Rapid construction of E.coli chassis with genome multi-position integration of isopentenol utilization pathway for efficient and stable terpenoid accumulation

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Abstract

The isopentenol utilization pathway (IUP) is potential in terpenoids synthesis. This study aimed to construct IUP-employed E. coli chassis for stably synthesizing terpenoids. As to effectiveness, promotor engineering strategy was employed to regulate IUP expression level, while ribosome-binding site (RBS) library of the key enzyme was constructed for screening the optimal RBS, followed by optimization of concentration of inducer and substrates, the titer of reporting production, lycopene, from 0.087 to 8.67 mg/OD600. As about stability, the IUP expression cassette was integrated into the genome through transposition tool based on CRISPR-associated transposases. Results showed that the strain with 13 copies produced 1.78-fold lycopene titer that of the controlled strain with IUP-harbored plasmid, and it exhibited stable expression after ten successions while the plasmid loss was observed in the controlled strain in the 3rd succession. This strategy provides valuable information for rapid construction of highly effective and stable chassis employing IUP for terpenoids production.

Rapid construction of *E.coli* chassis with genome multi-position integration of isopentenol utilization pathway for efficient and stable terpenoid accumulation

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ABSTRACT

The isopentenol utilization pathway (IUP) is potential in terpenoids synthesis. This study aimed to construct IUP-employed *E. coli*chassis for stably synthesizing terpenoids. As to effectiveness, promotor engineering strategy was employed to regulate IUP expression level, while ribosome-binding site (RBS) library of the key enzyme was constructed for screening the optimal RBS, followed by optimization of concentration of inducer and substrates, the titer of reporting production, lycopene, from 0.087 to 8.67 mg/OD_{600}. As about stability, the IUP expression cassette was integrated into the genome through transposition tool based on CRISPR-associated transposases. Results showed that the strain with 13 copies produced 1.78-fold lycopene titer that of the controlled strain with IUP-harbored plasmid, and it exhibited stable expression after ten successions while the plasmid loss was observed in the controlled strain in the 3rd succession. This strategy provides valuable information for rapid construction of highly effective and stable chassis employing IUP for terpenoids production.

KEYWORDS:

The isopentenol utilization pathway, Terpenoid biosynthesis, Genome integration, Stable expression

1. INTRODUCTION

Terpenoids are a large family of secondary metabolites that has diverse biological functions, and thus have a broad market prospect in food, pharmaceutical and cosmetic industries . Among them, lycopene serves as an excellent antioxidant applied in food, pharmaceutical and cosmetic industries [2]. Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are two precursors for terpenoids synthesis, the flux of which is closely related with the production . Nowadays, two natural terpenoids synthesis pathways, 2-C-methyl-D-erythritol 4-phosphate (MEP) and mevalonate (MVA) pathways, are commonly utilized .

IUP is a two-step artificial pathway directly supplying two C5 building blocks through added isoprenol/prenol. It is more competitive because it costs 2 adenosine triphosphates (ATP) compared with MEP and MVA pathway respectively having 7 and 6 steps and consuming 3ATP, 3 nicotinamide adenine dinucleotide phosphate (NADPH) and 3ATP, 2NADPH, which means it's more energy efficient. Besides, utilizing isoprenol/prenol as substrates instead of other carbon source like glucose or glycerol, IUP is characterized as partly orthogonal in aspect of metabolic substrates . Recently, IUP has exhibited great potential in synthesis of terpenoids in different hosts. For example, the introduction of IUP to*Saccharomyces cerevisiae* elevated the IPP/DMAPP pool by 147-fold compared with the native pathway . As to *Yarrowia lipolytica* , IUP contributed to more than 15.7-fold IPP/DMAPP that of using MVA only . In*E.coli* , the expression of IUP was capable of converting 2 g/L prenol to 1.5 g/L geranoids and 0.5 g/L limonene . It was also employed in *E.coli* , leading to 248 mg/L β-carotene and 364 mg/L R-(-)-linalool . Therefore, it is worth applying to greater extent.

It is worth noting that the expression form of IUP is an important aspect for optimization, mainly including plasmid expression or integration of the genome. Plasmid expression, however, brings about many problems. First, the expression level is not stable due to imprecise copy number controlled by several factors . Second, it adds extra burden to cells, leading longer lagged phase which further harms the productivity . Besides, the serious 'loss of plasmids' condition occurred in many cases. For example, during the fed-batch fermentation of astaxanthin, cells lost plasmids in the exponential phase and it results in unpromising production . Also, it adds up the fermentation cost, which isn't realistic in industrial production . In comparison, genome multi-position integration is relatively more advantageous . The lycopene synthetic stability of two strains, respectively employing plasmid system and genome integration, was compared. The accumulation of the former one decreased to only 3.3% after 21stgeneration without Cm^R while the latter one kept the same level, which highlighted the edge of genome expression compared with plasmid system . In recent years, editing strategies that employ CRISPR-associated transposases (CASTs) enabled genome programming and accelerated the process of construction of engineered cell factories which was once time-consuming and labor-intensive . With the help of these tools, strain library with various copies can be rapidly constructed and screened for the most suitable copy number for desired purpose. For instance, Zhang et al. developed multicopy chromosomal integration using the CASTs in *E. coli* , with which the glucose dehydrogenase expression cassettes were integrated into the BL21(DE3), increasing 2.6-fold enzymes than that of strain using pET24a . Meanwhile, the GDH activity of plasmid-carrying strain began falling from 24 h while the strain obtained by transposes demonstrated continuous and stable synthesis during 43 h fermentation period, finally reaching 2.3-fold that of plasmid-carrying strain . Furthermore, PtrCASR, an updated version, is relatively more effective, being able to integrate 15.4kb cargo with 100% integration into multi positions of the genome, with 12.5% of the total 16 strains tested obtaining 8 copies of cargo after a round of transposition .

In this study, lycopene was chosen as a model terpenoid compound. First, the feasibility of IUP pathway was verified. Then, the IUP expression cassette was optimized for effectively accumulation of lycopene, through promoter engineering, ribosome-binding site (RBS) screening and IPP Delta-isomerase (IDI) selection. Furthermore, the induction concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG), the ratio

and concentration of prenol/isoprenol was optimized, respectively. Besides, the IUP expression cassettes were rapidly integrated into the genome by the PtrCAST and strain with highest titer was selected followed by stability test.

2. MATERIALS AND METHODS

2.1 Construction of strains and plasmids

All the strains and plasmids in this study are listed in Table S1 and S2. *E. coli* DH5α was used for plasmid construction and BL21(DE3) was chosen as the host strain. All PCR amplifications were performed by PrimeSTAR Max DNA Polymerase (Vazyme, Nanjing, China). The plasmids which were used to express IUP pathway were constructed on the basis of pSU2718 (chloramphenicol-selectable). Plasmids harboring IUP under control of constitutive promotors were constructed through restriction enzyme digestion and ligation *(KpnI* , *BamHI*). To construct Plasmids harboring IUP under the control of inducible promotors, the DNA fragments and plasmid backbone were amplified by PCR and constructed through Gibson assembly. The constructed plasmid was introduced into DH5α by chemical transformation or electro-transformation and correct transformants were confirmed by colony PCR. Plasmids of IUP and enzymes for lycopene production were introduced to BL21(DE3) through electro-transformation.

2.2 Construction of RBS library

The RBS library of $ThiM_{Ec}$ was obtained by circular-PCR using degenerate primer including R (representing A or G) to introduce RBS library. The primes are listed in Table S3. The linear PCR segment with overlap region was transformed to DH5α. The transformers covered on plate were washed with fresh Luria Bertani (LB) and cultivated in LB medium for 12h. Then the plasmids were extracted and transformed to BL21(DE3) with pEBI. Several transformants on agar plates were picked and conserved, constituting the RBS library.

2.3 Culture and fermentation conditions

In 24-well plate fermentation and flask fermentation, all *E. coli*strains were cultivated and fermented in LB medium at 37 . The strains were all shaken at 220 rpm/min while growing in liquid medium.

24-well plates and flask fermentation were performed as follows: BL21 (DE3) strains containing pEBI and various IUP pathway plasmids of pRhaIUPnIDIsc were selected and transferred into 48-well plates/ tubes with 500 μL/3 mL LB liquid medium adding according antibiotics (chloramphenicol (Cm^R) 25 μg/mL and ampicillin (Amp^R) 100 μg/mL) for 12 h at 37 . Then, bacterial culture was transferred into 24-well plates/flask with 2 mL diluted to OD_{600}=0.1 and then cultivated in liquid LB medium at 37 . When OD_{600} reached 0.6-0.8, L-arabinose (Ara), L-rhamnose (Rha) and anhydrotetracycline (aTc) were added to a final concentration of 10mMol/L, 10mMol/L and 100 ng/mL respectively. When OD_{600} reached 2-4, 0.005˜ 0.1 mMol/L IPTG and 1˜3 g/L prenol/isoprenol was added and the fermentation continued for 24 h at 37 .

The methods for the fed-batch fermentation are referred to this study with modifications as follows: the fed-batch fermentation for lycopene production was performed in a 5 L bioreactor with a working volume of 2.4 L at 37 for 40 h. 10 mM/L Rha was added after 3 hours' cultivation and 2 g/L substrates (prenol: isoprenol=1:3) were added to the bioreactor at 6 h.

2.4 Fluorescence intensity measurement

The growth condition of cells was monitored by absorbance at 600 nm (OD_{600}) by microplate reader**.** 1 mL of cells were centrifuged at 12 000 rpm for 10 min and then was resuspended with 1 mL of phosphate-buffered saline (PBS), followed by transferring 200 μL of the resuspensions to 96-well Black Opti plate 96F plates. The fluorescence was detected with excitation wavelength at 485 nm and emission wavelength at 520 nm, with each group in triplicates.

2.5 Genome integration based on transposition

The ptrpDonor-IUP and pQCasTns-8array or pQCasTns-IS1were transformed into BL21(DE3), using transferring and induction condition as .

2.6 The subculture of strains and plasmid stability examination

Strain YZ72-5, harboring plasmid for IUP expression with chloramphenicol resistance, was cultivated in LB liquid media containing 25 μg/mL Cm^R in 37 for 12 h. Cells were sub-cultured for three generations, after each cultivation in LB liquid medium with 25 mg/mL Cm^R under 37 for 12 h, strains were scribed to the Cm^R-free LB solid plate. Then, strains were cultivated 37 for 12 h, obtaining single colonies. Afterwards, 100 single colonies were selected to grow on Cm^R-containing LB solid plate and Cm^R-free LB solid plate. After cultivation for 12 h at 37 °C, colonies growing on LB solid plate but not on LB with 25 μg/mL Cm^R were interpreted that the plasmid has been lost. Each experiment was carried out in three parallel groups. The plasmid stability was calculated as the number of samples with growth in LB with antibiotics/100× 100%.

Strain YZJ3-4 was plated on LB solid plate with 100 mg/mL Amp^R, which was then cultivated in 37 for 12 h, obtaining single colonies. Afterwards, strains on the plate were washed using fresh LB liquid medium, then they were subscribed to plates with 100 mg/mL Amp^R, which procedure was circulated. The times of "generation" means the times the process was carried out.

2.7 Extraction and quantification of lycopene

Acetone extraction and HPLC quantification method including the mobile phase composition was utilized as previously described with modification as follows. The analytes were separated on an Ultimate XB-C18 (150 mmx 4.6 mm, 3.5 μm, Welch, China), which was kept at 30 °C, and detected at 470 nm using a Shimadzu UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). The retention times of lycopene was 12 min Standard curves were generated using commercially available reference standards for lycopene (Macklin, China).

3. RESULTS AND DISCUSSION

3.1 Assembling IUP pathway in *E. coli* enables lycopene production

pC19IUP was first designed for expression of IUP pathway to test its ability to produce lycopene in*E. coli* . In this study, *IPK* from *Methanothermobacter thermautotrophicus* (IPK_{MTH})] and *ThiM* from *Escherichia coli*($ThiM_{Ec}$) are employed, which was accordingly under the control of the constitutive promoter P_{J23119}(BBa_J23119, high strength) (Figure 1B). To better balance the ratio of IPP and DMAPP, *idi* from *Escherichia coli*(idi_{Ec}) was also included. Downstream genes for lycopene production (*CrtE* , *CrtB* , *CrtI*) were co-expressed in pEBI driven by inducible promoter Ptrc(a gift from Yang). Two strains were obtained, the control strain YZ0 with only MEP pathway and strain YZ1 with both MEP and IUP pathway. These two strains were cultivated in LB medium for about 3 h when we added a lower concentration of IPTG (0.01 mM) in combination with 1g/L prenol as substrate. When comparing the lycopene titer of two strains after 24 hours' fermentation, YZ0 produced 0.5 mg/OD_{600} lycopene, which was nearly 6.2-fold of the control strain (0.086 mg/OD_{600}), verifying the feasibility of IUP pathway.

3.2 Optimization of IUP expression cassette based on promotor engineering and IDI selection

Studies have shown that promoters are valuable tool for controlling and regulating gene transcriptional level . Synthetic constitutive promoters like J231 family have been widely applied in gene controlling, however, exhibiting different activities according to genetic host background, and thus strength-varied promoters needed to be tested . Hence, promoter engineering strategy was conducted on two genes involved in IUP, $ThiM_{Ec}$ and IPK_{MTH} . To be specific, constitutive promotors and inducible promotor library was built respectively. Firstly, the constitutive promoter ofIPK_{MTH} was replaced with P_{J23106}(BBa_J23106, medium strength) and P_{J23109} (BBa_J23109, low strength). Based on these, we then inserted P_{J23119}, P_{J23106} and P_{J23109} to control*ThiM* , obtaining a promoter library (3*3=9 types) (Figure 2A). Strains harboring these plasmids were fermented and strain YZ3-1 had the highest amount of lycopene titer (0.59 mg/OD_{600}) (Figure 2B). L-rhamnose inducible promoter (P_{Rha}), arabinose inducible (P_{BAD}), tac promoter (P_{tac}) and anhydrotetracycline inducible promoter (P_{tet}) are commonly used inducible promoters for tunable heterologous gene expression in *E.coli* . Thus, the constitutive promoter P_{J23119} in pC19IUP was replaced with these 4 kinds of

inducible promotors, meanwhile, controlling both *ThiM*$_{Ec}$ and *IPK*$_{MTH}$ (Figure 2C) and thus four inducible IUP-harbored plasmids, pTacIUPidi, pTetIUPidi, pBADIUPidi, pRhaIUPidi were assembled, followed by being transformed with pEBI to BL21(DE3), creating strains YZ4, YZ5, YZ6 and YZ7, accordingly. After the same fermentation condition, 4 strains produced 1.1, 0.56, 0.58 and 1.96 mg/OD$_{600}$ lycopene (Figure 2D). Comparing strain YZ3-1 and YZ7, it was the P$_{\mathrm{Rha}}$ that was the most effective in controlling IUP for lycopene titer, reaching almost 22.8-fold that of the control strain without IUP. This may be due to the fact that the P$_{\mathrm{Rha}}$ has tight expression with almost none leakage enabling better cell growth in uninduced stage and increased IUP expression level in induced stage.

To further increase the lycopene titer, the ratio of prenol and isoprene was changed. This is because the block may occur during the C5 to C40 conversion process. It is worth noting that IPP and DMAPP are two key precursors of terpenoids and the former one is especially critical in generating farnesyl diphosphate (FPP), geranylpyrophosphate (GPP) and geranylgeranyl diphosphate (GGPP) . Besides, two isopentenol isomers (isoprenol/prenol) can turn into IPP and DMAPP accordingly through IUP pathway, so changing the ratio of these two substrates is a direct method of C5 supplement balance . Therefore, 1 g/L prenol was replaced with 1 g/L 1:1 prenol/isoprenol. Surprisingly, the accumulation of lycopene reached 2.3 mg/OD$_{600}$ (Figure 2E), demonstrating this limitation step and the poor performance of IDI$_{\mathrm{Ec}}$ we employed.

It's reported type II IDI behaves better than that of type I , thus we then replaced IDI$_{\mathrm{Ec}}$ with IDI$_{\mathrm{Bc}}$ and IDI$_{\mathrm{Sc}}$ of type II (Figure 2C). It came out that the lycopene production was respectively increased by 2.06 and 2.66-fold compared with the strain harboring original IDI$_{\mathrm{Ec}}$, the later reaching 4.42 mg/OD$_{600}$, 51.5-fold that of the control strain (Figure 2F). Base on this result, we decide to employ IDI$_{\mathrm{Sc}}$ in the following experiment.

Optimization of IUP expression cassette based on construction of RBS library of *ThiM*$_{Ec}$

ThiM$_{Ec}$ was reported being capable of catalyzing phosphorylation reaction, the overexpression of which combined with other optimizations brought about 200 mg/L geranoids . It is also the first enzyme in IUP utilizing substrates, determining the influx of IUP . According to the first part, it could be concluded that the lower transcriptional level of *IPK*$_{MTH}$ and higher transcriptional level of *ThiM*$_{Ec}$ benefited the lycopene respectively. Therefore, the online 'UTR designer' (*https://sbi.postech.ac.kr/utr_designer/#*) was turned to predict the expression level of two genes , it turned out that compared to *IPK*$_{MTH}$ (value=1283574.22),*ThiM*$_{Ec}$, in contrast, was in relatively low expression level (RBS0(GCAGGAGCA)value =40772.93). Thus, the RBS library of *ThiM*$_{Ec}$ (pRhaIUP-nRBSThiM-idisc) was constructed through circular PCR to screen the most suitable RBS for expression of *ThiM*$_{Ec}$ so as to increase lycopene titer (Figure 3B). In our study, 135 strains were randomly picked and fermented in 24-well plates, of which 11 candidates with higher lycopene accumulation were screened by the intense of red color compared with the control strain. The results demonstrated that strain YZ72-5 harboring the RBS5 (AAGGGGGGA) is the optimal, accumulating 6.5 mg/OD$_{600}$lycopene titer, reaching almost 1.5-fold of the control strain YZ72 (Figure 3C). To further estimate the expression strength of*ThiM*$_{Ec}$, we recorded its fluorescence value per unit cell with GFP using a reporter. It demonstrated that RBS5 contributes a higher translational level(3-fold) than RBS0 (Figure 3D). Meanwhile, the expression level of *ThiM*$_{Ec}$with RBS5 was also predicted using online 'UTR designer' mentioned above, the value increased to 861937.03, which showed the identical trend of out verification.

3.4 Optimization of fermentation condition for lycopene production

It had been reported that the concentration of IPTG has a significant effect on gene expression levels . Meanwhile, the excessive addition of IPTG is toxic to cells, which illustrated the importance of optimization . Therefore, the concentration of IPTG was tested, which controls the expression of *CrtE* , *CrtB* and *CrtI* catalyzing for synthesizing lycopene but is toxic to cells (Figure 4A). A concentration gradient (0.5 mM/L, 0.1 mM/L, 0.05 mM/L, 0.01 mM/L and 0.005 mM/L) was added respectively and it turned out that only strain adding 0.005 mM/L IPTG behaves better than the original strain adding 0.1mM IPTG, producing 6.86 mg/OD$_{600}$ lycopene (Figure 4B).

As illustrated before, two kinds of isopentenol substrates contributes to different C5 skeleton. Based on

previous ratio 1p/1i (prenol: isoprenol=1:1), the other different ratio: 100%p (p), prenol: isoprenol=5:1, 3:1; 1:1; 1:3 and 1:5 and 100%i (i) were also tested. It turned out that 1p/3i (prenol: isoprenol=1:3) is the most beneficial proportion to balancing IPP/DMAPP in cellular, leading to about 7.94 mg/OD_{600}lycopene, 1.4-fold of the original strain provided with 1p/1i (prenol: isoprenol=1:1) (Figure 4C). Next, various substrates concentration (1g/L, 2g/L and 3g/L) was added and it demonstrated that the best concentration is 2g/L since it is not only the most favorable for synthesizing lycopene (˜8.67 mg/OD_{600}) but also leads to the moderate toxicity to cells compared with that of 1g/L and 3g/L (Figure 4D).

3.5 Rapid construction of the multicopy IUP genome-harboring chassis through PtrCAST for lycopene production

Heterogeneous gene expressions usually relied on multiple compatible plasmids, however, it brings about expression instability due to unprecise copy number and higher fermentation cost because of antibiotic addition, which is also unrealistic and reliable in industrial production [31]. For instance, in the case of lycopene production of *E.coli* , 3 plasmids were employed to introduce MVA pathway and lycopene-related synthases, in which the longer lag phase and lower mean productivity was observed . In another case, only when adding more than 5-fold Km^R than normal level (50 mg/mL) can cells for astaxanthin production prevent plasmids loss during fed-batch condition .

In our study, the IUP expression cassette was initially placed on plasmid and might lead to similar problems mentioned above]. Therefore, the plasmid stability was first measured by succession in Cm^R-free medium and it showed that the plasmid stability decreased to lower 50% in the third transfer (Figure S1). To address this problem, the genome multi-position integration strategy was adopted. However, it was laborious and time-consuming, and it's really troublesome to construct a chassis in need of serial manipulation], though highly-effective CRISPR/Cas system has been widely utilized in engineering cell factories . Multicopy integration of chromosome based on CASTs, more convenient and effective, was applied to construct strain library and screening the most productive chassis . Importantly, the CAST from *Pseudoalteromonas translucida* KMM520 was employed to form a novel tool PtrCAST, performing multiple sites integration of larger cargo (˜15.4 kb) with higher efficiency .

Thanks to this tool, a gift from Yang, several copies of IUP expression cassette were successfully integrated into the genome of BL21(DE3). The transposition targeting 8 loci was conducted through ptrTnsQCas-8array, acquiring a mixed strain library with different IUP expression cassette copies (Figure 5A-5B). It could be concluded that the lycopene production performances of strains with different copy number varied (Figure S2). Then, the pEBI was transferred into the strain library, obtaining transformants which were selected for fermentation. Strains with crimson were detected and sequenced, of which strain YZJ3 with 7 copies of IUP expression cassette performed best but failed to reach the control strain YZ72-5 (Figure 5C and G). Therefore, the second transposition was performed on YZJ3 targeting IS1 (Figure 5D). The same procedure was followed mentioned above (Figure 5E-5F). Finally, strain YZJ3-4 of 13 copies in total was obtained, resulting in 12.2 mg/OD_{600}, 1.5-fold that of strain YZ72-5 using plasmids (Figure 5G). In fed-batch fermentation, it produced 15.43 mg/OD_{600}. Besides, we transferred this strain for ten generations and examined each 13 loci and it showed that the IUP expression cassettes were stably existed in each locus. Also, the fermentation was conducted and it showed the similar level with the first generation (Figure S3). It was reported that the genome position had relationship with genome position, so that adoption of PtrCAST could further screening for more suitable site-combination of IUP expression cassettes, lowing the copy number and enhancing the effectiveness of each cassette .

4 CONCLUSION

In conclusion, the feasibility of IUP was verified in *E.coli* , followed by serial optimization. To increase the titer of model terpenoid, lycopene, P_{Rha}, RBS5 and type II IDI from*S. cerevisiae* were screened for controlling IUP expression cassette, $ThiM_{Ec}$ and inter-converting of IPP/DMAPP, respectively. The best concentration of IPTG, isopentenol's ratio and concentration was 0.005 mMol/L, prenol: isoprenol=1:3 and 2 g/L. The optimal strain harboring plasmid system produced 8.67 mg/OD_{600} lycopene titer. However,

the plasmid loss was observed over 50% in the third cultivation. In comparison, the strain integrated 13 copy IUP expression cassettes in genome was constructed in 2 weeks, not only showing stability after 10 generations but also producing higher lycopene titer (15.43 mg/OD_{600} by fed-batch).

ABBREVIATIONS

IUP Isopentenol utilization pathway

MEP 2-C-methyl-D-erythritol 4-phosphate

MVA Mevalonate

CAST CRISPR-associated transposases

ATP Adenosine triphosphate

NADPH Nicotinamide adenine dinucleotide phosphate

IPP Isopentenyl diphosphate;

DMAPP Dimethylallyl diphosphate

IDI IPP Delta-isomerase

OD_{600} Optical density at a wavelength of 600 nm

RBS Ribosome-binding site

IPTG Isopropyl β-D-1-thiogalactopyranoside

HPLC High-performance liquid chromatography

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CONFLICT OF INTEREST

All authors declare no conflict of interest regarding the publication of this article.

DATA AVA ILABILITY STATEMENT

All data included in this study are available upon request by contact with the corresponding author.

REFERENCES

(1) Li, Z. J., Wang, Y. Z., Wang, L. R., Shi, T. Q., Sun, X. M., & Huang, H. (2021) Advanced strategies for the synthesis of terpenoids in *Yarrowia Lipolytica* . *Journal of Agricultural and Food Chemistry, 69* , 2367-2381.

(2) Shi, T., Li, Y., Zhu, L., Tong, Y., Yang, J., Fang, Y., Wang, M., Zhang, J., Jiang, Y., & Yang, S. (2021) Engineering the oleaginous yeast *Yarrowia lipolytica* for β-farnesene overproduction.*Biotechnology Journal, 16* , e2100097.

(3) Schempp, F. M., Drummond, L., Buchhaupt, M., & Schrader, J. (2018) Microbial cell factories for the production of terpenoid flavor and fragrance compounds. *Journal of Agricultural and Food Chemistry,66* , 2247-2258.

(4) Yang, J., Zhao, G., Sun, Y., Zheng, Y., Jiang, X., Liu, W., & Xian, M. (2012) Bio-isoprene production using exogenous MVA pathway and isoprene synthase in *Escherichia coli* . *Bioresource Technology, 104* , 642-647.

(5) Clomburg, J. M., Qian, S., Tan, Z., Cheong, S., & Gonzalez, R. (2019) The isoprenoid alcohol pathway, a synthetic route for isoprenoid biosynthesis. *PNAS, 116* , 12810-12815.

(6) Ma, Y., Zu, Y., Huang, S., & Stephanopoulos, G. (2023) Engineering a universal and Efficient platform for terpenoid synthesis in yeast.*PNAS, 120* , e2207680120.

(7) Luo, Z., Liu, N., Lazar, Z., Chatzivasileiou, A., Ward, V., Chen, J., & Zhou, J., Stephanopoulos, G. (2020) Enhancing isoprenoid synthesis in *Yarrowia Lipolytica* by expressing the isopentenol utilization pathway and modulating intracellular hydrophobicity.*Metabolic Engineering, 61* , 344-351.

(8) Ma, X., Liang, H., Pan, Q., Prather, K. L. J., Sinskey, A. J., Stephanopoulos, G., & Zhou, K. (2022) Optimization of the isopentenol utilization pathway for isoprenoid synthesis in *Escherichia coli* .*Journal of Agricultural and Food Chemistry, 70* , 3512-3520.

(9) Rouches V, M., Xu, Y., Cortes, L. B. G., & Lambert, G. (2022) A plasmid system with tunable copy number. *Nature Communication,13* , 3908.

(10) Zhu, F., Lu, L., Fu, S., Zhong, X., Hu, M., Deng, Z., & Liu, T. (2015) Targeted engineering and scale up of lycopene overproduction in*Escherichia coli* . *Process Biochemistry, 50* , 341-346.

(11) Park, S. Y., Binkley, R. M., Kim, W. J., Lee, M. H., & Lee, S. Y. (2018) Metabolic engineering of *Escherichia coli* for high-level astaxanthin production with high productivity. *Metabolic Engineering, 49* , 105-115.

(12) Kim, S. K., Kim, H., Woo, S. G., Kim, T. H., Rha, E., Kwon, K. K., Lee, H., Lee, S.G., & Lee, D.H. (2022) CRISPRi-based programmable logic inverter cascade for antibiotic-free selection and maintenance of multiple plasmids. *Nucleic Acids Research, 50* , 13155-13171.

(13) Wei, Y., Mohsin, A., Hong, Q., Guo, M., & Fang, H. (2018) Enhanced production of biosynthesized lycopene via heterogenous MVA pathway based on chromosomal multiple position integration strategy plus plasmid systems in *Escherichia coli* . *Bioresource Technology,250,* 382-389.

(14) Hussain, M. H., Hong, Q., Zaman, W. Q., Mohsin, A., Wei, Y. L., Zhang, N., Fang, H. Q., Wang, Z. J., Hang, H. F., Zhuang, Y. P., & Guo, M. J. (2021) Rationally optimized generation of integrated*Escherichia coli* with stable and high yield lycopene biosynthesis from heterologous mevalonate (MVA) and lycopene expression pathways.*Synthetic and Systems Biotechnology, 6* , 85-94.

(15) Vo, P. L. H., Ronda, C., Klompe, S. E., Chen, E. E., Acree, C., Wang, H. H., & Sternberg, S. H. (2021) CRISPR RNA-guided integrases for high-efficiency, multiplexed bacterial genome engineering. *Nature Biotechnology, 39* , 480-489.

(16) Vento, J. M., Crook, N., & Beisel, C. L. (2019) Barriers to genome editing with CRISPR in bacteria. *Journal of Industrial Microbiology & Biotechnology, 46* , 1327-1341.

(17) Zhang, Y.W., Sun, X.M., Wang, Q.Z., Xu, J.Q., Dong, F., Yang, S.Q., Yang, J.W., Zhang, Z.X., Qian, Y., Chen, J., Zhang, J., Liu, Y.M., Tao, R.S., Jiang, Y., Yang, J.J., & Yang, S. (2020) Multicopy chromosomal integration using CRISPR-associated transposases. *ACS Synthetic Biology, 9* , 1998-2008.

(18) Yang, S.Q., Zhang, Y.W., Xu, J.Q., Zhang, J., Zhang, J.Z., Yang, J.J., Jiang, Y., & Yang, S. (2021) Orthogonal CRISPR-associated transposases for parallel and multiplexed chromosomal integration.*Nucleic Acids Research, 49* , 10192-10202.

(19) Li, D., Li, Y., Xu, J. Y., Li, Q. Y., Tang, J. L., Jia, S. R., Bi, C. H., Dai, Z. B., Zhu, X. N., & Zhang, X. L. (2020) Engineering CrtW and CrtZ for improving biosynthesis of astaxanthin in *Escherichia coli* . *Chinese Journal of Natural Medicines. 18* , 666-676.

(20) Sri Wahyu Effendi, S., Lin, J.Y., & Ng, I.S. (2022) Simultaneous carbon dioxide sequestration and utilization for cadaverine production using dual promoters in engineered *Escherichia coli* strains.*Bioresource Technology, 363* , 127980.

(21) Jervis, A. J., Carbonell, P., Taylor, S., Sung, R., Dunstan, M. S., Robinson, C. J., Breitling, R., Takano, E., & Scrutton, N. S. (2019) SelProm: a queryable and predictive expression vector selection tool for*Escherichia coli* . *ACS Synthetic Biology, 8* , 1478-1483.

(22) Jin, L. Y., Nawab, S., Xia, M. L., Ma, X. Y., & Huo, Y. X. (2019) Context-dependency of synthetic minimal promoters in driving gene expression: a case study. *Microbial Biotechnology* , *12* , 1476-1486.

(23) Du, F., Liu, Y. Q.; Xu, Y. S.; Li, Z. J.; Wang, Y. Z.; Zhang, Z. X.; & Sun, X. M. (2021) Regulating the T7 RNA polymerase expression in*E. coli* BL21 (DE3) to provide more host options for recombinant protein production. *Microbial Cell Factory, 20* , 189.

(24) Cao, X., Yu, W., Chen, Y., Yang, S., Zhao, Z. K., Nielsen, J., Luan, H., & Zhou, Y. J. (2023) Engineering yeast for high-level production of diterpenoid sclareol. *Metabolic Engineering, 75* , 19-28.

(25) Pan, Q., Ma, X., Liang, H., Liu, Y., Zhou, Y., Stephanopoulos, G., & Zhou, K. (2023) Biosynthesis of geranate via isopentenol utilization pathway in *Escherichia coli* . *Biotechnology Bioengineering,120* , 230-238.

(26) Rad, S. A., Zahiri, H. S., Noghabi, K. A., Rajaei, S., Heidari, R., & Mojallali, L. (2012) Type 2 IDI performs better than type 1 for improving lycopene production in metabolically engineered *E. coli*strains. *World Journal of Microbiology & Biotechnology,28* , 313-321.

(27) Seo, S. W., Yang, J.S., Kim, I., Yang, J., Min, B. E., Kim, S., & Jung, G. Y. (2013) Predictive design of mRNA translation initiation region to control prokaryotic translation efficiency. *Metabolic Engineering, 15* , 67-74.

(28) Ma, H., Zhao, Y., Huang, W., Zhang, L., Wu, F., Ye, J., & Chen, G.Q. (2020) Rational flux-tuning of *Halomonas Bluephagenesis* for co-production of bioplastic PHB and ectoine. *Nature Communication, 11* , 3313.

(29) Ribeiro, V. T., Asevedo, E. A., Costa de Paiva Vasconcelos, L. T., Oliveira Filho, M. A., de Araujo, J. S., de Macedo, G. R., de Sousa Junior, F. C., & dos Santos, E. S. (2019) Evaluation of induction conditions for plasmid pQE-30 stability and 503 antigen of*Leishmania i. Chagasi* expression in *E. coli* M15.*Applied Microbial Biotechnology. 103* , 6495-6504.

(30) Klepsch, M. M., Persson, J. O., & de Gier, J.W. L. (2011) Consequences of the overexpression of a eukaryotic membrane protein, the human KDEL receptor, in *Escherichia coli* . *Journal of Molecular Biology, 407* , 532-542.

(31) Schmidt, C. M., Shis, D. L., Nguyen-Huu, T. D., & Bennett, M. R. (2012) Stable maintenance of multiple plasmids in *E. coli* using a single selective marker. *ACS Synthetic Biology, 1* , 445-450.

(32) Campbell, K., Xia, J., & Nielsen, J. The impact of systems biology on bioprocessing. (2017) *Trends in Biotechnology, 35* , 1156-1168.

(33) Bryant, J. A., Sellars, L. E., Busby, S. J. W., & Lee, D. J. (2014) Chromosome position effects on gene expression in*Escherichia coli* K-12. *Nucleic Acids Research, 42* , 11383-11392.

Figure captions

FIGURE 1 Construction and verification of IUP in *E. coli*for lycopene production. (A) Two steps catalysis from isoprenol and prenol to two building blocks IPP and DMAPP, followed by conversion to lycopene by *CrtEBI* . ADP: adenosine diphosphate; ATP: adenosine triphosphate; IP: isopentenyl phosphate; IPP: isopentenyl diphosphate; DMAP: dimethylallyl phosphate; DMAPP: dimethylallyl diphosphate; GPP: geranyl diphosphate; FPP: farnesyl diphosphate; GGPP: geranylgeranyl diphosphate. *ThiM* , *E. coli* kinase: hydroxyethylthiazole kinase; *IPK* isopentenol kinase; *idi* : IPP Δ-isomerase;*IspA* : FPP synthase; *CrtE* : GGPP synthase; *CrtB* : phytoene synthase; *CrtI* phytoene desaturase (B) YZ0: BL21(DE3) harboring

pEBI; YZ1: BL21(DE3) harboring pEBI and pC19IUP. (C) Lycopene titer of strain YZ0 with or without prenol and YZ1 with prenol.

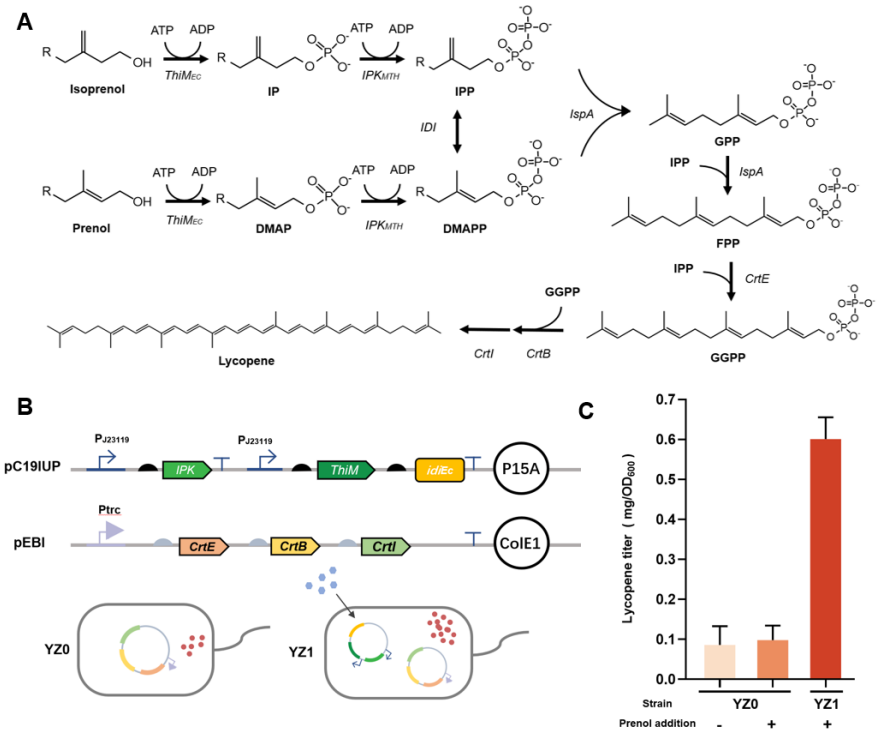
FIGURE 2 The optimization of ThiMEc andIPK_{MTH} by promoter engineering and IDI selection. (A) IPK_{MTH} and $ThiM_{Ec}$ are under control of constitutive promotors of three level: strong (***): PJ23119, medium (**): PJ23106 and low (*): PJ23109, respectively. (B) Lycopene titer of 9 strains in combination of constitutive promotors. (C) Both IPK_{MTH} and $ThiM_{Ec}$are under control of four kinds of inducible promotor: L-rhamnose monohydrate inducible promotor (P_{Rha}), Anhydrotetracycline hydrochloride inducible promotor (P_{tet}), L-Arabinose inducible promotor (P_{BAD}) and IPTG inducible promotor (P_{tac}). Type I IDI: *idi* from *E.coli* ; Type II IDI: *idi* from *Bacillus subtilis* and *idi* from*Saccharomyces cerevisiae* (D) Lycopene titer of strain YZ4˜7 referring to BL21(DE3), respectively harboring pEBI and IUP pathway controlled by P_{tac}, P_{tet}, P_{BAD} and P_{Rha}. Lycopene titer of strain YZ7 mixed with 1g/L prenol and 1g/L of combined prenol and isoprenol (1p/1i means the ratio of prenol and isoprenol is 1:1). (F) Lycopene titer of strains YZ7, YZ71 and YZ72, separately employing IDI_{Ec}, IDI_{Bc} and IDI_{Sc}.

FIGURE 3 Screening and verification of RBS of $ThiM_{Ec}$ to increase lycopene production. (A) The IUP expression frame after preliminary optimization. IPK_{MTH} ,$ThiM_{Ec}$ and idi_{Sc} are under control inducible promoter P_{Rha}. The streamline of best RBS screen and verification for $ThiM_{Ec}$. a) Circular PCR utilizing degenerate primer containing R(A/G) for construction of RBS library of $ThiM_{Ec}$. b) The RBS library of $ThiM_{Ec}$. was obtained by transferring PCR product to DH5α and extraction of mixed plasmids. c) The mixed library combined with pEBI was transferred to BL21(DE3), obtaining a series of single colonies. d) Randomly selection and fermentation of transformants e) Selection of cells with crimson followed by sequencing g) The ligation of $ThiM_{Ec}$. controlled by original RBS(RBS0) and selected RBS with GFP and fermented the according strains at 2 hours after induction) The test of cell density and expression level of $ThiM_{Ec}$ .with RBS0 and RBS5 through fluorescence by microplate reader. (B) The lycopene titer of 11 strains (YZ72-1˜11) randomly selected with crimson. Control strain YZ72 harbors IUP, of which the $ThiM_{Ec}$ .is controlled by RBS0. (C) The fluorescence value per unit cell of$ThiM_{Ec}$. with RBS0 and RBS5 (calculation: total fluorescence/OD_{600}).

FIGURE 4 The optimization of fermentation condition for lycopene production through IUP pathway. (A) Effects on transcription and cell growth of IPTG concentration. (B) Lycopene titer of strain YZ72-5 mixed with a series of concentration of IPTG (0.5, 0.1, 0.05, 0.01 and 0.005 mMol/L). (C) Lycopene titer of strain YZ72-5 provided with 0.005 mMol/L IPTG and 1g/L substrates of different ratio (p: 100% prenol; prenol: isoprenol=5:1, 3:1; 1:1; 1:3 and 1:5; i: 100% isoprenol). (D) Lycopene titer of strain YZ72-5 supplied with 0.005 mMol/L IPTG and different concentration (1g/L; 2g/L and 3g/L) of substrates, of which prenol: isoprenol is 1:3.

FIGURE 5 Rapid construction of the multicopy IUP genome-harboring chassis through PtrCAST for lycopene production (A) Transposition targeting 8 loci/IS1 loci on BL21(DE3); Verification strain library with different copies of IUP expression cassette; Transformation of pEBI into strain library and random selection of transformants for fermentation, followed by picking strains with crimson for detection and sequencing. (B) Lycopene titer of strain YZ72-5 with IUP in plasmids, YZJ3 with 7 copies IUP expression cassette in genome and YZJ3-4 with 13 copies IUP expression cassette in genome both cultivated in flask and fermenter. (C) Fed-batch fermentation profile of the strain YZJ3-4.

FIGURE 1



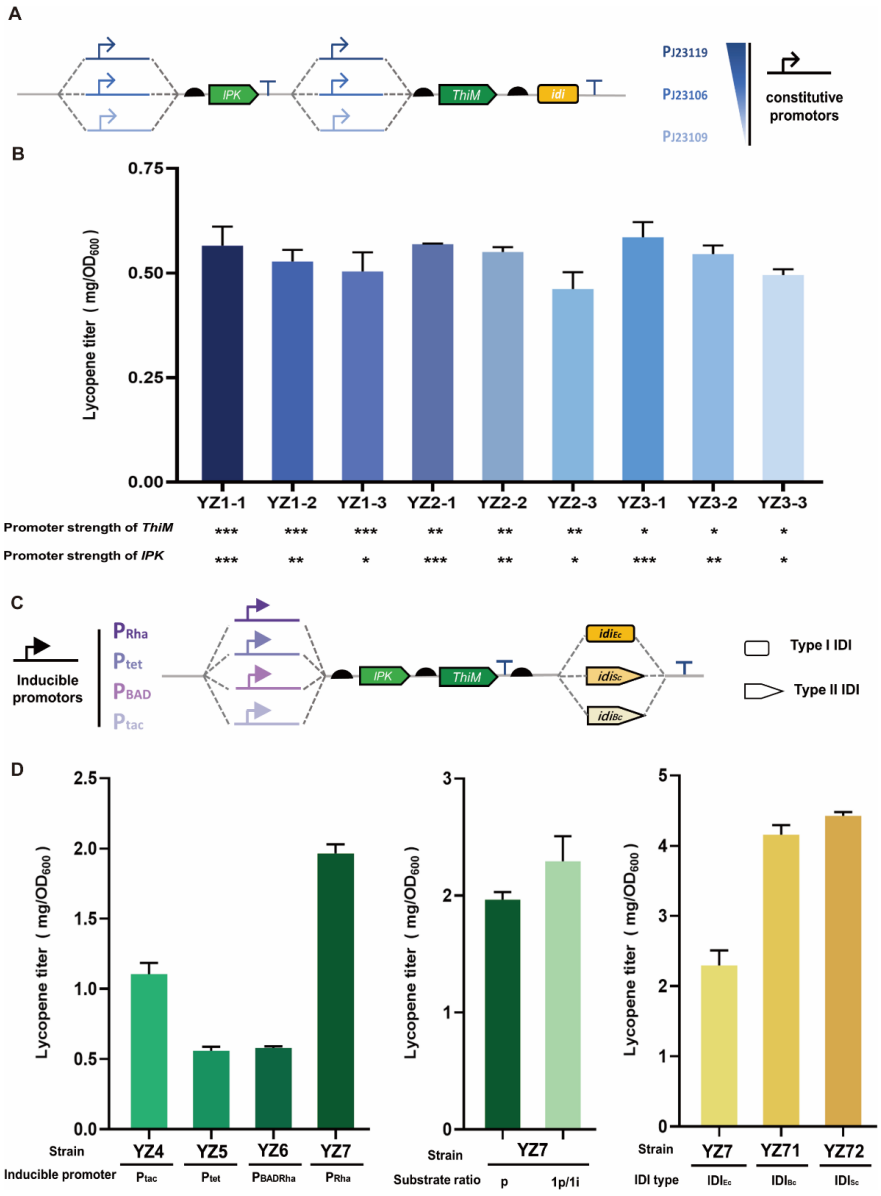


FIGURE 2

FIGURE 3

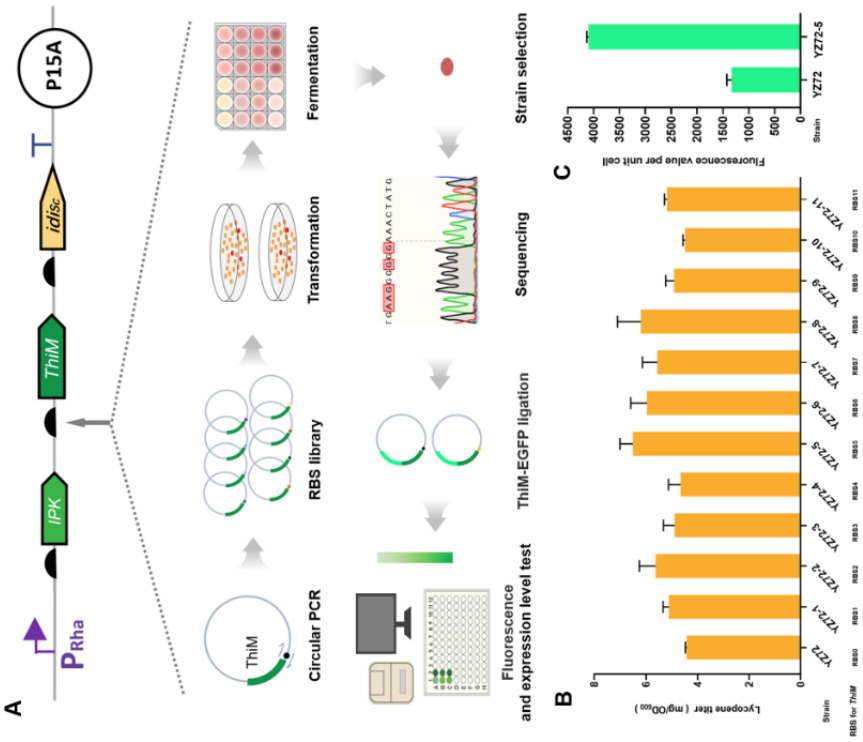


FIGURE 4

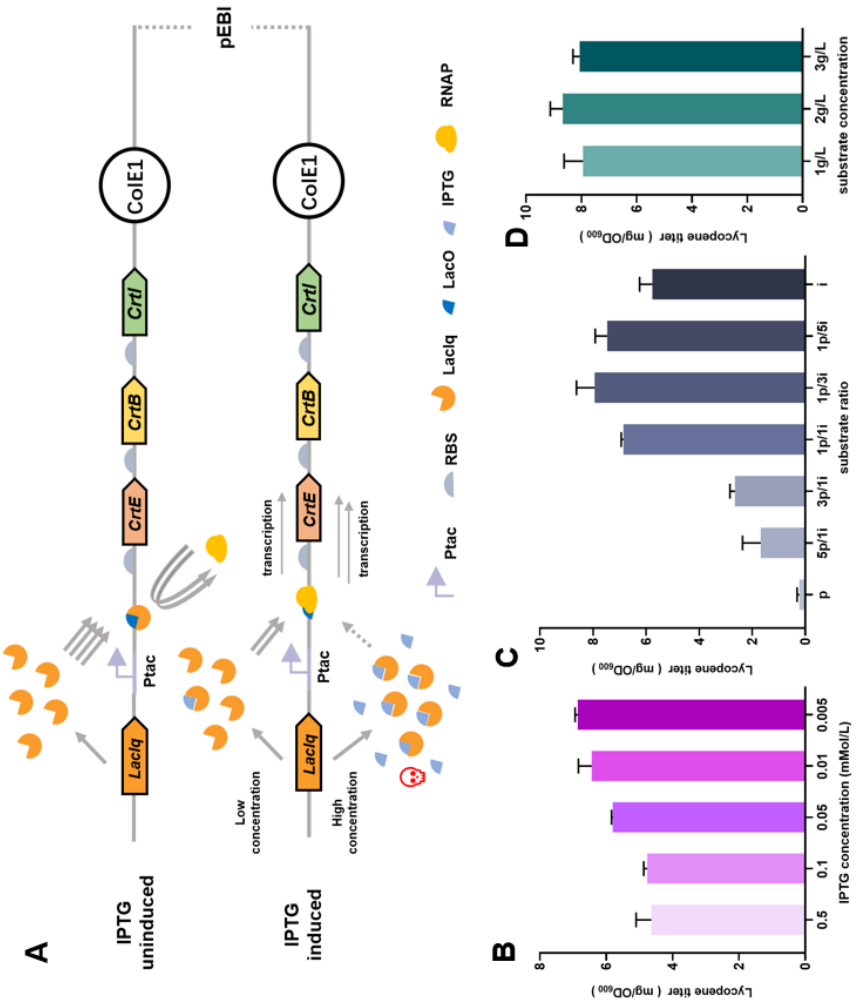


FIGURE 5