Synthetic and Systems Biotechnology 9 (2024) 176–185

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology

Original Research Article

Enhancement of polymyxin B1 production by an artificial microbial consortium of *Paenibacillus polymyxa* and recombinant *Corynebacterium glutamicum* producing precursor amino acids

Hui-Zhong Sun [a,c,1], Si-Yu Wei [a,c,1], Qiu-Man Xu [b,**], Wei Shang [a,c], Qing Li [a,c], Jing-Sheng Cheng [a,c,*], Ying-Jin Yuan [a,c]

[a] Frontiers Science Center for Synthetic Biology and Key Laboratory of Systems Bioengineering (Ministry of Education), School of Chemical Engineering and Technology, Tianjin University, Yaguan Road 135, Jinnan District, Tianjin, 300350, PR China
[b] Tianjin Key Laboratory of Animal and Plant Resistance, College of Life Science, Tianjin Normal University, Binshuixi Road 393, Xiqing District, Tianjin, 300387, PR China
[c] Department of Pharmaceutical, School of Chemical Engineering and Technology, Tianjin University, Yaguan Road 135, Jinnan District, Tianjin, 300350, PR China

ARTICLE INFO

Keywords:
Polymyxin
Co-culture
Paenibacillus polymyxa
Corynebacterium glutamicum
Medium optimization
Metabolic precursors

ABSTRACT

Polymyxin B, produced by *Paenibacillus polymyxa,* is used as the last line of defense clinically. In this study, exogenous mixture of precursor amino acids increased the level and proportion of polymyxin B1 in the total of polymyxin B analogs of *P. polymyxa* CJX518-AC (PPAC) from 0.15 g/L and 61.8 % to 0.33 g/L and 79.9 %, respectively. The co-culture of strain PPAC and recombinant *Corynebacterium glutamicum*-leu01, which produces high levels of threonine, leucine, and isoleucine, increased polymyxin B1 production to 0.64 g/L. When strains PPAC and *C. glu*-leu01 simultaneously inoculated into an optimized medium with 20 g/L peptone, polymyxin B1 production was increased to 0.97 g/L. Furthermore, the polymyxin B1 production in the co-culture of strains PPAC and *C. glu*-leu01 increased to 2.21 g/L after optimized inoculation ratios and fermentation medium with 60 g/L peptone. This study provides a new strategy to improve polymyxin B1 production.

1. Introduction

Infections caused by multidrug-resistant pathogens are a global public health crisis. Polymyxins synthesized by *Paenibacillus polymyxa* are lipopeptide antibiotics that are effective against the superbugs *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, etc. [1]. Polymyxin B and E are widely used in clinical practice. Polymyxin B1, B2, B3 and B1–1 are the most effective components of therapeutic drug polymyxin B, and their component ratios affect their pharmacokinetics, pharmacodynamics, and toxicity kinetics [2–4]. The European Pharmacopoeia stipulates that in clinical practice polymyxin B mixtures should comprise ≥80 % polymyxins B1–B3 and B1–1, in which B3 and B1–1 should not exceed 15 % and 6 %, respectively. Standardization of the ingredients of polymyxin mixtures currently available for clinical use must be stricter, or safer, one-component polymyxin lipopeptide products with clear pharmacokinetics must be developed [5]. Because the yield of polymyxin B is relatively low, it is necessary to increase the production of polymyxin B and the component proportion of polymyxin B1.

Polymyxin is synthesized by non-ribosome peptide synthetase (NRPS), which is encoded by a 40 kb gene cluster. The polymyxin gene cluster consists of five open reading frames: *pmxA*, *pmxB*, *pmxC*, *pmxD*,

Abbreviations: P. *polymyxa*, *Paenibacillus polymyxa*; PPAC, *P. polymyxa* CJX518-AC; *C. glutamicum*, *Corynebacterium glutamicum*; *C. glu*-thr, *C. glutamicum*-thr; *C. glu*-leu01, *C. glutamicum*-leu01; *C. glu*-cgb7, *C. glutamicum*-cgb7; *C. glu*-cgb11, *C. glutamicum*-cgb11; *E. coli*, *Escherichia coli*; 6-MOA, 6-methyloctanoic acid; L-Dab, L-2,4-diaminobutyric acid; L-leu, L-leucine; L-Ile, L-isoleucine; L-Thr, L-threonine; D-Phe, D-phenylalanine.

Peer review under responsibility of KeAi Communications Co., Ltd.

* Corresponding author. Frontiers Science Center for Synthetic Biology and Key Laboratory of Systems Bioengineering (Ministry of Education), School of Chemical Engineering and Technology, Tianjin University, Yaguan Road 135, Jinnan District, Tianjin, 300350, PR China.
** Corresponding author.
E-mail addresses: qmxu0929@126.com (Q.-M. Xu), jscheng@tju.edu.cn (J.-S. Cheng).
[1] Hui-Zhong Sun and Si-Yu Wei contributed equally to this work.

https://doi.org/10.1016/j.synbio.2024.01.015
Received 21 June 2023; Received in revised form 24 December 2023; Accepted 31 January 2024
Available online 1 February 2024

and *pmxE*. Among them, *pmxC* and *pmxD* encode transport proteins that transport polymyxin to the extracellular space, while *pmxA*, *pmxB*, and *pmxE* encode polymyxin synthase [6]. PmxA, PmxB, and PmxE consist of 10 modules (Fig. 1A), each with selective specificity for amino acid substrates. Specifically, the starter condensation domain has selective specificity for fatty acid chains [7,8]. Polymyxin B synthase selects and utilizes fatty acids including 6-methyloctanoic acid (6-MOA), 6-methylheptanoic acid, and octoic acid, as well as amino acids including L-2, 4-diaminobutyric acid (L-Dab), L-leucine (L-leu), L-isoleucine (L-Ile), L-threonine (L-Thr), and D-phenylalanine (D-Phe) in modular order to synthesize cyclic lipopeptide compounds. Polymyxins B1, B2, B3, and B1-1 differ in the fatty acyl moiety at the N-terminus (B1: 6-methyloctanoyl; B2: 6-methylheptanonyl; B3: octanoyl), or an amino acid within the heptapeptide ring (B1, position 7: L-Leu; B1-1, position 7: L-Ile) [9] (Fig. 1A).

Increasing the supply of important precursors is an effective way to enhance the production of lipopeptides such as surfactin [10], fengycin [11], iturin [12], and daptomycin [13]. The lack of precursor fatty acids and amino acids decreases lipopeptide synthesis [14]. However, the effects of amino acid precursors on polymyxin production are inconsistent. For example, exogenous addition of precursor amino acids, such as L-Dab, L-Thr, L-Leu or L-Ile, inhibits the synthesis of polymyxin E by *P. polymyxa* [15]. Polymyxin D1 and D2 levels are significantly increased in the presence of L-glutamate and glycine [16]. The impact of amino acid supplementation on polymyxin production is highly strain specific and dependent on the growth media [16]. Surfactin production can be increased by optimizing culture medium and fermentation strategy [17]. A combination of precursor amino acids can promote polymyxin synthesis. Thus, optimizing culture medium and fermentation conditions is necessary for improving polymyxin production.

The construction of a co-culture systems is a feasible strategy to provide key precursors or nutrients for producing valuable chemical products [18–20]. *Corynebacterium glutamicum* has been used industrially to produce natural amino acids [21] and amino acid derivatives [22]. The co-culture of *Bacillus* strains and *C. glutamicum* is based on the amino acid feeding mechanism of *C. glutamicum*, such as the co-culture of recombinant *C. glutamicum* producing high proline, with *Bacillus amyloliquefaciens* to enhance the production of lipopeptides [23]. Similarly, the co-culture of *C. glutamicum* and *Bacillus subtilis* with enhanced amino acids transporters to improve the titer of fengycin [24]. Another example is the co-culture system of *E. coli* and *C. glutamicum*, which not only relies on high level lysine produced by *C. glutamicum* to establish a commensalism [25], but also can enhance the growth of *E. coli* by using fructose metabolized from *C. glutamicum* as a carbon source [26]. Additionally, yeast has been shown to utilize the metabolic by-products of *C. glutamicum* to further improve the L-Ornithine production during the co-culture fermentation process [27].

Previous work has demonstrated that *P. polymyxa* CJX518 produces several polymyxin B homologues, including 41.36 % B1, 18.25 % B1-1, 23.4 % B2, and 16.99 % B3, whereas the recombinant *P. polymyxa* CJX518-AC (PPAC) significantly increased the component ratio of polymyxin B1 [28]. This study investigated the effects of simultaneous supplies of exogenous multi-precursor amino acids on polymyxin B1 production and polymyxin B1 ratio in polymyxin B homologues of strain PPAC by direct addition or artificial consortium. A recombinant *C. glutamicum* was used herein for producing higher levels of the precursor amino acids for polymyxin B1 synthesis. To improve the production of polymyxin B1, an artificial consortium of strains PPAC and recombinant *C. glutamicum* was constructed, and optimization of fermentation conditions was also carried out.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture media

The strains and plasmids used in this study are listed in Table S1.

E. coli DH5α was used for plasmid construction and amplification, and was also used as indicator for antibacterial experiment. *E. coli* DH5α cells cultured in Luria Betani (LB) medium containing 5.0 g/L yeast extract, 10.0 g/L peptone, and 10.0 g/L NaCl. *C. glutamicum* ATCC

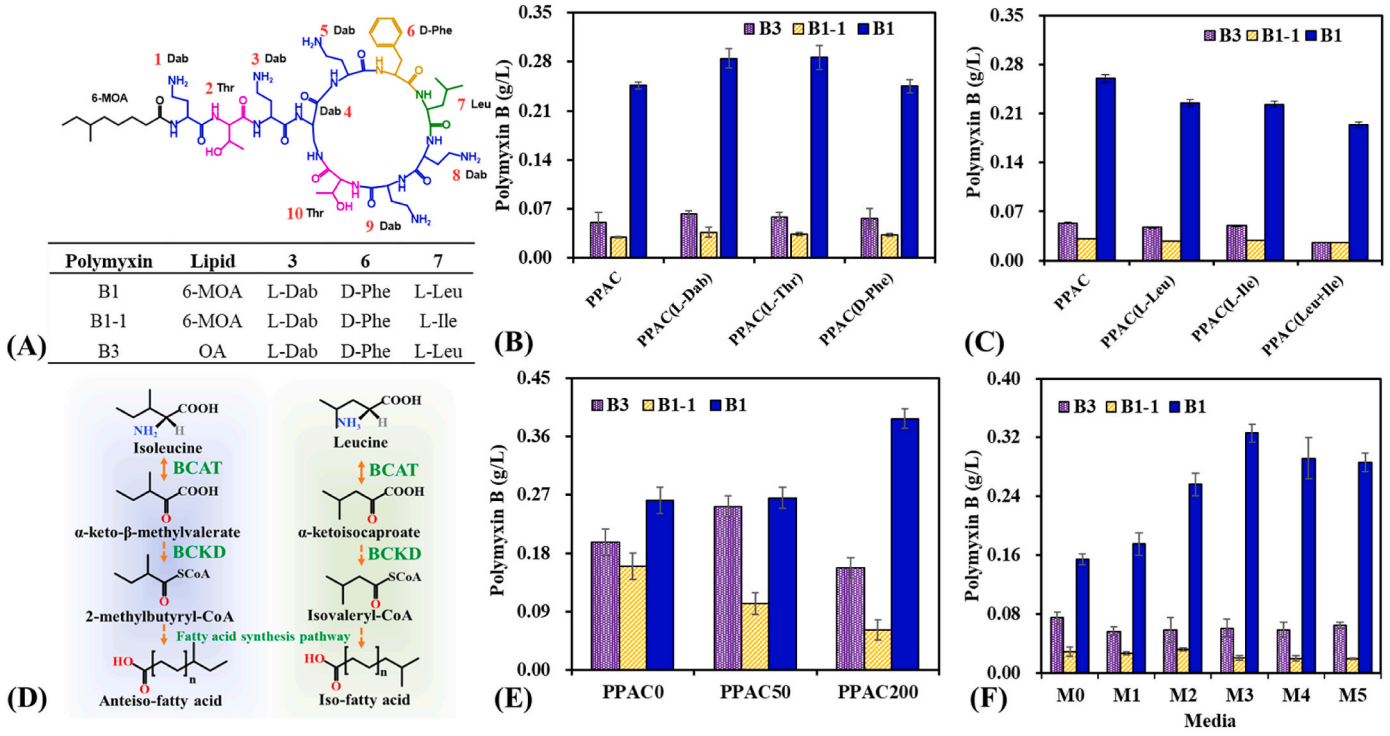

Fig. 1. Effect of exogenous supply of precursor substrate on polymyxin B level. (A) Chemical structure of polymyxin B, (B) Supply of 0.8 g/L L-Dab, 0.8 g/L L-Thr, and 0.8 g/L D-Phe, (C) Supply of 0.8 g/L L-Leu and 0.8 g/L L-Ile, (D) Fatty acid metabolism pathway, (E) Supply of 2-methylbutyric acid, (F) Supply of combined amino acids.

13032 was used as the parental strain for genetic engineering and cultured in 74.0 g/L brain heart infusion broth. The strain was also used for co-culture with strain PPAC to produce polymyxin.

The seed medium of strain PPAC contained 50.0 g/L glucose, 4.0 g/L peptone, 2.0 g/L yeast extract, 1.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4•7H_2O$, and 0.5 g/L NaCl at neutral pH. The seed medium of *C. glutamicum* contained 30.0 g/L glucose, 5.0 g/L peptone, 4.0 g/L K_2HPO_4, 2.0 g/L $MgSO_4•7H_2O$, 0.3 mg/L vitamin B1 (VB_1), 0.2 mg/L vitamin B7 (VB_7), 30.0 mg/L $FeSO_4•7H_2O$, and 30.0 mg/L $MnSO_4•H_2O$ at neutral pH.

This study used fermentation media I including media for pure culture of strains PPAC or *C. glutamicum*, media II for the co-culture of strains PPAC and *C. glutamicum*, and optimization media for the co-culture.

The medium for the pure culture of strain PPAC (PP medium) contained 60.0 g/L glucose, 1.0 g/L yeast extract, 10.0 g/L peptone, 1.0 g/L NaCl, 2.0 g/L K_2HPO_4, 0.7 g/L $MgSO_4•7H_2O$, 15.0 g/L $(NH_4)_2SO_4$, 0.5 g/L KCl, 0.3 mg/L $FeSO_4•7H_2O$, and 5.0 mg/L $MnSO_4•H_2O$ at pH 7.2, was described [28].

The medium for the pure culture of *C. glutamicum* contained 60.0 g/L glucose, 15.0 g/L yeast extract, 5.0 g/L peptone, 4.0 g/L K_2HPO_4, 2.0 g/L $MgSO_4•7H_2O$, 0.5 mg/L VB_1, 0.3 mg/L VB_7, 30.0 mg/L $FeSO_4•7H_2O$, and 30.0 mg/L $MnSO_4•H_2O$ at natural pH.

Based on the media for the pure culture of strain PPAC or *C. glutamicum*, the medium for the co-culture of strains PPAC and *C. glutamicum* (*C. glu*-PP medium) contained 60.0 g/L glucose, 15.0 g/L yeast extract, 20.0 g/L peptone, 4.0 g/L K_2HPO_4, 15.0 g/L $(NH_4)_2SO_4$, 2.0 g/L $MgSO_4•7H_2O$, 30.0 mg/L $FeSO_4•7H_2O$, and 30.0 mg/L $MnSO_4•H_2O$, 0.5 mg/L VB_1, 0.3 mg/L VB_7, 1.0 g/L NaCl, and 0.5 g/L KCl at pH 7.2.

For optimization media (*C. glu*-PP80 and CP-M) of the co-culture media, 60.0 g/L glucose was changed to 80.0 g/L glucose or 50.0 g/L maltodextrin, respectively.

2.2. Fermentation procedure

The seed of strain PPAC (1 mL) was prepared by inoculating into 200 mL seed medium in a 500-mL shake flask and cultured for 24 h at 180 rpm and 30°C. The activated stock seed of *C. glutamicum* (500 μL) was inoculated into in a 250-mL baffled shake flask containing 50 mL seed medium and cultured for 24 h at 180 rpm and 30°C. Fermentation was performed by inoculating seeds of strains PPAC and/or *C. glutamicum* with OD_{600} into 80 mL fermentation medium and incubating for 72 h at 30°C and 180 rpm.

2.3. Construction of metX deletion strain C. glutamicum

The pK18mobsacB plasmid was used as a vector to construct *metX* deletion plasmid pK18-Δ*metX*. The target plasmid achieved knockout of the *metX* gene on the genome of *C. glutamicum* through homologous recombination. The primers used in this study are listed in Table S2.

The plasmid pK18mobsacB was linearized using *Bam*H I and *Eco*R I. The upstream and downstream homologous arms of gene *metX* were amplified from the *C. glutamicum* genome and ligated with the linearized vector. The plasmid was transformed into the *E. coli* competent cells. The cells were plated on LB plates containing 50 μg/mL kanamycin, cultured for 24 h, and screened. Finally, colonies were verified via polymerase chain reaction (PCR) using the primers JP-PK18-F and JP-PK18-R. A 1401-bp band confirmed that the *metX* plasmid was successfully constructed (Fig. S1A).

The plasmid pK18-Δ*metX* was electroporated into competent cells of *C. glutamicum* ATCC13032 with a 1 cm electroporation cup at 1800 V. Chromosomal editing was performed by selecting cells that were kanamycin resistant and sucrose nonresistant cells [29]. The recombinant strain *C. glutamicum*-thr (*C. glu*-thr) was verified by PCR with primers *metX*-F-SE and *metX*-R-SE (Fig. S1B).

2.4. Analytical methods

2.4.1. Extraction and detecting of polymyxin B

The fermentation broth was centrifuged at 12,000 rpm for 10 min. An equal volume of ethyl acetate was added to the cell-free supernatant and extracted for 2 h. After stewing and layering were complete, the upper organic phase was removed and evaporated at 42°C to obtain a yellow powder, which comprised a crude extract of lipopeptides, including polymyxin. The powder was dissolved in methanol and filtered through a 0.22-μm nylon membrane for quantitative analysis. Polymyxin B was analyzed using a reversed-phase C18 column (4.6 × 250 mm, 4.6 μm; Alliance, USA). High-performance liquid chromatography (HPLC) was used to analyze polymyxin B, as described by Yuan et al. [28].

2.4.2. Assay of amino acids

Phenyl isothiocyanate (PITC), triethylamine, and n-hexane were purchased from Aladdin (Shanghai, China). The amino acids in the fermentation broth were quantified using the PITC derivatization method described by Chen et al. [30]. Then amino acids composition was determined by HPLC using a 4.6 mm × 250 mm Venusil AA column, as described by Chen et al. [24].

2.4.3. Determination of glucose by HPLC

The fermentation broth was centrifuged at 12,000×*g* for 10 min and filtered through a 0.22-μm aqueous membrane. Then, a 10-μL aliquot was examined with reversed-phase HPLC using an Aminex HPX-87H ion exclusion column (7.8 mm × 300 mm, Bio-Rad) and a differential refractive index detector. The mobile phase was 5.0 mM sulfuric acid in water at a flow rate of 0.6 mL/min, and the column temperature was maintained at 65°C [17]. The retention time of glucose was 9.3 min.

2.5. Antibacterial activity assay

The fermentation broth of strain PPAC was centrifuged at 12000×*g* for 10 min to pellet cells and filtered through a sterile 0.22-μm filter. The antibacterial activity of the supernatant was analyzed using *E. coli* DH5α as indicators, which were cultured in LB medium at 37°C and plated on LB agar. A sterile Oxford cup was placed on the surface of LB agar, and 200 μL of sterile supernatant was added to the Oxford cup. The supernatant was allowed to stand at 37°C to be absorbed by the agar. The Oxford cup was removed and inverted for 24 h.

2.6. RNA extraction and qRT-PCR analysis

qRT-PCR was performed to detect differences in the expression of genes involved in polymyxin biosynthesis. After 48 h of fermentation, strain PPAC cells were harvested through centrifugation at 6000 rpm for 5 min. Total RNA was extracted using the RNAprep Pure Cell/Bacteria Kit (TianGEN, China). The quantity and purity of total RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). cDNA was obtained by reverse transcription of total RNA using the SPARKscript II 1st Strand cDNA Synthesis Kit (SparkJade, China). The 16 S rRNA gene, amplified using the primers 16SF (5′-GAGAAGAAAGCCCCGGCTAA-3′) and 16SR (5′-ACCAGACTTAAAGAGCCGCC-3′), served as an internal reference. Primers for amplifying target genes are listed in Table S3 qRT-PCR was performed using LightCycler 480 (Roche, USA) with 2 × SYBR Green qPCR Mix (SparkJade, China), and data were analyzed according to Yu et al. [15].

2.7. Statistical analysis

All experiments were performed in triplicate. Statistical analysis was performed using SPSS version 27 software (USA). Differences with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ were labeled with one, two, and three asterisks, respectively.

3. Results and discussion

3.1. Effects of exogenous precursors on polymyxin synthesis

3.1.1. Supply of individual precursor amino acids

The synthesis of polymyxin B requires L-Dab, L-Leu, L-Thr, and D-Phe as precursor amino acids (Fig. 1A). As shown in Fig. 1B and C, the effects of amino acids on polymyxin B level of strain PPAC were studied by exogenously adding structural amino acids of polymyxin B. Compared to the control without exogenous amino acids, the individually addition of 0.8 g/L L-Dab and 0.8 g/L L-Thr significantly improved the polymyxin B production of strain PPAC, while 0.8 g/L L-Leu and 0.8 g/L L-Ile obviously reduced the production of polymyxin B of strain PPAC in PP medium. In addition, polymyxin B production was not significant change when supplied with D-Phe. It is because that the epimerization domain in the polymyxin B gene cluster alters the configuration of L-Phe, and D-Phe is involved in the structure of polymyxin B [31], while exogenous D-Phe did not possibly be incorporated into the structure of polymyxin B.

Moreover, the co-addition of 0.8 g/L of L-Leu and 0.8 g/L of L-Ile further reduced the polymyxin B, and raised the ratio of B1 in polymyxin B from 75.3 % to 79.0 %. The competitive product B1-1 of polymyxin B1 requires L-Ile at position 7 of the chemical structure, while polymyxin B1 is L-Leu (Fig. 1A), indicating that L-Ile will be used competitively in the synthesis of polymyxin B1 and B1-1, and the simultaneous addition of L-Leu may increase polymyxin B1 synthesis to inhibit the synthesis of B1-1. Consistently, it has been demonstrated that addition of precursor amino acids during fermentation inhibit polymyxin E production, which probably affects the expression of its biosynthesis-related genes [15]. Meanwhile, it is also reported that the production of polymyxin D1 and D2 was stimulated by L-alanine, L-arginine, L-histidine, L-cysteine, L-asparagine, L-glutamine, L-Ser, and L-Thr in a concentration-dependent manner [16]. Taken together, the impact of amino acid supplementation on polymyxin production is highly strain-specific and dependent on growth media [15,16].

The production of polymyxin B is affected by the expression of its biosynthesis-related genes in strain PPAC. Polymyxin B is biosynthesized by NRPS, which comprises the synthetase PmxA, PmxB, PmxC, PmxD, and PmxE, encoded by *pmxA*, *pmxB*, *pmxC*, *pmxD*, and *pmxE*, respectively. As shown in Fig. S2, compared to the M0 control, the relative expression levels of *pmxA–pmxE* decreased when 0.8 g/L of L-Leu, 0.8 g/L of L-Ile, or 0.8 g/L of L-Leu and 0.8 g/L of L-Ile were added to the medium. Conversely, the relative expression levels of *pmxA-pmxE* genes increased when individually supplemented with 0.8 g/L L-Dab and 0.8 g/L L-Thr, while *pmxC* showed a decrease when supplemented with 0.8 g/L L-Thr. In particular, L-Ile, exogenous L-Leu, and L-Dab had the same trend of influence on the *pmxA–pmxE* genes, suggesting that the exogenous amino acids did not directly affect the expression ability of *pmxA–pmxE* but influenced the gene cluster of polymyxin through potential signaling and regulatory events.

NRPS is activated by 4′-phosphopantetheinyl transferase (Sfp), encoded by *sfp* [32], and L-Dab is synthesized from L-asp-semialdehyde (L-Asp) by EctB which is encoded by *ectB* [33]. The unchanged expression levels of *sfp* and *ectB* suggest that the exogenous supplementation of amino acids did not significantly affect the transcription of *sfp* and *ectB* genes. The relative expression levels of *abrB* increased with the addition of L-Ile and L-Dab. The *pmxA* expression is negatively regulated by the DNA-binding protein, AbrB encoded by *abrB* [33]. However, the expression of *abrB* is negatively regulated by the DNA-binding protein, Spo0A encoded by *spo0A* [34]. The relative expression levels of *spo0A* increased with the addition of L-Leu, L-Ile, L-Dab, and co-addition of L-Leu and L-Ile, resulting in the transcription repression of *abrB*.

3.1.2. Supply of 2-methylbutyric acid

The synthesis of polymyxin B1 requires 6-MOA as a precursor fatty acid, which is metabolized from L-Ile (Fig. 1D); 2-methylbutyric acid is an intermediate of this metabolic pathway. Compared to the PPAC0 control, the level and ratio of polymyxin B1 increased with the exogenous addition of 200 μg/mL 2-methylbutyric acid in PPAC200 (Fig. 1E). The addition of 200 μg/mL 2-methylbutyric acid increased the level of polymyxin B1 from 0.26 g/L to 0.39 g/L and the ratio from 42.31 % to 63.9 %, confirming the precursor role of 6-MOA. In addition, the addition of 2-methylbutyric acid had a negative effect on the growth of strain PPAC (Fig. S3A). Strengthening the production of 6-MOA can promote polymyxin B1 synthesis. However, present gene editing cannot enhance the metabolic pathway of 6-MOA due to the limitations of the unclear restriction-modification system in strain PPAC. Thus, enhancing the 6-MOA synthesis by exogenous supplementation of L-Ile to the medium.

3.1.3. Supply of five precursor amino acids mixture

To increase both the proportion and production of polymyxin B1, a mixture of L-Dab, L-Thr, L-Leu, L-Ile, and D-Phe was added to the PP medium. Considering that the amount of L-Dab, L-Thr, L-Leu, L-Ile, and D-Phe is in the ratio 6:2:1:1:1 in the structure of polymyxin B1, amino acids were proportionally supplied. Group M0 was not supplied exogenously with the amino acids, whereas group M1 received 2.4 g/L of L-Dab, 0.4 g/L of L-Leu, 0.8 g/L of L-Thr, 0.4 g/L of L-Ile, and 0.4 g/L of D-Phe. The amino acid supply of groups M2-5 was 2–5 times that of group M1, respectively. The biomass of groups M3-5 decreased due to excess amino acids added (Fig. S3B). The level and ratio of polymyxin B1 positively correlated with the addition of combined amino acids, increasing from 0.15 g/L and 61.8 % in group M0 to 0.33 g/L and 79.9 % in group M3, respectively. In contrast, the level and ratio of polymyxin B1 decreased with the further increase of amino acids in groups M4 and M5 (Fig. 1F). Therefore, the optimal amounts of amino acids were 7.2 g/L L-Dab, 1.2 g/L L-Leu, 2.4 g/L L-Thr, 1.2 g/L L-Ile, and 1.2 g/L D-Phe.

The relative transcriptional levels of biosynthesis-related genes in different media M0, M1, and M3 were further analyzed. As shown in Fig. S4, compared to the control medium M0, the relative expression of *pmxA*, *pmxB*, *pmxC*, and *pmxD* increased in the medium M3. Relative expression of *ectB*, *sfp*, *spo0A*, and *abrB* in the media M0, M1, and M3 was not significantly different. Therefore, an increase in polymyxin production by exogenous amino acid mixtures is partly correlated with increased expression of the NRPS gene cluster.

3.2. Construction of a co-culture system for polymyxin B production

3.2.1. Optimization of inoculation time of strains PPAC and C. glutamicum

To increase polymyxin B1 production with amino acid precursors, *C. glutamicum* ATCC 13032 and PPAC were co-cultivated (Fig. 2A). The inoculation ratio of strains PPAC and *C. glutamicum* ATCC 13032 was 0.5:0.25 (at OD_{600}). The effect of the inoculation time of strains *C. glutamicum* ATCC 13032 and PPAC on polymyxin B1 production was studied. Strain PPAC was first inoculated in PP medium for 0, 4, and 8 h before *C. glutamicum* ATCC 13032 inoculated in above co-culture, respectively. Polymyxin B1 production was the highest (0.28 g/L) than that simultaneous inoculation (at 0 h) of strains PPAC and *C. glutamicum* ATCC 13032, increasing 0.16-fold compared with that in pure culture (Fig. 2B). Microscopic analysis of 12- and 48-h fermentation samples showed that *C. glutamicum* ATCC 13032 was relatively scarce in the co-culture (Fig. S5A). Cellular growth trends in the co-culture and pure culture were almost consistent, and the highest biomass of *C. glutamicum* ATCC 13032 was ~11.8 (OD_{600}) at 96 h (Fig. 2C1), indicating that the biomass of *C. glutamicum* ATCC 13032 and strain PPAC can be improved by optimizing the culture medium.

In *C. glu*-PP medium, polymyxin production in strain PPAC at an initial inoculation OD_{600} of 0.5 was higher than that at OD_{600} 2.0, indicating that an excessive initial inoculation dose inhibits the production of polymyxin B1. Strain *C. glutamicum* ATCC 13032 was first inoculated in PP medium for 0, 4, and 8 h before strain PPAC inoculated in above co-culture, respectively. The highest production of polymyxin B1 in the co-culture was 0.54 g/L when strains PPAC and *C. glutamicum*

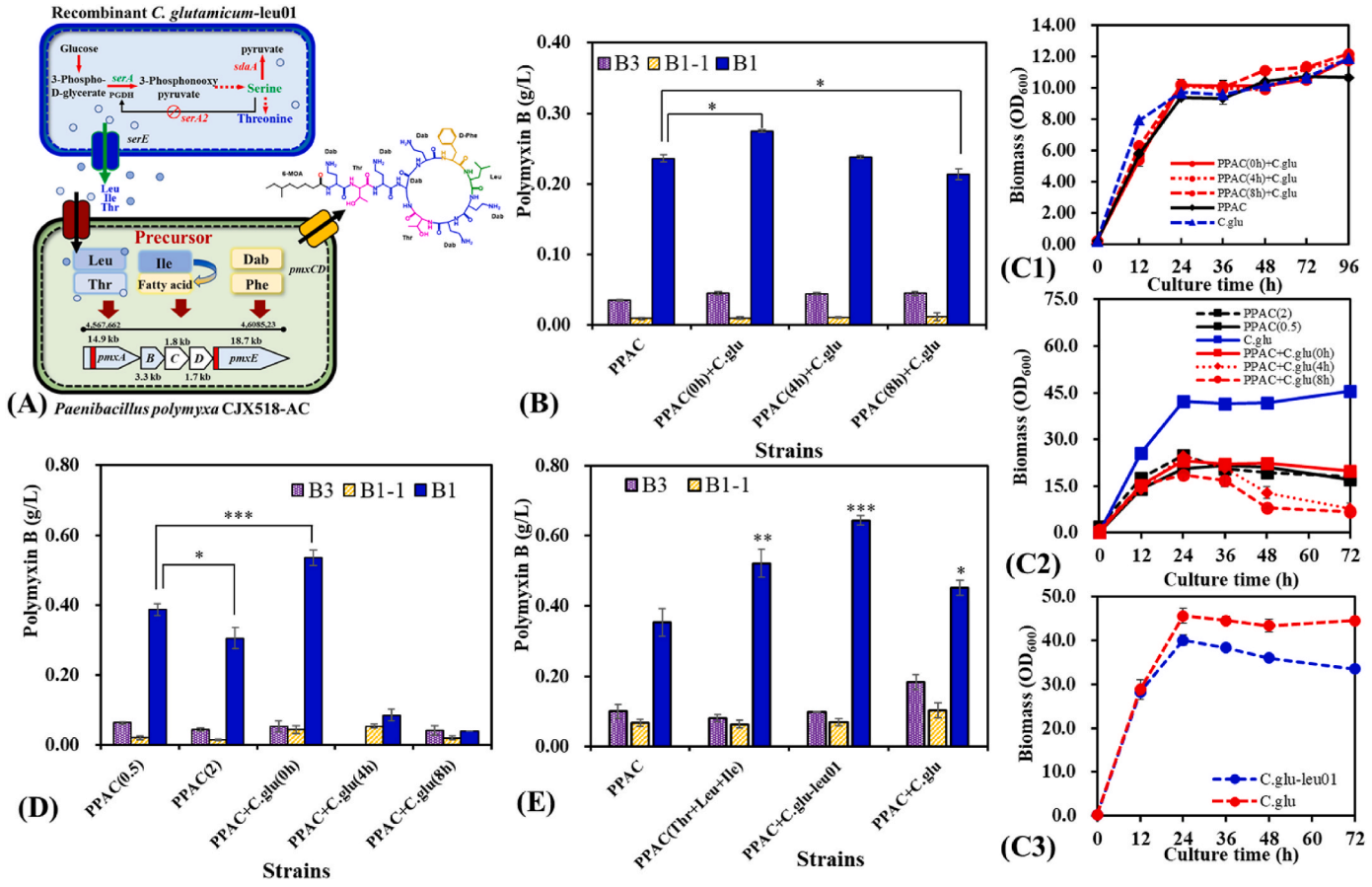

Fig. 2. Effects of inoculation time on biomass and polymyxin B production after co-culture of strains PPAC and *Corynebacterium glutamicum*. (A) Diagram of strains PPAC and *C. glu*-leu01, (B) Polymyxin B production after inoculation of strain PPAC seed into the culture medium before inoculation of *C. glutamicum* seed, (C) Growth curves of the pure culture of strain PPAC, co-culture, and *C. glutamicum* pure culture, (D) Polymyxin production after inoculation of *C. glutamicum* seed into the culture medium before inoculation of strain PPAC seed, (E) Polymyxin B production in different co-culture systems, PPAC(Thr + Leu + Ile) means adding 0.41 g/L L-Thr, 0.55 g/L L-Leu, and 0.40 g/L L-Ile to the pure culture of strain PPAC. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ATCC 13032 were the simultaneously inoculated into the co-culture at 0 h, 0.38-fold higher than that in the pure culture (0.39 g/L) (Fig. 2D). The lowest production of polymyxin B1 was obtained in the co-culture when the seed of *C. glutamicum* ATCC 13032 was first cultured for 4 and 8 h before strain PPAC inoculation.

Microscopic analysis of 48-h fermentation samples after strain PPAC inoculated into the co-culture system showed that the presence of both strain PPAC and *C. glutamicum* (Fig. S5B). The total biomass after 24 h significantly decreased when strain *C. glutamicum* ATCC 13032 was first inoculated in PP medium for 4 and 8 h before strain PPAC. It is likely that strain PPAC cells is inhibited and further result in decrease of polymyxin B1 synthesis. One possible reason is that a large amount of nutrients was exhausted in the priority inoculation of *C. glutamicum* ATCC 13032 (Fig. 2C2). Therefore, the inoculation size ratio (0.50: 0.25) and simultaneous cultivation of strains PPAC and *C. glutamicum* ATCC 13032 in the co-culture was carried out in following work, respectively.

3.2.2. Construction of co-culture system with strains PPAC and recombinant C. glutamicum

Previous work has been confirmed that recombinant strain *C. glutamicum*-leu01 (*C. glu*-leu01) enhanced the expression of *serE* gene, which is responsible for the efflux of L-Ser and L-Thr [35]. It was also found that the L-Thr level of strain *C. glu*-leu01 reached 0.41 g/L at 72 h in *C.glu*-PP medium. To further increase the supply of precursor amino acids, recombinant strain *C. glutamicum*-leu01 (*C. glu*-leu01), producing higher levels of L-Thr, L-Leu, and L-Ile, was used in the co-culture of strain PPAC. Due to the metabolic burden of recombinant strain *C. glu*-leu01, the biomass of strain *C. glu*-leu01 was lower than that of *C. glutamicum* ATCC 13032 after 12 h of cultivation (Fig. 2C3). The overexpression of *sdaA* gene with P*tuf* promoter enhanced the metabolic pathway of L-Ser conversion to pyruvate, providing additional essential precursors for the synthesis of L-Leu and L-Ile, resulting in the levels of L-Leu and L-Ile reaching 0.55 g/L and 0.40 g/L, respectively.

As shown in Fig. 2E, the production of polymyxin B1 in the co-culture with strain *C. glu*-leu01 increased to 0.64 g/L, a 0.68-fold higher than that in the pure culture of strain PPAC. It is possible related to increases of L-Thr, L-Leu, and L-Ile syntheses by strain *C. glu*-leu01, which results in enhance of polymyxin B1 synthesis. To simulate effects of the amino acid accumulation of *C. glu*-leu01 on polymyxin B1 production and potential relationship between strains *C. glu*-leu01 and PPAC in the co-culture, 0.41 g/L L-Thr, 0.55 g/L L-Leu, and 0.40 g/L L-Ile were simultaneously added into the pure culture of strain PPAC. However, the enhancement in polymyxin B1 production was lower than that in the co-culture of strains PPAC and *C. glu*-leu01. Considering that the co-culture of *C. glutamicum* ATCC 13032 can also improve the production of polymyxin B1, while the amino acids efflux capacity of *C. glutamicum* ATCC 13032 is limited. It is inferred that the enhanced polymyxin B production in the co-culture could be due to the ability of strain PPAC to utilize other metabolites of *C. glutamicum*, such as residual sugar metabolic intermediates, hetero-acids under improvement of ε-Poly-L-lysine production by co-culture [27].

When lipopeptide production was enhanced by co-culture or the exogenous addition of nutrients, transcriptome analysis revealed overexpression of genes related to precursor metabolic pathways, which is

closely involved in the up-regulated of key enzyme genes responsible for lipopeptide synthesis, and NRPS [36,37]. Particularly, quorum sensing-related genes *oppA* and *mppA* had significant up-regulation, and then activated ComP to promote lipopeptide synthesis [37].

To further explore the metabolic relationship between strains PPAC and *C. glu*-leu01, the relative expressions of genes related to polymyxin biosynthesis in the pure culture and co-culture of strain PPAC were also investigated (Fig. S6). Compared to the pure culture of strain PPAC, the relative expression of *pmxA–pmxE* in the co-culture of strains PPAC and *C. glu*-leu01 was upregulated in the PP or *C. glu*-PP media. Increased expression of *pmxA*, *pmxB*, and *pmxE* can promote the biosynthesis of polymyxin synthase, and increased expression of *pmxC* and *pmxD* can promote the efflux of polymyxin [6]. In this work, the relative expression levels of *pmxE*, *pmxD*, and *spo0A* in the coculture system in *C. glu*-PP medium was higher than that in PP medium, indicating that medium optimization affects the expression of polymyxin biosynthesis genes.

3.3. Culture conditions optimization of strains PPAC and recombinant *C. glutamicum* in the co-culture

Fermentation parameters are crucial to produce lipopeptides, and its high productivity can reduce production costs [38]. The growth of a strain and its ability to produce valuable products is affected by oxygen supply [39]. The use of baffled shake flasks and conventional shake flasks and the flask-shaker speed of the flask are crucial for ensuring oxygen supply for aerobic strains [40,41]. Different flask-shaker speed (120–220 rpm) were studied to explore the relationship between strain growth and polymyxin production. Compared to 120 rpm, polymyxin B1 production at 140, 160, and 180 rpm increased, with an increase in biomass (Fig. 3A and B). However, polymyxin production began gradually decrease when speed exceeded 180 rpm. At optimal flask-shaker speed 180 rpm, the production and ratio of polymyxin B were 0.71 g/L and 72.3 %, respectively. The biomass of the two strains in the coculture or pure culture improved with increased flash-shaker speed, with the highest at 220 rpm (Fig. 3B).

The types and concentrations of carbon and nitrogen sources play impact roles in the synthesis of natural products [42]. To further improve polymyxin B1 production, the co-culture of strains *C. glu*-leu01 and PPAC was inoculated into different media, including *C. glu*, *C. glu*-PP, *C. glu*-PP80, and CP-M media. Among them, the contents of nitrogen, metal ions, and other nutrients in *C. glu* medium were lower than those in the other media. In addition, compared with 60 g/L glucose contained in *C. glu* and *C. glu*-PP as carbon source, *C. glu*-PP80 contained 80 g/L glucose and CP-M contained 50 g/L maltodextrin. The highest production of polymyxin B1 in the co-culture with *C. glu*-PP medium was 0.97 g/L under the shake-flask fermentation (Fig. 3C1). The highest production of polymyxin B1 was 0.77 g/L in *C. glu*-PP80 medium under the baffled shake-flask fermentation (Fig. 3C2). The biomass of the co-culture in different media in the baffled shake-flasks was higher than that in the shake-flasks before 36 h (Figs. S7A and B). The change of glucose content in the culture media (Fig. 3D) indicated that the glucose consumption in baffled shake-flasks at 24 h was higher than that in shake flasks. Glucose in the baffled shake-flask fermentation was completely consumed at 24 h (except in *C. glu*-PP80 medium), whereas it in the shake-flask fermentation was used up till 36 h. The non-positive correlation between the trend for biomass and polymyxin B1 production is due to the higher consumption of glucose in baffled shake flasks than shake flasks. This result is similar to the study by Saat et al. [41], who found that biomass increase and substrate consumption in baffled shake flasks were higher in the shake flasks. Taken together, the fermentation conditions for the co-culture of strains PPAC and *C. glu*-leu01 were as follows: shake-flask fermentation at 180 rpm in *C. glu*-PP medium.

3.4. Effect of peptone supply in culture medium on polymyxin B1 production

Polymyxin is a cyclic lipopeptide compound formed by the

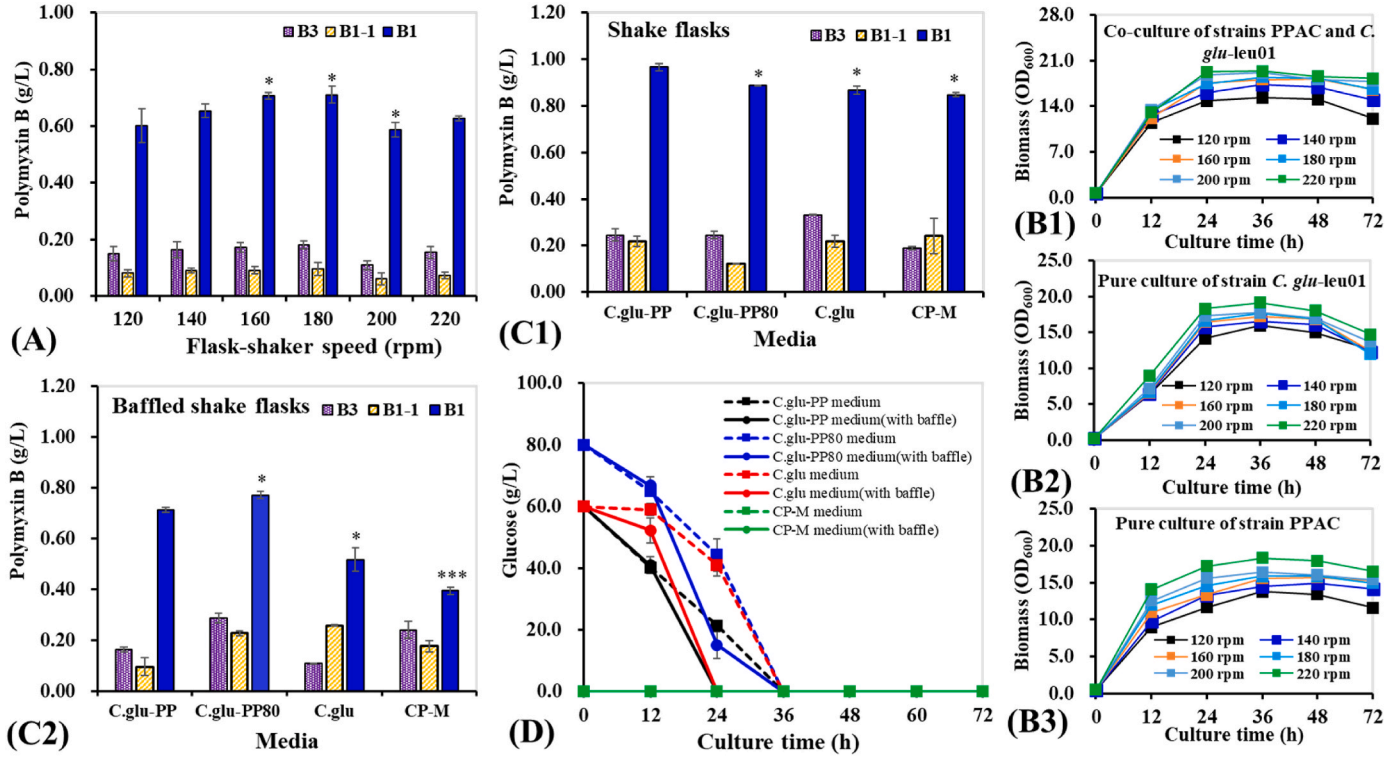

Fig. 3. Effects of fermentation conditions on polymyxin B production, glucose consumption, and biomass. (A) Production of polymyxin B under different flask-shaker speeds, (B) Growth curve of the pure culture of strain PPAC, co-culture, and *C. glutamicum* pure culture, (C) Baffled shake-flask and shake-flask fermentation, (D) Glucose content in medium. $^{*}p < 0.05$ and $^{***}p < 0.001$.

condensation and cyclization of fatty acid chains and amino acids [6]. Therefore, a nitrogen source is crucial for polymyxin synthesis by strain PPAC. A nitrogen source is required not only for amino acid synthesis and polymyxin production but also for cell growth. In *C. glu*-PP medium, peptone is the main organic nitrogen source. As the supply of peptone in the culture medium increased, the biomass of strain PPAC in pure culture or co-culture with strain *C. glu*-leu01 increased, whereas the biomass of strain *C. glu*-leu01 did not increase significantly (Fig. 4A). The increase in biomass in the co-culture was mainly affected by the growth of strain PPAC. Peptone (40–60 g/L) is effective for the growth of strain PPAC. The supply of peptone plays an essential role in the growth [43] and production [44,45] of *P. polymyxa*, and the optimal peptone supply varies for different strains. Kaziūnienė et al. [43] has reported that the biomass of *Paenibacillus* sp. MVY-024 is 36-fold higher than that without nitrogen supply when 10 g/L peptone was used as a nitrogen source. The lipopeptide production in *Paenibacillus polymyxa* Cp-S316 has increased 3.05-fold with the addition of 17.5 g/L peptone [44].

The production of polymyxin B1 in the pure culture or coculture of strain PPAC was the highest when 60.0 g/L peptone was supplied (Fig. 4B) (1.40 g/L and 1.54 g/L, respectively). Compared to the original culture medium (supplied with 20.0 g/L peptone), the polymyxin B1 production of strain PPAC in the pure culture and co-culture increased by 0.87- and 0.60-fold, respectively. The lesser the amount of peptone supplied, the lower the production of polymyxin B1. When 2.0 g/L peptone was added to the culture medium, only 0.10 and 0.11 g/L polymyxin B1 was produced in the pure culture and co-culture of PPAC, respectively. The production of polymyxin B1 in the co-culture was higher than that in the pure culture of strain PPAC with different amounts of peptone supplied.

The antibacterial activities of fermentation supernatants of strain PPAC in the pure culture and co-culture against *E. coli* DH5α were detected when media contained 5.0, 10.0, 40.0, and 60.0 g/L peptone, respectively. It was found that, whether in pure culture or in the co-culture, antibacterial activities of fermentation supernatants of strain PPAC with high concentrations of peptone (40.0 and 60.0 g/L) were higher than that with low concentrations of peptone (5.0 and 10.0 g/L) (Fig. S8A).

Exogenous mixtures of multi-precursor amino acids, including 2.4 g/L of L-Thr, 1.2 g/L of L-Leu, 1.2 g/L of L-Ile, and 4.2 g/L of L-Dab, were added to *C. glu*-PP medium containing 40.0 or 60.0 g/L peptone and polymyxin B1 production was investigated. The production of polymyxin B1 in the co-culture of strains PPAC and *C. glu*-leu01 increased to 1.23 and 1.71 g/L when 40.0 and 60.0 g/L of peptone was supplied, respectively (Fig. 5A). As shown in Fig. 5B, culture medium with 60.0 g/L peptone of strain PPAC pure culture contained 0.42 g/L of L-Thr, 0.14 g/L of L-Leu, 0.12 g/L of L-Ile, and 0.08 g/L of L-Dab at 72 h, whereas strain *C. glu*-leu01 contained 0.40 g/L of L-Thr, 0.32 g/L of L-Ile, and 0.51 g/L of L-Leu at 72 h (Fig. 5C). Thus, the increase in polymyxin B1 level is relevant to the consumption of exogenous amino acids, and the supply of L-Thr, L-Leu, and L-Ile is insufficient.

3.5. *Effect of co-culture systems of strain PPAC with multiple recombinant strains C. glutamicum*

To increase L-Thr production by *C. glutamicum*, a recombinant *C. glutamicum* strain with *metX* knockout was constructed. Gene *metX* encodes homoserine acetyltransferase, a key enzyme in the methionine synthesis pathway. The plasmid pK18-Δ*metX* was electroporated into *C. glutamicum* ATCC 13032, and *metX* was knocked out by homologous recombination to construct strain *C. glu*-thr. Compared to *C. glutamicum* ATCC 13032, the level of L-Thr in strain *C. glu*-thr was 1.10 g/L in *C. glu* medium, a 1.5-fold increase. Both *C. glutamicum*-cgb7 (*C. glu*-cgb7) and

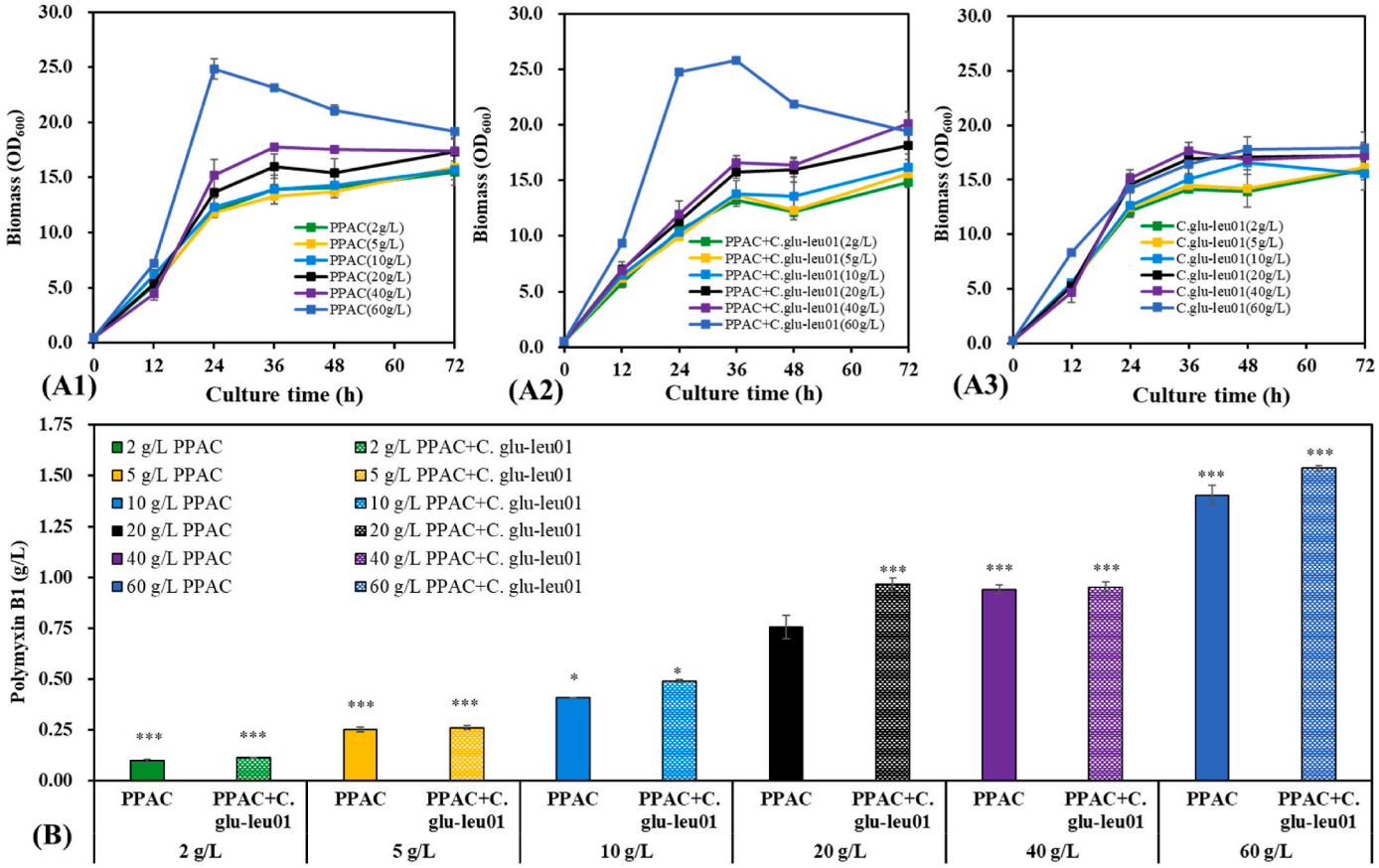

Fig. 4. Effect of peptone supply on polymyxin B1. (A) Growth curve, (B) The production of polymyxin B1. *$p < 0.05$ and ***$p < 0.001$.

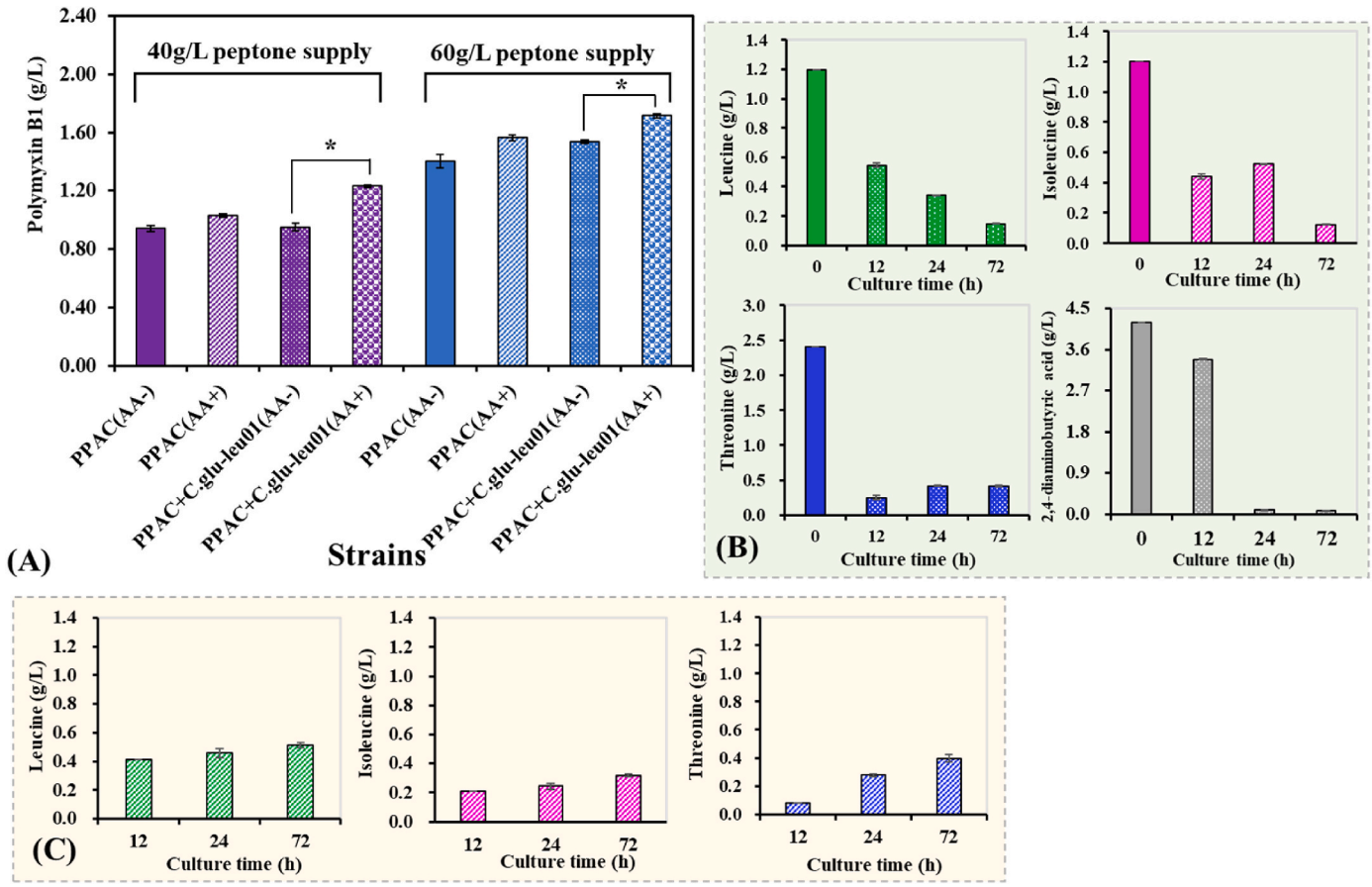

Fig. 5. The effect of precursor amino acid supply on the co-culture of PPAC and *C. glu*-leu01. (A) Polymyxin B1 production, (B) Amino acid levels in the pure culture of strain PPAC, (C) Amino acid levels in the pure culture of strain *C. glu*-leu01 without precursor supply. $*p < 0.05$.

C. glutamicum-cgb11 (*C. glu*-cgb11) increased L-Thr metabolism. The effects of strains *C. glu*-leu01, *C. glu*-thr, *C. glu*-cgb7, and *C. glu*-cgb11 co-cultivated with strain PPAC on the level of polymyxin B1 were further studied (Fig. 6A). Compared to strain PPAC in the pure culture (1.27 g/L), the level of polymyxin B1 significantly increased to 1.58, 1.57, and 1.55 g/L when strain PPAC was co-cultivated with strains *C. glu*-leu01, *C. glu*-cgb7, and *C. glu*-thr, respectively (Fig. 6B). The highest level of L-Thr in strain *C. glu*-cbg7 was 0.43 g/L at 72 h, and the highest levels of L-Leu and L-Ile in strain *C. glu*-leu01 were 0.55 and 0.36 g/L at 72 h, respectively (Fig. 6C). However, the production of amino acids was insufficient in fermentation medium than in *C. glu* medium, and the ability of *C. glutamicum* to synthesize amino acids was greatly affected by the composition of the medium and the culture environment (especially oxygen supply) [46,47].

Isopropyl-β-D-thiogalactopyranoside (IPTG) is an inducer of overexpression of plasmid pEC-*serACB* present in strain *C. glu*-leu01, which promotes the synthesis of L-Ser. The more the supply of IPTG, the lower the level of polymyxin B1 (Fig. 6B). Both biomass (Fig. S7C) and polymyxin B1 production (Fig. 6B) for strain PPAC pure culture with 0.3 mM IPTG showed no change, indicating that IPTG had no effect on strain PPAC. The growth curves of strain *C. glu*-leu01 with the addition of IPTG were consistent with the control (Fig. S7D), indicating that the addition of IPTG did not affect the growth of strain *C. glu*-leu01. The induction of strain *C. glu*-leu01 by IPTG promoted the synthesis of L-Ser. Combined with Fig. 6C, the levels of L-Thr, L-Leu, and L-Ile for strain *C. glu*-leu01 decreased, the strengthening of L-Ser synthesis pathway inhibits the metabolism of L-Thr, L-Leu, and L-Ile. The decrease in the supply of precursor amino acids reduced polymyxin B1 yield.

3.5.1. Optimization of inoculation ratio on the co-culture system

The co-culture of strains PPAC and *C. glu*-leu01 produced the highest level of polymyxin B1 in various co-culture systems. To further optimize inoculation ratio, seeds of strains PPAC and *C. glu*-leu01 were inoculated into *C. glu*-PP medium containing 60.0 g/L peptone at initial OD_{600} ratios of 0.5:0.125, 0.5:0.25, 0.5:0.5, 0.5:1, and 0.5:2. Compared to the PPAC pure culture, the level of polymyxin B1 increased at inoculation ratios 0.5:0.25, 0.5:0.5, 0.5:1, and 0.5:2. The highest level of polymyxin B1 was 2.21 g/L at inoculation ratio 0.5:1, which is 0.74-fold higher than that of the PPAC pure culture (Fig. 6D). Different inoculation ratios in a co-culture system containing vitamin B2-producing *E. coli* and *P. polymyxa* affected acetoin production [17]. Consistently, the production of polymyxin B1 has increased at optimized inoculation time and ratio in this study. Inoculation time and ratios of strains could affect the absorption and utilization of carbon and nitrogen sources, and thus affect production [24,48].

4. Conclusion

The supply of multi-precursor amino acids effectively increased the level of polymyxin B1 to 0.33 g/L, which is approximately 79.9 % of the total production of polymyxin B. Recombinant *C. glutamicum* under co-culture provided high levels of L-Thr, L-Leu, and L-Ile for polymyxin B synthesis. Optimization of fermentation media and the time and ratio of inoculation in the co-culture increased polymyxin B1 levels to 2.21 g/L, a 13.4-fold increase than that of pure culture. This study provides a new strategy to improve polymyxin B1 production.

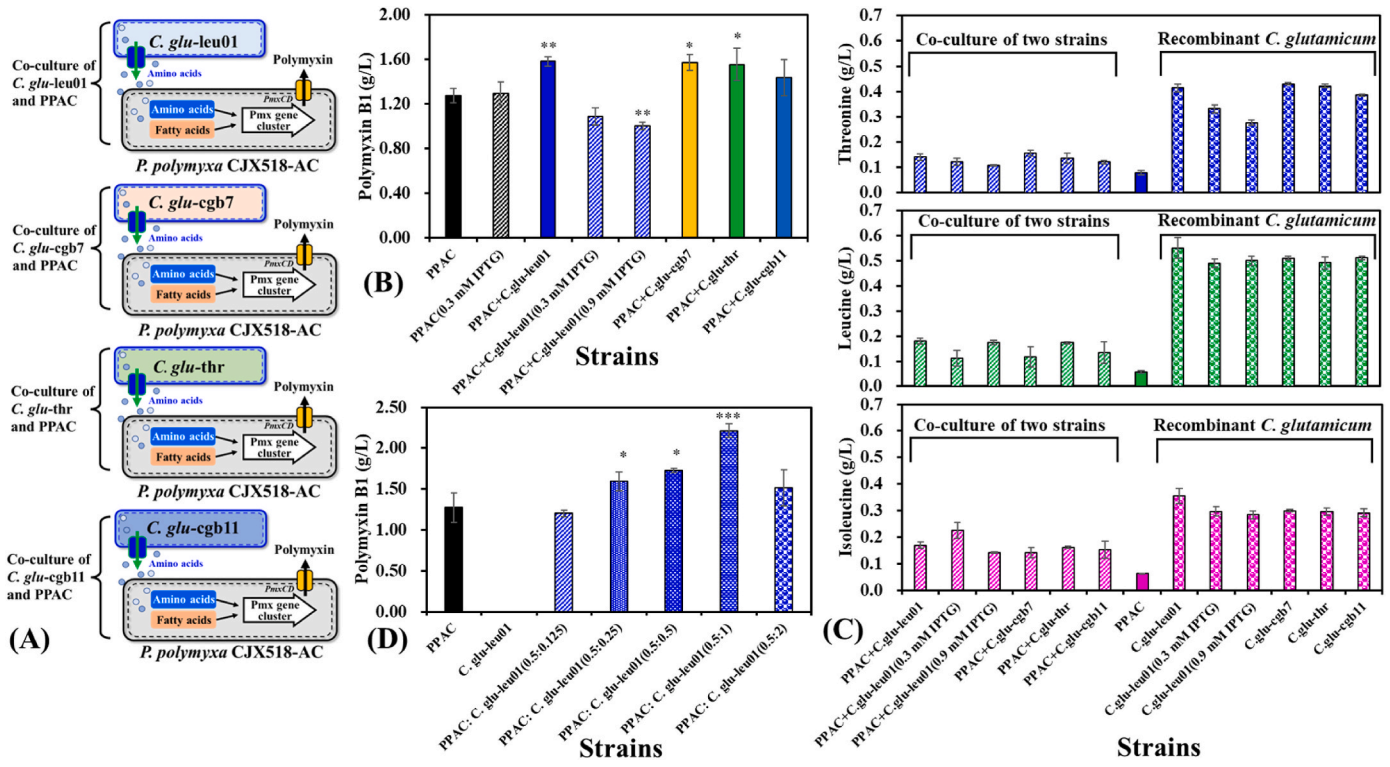

Fig. 6. Changes in polymyxin B1 and amino acid levels of multiple co-culture systems containing PPAC and recombinant *C. glutamicum*. (A) Diagram of multiple co-culture systems, (B) The production of polymyxin B1 in different co-culture system containing recombinant *C. glutamicum*, (C) Amino acid levels, (D) Effect of inoculation ratio on polymyxin B1 production. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Declaration of competing interest

No conflict of interest exists in the submission of this manuscript. The manuscript has not been published in part or in full elsewhere and is not under consideration for publication elsewhere. It is original and suitable for publishing in Synthetic and Systems Biotechnology. All the authors are aware of, and accept responsibility for, the manuscript.

CRediT authorship contribution statement

Hui-Zhong Sun: Implementing experiments, Formal analysis, Writing – original draft. **Si-Yu Wei:** Taking part in some experiments, Writing – review & editing. **Qiu-Man Xu:** Funding acquisition, Writing – review & editing. **Wei Shang:** Providing experimental assistance, Writing – review & editing. **Qing Li:** Providing experimental assistance. **Jing-Sheng Cheng:** Funding acquisition, Project administration, Supervision, Writing – review & editing. **Ying-Jin Yuan:** Funding acquisition, Project administration.

Acknowledgments

The authors are grateful for the financial supports from the National Key R&D Program of China (2018YFA0902200) and the National Natural Science Foundation of China (Program: 21878224).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.01.015.

References

[1] Brown P, Dawson MJ. Development of new polymyxin derivatives for multi-drug resistant Gram-negative infections. J Antibiot (Tokyo) 2017;70(4):386–94. https://doi.org/10.1038/ja.2016.146.

[2] Velkov T, Roberts KD, Nation RL, Thompson PE, Li J. Pharmacology of polymyxins: new insights into an 'old' class of antibiotics. Future Microbiol 2013;8(6):711–24. https://doi.org/10.2217/fmb.13.39.

[3] Li J, Milne RW, Nation RL, Turnidge JD, Smeaton TC, Coulthard K. Use of high-performance liquid chromatography to study the pharmacokinetics of colistin sulfate in rats following intravenous administration. Antimicrob Agents Chemother 2003;47(5):1766–70. https://doi.org/10.1128/AAC.47.5.1766-1770.2003.

[4] Zeitlinger MA, Derendorf H, Mouton JW, Cars O, Craig WA, Andes D, Theuretzbacher U. Protein binding: do we ever learn? Antimicrob Agents Chemother 2011;55(7):3067–74. https://doi.org/10.1128/AAC.01433-10.

[5] Sivanesan S, Roberts K, Wang J, Chea SE, Thompson PE, Li J, Nation RL, Velkov T. Pharmacokinetics of the individual major components of polymyxin B and colistin in rats. J Nat Prod 2017;80(1):225–9. https://doi.org/10.1021/acs.jnatprod.6b01176.

[6] McErlean M, Overbay J, Van Lanen S. Refining and expanding nonribosomal peptide synthetase function and mechanism. J Ind Microbiol Biotechnol 2019;46 (3–4):493–513. https://doi.org/10.1007/s10295-018-02130-w.

[7] Kraas FI, Helmetag V, Wittmann M, Strieker M, Marahiel MA. Functional dissection of surfactin synthetase initiation module reveals insights into the mechanism of lipoinitiation. Chem Biol 2010;17(8):872–80. https://doi.org/10.1016/j.chembiol.2010.06.015.

[8] Liu Q, Fan W, Zhao Y, Deng Z, Feng Y. Probing and engineering the fatty acyl substrate selectivity of starter condensation domains of nonribosomal peptide synthetases in lipopeptide biosynthesis. Biotechnol J 2020;15(2):e1900175. https://doi.org/10.1002/biot.201900175.

[9] Li S, Zhang R, Wang Y, Zhang N, Shao J, Qiu M, Shen B, Yin X, Shen Q. Promoter analysis and transcription regulation of *fus* gene cluster responsible for fusaricidin synthesis of *Paenibacillus polymyxa* SQR-21. Appl Microbiol Biotechnol 2013;97 (21):9479–89. https://doi.org/10.1007/s00253-013-5157-6.

[10] Coutte F, Niehren J, Dhali D, John M, Versari C, Jacques P. Modeling leucine's metabolic pathway and knockout prediction improving the production of surfactin, a biosurfactant from *Bacillus subtilis*. Biotechnol J 2015;10(8):1216–34. https://doi.org/10.1002/biot.201400541.

[11] Gao GR, Hou ZJ, Ding MZ, Bai S, Wei SY, Qiao B, Xu QM, Cheng JS, Yuan YJ. Improved production of fengycin in *Bacillus subtilis* by integrated strain engineering strategy. ACS Synth Biol 2022;11(12):4065–76. https://doi.org/10.1021/acssynbio.2c00380.

[12] Vahidinasab M, Adiek I, Hosseini B, Akintayo SO, Abrishamchi B, Pfannstiel J, Henkel M, Lilge L, Voegele RT, Hausmann R. Characterization of *Bacillus velezensis*

UTB96, demonstrating improved lipopeptide production compared to the strain *B. velezensis* FZB42. Microorganisms 2022;10(11):2225. https://doi.org/10.3390/microorganisms10112225.
[13] Ding L, Zhang S, Guo W, Chen X. Exogenous indole regulates lipopeptide biosynthesis in Antarctic *Bacillus amyloliquefaciens* Pc3. J Microbiol Biotechnol 2018;28(5):784–95. https://doi.org/10.4014/jmb.1712.12014.
[14] Liao G, Wang L, Liu Q, Guan F, Huang Y, Hu C. Manipulation of kynurenine pathway for enhanced daptomycin production in *Streptomyces roseosporus*. Biotechnol Prog 2013;29(4):847–52. https://doi.org/10.1002/btpr.1740.
[15] Yu Z, Guo C, Qiu J. Precursor amino acids inhibit polymyxin E biosynthesis in *Paenibacillus polymyxa*, probably by affecting the expression of polymyxin E biosynthesis-associated genes. BioMed Res Int 2015:690830. https://doi.org/10.1155/2015/690830.
[16] Galea CA, Han M, Zhu Y, Roberts K, Wang J, Thompson PE, J L, Velkov T. Characterization of the polymyxin D synthetase biosynthetic cluster and product profile of *Paenibacillus polymyxa* ATCC 10401. J Nat Prod 2017;80(5):1264–74. https://doi.org/10.1021/acs.jnatprod.6b00807.
[17] Liu L, Xu QM, Chen T, Cheng JS, Yuan YJ. Artificial consortium that produces riboflavin regulates distribution of acetoin and 2,3-butanediol by *Paenibacillus polymyxa* CJX518. Eng Life Sci 2017;17:1039–49. https://doi.org/10.1002/elsc.201600239.
[18] Willenbacher J, Yeremchuk W, Mohr T, Syldatk C, Hausmann R. Enhancement of Surfactin yield by improving the medium composition and fermentation process. Amb Express 2015;5(1):145. https://doi.org/10.1186/s13568-015-0145-0.
[19] Ganesan V, Li Z, Wang X, Zhang H. Heterologous biosynthesis of natural product naringenin by co-culture engineering. Synth Syst Biotechnol 2017;2(3):236–42. https://doi.org/10.1016/j.synbio.2017.08.003.
[20] Li J, Qiu Z, Zhao GR. Modular engineering of *E. coli* coculture for efficient production of resveratrol from glucose and arabinose mixture. Synth Syst Biotechnol 2020;7(2):718–29. https://doi.org/10.1016/j.synbio.2022.03.001.
[21] Ma Q, Zhang Q, Xu Q, Zhang C, Li Y, Fan X, Xie X, Chen N. Systems metabolic engineering strategies for the production of amino acids. Synth Syst Biotechnol 2017;2(2):87–96. https://doi.org/10.1016/j.synbio.2017.07.003.
[22] Zha J, Zhao Z, Xiao Z, Eng T, Mukhopadhyay A, Koffas MA, Tang YJ. Biosystem design of *Corynebacterium glutamicum* for bioproduction. Curr Opin Biotechnol 2023;79:102870. https://doi.org/10.1016/j.copbio.2022.102870.
[23] Gao GR, Wei SY, Ding MZ, Hou ZJ, Wang DJ, Xu QM, Cheng JS, Yuan YJ. Enhancing fengycin production in the co-culture of *Bacillus subtilis* and *Corynebacterium glutamicum* by engineering proline transporter. Bioresour Technol 2023;383:129229. https://doi.org/10.1016/j.biortech.2023.129229.
[24] Chen XY, Sun HZ, Qiao B, Miao CH, Hou ZJ, Xu SJ, Xu QM, Cheng JS. Improved the lipopeptide production of *Bacillus amyloliquefaciens* HM618 under co-culture with the recombinant *Corynebacterium glutamicum* producing high-level proline. Bioresour Technol 2022;349:126863. https://doi.org/10.1016/j.biortech.2022.126863.
[25] Vortmann M, Stumpf AK, Sgobba E, Dirks-Hofmeister ME, Krehenbrink M, Wendisch VF, Philipp B, Moerschbacher BM. A bottom-up approach towards a bacterial consortium for the biotechnological conversion of chitin to L-lysine. Appl Microbiol Biotechnol 2021;105(4):1547–61. https://doi.org/10.1007/s00253-021-11112-5.
[26] Sgobba E, Stumpf AK, Vortmann M, Jagmann N, Krehenbrink M, Dirks-Hofmeister ME, Moerschbacher B, Philipp B, Wendisch VF. Synthetic *Escherichia coli-Corynebacterium glutamicum* consortia for l-lysine production from starch and sucrose. Bioresour Technol 2018;260:302–10. https://doi.org/10.1016/j.biortech.2018.03.113.
[27] Pan L, Zhang CJ, Yuan XY, Zhang Y, Chen XS, Tian CZ, Zhang ZS, Tian MQ, Liao AM, Yu GH, Hui M, Zeng X, Huang JH. Improvement of ε-Poly-L-lysine production by co-culture fermentation strategy. Fermentation 2023;9:626. https://doi.org/10.3390/fermentation9070626.
[28] Yuan Y, Xu QM, Yu SC, Sun HZ, Cheng JS, Yuan YJ. Control of the polymyxin analog ratio by domain swapping in the nonribosomal peptide synthetase of *Paenibacillus polymyxa*. J Ind Microbiol Biotechnol 2020;47(6–7):551–62. https://doi.org/10.1007/s10295-020-02275-7.
[29] Cheng F, Yu H, Stephanopoulos G. Engineering *Corynebacterium glutamicum* for high-titer biosynthesis of hyaluronic acid. Metab Eng 2019;55:276–89. https://doi.org/10.1016/j.ymben.2019.07.003.
[30] Chen Q, Wang Q, Wei G, Liang Q, Qi Q. Production in *Escherichia coli* of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) with differing monomer compositions from unrelated carbon sources. Appl Environ Microbiol 2011;77(14):4886–93. https://doi.org/10.1128/AEM.00091-11.
[31] Payne JA, Schoppet M, Hansen MH, Cryle MJ. Diversity of nature's assembly lines-recent discoveries in non-ribosomal peptide synthesis. Mol Biosyst 2016;13(1):9–22. https://doi.org/10.1039/c6mb00675b. 20.
[32] Sunbul M, Marshall NJ, Zou Y, Zhang K, Yin J. Catalytic turnover-based phage selection for engineering the substrate specificity of Sfp phosphopantetheinyl transferase. J Mol Biol 2009;387(4):883–98. https://doi.org/10.1016/j.jmb.2009.02.010.
[33] Park SY, Choi SK, Kim J, Oh TK, Park SH. Efficient production of polymyxin in the surrogate host *Bacillus subtilis* by introducing a foreign *ectB* gene and disrupting the *abrB* gene. Appl Environ Microbiol 2012;78(12):4194–9. https://doi.org/10.1128/AEM.07912-11.
[34] Fujita M, González-Pastor JE, Losick R. High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. J Bacteriol 2005;187(4):1357–68. https://doi.org/10.1128/JB.187.4.1357-1368.2005.
[35] Zhang X, Gao Y, Chen Z, Xu G, Zhang X, Li H, Shi J, Koffas MAG, Xu Z. High-yield production of L-serine through a novel identified exporter combined with synthetic pathway in *Corynebacterium glutamicum*. Microb Cell Fact 2020;19(1):115. https://doi.org/10.1186/s12934-020-01374-5.
[36] Lu H, Xu H, Yang P, Bilal M, Zhu S, Zhong M, Zhao L, Gu C, Liu S, Zhao Y, Geng C. Transcriptome analysis of *Bacillus amyloliquefaciens* reveals fructose addition effects on fengycin synthesis. Genes 2022;13(6):984. https://doi.org/10.3390/genes13060984. 31.
[37] Liu W, Wang J, Zhang H, Qi X, Du C. Transcriptome analysis of the production enhancement mechanism of antimicrobial lipopeptides of *Streptomyces bikiniensis* HD-087 by co-culture with *Magnaporthe oryzae* Guy11. Microb Cell Fact 2022;21(1):187. https://doi.org/10.1186/s12934-022-01913-2. 10.
[38] Biniarz P, Coutte F, Gancel F, Łukaszewicz M. High-throughput optimization of medium components and culture conditions for the efficient production of a lipopeptide pseudofactin by *Pseudomonas fluorescens* BD5. Microb Cell Fact 2018;17(1):121. https://doi.org/10.1186/s12934-018-0968-x.
[39] Losen M, Frölich B, Pohl M, Büchs J. Effect of oxygen limitation and medium composition on *Escherichia coli* fermentation in shake-flask cultures. Biotechnol Prog 2004;20(4):1062–8. https://doi.org/10.1021/bp034282t.
[40] Meier K, Klockner W, Bonhage B, Antonov E, Regestein L, Buchs J. Correlation for the maximum oxygen transfer capacity in shake flasks for a wide range of operating conditions and for different culture media. Biochem Eng J 2016;109:228–35. https://doi.org/10.1016/j.bej.2016.01.014.
[41] Saat MN, Annuar MS, Alias Z, Chuan LT, Chisti Y. Modeling of growth and laccase production by *Pycnoporus sanguineus*. Bioproc Biosyst Eng 2014;37(5):765–75. https://doi.org/10.1007/s00449-013-1046-8.
[42] Wang Q, Ye H, Sen B, Xie Y, He Y, Park S, et al. Improved production of docosahexaenoic acid in batch fermentation by newly-isolated strains of *Schizochytrium* sp. and Thraustochytriidae sp. through bioprocess optimization. Synth Syst Biotechnol 2018;3(2):121–9. https://doi.org/10.1016/j.synbio.2018.04.001.
[43] Kaziūnienė J, Mažylytė R, Krasauskas A, Toleikienė M, Gegeckas A. Optimizing the growth conditions of the selected plant-growth-promoting rhizobacteria *Paenibacillus* sp. MVY-024 for industrial scale production. Biology 2022;11(5):745. https://doi.org/10.3390/biology11050745.
[44] Wang ZW, Liu XL. Medium optimization for antifungal active substances production from a newly isolated *Paenibacillus* sp. using response surface methodology. Bioresour Technol 2008;99(17):8245–51. https://doi.org/10.1016/j.biortech.2008.03.039.
[45] Li O, Lu C, Liu A, Zhu L, Wang PM, Qian CD, Jiang XH, Wu XC. Optimization and characterization of polysaccharide-based bioflocculant produced by *Paenibacillus elgii* B69 and its application in wastewater treatment. Bioresour Technol 2013;134:87–93. https://doi.org/10.1016/j.biortech.2013.02.013.
[46] Coello N, Montiel E, Concepcion M, Christen P. Optimisation of a culture medium containing fish silage for L-lysine production by *Corynebacterium glutamicum*. Bioresour Technol 2002;85(2):207–11. https://doi.org/10.1016/s0960-8524(02)00084-6.
[47] Liu Q, Zhang J, Wei XX, Ouyang SP, Wu Q, Chen GQ. Microbial production of L-glutamate and L-glutamine by recombinant *Corynebacterium glutamicum* harboring *Vitreoscilla hemoglobin* gene *vgb*. Appl Microbiol Biotechnol 2008;77(6):1297–304. https://doi.org/10.1007/s00253-007-1254-8.
[48] Wen Z, Wu M, Lin Y, Yang L, Lin J, Cen P. Artificial symbiosis for acetone-butanol-ethanol (ABE) fermentation from alkali extracted deshelled corn cobs by co-culture of *Clostridium beijerinckii* and *Clostridium cellulovorans*. Microb Cell Fact 2014;13(1):92. https://doi.org/10.1186/s12934-014-0092-5.