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Original Research Article

Multivariate modular metabolic engineering and medium optimization for vitamin B_{12} production by *Escherichia coli*

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ABSTRACT

Vitamin B_{12} is a complex compound synthesized by microorganisms. The industrial production of vitamin B_{12} relies on specific microbial fermentation processes. *E. coli* has been utilized as a host for the *de novo* biosynthesis of vitamin B_{12}, incorporating approximately 30 heterologous genes. However, a metabolic imbalance in the intricate pathway significantly limits vitamin B_{12} production. In this study, we employed multivariate modular metabolic engineering to enhance vitamin B_{12} production in *E. coli* by manipulating two modules comprising a total of 10 genes within the vitamin B_{12} biosynthetic pathway. These two modules were integrated into the chromosome of a chassis cell, regulated by T7, J23119, and J23106 promoters to achieve combinatorial pathway optimization. The highest vitamin B_{12} titer was attained by engineering the two modules controlled by J23119 and T7 promoters. The inclusion of yeast powder to the fermentation medium increased the vitamin B_{12} titer to 1.52 mg/L. This enhancement was attributed to the effect of yeast powder on elevating the oxygen transfer rate and augmenting the strain's isopropyl-β-D-1-thiogalactopyranoside (IPTG) tolerance. Ultimately, vitamin B_{12} titer of 2.89 mg/L was achieved through scaled-up fermentation in a 5-liter fermenter. The strategies reported herein will expedite the development of industry-scale vitamin B_{12} production utilizing *E. coli*.

1. Introduction

Vitamin B_{12}, also known as cobalamin, is a water-soluble vitamin that contains a metallic element [1]. It has been widely used in various sectors such as pharmaceuticals, nutraceuticals, food, and animal feed additives. Methylcobalamin and adenosylcobalamin represent the natural forms of vitamin B_{12} are methylcobalamin and adenosylcobalamin, synthesized by prokaryotes through aerobic, anaerobic, or salvage pathways [2]. Hence, the industrial production of vitamin B_{12} primarily depends on microbial fermentation, with *Pseudomonas denitrificans* and *Propionibacterium* species serving as the principal production hosts [3]. Through the optimization of fermentation media for natural strains and the implementation of physical mutagenesis and overexpression of relevant gene clusters, researchers have attained vitamin B_{12} titers reaching up to 171.2 mg/L [4–6]. Nonetheless, conventional vitamin B_{12} producers frequently encounter various drawbacks such as overproduction of by-products, extended fermentation cycles, high rates of spontaneous mutation, and limited genetic engineering tools. To tackle these challenges, previous studies have investigated the utilization of engineered *E. coli* for vitamin B_{12} production [7]. However, despite these efforts being encouraging, the vitamin B_{12} titers of engineered *E. coli* strains still fall short of the levels required for industrial applications. Hence, further research and development are warranted to attain higher vitamin B_{12} production titers through microbial

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fermentation.

In microbial metabolism, the re-regulation of endogenous microbial metabolism through genetic manipulation can often lead to imbalances in metabolic pathways. This is because the introduction of exogenous genes or metabolic pathways can result in the accumulation of intermediates and feedback inhibition of enzymes, ultimately reducing the efficiency of product formation [8,9]. To achieve the maximum yield and titer of the final product, it is essential to identify and optimize the metabolic state of the strain to eliminate any metabolic bottlenecks [10]. Coordinating genes involved in the same metabolic pathway is crucial for maximizing the yield of the target product. Promoter engineering, which involves using promoters of different strengths to express target genes, allows for the precise regulation of gene expression. This approach has been successfully used in the production of valerenadiene and N-acetylglucosamine [11,12].

Multivariate modular metabolic engineering is a promising strategy that involves dividing metabolic pathways into different modules, controlling the expression levels of each module, and assembling multiple modules to generate strain libraries [13,14]. This strategy is particularly useful for coordinating complex multi-gene biosynthetic pathways, such as those involved in the production of fatty acids [14], isoprenes [15], ornithine [16], and tyrosine [17]. Enzyme expression can be regulated at three levels, including transcriptional, post-transcriptional, and post-translational levels [18], among which artificial regulation at the transcriptional level has been most frequently used due to its convenient operation.

In the context of utilizing *E. coli* as a microbial cell factory for vitamin B_{12} production, the imbalance in metabolic flux imbalance poses a significant challenge that impedes its efficiency [7]. The instability of intermediates in the biosynthetic pathway and the lack of standardized chemicals for these intermediates render it difficult to identify precise metabolic bottlenecks. To tackle this challenge, multivariate modular metabolic engineering presents a promising strategy for optimizing vitamin B_{12} production modules. This approach allows for the fine-tuning of the expression levels of different modules without necessitating detailed knowledge of precise metabolic bottlenecks. Through the utilization of different promoters, it might be possible to achieve a balance in the expression levels of genes upstream and downstream in the pathway, thereby enhancing vitamin B_{12} production. Previously, the majority of pathway genes in engineered strains were controlled by T7 promoters [3]. The application of multivariate modular metabolic engineering holds the potential to significantly enhance vitamin B_{12} production and facilitate its industrial applications.

In this study, the multivariate modular metabolic engineering strategy was applied to optimize the lower section of the vitamin B_{12} biosynthetic pathway in *E. coli*, with the aim of eliminating metabolic

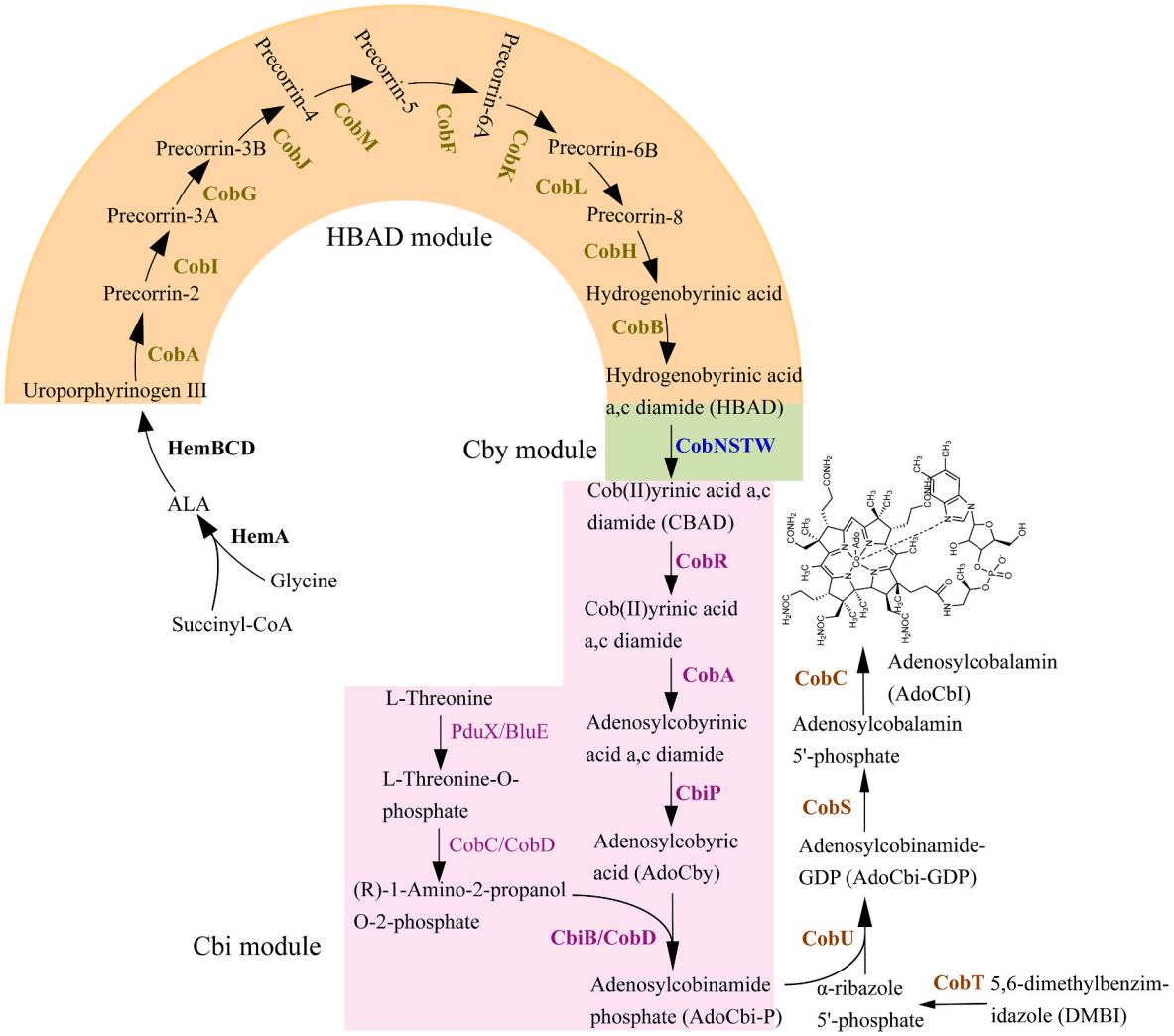

Fig. 1. The biosynthetic pathway of adenosylcobalamin and the modules that were designed for this study. The olive-labeled genes denote the HBAD module, the blue-labeled genes denote the Cby module, the purple-labeled genes denote the Cbi module, and the brown-labeled genes indicate the native biosynthetic pathway in *E. coli*.

imbalance. The Cby module, responsible for synthesizing co(II)byrinic acid a,c-diamide (CBAD) from hydrogenobyrinic acid a,c-diamide (HBAD), and the Cbi module, responsible for synthesizing adenosylcobalamin phosphate (AdoCbi-P) from CBAD and L-threonine, were optimized combinatorially at the transcriptional level (Fig. 1). Organic nitrogen sources were additionally incorporated into the fermentation medium to enhance IPTG tolerance of the final strain. The results of this study indicate that the multivariate modular metabolic engineering approach can significantly boost vitamin B_{12} production in *E. coli*. By balancing the expression levels of different modules, the study achieved the highest reported heterologous production of vitamin B_{12} in a 5-L fermenter. This study represents a noteworthy advancement towards the industrial production of vitamin B_{12} utilizing *E. coli* as a microbial cell factory. Furthermore, the metabolic engineering and fermentation conditions optimization conducted in this study are transferable to other strains to enhance vitamin B_{12} production. This methodology holds promise for augmenting vitamin B_{12} production across diverse microbial cell factories, thereby facilitating its industrial applications.

2. Materials and methods

2.1. Strains and plasmids

The strains and plasmids utilized in this study are detailed in Supplementary Table S1. *E. coli* DH5α served as the host strain for all routine cloning manipulations. The primers employed in this study are listed in Supplementary Table S2, and were prepared by Tsingke Biological Technology Co. Ltd. PCR amplifications were conducted utilizing Q5 high-fidelity DNA polymerase from New England Biolabs (NEB) according to the manufacturer's protocols.

The HBAD-producing strain FH511 was used as the starting strain. The plasmids pCas9gB-hsdR-RC and pDonor01-hsdR-RccobB-T7hyb1 were constructed according to a recent study [19], and then they were employed to insert a strong T7hyb1 terminator behind the *RccobB* gene, generating the JH18 strain. The Cby and Cbi modules were assembled using the MetClo DNA assembly method [20], and then integrated into the chromosome of the chassis cell (the JH18 strain) by standardized iterative genome editing (SIGE) [19] or CRISPR/Cas9 [21]. The details are described below. The tandem rrnB T1 and rrnB T2 terminators were inserted behind the cloning site of pMXLK_pFa plasmid and pMXLC_pFa via Gibson assembly [22], resulting in pMXLKT_pFa and pMXLCT_pFa, respectively. The DNA assembly module from pMXLC_pFa was cloned to the pUC19 vector, generating pUC19pFa. The annealed oligos for the J23106 promoter were cloned into the pMXLKT_pFa and pMXLCT_pFa via Golden Gate, creating pMXLKT_pFa-J23106 and pMXLCT_pFa-J23106, respectively. The pMXLKT_pFa-J23119 plasmid was constructed similarly. A codon-optimized *SmcobN* gene was cloned to the pMXLC_aFb plasmid, resulting in pMXLC-aFb-SmcobN, and then a class II terminator was removed via site-directed mutagenesis [23], generating pMXLC-aFb-SmcobN-mut. Homologous arms for recombination at *yghX* were amplified using the *E. coli* K12 MG1655 genome as a template and then cloned to the MetClo level 0 vector. All building blocks for the Cby and Cbi modules, including promoters, coding genes, terminators, were assembled on the MetClo vector using hierarchical DNA assembly. Next, the Cby and Cbi modules under control of promoters with gradient strengths were assembled on the pDonor01 vector. The spacer targeting *yghX* was integrated into the pCas9gB plasmid, resulting in pCas9gB-yghX. These plasmids derived from pCas9gB and pDonor01 were used for genome editing as previously described [19] to obtain recombinant strains including CFT01, CFT03, CFT05, CFT06, CFT07, CFT09, CFT10, CFT11, and CFT12 for vitamin B_{12} production.

The pET-28a-J23119-sfGFP and pET-28a-J23106-sfGFP plasmids were generated by inverse PCR using the pET-28a-sfGFP plasmid as a template, followed by digestion with DpnI, phosphorylation, and ligation. Homologous arms were cloned into the pCas9 backbone to replace the J23106 promoter with the J23119 promoter, resulting in pCas9-J23119-PacbiB. The plasmid was transformed into CFT01, CFT05, and CFT07, to replace the J23106 promoter with the J23119 promoter using CRISPR/Cas9, generating CFT02, CFT04, and CFT08 [21]. The edited strains were validated through colony PCR and Sanger sequencing. The pCas9-hsdR-GAP-RccobA backbone was amplified with inverse primers, phosphorylated using polynucleotide kinase, and subsequently circularized to form the plasmid pCas9-hsdR-MCS-RccobA. The annealed oligonucleotides corresponding to the promoters PthrC3_8 [24], pSH045 [25], Kosuri [26], P50061 (designed by Promoter Calculator [27]), and P76128 (designed by Promoter Calculator [27]) as detailed in Supplementary Table S3 were inserted into the plasmid pCas9-hsdR-MCS-RccobA via Golden Gate, obtaining pCas9-hsdR-PthrC3_8-RccobA, pCas9-hsdR-pSH045-RccobA, pCas9-hsdR-Kosuri-RccobA, pCas9-hsdR-P50061-RccobA, pCas9-hsdR-P76128-RccobA. The plasmids were individually transformed into the FH385 strain, respectively, to replace the T7 promoter with respective promoters by CRISPR/Cas9 [21].

2.2. Medium and growth conditions

Bacterial strains are cultivated in Luria-Bertani (LB) broth or agar plates at 37 °C. Antibiotics are supplemented as required, with final concentrations of ampicillin at 100 mg/L, chloramphenicol at 34 mg/L, and kanamycin at 50 mg/L.

The MR or MR01 medium is employed for vitamin B_{12} production in shake flasks. Each liter of MR medium, as modified from a previous study [28], comprised the following components: 6.67 g KH_2PO_4, 4 g $(NH_4)_2HPO_4$, 0.8 g $MgSO_4{\cdot}7H_2O$, 0.8 g citric acid, 20 mg $CoCl_2{\cdot}6H_2O$, 90 mg 5,6-dimethylbenzimidazole (DMBI), 2 g glycine, 10 g succinic acid, 5 g betaine, 5 mL trace metal solution, and 20 g glycerol serving as the carbon source. The trace metal solution (per liter) is composed of the following components: 0.5 mol HCl, 10 g $FeSO_4{\cdot}7H_2O$, 2 g $CaCl_2$, 2.2 g $ZnSO_4{\cdot}7H_2O$, 0.5 g $MnSO_4{\cdot}4H_2O$, 1 g $CuSO_4{\cdot}5H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.02 g $Na_2B_4O_7{\cdot}10H_2O$. The MR01 medium represents an adaptation of the MR medium characterized by a decreased betaine concentration of 0.5 g/L. The MR02 medium is another variant derived from the MR01 medium, incorporating an additional 5 g/L yeast powder (Solarbio, China).

Pre-cultures cultivated overnight in LB broth were inoculated into shake flasks containing 30 mL of MR medium. These cultures were cultivated at 37 °C on a rotary shaker set at 220 rpm. Upon reaching an optical density of approximately 0.8 at 600 nm (OD600), induction was initiated by the addition of 1 mM IPTG to the cultures. Subsequently, the cultures were then incubated at 32 °C and 220 rpm for 40 h. Additional organic nitrogen sources, such as casein acid hydrolysate, peptone (Solabio), yeast powder (Solabio), yeast extract powder (Solabio), yeast extract FM902 (Angel, China), peptone FP103 (Angel, China), and soy peptone FP410 (Angel, China), were supplemented to the MR01 medium at final concentrations of either 2 g/L or 5 g/L as required.

2.3. Analytical methods

Vitamin B_{12} was quantified as previously described [3], with the following modification: a 20 mL sample of bacterial pellet from shake flask cultures was collected from the fermentation broth and resuspended in ddH_2O to a final volume of 2 mL. 200 μL of 8% $NaNO_2$ (w/v) and 200 μL of glacial acetic acid were added to the resuspended sample. The mixture was autoclaved at 100 °C for 30 min. After natural cooling, the samples were centrifuged at 4,000 rpm for 10 min. The supernatant was collected, filtered through a 0.22 μm aqueous membrane, and injected into an Agilent 1260 series HPLC equipped with a reversed-phase TC C-18 column (4.6 × 250 mm, 5 μm; Agilent, Inc., USA) and a 361 nm DAD detector. The mobile phase consisted of 30% methanol and 70% ddH_2O at a flow rate of 0.8 mL/min, and the column temperature was maintained at 35 °C. For the sample from the bioreactor, 100 μL of 8% $NaNO_2$ (w/v) and 100 μL of glacial acetic acid were added to 1 mL of cultures from the bioreactor. The subsequent steps

were the same as those for the shake flask samples. Acetate in the supernatant of the fermentation culture was detected by HPLC (Agilent 1260) with a Bio-Rad HPX-87H Aminex ion-exclusion (Bio-Rad Laboratories, USA) column connected to RI and UV detectors at 65 °C. The column was eluted with 5 mM sulphuric acid at a flow rate of 0.5 mL/min for 25 min. Furthermore, optical density was measured using a spectrophotometer (AOELAB UV-1000, China) at a wavelength of 600 nm. Glycerol content during fermentation was determined using a biosensor analyzer (SIEMAN M − 1000). GFP fluorescence was quantified with a Synergy HTX plate reader (BioTek, USA) and normalized to the OD_{600} values for analysis. The excitation/emission wavelengths were set at 485/528 nm.

2.4. Shake flask fermentations in RAMOS

Pre-cultures were grown in test tubes containing 5 mL of LB medium. Batch cultivations were performed using modified Erlenmeyer shake flasks with various filling volumes (40, 30, or 20 mL) in the RAMOS® parallel fermenter system (HiTec Zang, Herzogenrath, Germany). The system included a rinsing phase of 20 min and a measuring phase of 10 min, resulting in a total measuring cycle of 30 min. The fermentation batches were incubated without an oxygen supply. The flow rate of rinsing phase and the oxygen content of dry gas were set at 10 mL/min and 20.95 %, respectively. An oxygen calibration test and a leakage test were performed before each measurement. Pre-cultures were transferred to shake flasks containing the MR01 or MR02 medium with a starting OD_{600} at 0.05. The fermentation conditions were: a shaking frequency of 250 or 300 rpm and an incubation temperature of 32 °C. When the OD_{600} reached 0.8, 1 mM IPTG was added to induce the expression of genes involved in vitamin B_{12} biosynthesis, and then the cells were allowed to grow for 40 h.

2.5. Fed-batch fermentation in a bioreactor

A single colony of CFT10 was grown in 5 mL of LB medium in a glass test tubeand incubated overnight at 37 °C with shaking at 200 rpm. The initial seed culture was then transferred into a 250 mL shake flask containing 50 mL of MR medium, following the previously described method [28]. The flask was incubated at 37 °C with shaking at 200 rpm. Subsequently, the seed culture was transferred into a 5 L bioreactor (Baoxing, Shanghai, China) containing 2 L of medium. The inoculation ratio was set to 10% (v/v). The pH was automatically maintained at 7.0 by adding phosphoric acid (30%, v/v) and NH_4OH (25%, v/v). The stirrer speed was adjusted to maintain dissolved oxygen levels above 40%. The aeration rate was maintained at 3 L/min throughout the fermentation process. The initial agitation speed and temperature were set at 300 rpm and 37 °C, respectively. When the optical density reached approximately 10 (12–14 h post-inoculation), the cells were induced with 1 mM IPTG, and the culture temperature was adjusted to 32 °C. To maintain the glycerol concentration below 10 g/L, a 40% glycerol solution was added at an appropriate rate. Periodic sampling (2 mL) was conducted to analyze the biomass, residual sugars, and vitamin B_{12} content during the fermentation process.

3. Results and discussion

3.1. Multivariate modular metabolic engineering of vitamin B_{12} biosynthetic pathway

The vitamin B_{12} biosynthetic pathway initiates with δ-aminolevulinate (ALA) and encompasses 31 genes. We previously engineered various strains that express the HBAD module, Cby module, and Cbi module under the control of the T7 promoter in *E. coli* [3]. However, the accumulation of HBAD indicated that metabolic imbalances across these modules were responsible for the limited production of vitamin B_{12}; consequently, reorganizing the Cby module and Cbi module could enhance the vitamin B_{12} titer [19]. To address this issue, we applied multivariate modular metabolic engineering to determine the optimal combination of these modules. While previous studies mostly focused on optimizing plasmid-based module expression, the use of antibiotic-resistant markers in plasmid maintenance systems is increasingly unacceptable in various industrial biotechnology fields such as medicine, therapeutics, and agriculture [29]. The avoidance of antibiotics in production not only reduces costs but also complies with regulatory requirements. Furthermore, expressing genes in the host chromosome can reduce the metabolic burden associated with plasmids. Consequently, our objective was to integrate all pertinent genes into the host chromosome to create a plasmid-free strain.

To reduce the number of strains that required engineering, the Cby module and Cbi module were first targeted for optimization due to the presence of metabolic bottlenecks between them [3]. Three promoters, namely T7, J23119, and J23106, characterized by high, medium, and low strengths as confirmed by the *sfGFP* reporter gene described in Supplementary Table S4, respectively, were employed to combinatorially drive these two modules. However, we encountered a technical challenge when attempting to clone the J23119 promoter into the pMXLC_pFa vector. Mutations consistently occurred in the promoter region. To mitigate this issue, we attempted to incorporate terminators into the pMXLC_pFa vector and even replaced the original p15A origin with a high-copy-number ColE1 origin of replication to create pMXLCT_pFa and pUC19pFa vectors. However, these modifications did not resolve the problem, possibly due to the robustness of the J23119 promoter negatively impacting plasmid replication. Given these challenges, we employed CRISPR/Cas9 technology to directly edit two pre-engineered strains (CFT01 and CFT05) and replaced the J23106 promoter driving the Cbi module with the J23119 promoter. This approach circumvented the plasmid-related issues encountered earlier. Eventually, we generated a total of 9 plasmid-free strains starting from the JH18 strain. The schematic of these strains is shown in Fig. 2a. Specifically, the HBAD module containing an operon composed of 10 genes driven by the T7 promoter was integrated at the *hsdR* locus, while the Cby and Cbi modules containing two operons composed of 4 and 6 genes driven by corresponding promoters were integrated at the *yghX* and *yjbI* loci, respectively.

Fig. 2b displays the shake flask fermentation results for the strains. The expression levels of the Cbi module, regulated by promoters ranging from J23106 to T7, led to corresponding increases in vitamin B_{12} titer, suggesting that the Cbi module ought to be overexpressed. A vitamin B_{12} titer of 0.96 mg/L was achieved with the Cby and Cbi modules driven by J23119 and T7 promoters, respectively. To determine whether further overexpression of the Cbi module would enhance vitamin B_{12} production, two alternative versions of the Cbi module, each harboring different operons and driven by one or two T7 promoters (unpublished), were engineered in conjunction with the Cby module driven by either the J23119 or J23106 promoter, generating four strains. The CFT10 strain, harboring the Cby module driven by the J23119 promoter alongside the single-operon variant of the Cbi module driven by the T7 promoter, attained a vitamin B_{12} titer of 1.36 mg/L (depicted in Fig. 2c). Integration of two copies of the Cbi module led to a significant reduction in vitamin B_{12} titer to 0.33 mg/L maybe due to transcription competition between the Cbi module and the HBAD module, suggesting that the current number of Cbi modules is optimal. We then attempted to replace the T7 promoter of the HBAD module with various strong promoters such as PthrC3_8, pSH045, Kosuri, P50061, and P76128. None of these strains were able to produce HBAD, possibly due to the large 9.4 kb operon's requirement for the strongest T7 promoter for expression.

3.2. Effect of supplementing organic nitrogen sources on vitamin B_{12} biosynthesis

The expression of 28 heterologous genes in a single host requires a significant consumption of amino acids, which influences the medium

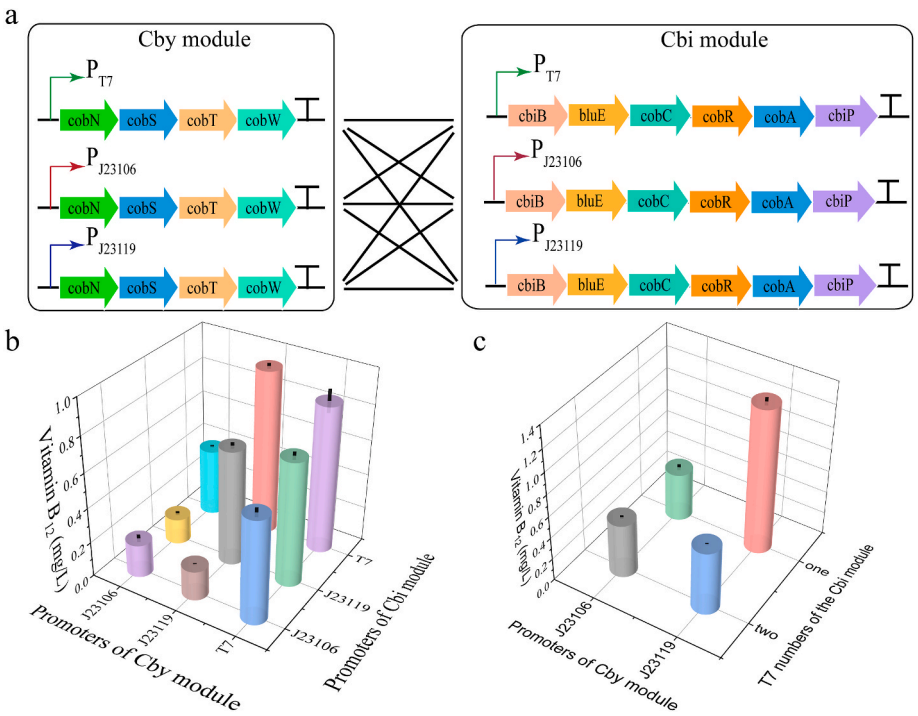

Fig. 2. Optimization of vitamin B_{12} production through engineering promoters of the Cby and Cbi modules. a. Schematic of modular regulation approach for optimization of the vitamin B_{12} biosynthesis pathway. b. The chromosomally integrated Cby and Cbi modules were modulated using three promoters with different strengths: T7, J23119, and J23106. These promoters were utilized to adjust the expression levels of the upstream and downstream pathways. c. Combinatorial optimization of Cby and Cbi modules for further vitamin B_{12} production. The Cby module was controlled by either the J23119 or J23106 promoter, while two versions of the Cbi module containing distinct operons were each driven by either one or two T7 promoters. Error bars indicate the standard deviation from three biological replicates.

composition on cell growth and vitamin B_{12} biosynthesis [3]. To obtain consistent and reproducible data, we initially utilized a chemically defined medium. This choice is based on the fact that synthetic media allow for precise formulation, thereby minimizing potential interactions that might confound experimental results. Furthermore, synthetic media provide enhanced downstream product purification compared to complex media [30]. Nevertheless, synthetic media can present formulation challenges and often lead to slow growth rates, extended lag phases, and limited strain tolerance due to their restricted nutrient composition. During fermentation in shake flasks and bioreactors, cell lysis was occasionally observed following IPTG induction (data not shown). Given that a reduced IPTG concentration may result in diminished gene expression, we aimed to improve strain tolerance using a richer medium. Nitrogen constitutes an essential component for microbial growth [31]. Furthermore, the strategic addition of organic nitrogen sources to synthetic media can shorten the lag phase and enhance strain tolerance. We

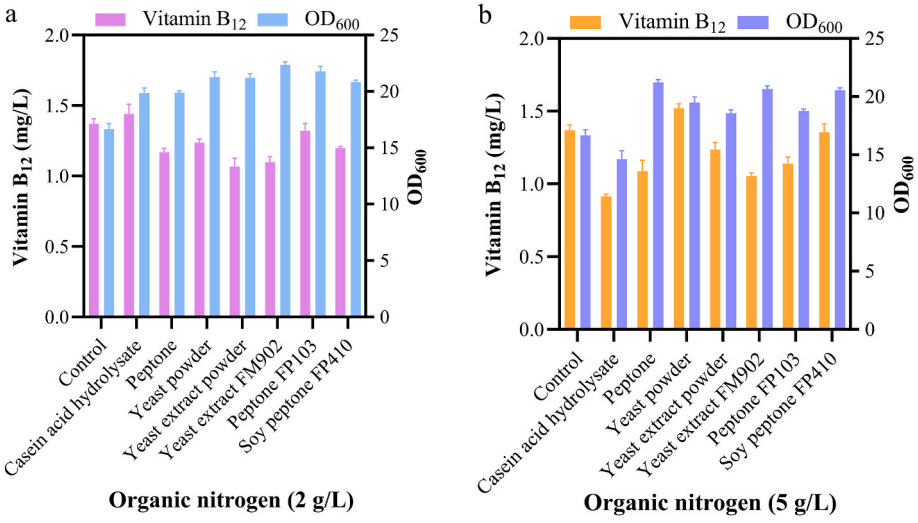

Fig. 3. Optimization of the incorporation of organic nitrogen sources into fermentation media to enhance vitamin B_{12} production. a. Vitamin B_{12} production and cell growth under control conditions with inorganic nitrogen $(NH_4)_2HPO_4$ and supplementation with organic nitrogen sources at a concentration of 2 g/L are shown. b. Vitamin B_{12} production and cell growth under control conditions with inorganic nitrogen $(NH_4)_2HPO_4$ and supplement of organic nitrogen sources at 5 g/L. Error bars indicate the standard deviation of three biological replicates.

evaluated the impact of supplementing with various organic nitrogen sources, namely casein acid hydrolysate, peptone (Solabio), yeast powder (Solabio), yeast extract powder (Solabio), yeast extract FM902 (Angel), peptone FP103 (Angel), and soy peptone FP410 (Angel), which mainly contain a mixture of amino acids, oligopeptides, and vitamins.

The study found that adding 2 g/L of all organic nitrogen sources increased the OD_{600} value, compared to the basic medium (Fig. 3a). Among all the nitrogen sources tested, yeast extract FM902 was the most beneficial for strain growth. It is worth noting that only the addition of 2 g/L casein acid hydrolysate increased the vitamin B_{12} titer, compared to the control. However, adding other nitrogen sources slightly decreased the vitamin B_{12} titer. When 5 g/L of all organic nitrogen sources, with the exception of casein acid hydrolysate were added, an increase in OD_{600} was also increased (Fig. 3b). Surprisingly, the addition of 2 g/L and 5 g/L casein acid hydrolysate had differing effects on the vitamin B_{12} titer, which may be attributed to the high acetate concentration arising from excess organic nitrogen source (Supplementary Fig. S1). This indicates that the proportion of organic nitrogen sources is of critical importance. The optimal vitamin B_{12} titer of 1.52 mg/L was achieved with the addition of 5 g/L yeast powder (MR02 medium). In conclusion, the study demonstrates that the type and proportion of organic nitrogen sources play a critical role in strain growth and vitamin B_{12} production. The findings can guide future research on optimizing media composition for microbial fermentation processes.

3.3. Comparison of the respiration behavior of vitamin B_{12} producer cultured in two media

To assess the impact of yeast powder supplementation on subsequent fermentation in the bioreactor, we conducted online monitoring of the oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR) for the CFT10 strain using the respiratory activity monitoring system (RAMOS). This system has been widely used for aerobic processes [32,33]. Semi-continuous online monitoring of oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR) enables the identification of factors significantly impacting the culture, such as oxygen transfer limitation, pH influence, medium limitation, and dioxygen growth [34,35].

In Fig. 4a, two distinct media were compared utilizing parallel flasks, each with 40 mL liquid volume, and a shaking frequency of 250 rpm. The observed plateau of the OTR from 19 h to 30.5 h in the MR01 medium corresponds to the maximum OTR (OTRmax), indicative of oxygen limitation during this fermentation phase for the strain. Similarly, oxygen limitation in the MR02 medium was evident from 11 h to 29.5 h. Incorporation of an organic nitrogen source into the MR02 medium promoted an earlier attainment of oxygen respiration limit for the strain, at approximately 11 h of fermentation, as opposed to the MR01 medium where a prolonged lag period preceded reaching the oxygen respiration limit at roughly 19 h (Fig. 4a). Despite these distinctions, both operating conditions yielded comparably maximum OTRs levels. At approximately 30.5 h, a 10 mL sample was extracted from the measuring flasks, resulting in an oscillation of the OTR observed at roughly 31 h in both media. The maximum OTRs in the MR01 and MR02 media escalated to 0.02660 and 0.03175 mol/L/h, respectively. During the initial 18 h, the carbon dioxide transfer rate (CTR) exhibited an exponential rise. However, subsequent to 30.5 h in the MR01 medium and 31 h in the MR02 medium, the CTR profiles commenced a marked decline, attributed to the depletion of carbon or nitrogen source.

To mitigate the risk of oxygen limitation during fermentation, ensuing experiments were conducted with reduced liquid filling volumes in the shake flasks. As the filling volumes were reduced, the maximum OTRs showed an increase (Fig. 4b). Notably, when the filling volume reached 20 mL and the shaking frequency was increased to 300 rpm, the oxygen respiration limit was no longer observed in both media (Fig. 4b). With a 20 mL liquid volume, the OTR showed an exponential increase following a short lag phase. The OTRmax rose from 0.03185 mol/L/h in the MR01 medium to 0.04895 mol/L/h in the MR02 medium. Intriguingly, cultivation in MR01 medium with a 20 mL filling volume led to a marked decrease in both OTR and CTR after 18.5 h post-IPTG addition, indicating the strain's sensitivity to IPTG toxicity in this medium (Fig. 4c). Following 19.5 h, OTR and CTR recovered briefly before declining once more, indicating the end of the fermentation process. Conversely, IPTG addition to the MR02 medium elicited no notable fluctuations, suggesting that the yeast powder supplementation enhanced the strain's IPTG tolerance. In summary, our findings indicate that the addition of yeast powder supplementation augments the strain's OTR and IPTG tolerance, which is pivotal for the prospective industrial-scale fermentation synthesis of vitamin B_{12}. This discovery has substantial implications for the scaling up of production and the optimization of fermentation conditions.

3.4. Fed-batch fermentation for vitamin B_{12} production

To further evaluate the effects of metabolic engineering and medium optimization on the production of vitamin B_{12}, fed-batch fermentations of CFT10 were conducted in a 5-L bioreactor. The fermentation results indicated that the bacterial biomass in the MR02 medium rapidly increased following a brief lag phase, reaching a peak of 57.8 at 18 h. This suggested that the strain was insensitive to IPTG (Fig. 5). The medium contained glycerol and yeast powder, with the latter serving as a combined source of both carbon and nitrogen. These carbon sources were consumed sequentially, leading to a short diauxie period during exponential growth. Vitamin B_{12} production paralleled the increase in biomass, indicative of a coupling between vitamin B_{12} biosynthesis and cell growth. The maximum titer of vitamin B_{12} reached was 2.89 mg/L at 24 h.

4. Conclusions

In this study, we successfully employed the multivariate modular metabolic engineering strategy to optimize the Cby and Cbi modules in the distal part of the vitamin B_{12} biosynthetic pathway. By expressing these modules under the control of constitutive promoters J23119 and T7, we attained the highest vitamin B_{12} titer recorded to date. Furthermore, by optimizing the fermentation medium, especially the nitrogen sources, we were able to further enhance vitamin B_{12} production even further. The addition of yeast powder not only elevated the oxygen transfer rate (OTR) of the final strain but also alleviated the toxicity of IPTG. The vitamin B_{12} titer achieved in this study, 2.89 mg/L in a 5-liter fermenter, represents the highest yield reported for heterologous production of this essential cofactor in *E. coli*. These findings undoubtedly pave the way for future industrialization of vitamin B_{12} production in this bacterium. In summary, our results demonstrate that multivariate modular metabolic engineering constitutes a powerful tool for enhancing vitamin B_{12} biosynthesis in *E. coli*. This approach can be harnessed to other biosynthetic pathways to improve their efficiency and yield, thus advancing the field of metabolic engineering and synthetic biology.

5. Availability of data and materials

All data generated or analyzed during this study are included in this research article.

CRediT authorship contribution statement

Feitao Chen: Conceptualization, Investigation, Formal analysis, Data curation, Methodology, Writing – original draft. **Huan Fang:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Jianghua Zhao:** Methodology, Resources. **Pingtao Jiang:** Methodology, Resources. **Huina Dong:** Resources. **Ying Zhao:**

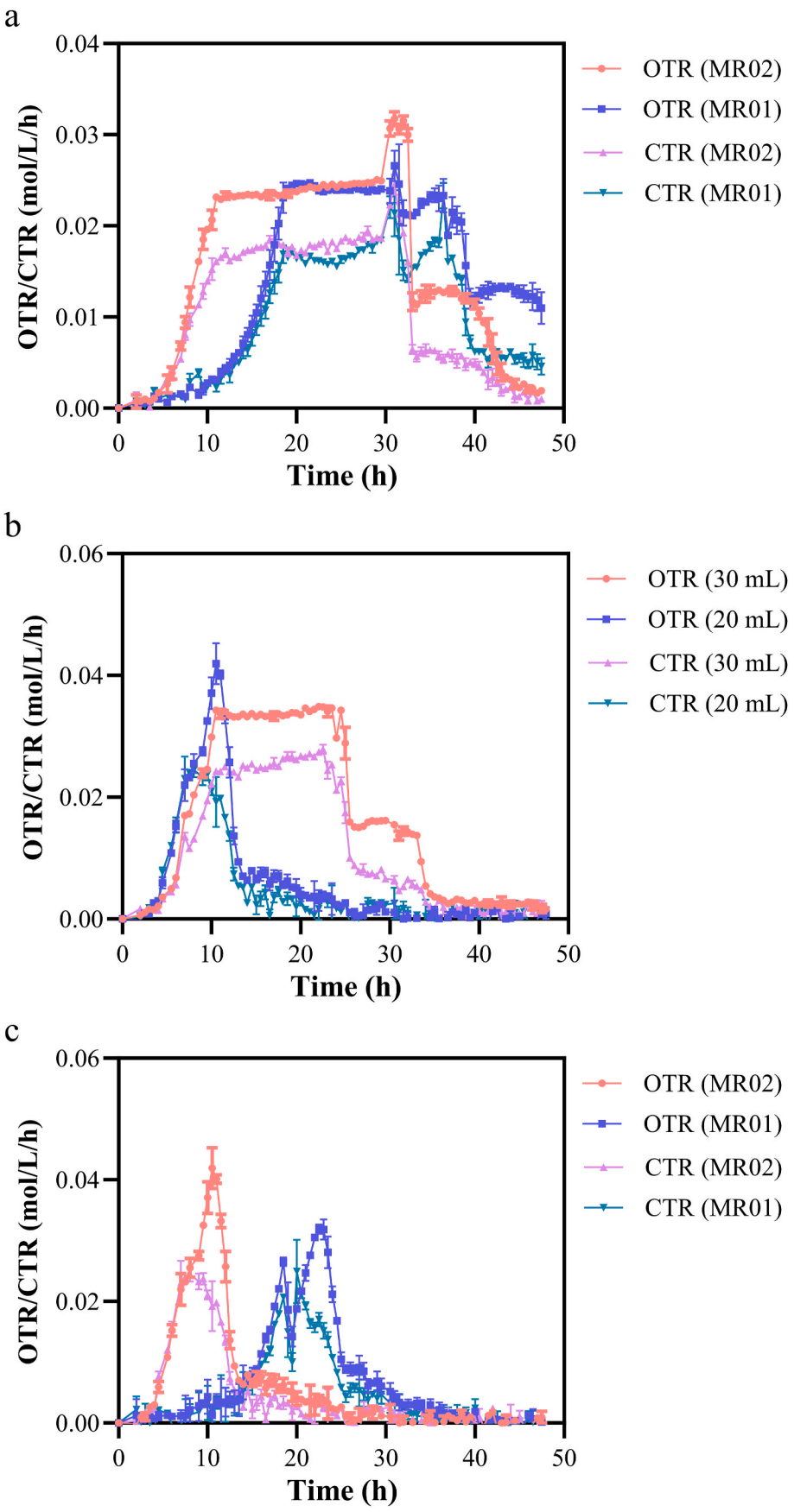

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Fig. 4. Characterization of CFT10 with different respiration behaviors under different fermentation conditions. a. The oxygen and carbon dioxide transfer rates of strain CFT10 strain were measured in the RAMOS system with a filling volume of 40 mL, using either the MR01 or MR02 medium. b. The oxygen and carbon dioxide transfer rates of CFT10 were measured in the RAMOS system using different filling volumes of the MR02 medium. c. The oxygen and carbon dioxide transfer rates of strain CFT10 were measured in the RAMOS system with a filling volume of 20 mL, using either the MR01 or MR02 medium. Error bars indicate the standard deviation of two biological replicates.

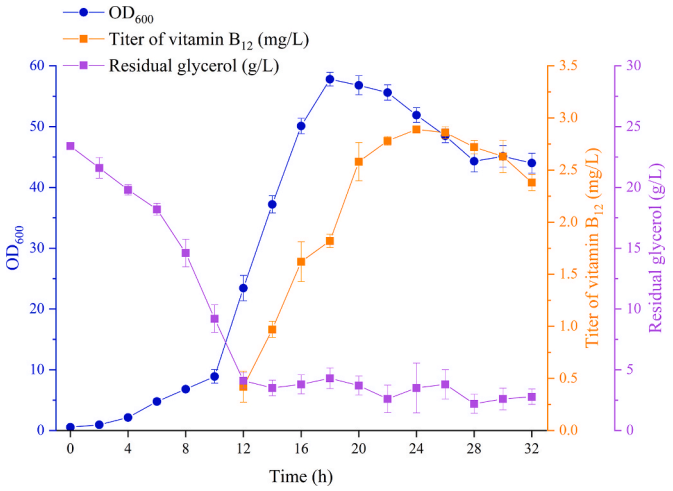

Fig. 5. Fed-batch production of vitamin B_{12} using CFT10 in a 5-L fermenter. The time courses of vitamin B_{12} titer, OD_{600}, and glycerol concentration are presented. Error bars indicate the standard deviation of two biological replicates.

Resources. **Huiying Wang:** Resources. **Tongcun Zhang:** Supervision. **Dawei Zhang:** Conceptualization, Data curation, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.03.017.

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