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Systematic Engineering of *Escherichia coli* for Efficient Production of Cytidine 5′-Monophosphate

Min Zhou,* Liyuan Wei, Chongzhi Wu, Wei Chen,* and Zhengju Tang*

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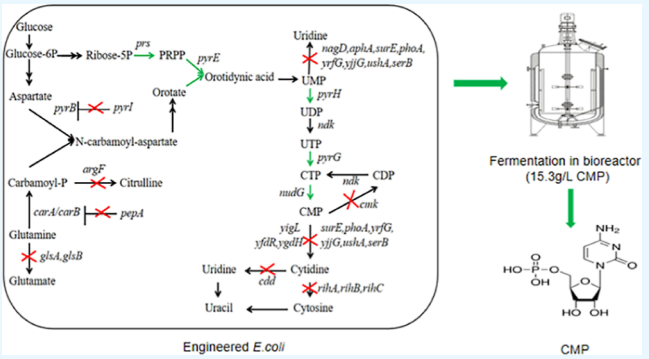
ABSTRACT: Cytidine 5′-monophosphate (CMP) was widely applied in the food and pharmaceutical industries. Currently, CMP is mainly produced by enzyme catalysis. However, the starting materials for enzyme catalysis were relatively expensive. Therefore, seeking a low-cost production process for CMP was attractive. In this study, *Escherichia coli* (*E. coli*) was systematically modified to produce CMP. First, a the cytidine-producing strain was constructed by deleting *cdd*, *rihA*, *rihB*, and *rihC*. Second, the genes involved in the pyrimidine precursor competing pathway and negative regulation were deleted to increase cyti dine biosynthesis. Third, the deletion of the genes that caused the loss of CMP phosphatase activity led to the accumulation of CMP, and the overexpression of the rate-limiting step genes and feedback inhibition resistance genes greatly increased the yield of CMP. The yield of CMP was further increased to 1013.6 mg/L by blocking CMP phosphorylation. Ultimately, the yield of CMP reached 15.3 g/L in a 50 L bioreactor. Overall, the engineered *E. coli* with a high yield of CMP was successfully constructed and showed the potential for industrial production.



INTRODUCTION

Cytidine 5′-monophosphate (CMP) is a nucleoside phosphate that is comprised of a ribonucleoside and one phosphate group and is widely applied in food[1] and pharmaceutical industries.[2] CMP is used as the starting material to produce cytidine 5′-triphosphate[1] and CDP-choline,[3] which are widely used in recovery from acute brain injury and consciousness disturbance after brain surgery.[4]

CMP can be obtained by RNA hydrolysis,[5] but the hydrolyzed products are mixtures of four nucleotides, which increases the difficulty of the extraction process and production costs. Enzyme catalysis is an alternative approach to producing CMP. In recent years, great progress has been achieved in the enzymatic synthesis of CMP. Uridine-cytidine kinase and acetate kinase (ACK) were overexpressed to synthesize CMP from cytidine, and guanosine 5′-triphosphate (GTP) was added as phosphate donors that were regenerated by ACK from acetyl phosphate. The yield of CMP reached 97%.[6] However, GTP and acetyl phosphate were expensive, which led to this enzymatic process being unconducive to industrial production. A polyphosphate-based adenosine triphosphate (ATP) regeneration system has several advantages, including low cost and high stability, which makes it of special interest for the enzyme synthesis of CMP. McPPK2 from *Meiothermus cerbereus* exhibited high specific activity and was used to accomplish ATP regeneration, and the combination of McPPK2 with uridine-cytidine kinase from *Lactobacillus helveticus* produced 143.5 mM CMP.[7] In another study, both cytidine kinase (CK) and polyphosphate kinase (PPK) from extremophiles were applied to produce CMP, and ATP as phosphate donor was regenerated by PPK from polyphosphate. Under the optimal conditions, 96 mM CMP was produced within 6 h, and the yield reached nearly 100%.[8] However, the activity of the CK in this method was not high, resulting in long reaction times and low productivity. CK from *Phorcysia thermohydrogeniphila* was iteratively mutated. The mutant increased the maximum productivity by 4.0 times, and 335.0 mM CMP was produced within 5 h.[9]

The strategy of enzyme immobilization was also used to improve enzyme activity, stability, and decrease production costs by recycling in CMP production.[10,11]

D403 metal chelate resin adsorbing Ni^{2+} was used as an immobilized carrier, 60 mmol/L cytidine and 0.5 mmol/L ATP were used as substrates for CMP production, and the average yield of CMP reached 91.2% in the first five batches of continuous catalytic reaction.[11] In another study, uridine-cytidine kinase and ACK were coimmobilized on ε-polylysine-functionalized sepharose, and the residual activity of the

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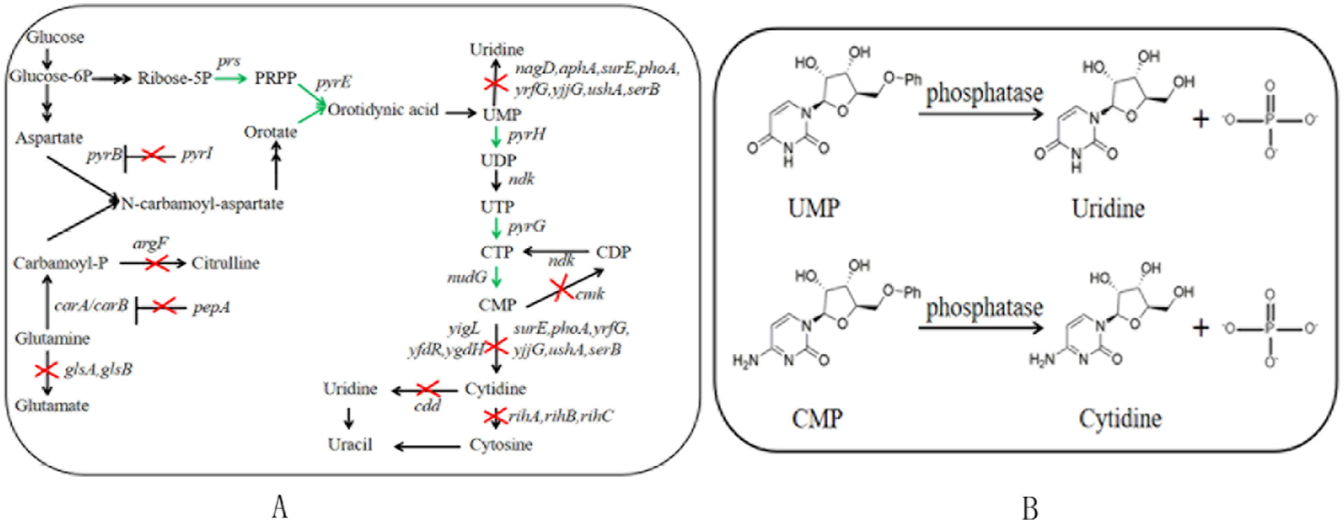

Figure 1. Scheme of construction of the CMP production strain. (A) CMP biosynthesis pathway. Single arrows represent one-step biosynthesis, double arrows represent multistep biosynthesis, green arrows indicate gene overexpression, and "X" indicates deletion of the corresponding gene. (B) Reaction equations catalyzed by phosphatase.

coimmobilized enzymes was higher than 70.6% after being recycled 10 times.[10]

Although progress has been achieved in the enzymatic synthesis of CMP, the starting materials, cytidine and ATP, are still relatively expensive for industry production. Therefore, seeking more economical production processes remained attractive. Cytidine can be produced by *Escherichia coli* (*E. coli*) fermentation with a high yield.[12] Because CMP was the biosynthesis precursor of cytidine, CMP could accumulate by blocking CMP catabolism. Furthermore, living cell fermentation can continuously generate enzymes and ATP, which can avoid the addition of expensive precursors used in enzymatic synthesis. In this study, several strategies were used to engineer *E. coli* for the efficient production of CMP (Figure 1). First, a cytidine-producing strain was constructed. Second, the genes involved in precursor competing pathway and negative regulation in pyrimidine biosynthesis were deleted to increase the yield of cytidine. Then, the genes related to CMP catabolism were deleted to accumulate CMP. Finally, the feedback inhibition tolerance genes and the rate-limiting step genes were overexpressed to increase the yield of CMP. After shake flask fermentation, 1013.6 mg/L of CMP was produced by the optimal strain, and the yield of CMP reached 15.3 g/L in a 50 L bioreactor by fed-batch fermentation.

RESULTS AND DISCUSSION

Engineered Pyrimidine Biosynthesis Pathway to Increase CMP Production. There are several pyrimidine products in the pyrimidine biosynthesis pathway,[13] and cytidine is the hydrolysis product of CMP which is not significantly accumulated in the fermentation broth of *E. coli* MG1665 (Figure 2). In order to better evaluate the effects of genome modifications, the strain which could accumulate cytidine as a major end product was first constructed by deleting *cdd*, *rihA*, *rihB*, and *rihC* on the base of MG1665,[12] and the resulting strain was named CP4. After flask fermentation, 4.5 mg/L of cytidine was accumulated in the fermentation broth of CP4, while no cytidine was detected in the fermentation broth of MG1665. Meanwhile, CMP was not

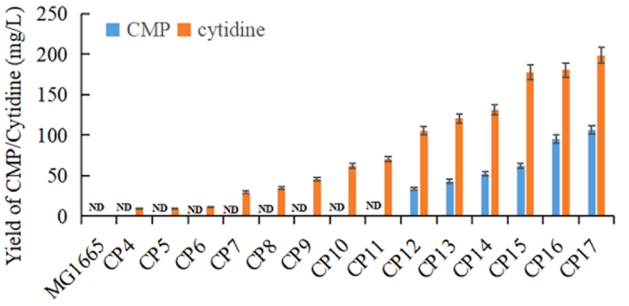

Figure 2. Enhancing the yield of CMP by engineering the pyrimidine biosynthesis pathway. ND: not detectable.

detected in the fermentation broth of CP4 and MG1665 (Figure 2).

There are several genes affecting pyrimidine intermediate biosynthesis. *glsA* and *glsB* catalyze the deamidation of L-glutamine, which is one of the CMP starting precursor.[14] The aminopeptidase gene *pepA* functions in pyrimidine-mediated repression of *carAB* transcription, which leads to a decrease in the yield of pyrimidine.[15] Carbamoyl-P is another key precursor of pyrimidine, and deletion of *argF* eliminates the competition for carbamoyl-P in uridine biosynthesis.[16] Uridine 5′-triphosphate (UTP) has a feedback inhibition effect on expression of the *pyrB-pyrI* operon, and *pyrI* functions as the negative regulator.[17] *nagD*[18] and *aphA*[19] were reported to have phosphatase activity against uridine monophosphate (UMP). Then, the aforementioned genes, namely, *glsA*, *glsB*, *pepA*, *argF*, *pyrI*, *nagD*, and *aphA*, were sequentially knocked out, and the resulting strain was named CP5, CP6, CP7, CP8, CP9, CP10, and CP11, respectively. The cytidine yields of CP7, CP9, and CP10 improved greatly (Figure 2), which illustrated that the deletions of *pepA*, *pyrI*, and *nagD* were important for improving cytidine biosynthesis. Other genes, except for *argF*, had no significant effect on increasing the cytidine yield. Finally, the cytidine yield of CP11 increased to 68.2 mg/L, which was 15 times higher than that of CP4 (Figure 2). However, CMP was still not detected. Meanwhile, the deletions of *pepA*, *pyrI*, and *nagD* had a deleterious effect on growth (Figure 3).

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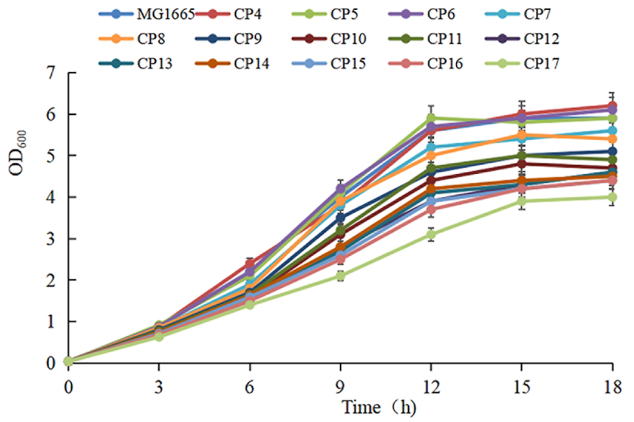

Figure 3. Growth curves of *E. coli*.

surE,[6,20] *phoA*,[21] *yrfG*,[22] *yjjG*,[6,20] *ushA*,[6,23] and *serB*[22] have been reported to show the activities of both UMP phosphatase and CMP phosphatase. These genes were sequentially knocked out, and the resulting strains were named CP12, CP13, CP14, CP15, CP16, and CP17, respectively. CMP was detected in the fermentation broth of CP12, and the yield reached 33.7 mg/L (Figure 2), which suggested that *surE* had strong hydrolytic activity against CMP. The deletions of *phoA* and *ushA* greatly increased the accumulation of CMP (Figure 2). The deletion of other genes can improve the yield of CMP, but it is not very significant. The deletions of these genes not only increased CMP production but also increased cytidine production (Figure 2). The cytidine yield of CP17 increased to 198.6 mg/L, which was 2.8-fold that of CP11 (Figure 2). Furthermore, the CMP yield of CP17 increased to 106.7 mg/L (Figure 2). The ratio of CMP to cytidine increased from 0.32 in the fermentation broth of CP12 to 0.54 in the fermentation broth of CP17. Meanwhile, the growth curves of CP12 and CP17 showed that the deletion of *surE* and *serB* led to impaired growth (Figure 3).

The fermentation results of CP12−CP17 illustrated that the deletion of these genes processing both UMP phosphatase and CMP phosphatase activities can improve the biosynthesis and accumulation of CMP. However, the accumulation of cytidine illustrated that there were some other genes involved in CMP metabolism.

Overexpression of Rate-Limiting Genes and Feedback-Resistant Genes. Beside the genes related to the precursor competing pathway and negative regulation, there are also some genes related to feedback inhibition, which negatively affect pyrimidine biosynthesis. Phosphoribosyl pyrophosphate (PRPP) synthetase gene (*prs*) catalyzes ribose-5-phosphate and ATP to generate PRPP, which is another important precursor for the biosynthesis of CMP, and the activity of PRS is inhibited by PRPP and GTP.[24] *Baprs*^{m} mutation from *Bacillus amyloliquefaciens* was reported to eliminate feedback inhibition.[25] UMP kinases (*pyrH*) and CTP synthetase (*pyrG*) suffered from feedback inhibition by UTP and CTP, respectively,[26,27] and the mutants *pyrH*^{m} and *CgpyrG*^{m} could desensitize the feedback inhibition.[26,28] *pyrE* and *nudG* were the rate-limiting step genes in cytidine biosynthesis.[12] To improve the yield of CMP, these genes, *Baprs*^{m}, *pyrE*, *pyrH*^{m}, *CgpyrG*^{m}, and *nudG*, were sequentially cloned into pET30a(+) under the T7 promoter to overexpression (Figure S1), yielding the plasmids pET30prs, pET30prs-pyrE, pET30prs-pyrE-pyrH, pET30prs-pyrE-pyrH-pyrG, and pET30prs-pyrE-pyrH-pyrG-nudG, respectively. Then, the five plasmids were sequentially transformed into CP17, yielding strains CP25, CP26, CP27, CP28, and CP22, respectively. Protein electrophoresis analysis of the CP22 supernatants showed that the five genes were correctly overexpressed (Figure S2). The CMP yield of CP25 increased by 34% compared with CP17 (Figure 5), which suggested that

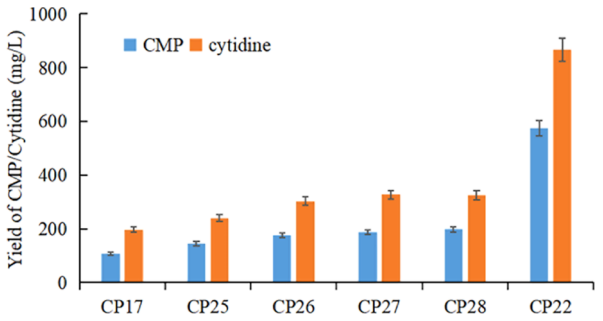

Figure 4. Enhancing the yield of CMP by overexpressing rate-limiting genes and feedback resistant genes.

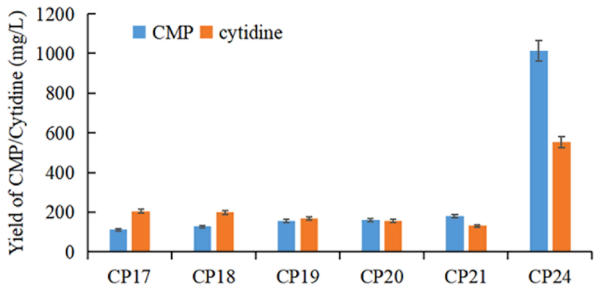

Figure 5. Enhancing the yield of CMP by deleting CMP catabolism-related genes.

the overexpression of *Baprs*^{m} can effectively increase CMP biosynthesis. The CMP yield of CP26 increased to 175.7 mg/L (Figure 4). However, the CMP yields of CP27 and CP28 did not increase significantly, which indicated that pyrH and pyrG were not severely inhibited. Because *pyrH*^{m} and *CgpyrG*^{m} were correctly expressed (Figure S1), it was speculated that the intracellular UTP and CTP may not be accumulated to a relatively high concentration. The CMP yield of CP22 increased to 573.4 mg/L, which was 2.9-fold that of CP28, suggesting that *nudG* was the critical rate-limiting gene (Figure 4).

Knockout of CMP Catabolism-Related Genes. The accumulation of cytidine in the fermentation broth of CP22 illustrated that CMP catabolism genes needed to be knocked out to further increase the accumulation of CMP. *yigL*,[22] *ygdH*,[29] and *yfdR*[30] were reported to process the phosphatase activity against CMP. The three genes were sequentially knocked out, and the resulting strains were named CP18, CP19, and CP20. Cytidylate kinase (*cmk*)-catalyzed phosphoryl transfer from ATP to CMP, resulting in the depletion of CMP,[31] which was unconducive for CMP accumulation. Therefore, *cmk* was deleted on the basis of CP20 to further increase the yield of CMP, and the resulting strain was named CP21. Then, the plasmid pET30prs-pyrE-pyrH-pyrG-nudG was transformed into CP21, yielding the strain CP24. Except for *yfdR*, the deletion of the other three genes significantly increased the CMP yield, and the ratio of CMP to cytidine

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increased from 0.54 in the fermentation broth of CP17 to 1.83 in the fermentation broth of CP24. The CMP yield of CP24 reached 1013.6 mg/L, and the yield of cytidine was 553.9 mg/L (Figure 5). The accumulation of cytidine indicated that there were some unknown genes involved in CMP dephosphorylation. The growth curves showed that the deletion of *yigL* had a growth defect (Figure 6). Compared with MG1665, the final mutant strain CP24 grew slower, and the logarithmic growth period was extended (Figures 3 and 6), which was possibly caused by a heavier metabolic burden and the destruction of pyrimidine homeostasis.[32]

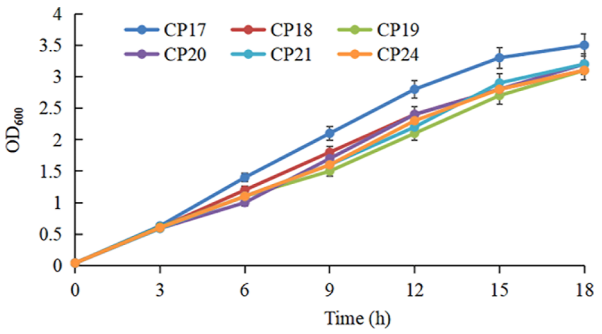

Figure 6. Growth curves of *E. coli*.

Fed-Batch Fermentation of CMP in a 50 L Bioreactor. The strain CP24 exhibited the highest yield of CMP in shake flasks and was used in fed-batch fermentation. As shown in Figure 7, during the growth stage, the 10 g/L glucose initially used was almost exhausted after 3 h, and glucose was then fed to maintain the concentration below 0.3%. After 4 h of cultivation, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the fermentation broth to induce protein overexpression. After induction for 2 h, 1.2 g/L CMP was detected in the fermentation broth. In the initial 21 h, the cells grew exponentially, and the yield of CMP also increased exponentially at the same time. After that, cell growth entered a plateau period, but CMP continued to accumulate, exhibiting a cell-growth-independent production profile. At 45 h, the yield of CMP reached a maximum of 15.3 g/L.

CONCLUSIONS

In this study, the engineered *E. coli* strain with high CMP production was successfully constructed. Deletions of the genes involved in the pyrimidine precursor competing pathway and negative regulation can increase cytidine biosynthesis. The deletions of CMP phosphatase genes led to the accumulation of CMP, and the overexpression of the rate-limiting step genes and feedback-inhibition resistance genes greatly increased the yield of CMP. The blocking of CMP phosphorylation further increased the yield of CMP. Ultimately, the yield of CMP reached 15.3 g/L in a 50 L bioreactor. To our knowledge, this is the first report that CMP was directly produced by *E. coli* fermentation, which showed the potential of industrial production.

MATERIALS AND METHODS

Bacterial Strain, Plasmids, and Reagents. The bacterial strains and plasmids used in this study are listed in Supporting Information Table S1. DNA polymerase, one-step cloning kits, and 2 × rapid Taq Master Mix were purchased from Tolobio Bio, Inc. (Shanghai, China). All genes were synthesized by Genscript Bio, Inc. (Nanjing, China). Oligonucleotide primers were synthesized by Sangon Biotech (Shanghai, China). All other chemicals and reagents were purchased from Aladdin (Shanghai, China).

Fermentation Conditions for CMP Production. *E. coli* cells were cultivated at 37 °C in Luria−Bertani (LB) medium for seed preparation. Kanamycin (50 mg/L) was added as needed. The flask fermentation medium formula is as follows: 4 g of glycerol, 24 g of yeast extract, 20 g of tryptone, 2.3 g of KH_2PO_4, 12.5 g of K_2HPO_4, and 1000 mL of water. IPTG was added to the medium at a final concentration of 0.1 mM when the OD_{600} reached 0.6.

The fed-batch fermentation medium formula is as follows: 10 g of glucose, 10 g of yeast extract, 10 mL of corn steep liquor, 1.35 g of KH_2PO_4, 2.5 g of $(NH_4)_2SO_4$, 1.1 g of citric acid, 1 g of $MgSO_4$, 15 mg of $MnSO_4$, and 2 mg of V_{B1} in 1000 mL of water. The pH was maintained at 7.0 by adding an NH_4OH solution. The dissolved oxygen was greater than 25% by adjusting the aeration and agitation rates.

Construction of Recombinant Plasmids. The primers used for gene cloning are shown in Supporting Information Table S2. 2× Ezmax Universal CloneMix (Tolobio Bio) was used for plasmid construction. *prs*^{m} was synthesized and colonized into pET30a (+) at NdeI and *Bgl*II by GenScript Bio Inc. (Nanjing, China), resulting in pET30prs. *pyrE* DNA fragments were amplified from *E. coli* MG1665 and cloned into pET-30prs at *Bgl*II, resulting in pET30prs-pyrE. The *pyrH*^{m} DNA fragments were amplified by PCR and cloned into pET30prs-pyrE at EcoRV, resulting in pET30prs-pyrE-pyrH.

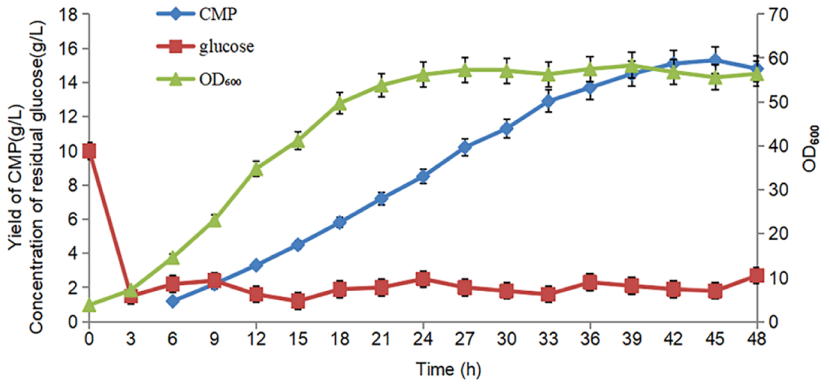

Figure 7. Fed-batch fermentation profiles of CP24 in a 50 L bioreactor.

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CgpyrG^{m} was synthesized by GenScript Bio Inc. (Nanjing, China) and was cloned into pET30prs-pyrE-pyrH at *Bam*HI, resulting in pET30prs-pyrE-pyrH-pyrG. *nudG* was amplified from *E. coli* MG1655 and was coloned at SacI of pET30prs-pyrE-pyrH-pyrG, resulting in pET30prs-pyrE-pyrH-pyrG-nudG. The RBS sequence (aaaggaggatatacat) was inserted between two successive genes. The sequences of overexpression genes are presented in Supporting Information Table S3.

Gene Deletion in *E. coli* Genome. Gene deletion was conducted using the CRISPR−Cas9 system.[33] The primers used for gene deletion are shown in the Supporting file: Table S2. The gRNA plasmid was amplified by PCR from plasmid pTargetF, and the PCR products were transformed into competent *E. coli* DH5α cells after DpnI digestion at 37 °C for 1 h. To construct the full-length DNA fragment for homologous recombination, two pairs of primers were used for amplifying the upper and lower homologous arms of the target gene, and the homologous recombination fragments were obtained by overlapping PCR. The pTargetF plasmid and homologous recombination fragment were transferred to *E. coli* MG1665 carrying the plasmid pCas. The cells were screened with spectinomycin and kanamycin at 30 °C. The gene deletions were verified by colony PCR. The correct mutant strain was inoculated into 3 mL of LB medium cultured at 30 °C, and IPTG was added to the medium at a final concentration of 0.1 mM to cure the pTargetF derivative. The cells sensitive to spectinomycin were used in the next round of gene knockout.

Protein Expression and SDS−PAGE Analysis. CP22 harboring overexpression plasmids were cultured in LB medium containing 50 mg/L kanamycin at 37 °C for 6 h until the OD_{600} reached 0.6. Then, IPTG was added to a final concentration of 0.1 mM to induce gene expression. The culture was harvested by centrifugation (6000 rpm, 5 min). The cell pellet was re-suspended in lysis buffer (20 mM phosphate buffer, pH 8.0, 300 mM NaCl). The cells were lysed by sonication. Then, the lysis solution was centrifuged at 15,000 rpm and 4 °C for 15 min. The supernatant was separated by SDS−PAGE (12% polyacrylamide gel).

Analytical Methods. Biomass was estimated by measuring the optical density at 600 nm. The concentration of CMP was detected by high-performance liquid chromatography (Agilent 1260 Series) on a C18 column (150 mm × 4.6 mm, Agilent) and monitored at 260 nm. Analysis was performed at 30 °C, and the mobile phase was composed of 50 mM monopotassium phosphate and methanol (90/10, v/v) at a flow rate of 1.0 mL/min.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c07713.

Schematic diagram of the plasmid construction; SDS-PAGE analysis of the supernatant of genes overexpressed in *E. coli*; bacterial strains and plasmids used in this study; primers used for plasmid construction and gene knockout; and sequences of genes involved in knockout and overexpression (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Min Zhou − *Institute of Biopharmaceuticals, School of Pharmaceutical Sciences, Taizhou University, Taizhou 318000, China*; orcid.org/0009-0007-2312-1867; Email: zhoumin@tzc.edu.cn

Wei Chen − *Hangzhou Hizyme Biotech Co., Ltd., Hangzhou 310011, China*; Email: doctor.chen1989@foxmail.com

Zhengju Tang − *Taizhou Central Hospital (Taizhou University Hospital), Taizhou 318000, China*; Email: tangzj@tzzxyy.com

Authors

Liyuan Wei − *Institute of Biopharmaceuticals, School of Pharmaceutical Sciences, Taizhou University, Taizhou 318000, China*

Chongzhi Wu − *Institute of Biopharmaceuticals, School of Pharmaceutical Sciences, Taizhou University, Taizhou 318000, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c07713

Author Contributions

M.Z. conceived the study, structured the work, conducted fermentation experiments in bioreactors, and wrote the manuscript. C.W. constructed plasmids and strains. L.W. conducted gene deletions and fermentation experiments in shake flasks. W.C. conceived the study and wrote the manuscript. Z.T. contributed to manuscript revision and data analysis.

Notes

The authors declare no competing financial interest.

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