully induced even at a low concentration of inducer. With the increase of IPTG concentration, the band intensity increased gradually: moderate expression at 0.5 mM (Lane 3), strong expression at 1 mM (Lane 4), and close to the maximum expression level at 5 mM and 10 mM (lanes 5 and 6). The expression levels of these two enzymes showed a significant IPTG concentration-dependent pattern, and reached saturation between 5 and 10 mM.

Because the molecular weights of TDC and TPH are almost the same, their migration bands on the gel are very close, and it is difficult to completely separate them. However, under the IPTG concentration gradient, the band intensity of about 55 kDa region gradually increased, which confirmed the coexpression of the two enzymes. Under all induction conditions, the total protein content in the cells remained relatively stable, which showed that under the test conditions, the host cells could withstand the metabolic burden caused by the expression of double enzymes without significant growth inhibition.

4. Discussion

We constructed the co expression system of TDC and TPH in *E. coli* as a basic step for the development of serotonin producing probiotics. The use of ptrc99k plasmid ensures that strict transcriptional inhibition can be achieved under non induced conditions, which is particularly important when heterologous enzymes bring metabolic burden to the host. Kanamycin resistance

ensured that the plasmid could be stably maintained during continuous passage. However, BL21 (DE3) is a laboratory strain optimized for protein overexpression, rather than a probiotic candidate strain. If used for oral administration, the expression cassette needs to be transferred to strains with established safety characteristics, such as *Escherichia coli* nissle 1917 or selected lactic acid bacteria.

There are still a number of technical challenges before converting this construct into therapeutic applications. We have not evaluated the catalytic activity of the expressed enzyme, nor have we quantitatively analyzed 5-HT or its precursor 5-HTP in the medium. In the absence of such functional data, this biosynthetic pathway has not been verified at the metabolic level. Other factors to be considered include improving the expression level through codon optimization, increasing the availability of intracellular tryptophan, and protecting bacterial cells from gastric acid and bile salt damage after ingestion. If these obstacles can be overcome, a strain that can be administered orally and produce 5-HT in the intestinal mucosa, by targeting the local intestinal serotonin pool rather than systemic circulation, will be able to supplement the existing antidepressants.

5. Conclusion

We successfully constructed pE. coli in a concentration dependent manner. Restriction enzyme digestion verified the correctness of plasmid assembly, and SDS-PAGE electrophoresis confirmed the presence of protein bands consistent with the expected molecular weight of the two enzymes. Although a lot of adaptive adjustments are still needed before the system is applied to real probiotic products, this work provides a starting point for the production of microbial serotonin. The idea of local supply of serotonin by intestinal colonized bacteria is still a promising way, which is worth further study.

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