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Review Article

Microbial synthesis of bacitracin: Recent progress, challenges, and prospects

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ABSTRACT

Microorganisms are important sources of various natural products that have been commercialized for human medicine and animal healthcare. Bacitracin is an important antibacterial natural product predominantly produced by *Bacillus licheniformis* and *Bacillus subtilis*, and it is characterized by a broad antimicrobial spectrum, strong activity and low resistance, thus bacitracin is extensively applied in animal feed and veterinary medicine industries. In recent years, various strategies have been proposed to improve bacitracin production. Herein, we systematically describe the regulation of bacitracin biosynthesis in genus *Bacillus* and its associated mechanism, to provide a theoretical basis for bacitracin overproduction. The metabolic engineering strategies applied for bacitracin production are explored, including improving substrate utilization, using an enlarged precursor amino acid pool, increasing ATP supply and NADPH generation, and engineering transcription regulators. We also present several approaches of fermentation process optimization to facilitate the industrial large-scale production of bacitracin. Finally, the challenges and prospects associated with microbial bacitracin synthesis are discussed to facilitate the establishment of high-yield and low-cost biological factories.

1. Introduction

Natural products played the critical roles in maintaining human health, and have been broadly applied in biomedicine, cosmetics, agriculture and chemical industry [1]. Natural products comprise a large range of compounds with various biological activities, and mostly originate from plants, marine animals, and microorganisms [2]. In recent years, microbial synthesis of natural products has attracted widespread attentions, owing to their short fermentation periods, mild reaction conditions, and sustainability [3]. However, the ability of most wild microorganisms to produce natural products is fairly poor, and traditional strain screening techniques are usually inefficient, thereby failing to meet the requirements for commercialization, and restricting further industrial production [4,5]. With the expansion of synthetic biology and metabolic engineering, large amounts of biological information have been analyzed, thus evolving the concepts of "building to understand" to "building to apply," and facilitating advances benefiting humanity. Through advanced synthetic biology technologies, the Institute of Microbiology, Chinese Academy of Sciences, has increased the avermectin yield by 1000 times to 9 g/L, thereby leading to the rapid development of avermectin industry [6]. In addition, a multivariate modular approach has been developed to increase the green surfactin titer to 12.8 g/L in *Bacillus subtilis* 168, with a sucrose yield of 65.0 mmol/mol [7]. Future researches are expected to overcome the problem, in which more natural products are being discovered yet fewer industrial applications.

Bacitracin, originally isolated from *Bacillus licheniformis* and *B. subtilis*, is a broad-spectrum cyclopeptide antibiotic complex comprising diverse amino acids, including L-histidine, L-asparagine, L-lysine (L-Lys), D-ornithine (D-Orn), D-aspartate (D-Asp), D-phenylalanine

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(D-Phe), D-glutamate (D-Glu), L-cysteine (L-Cys), L-leucine (L-Leu), L-isoleucine (L-Ile), and L-valine (L-Val) [8–10]. Bacitracin contains more than ten components (including bacitracin A, B_1, B_2, B_3, D_1, D_2, D_3.) that differ by one or two constituent amino acids, among which, bacitracin A shows the strongest biological activity [11]. Bacitracin possesses excellent characteristics of broad antimicrobial spectrum, rapid excretion rate, low livestock absorption, not easy to develop resistance and it restrains the synthesis of cell wall of most gram-positive and some of gram-negative bacteria. Thus, bacitracin has been applied extensively in the animal feed and medical industries for more than 50 years [12]. In 2017, the global market for bacitracin products reached 312 million dollars, and methylene salicylic acid bacitracin owned the largest market share.

Although the industrialization of bacitracin is relatively mature, high costs, low yields, and complex waste disposal procedures hinder its further applications [13]. Recently, various methods have been developed to engineer *Bacillus* for enhanced production of bacitracin (see Table 1). This review aims to provide deeper insights into recent progress in microbial synthesis of bacitracin, including the underlying mechanisms associated with bacitracin biosynthesis, metabolic engineering strategies in pathway modification, and optimization of fermentation processes. In addition, we discuss the challenges affecting the large-scale production of bacitracin and future prospects for microbial production of bacitracin.

2. Regulation and associated mechanisms of bacitracin biosynthesis in *bacillus*

Similar to other polypeptides produced by *Bacillus*, bacitracin is synthesized by nonribosomal peptide synthetases (NRPSs) through a thiotemplate mechanism [14]. The bacitracin synthetase gene cluster comprises *bacABC* operon and type II thioesterase gene *bacT*, and the function of latter has not been well analyzed [15]. Recently, Wu et al. demonstrated that *bacT* expression positively correlated with bacitracin synthesis according to transcriptomic analysis, and overexpression of *bacT* increased bacitracin A yield by 37.50% [16]. The bacitracin synthetase gene cluster *bacABC* is located downstream of *bacT*, among which, BacA (598 kDa) activates and polymerizes four amino acids (L-Ile, L-Leu, L-Cys, and D-Glu) in the tail of bacitracin, BacB (291 kDa) and BacC (723 kDa) are responsible for the activation and amidation of remaining seven amino acids [17,18] (Fig. 1). In addition, bacitracin contains four D-type amino acids (D-Glu, D-Orn, D-Phe, and D-Asp), which are obtained via isomerization of corresponding L-type amino acids. The biosynthesis of bacitracin is regulated by complex mechanisms. Wang et al. revealed that a feedback loop was formed by global transcription factors Spo0A and AbrB to regulate bacitracin biosynthesis in *B. licheniformis* DW2 [19]. Specifically, as shown in Fig. 1, AbrB repressed transcription of bacitracin synthase operonby directly binding the *bacABC* promoter, and Spo0A promoted bacitracin production by repressing *abrB* expression. After the knockout of *abrB* in *B. licheniformis* DW2, bacitracin produced by per colony-forming unit was increased by 17.5%. In addition, the Spo0A-AbrB regulation model was also illustrated in the biosynthesis of other antibiotics, such as bacilysin and polymyxin [20,21].

During the long-term evolution, bacteria have developed the efficient stress response systems to survive in adverse environments [22]. ATP-binding cassette (ABC) transporters and neighboring two-component systems (TCSs) are considered as the most sensitive and efficient modules for resisting antimicrobial peptides in many Firmicutes bacteria [23]. In *B. licheniformis* 9945a, bacitracin ABC transporter BcrABC comprises the ATP-binding protein BcrA (306 kDa) and two transmembrane proteins, BcrB (208 kDa) and BcrC (203 kDa) [24]. The genes *bacR* and *bacS* encoding for TCS BacRS are located between gene clusters *bacABC* and *bcrABC* [25]. The BacRS system modulates the

Table 1
Summary of metabolic engineering strategies for bacitracin production in *B. licheniformis*.

Strains and genotypes	Strategies	Effects	References
Strengthening utilization ratio of substances			
B. licheniformis DW2Δ*PaprA*	Enhancing the consumption ratio of soybean meal	Soybean meal utilization ratio increased by 28.86%; bacitracin titer increased by 18.92%	Cai et al., 2019a
Strengthening precursor amino acids			
B. licheniformis DW2-ASP10 (Δ*yveA*,Δ*aspA*,Δ*malS*, *aspD**, *ansB**, *pycA**, *ecaA**)	Strengthening the supply of aspartic acid	Aspartic acid titer increased by 394.47%; bacitracin titer increased by 40.10%;	Zhu et al., 2021
B. licheniformis DW2-LYS5 (Δ*yaaO*,Δ*lysE*,*ddh**, *pycA**, *lysA**, *lysP**, *yvsH**)	Strengthening lysine supply	Lysine titer increased by 52.78%; Bacitracin titer increased by 28.95%;	Wu et al., 2019
B.licheniformis DW2Δ*proB*Δ*proJ*Δ*argR:ppnk1*	Strengthening ornithine supply	Ornithine titer increased by 71.40%; Bacitracin titer increased by 16.58%;	Yu et al., 2019
B.licheniformis DW2-CYS4 (*cysK**, *cysE**, *cysP**, *tcyP**)	Strengthening cysteine supply	Cysteine titer increased by 46.36%; bacitracin titer increased by 21.10%;	Li et al., 2021
B.licheniformis DW2-KENPND (Δ*metN*,Δ*speD*,Δ*mtnN*, *metK**, *metH**, *metP**)	Strengthening S-Adenosylmethionine supply	SAM titer increased by 156%; bacitracin titer increased by 28.97%;	Cai et al., 2020a
B.licheniformis DW-BCAA6 (Δ*lrpC*, *ilvBN*^{fbr}*, *leuA*^{fbr}*,*ybgE**, *yvbW**, *braB**)	Strengthening the supplies of branched chain amino acids	Isoleucine, leucine and valine increased by 226%, 190% and 72%, respectively; bacitracin titer increased by 36.52%	Cai et al., 2020b
B.licheniformis DW2Δ*lrp:brnQ*	Strengthening the supplies of branched chain amino acids	Isoleucine, leucine and valine increased by 176.04%, 162.04%, 42.77%, respectively; bacitracin titer increased by 22.42%	Zhu et al., 2018
B.licheniformis DW2Δ*yhdG*	Strengthening the supplies of branched chain amino acids	Isoleucine, leucine and valine increased by 627%, 590%, 536%, respectively; bacitracin titer increased by 22.42%	Li et al., 2018
Strengthening ATP supply and NADPH generation			
B.licheniformis DW2-CQD (Δ*cydB*, *qoxA**,*dcK**)	Strengthening ATP supply	ATP content increased by 49.32%; bacitracin titer increased by 21.66%	Zhang et al., 2020
B.licheniformis BL2ST6 (*vgb**, *bacT**)	Strengthening oxygen availability and type II thioesterase	Bacitracin A titer increased by 36.84%	Wu et al., 2021
B.licheniformis DW2:*icd*	Increased the flux through TCA cycle	ATP content increased by 73.7%; bacitracin titer increased by 11.5%	Liu et al., 2018
B.licheniformis DW2:*zwf*	Strengthening NADPH generation	NADPH content increased by 61.24%; bacitracin titer increased by 12.43%	Zhu et al., 2019
Transcription factor engineering			
B. licheniformis DW2Δ*abrB*	Over-expression of *bacA* by inactivation of AbrB	Bacitracin titer increased by 17.50%;	Wang et al., 2017
B.licheniformis DW2-CNCTGP (Δ*ccpC*,Δ*ccpN*,Δ*phoP*, *tnrA**, *codY**, *glnR**)	Transcription factor engineering	ATP content increased by 60.65%; NADPH content increased by 54.74%; bacitracin titer increased by 35.72%	Cai et al., 2019b

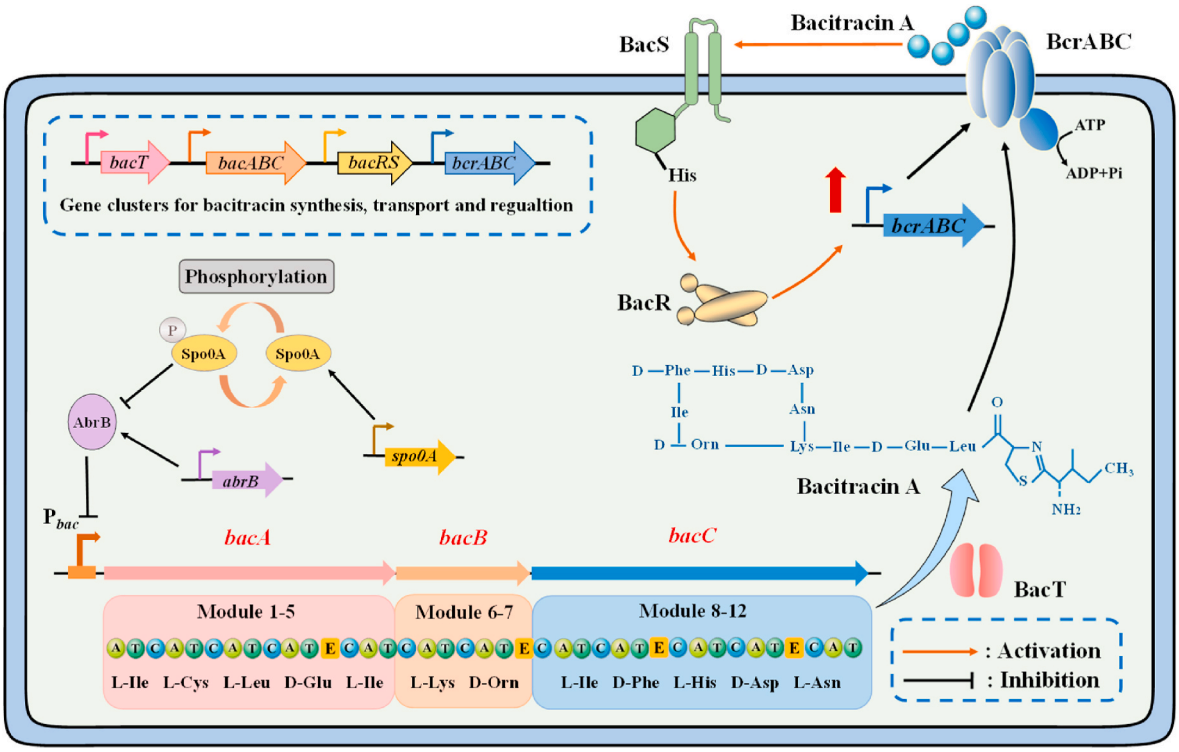

Fig. 1. Schematic representation of bacitracin biosynthesis, transport and regulatory network (Take bacitracin A as an example). *bacABC*, encoding for bacitracin synthetases; *bacT*, encoding for type II thioesterase; *bacRS*, encoding for two-component regulatory system that composed of a response regulator (BacR) and a sensory kinase (BacS); *bcrABC*, encoding for bacitracin ATP binding cassette (ABC) transporter system; *spo0A*, encoding for stage 0 sporulation protein A; *abrB*, encoding for global transcription regulator AbrB.

activation of *bcrABC* and its own expression, after sensing the accumulation of bacitracin [26] (Fig. 1). Thus, BacRS and BcrABC play critical roles in conferring resistance against bacitracin. Podlesek et al. have enhanced bacitracin resistance by more than 150% through increasing the copy number of *bcrABC* cluster in *B. licheniformis* ATCC 10716, thus also benefited bacitracin synthesis [27]. In addition, the bacitracin resistance network in *B. subtilis* involves four signal transduction mechanisms comprising TCSs BceRS, YvcPQ, and LiaRS, and the extracytoplasmic functioning SigM [28]. The TCS BceRS and its relevant ABC transporter BceAB directly and efficiently mediate the response of hosts to bacitracin, and YvcPQ is cross-activated by paralogous BceRS–BceAB system [29,30]. LiaRS and SigM function only under the condition with high concentration of bacitracin [31,32]. However, no studies have focused on the manipulation of resistance mechanism of *B. subtilis* for enhanced production of bacitracin.

3. Metabolic engineering for efficient production of bacitracin

The traditional microbial breeding methods for the industrial production of target metabolite mainly involve random mutagenesis and selection, however, these time-consuming methods usually lead to uncertain genotypic and phenotypic alterations [33]. The genomics revolution of early 21st century gave rise to the second biotechnology revolution, which contributed to the in-depth understanding of metabolic pathways and regulatory networks in microorganisms [34,35]. Thus, rational metabolic engineering has gradually become an indispensable approach for developing hyper-producers.

After engineering *Bacillus* to efficiently produce bacitracin for nearly 20 years, our group has proposed four main tactics: access, pull, drive, and regulate. The access tactic involves modifying the pathways that allow substrates, such as glucose and maltose, to cross the cell membrane. In particular, enough proteases must be secreted to hydrolyze the raw materials into small peptides or amino acids. The pull tactic refers to "pulling" metabolic flux toward the critical precursor amino acids serving as building blocks for bacitracin synthesis. The drive tactic involves improving the driving force to increase bacitracin synthesis, and mainly involves ATP supply and NADPH generation. The regulate tactic involves the systematic and dynamic manipulation of complicated metabolic pathways, e.g., simultaneous rewiring of several global transcription factors to facilitate bacitracin production. The order of implementing these tactics is flexible, owing to the different characteristics of bacitracin producing strains.

3.1. Strategies for improving the utilization ratios of substrates

Improving the utilization ratio of raw material aids in increasing the metabolite production effectively, thus decreasing the costs of industrial large-scale fermentation [36]. For instance, the glucose transport pathway was rewired by blocking the phosphotransferase system and reinforcing the expression levels of glucose/H^+ symporter GlcP and glucokinase GlcK in *B. subtilis*, thereby increasing N-acetylglucosamine yield by 73.84% [37]. In addition, the inhibitory effect of transcription factor SugR on phosphotransferase system was relieved, and glucose uptake and L-Leu yield were significantly increased in *Corynebacterium glutamicum* [38]. Moreover, Zhan et al. have strengthened the glycerol utilization pathway in *B. licheniformis*, and consequently increased poly-γ-glutamic acid yield by 42.96% [39]. Nevertheless, only several studies have focused on improving the utilization of substrates for bacitracin production.

The medium for bacitracin production contains 4.5% corn starch, 10% soybean meal, 0.6% $CaCO_3$, and 0.1% $(NH_4)_2SO_4$, with natural pH. Corn starch and soybean meal are commonly used raw materials for microbial production of antibiotics [40,41]. Corn starch is a polysaccharide molecule that is largely unable to cross cell membranes. Microorganisms generally secrete α-amylases to extracellular environment for starch degradation, and the obtained maltodextrins and

maltose are imported by ABC transport systems or phosphotransferase system [42,43]. Previous studies have shown that the ABC uptake systems for maltose and maltodextrins are highly conserved in bacteria [44]. Thus, several strategies have been developed to engineer starch utilization pathways for target metabolite production. For example, the expression of maltose ABC transporter MalEFG was up-regulated in *Streptomyces avermitilis*, and ivermectin yield was increased by 3.3 times, with 10% reduction of fermentation period [45]. Our team has also systematically optimized the starch utilization modules in *B. licheniformis* through strengthening the transmembrane transport of starch enzymatic hydrolysates by up-regulating the expression of permease MdxEFG, as well as MalP and GlcU, and promoting the degradation of enzymatic hydrolysates to central metabolic pathway, thereby enhancing bacitracin production in the fermentation medium with low content of corn starch (Unpublished results) (Fig. 2A).

Defatted soybean meal contains abundant proteins (approximately 45%), and has an excellent amino acid profile, and it is an important resource for precursor amino acids in bacitracin synthesis [46]. Subtilisin, an important industrial protease encoded by gene *aprE* in *B. licheniformis*, plays a critical role in the degradation and utilization of soybean meal [47]. To verify the effect of subtilisin enhancement on bacitracin synthesis, Cai et al. have strengthened the expression of gene *aprE* by repressing antisense sRNA *aprA* in *B. licheniformis* DW2, thereby increasing the soybean meal utilization ratio by 28.86% and bacitracin yield by 18.92%, respectively [48](Fig. 2A). In addition, a compound mutagenesis process involving atmospheric pressure room temperature plasma, auxiliary ethyl methanesulfonate, nitrosoguanidine, and ultraviolet mutagenesis was established to obtain a bacitracin-overproducing strain. The results of comparative genomics indicated that mutations in the oligopeptide permease OppB and serine protease DegP/HtrA might be beneficial for bacitracin production [49]. Therefore, heterologous expression of secreted high-efficiency proteolytic enzymes is considered as a promising strategy for increasing bacitracin synthesis.

3.2. Strategies for improving the supplies of precursor amino acids

The availability of sufficient precursor amino acids is an essential requirement for bacitracin synthesis, but the capabilities of amino acids synthesis are fairly weak in *B. licheniformis*, in addition, the amino acid composition of soybean meal differs from that of bacitracin, which hindered the high-level production of bacitracin. In general, three approaches are applied to increase the intracellular accumulation of precursor amino acids: modifying biosynthetic modules, manipulating amino acid transporters, and weakening degradation pathways [50]. Firstly, the exogenous addition of bacitracin precursor amino acids L-Cys, L-Ile, L-Leu, L-Lys, L-Asp, L-Orn, and S-adenosylmethionine (SAM) benefited bacitracin synthesis, indicating that these above amino acids may be the limiting factors in bacitracin synthesis [51–54].

L-Asp is an important central metabolite that links carbon and nitrogen metabolism. By manipulating the oxaloacetate metabolic pathway, strengthening the L-Asp synthesis pathway, and deleting the ammonia lyase gene *aspA*, researchers have increased the intracellular L-Asp pool by 233.16%. Moreover, the transporter YveA was identified as a L-Asp exporter in *B. licheniformis* DW2, and deletion of *yveA* led to a 15.69% increase of intracellular L-Asp concentration, and bacitracin yield was increased to 1059.86 U/mL in the final strain DW2-ASP10 [52]. L-Lys, a member of L-aspartate family amino acids, is derived from L-Asp through eight reaction steps in *B. licheniformis* [55].To

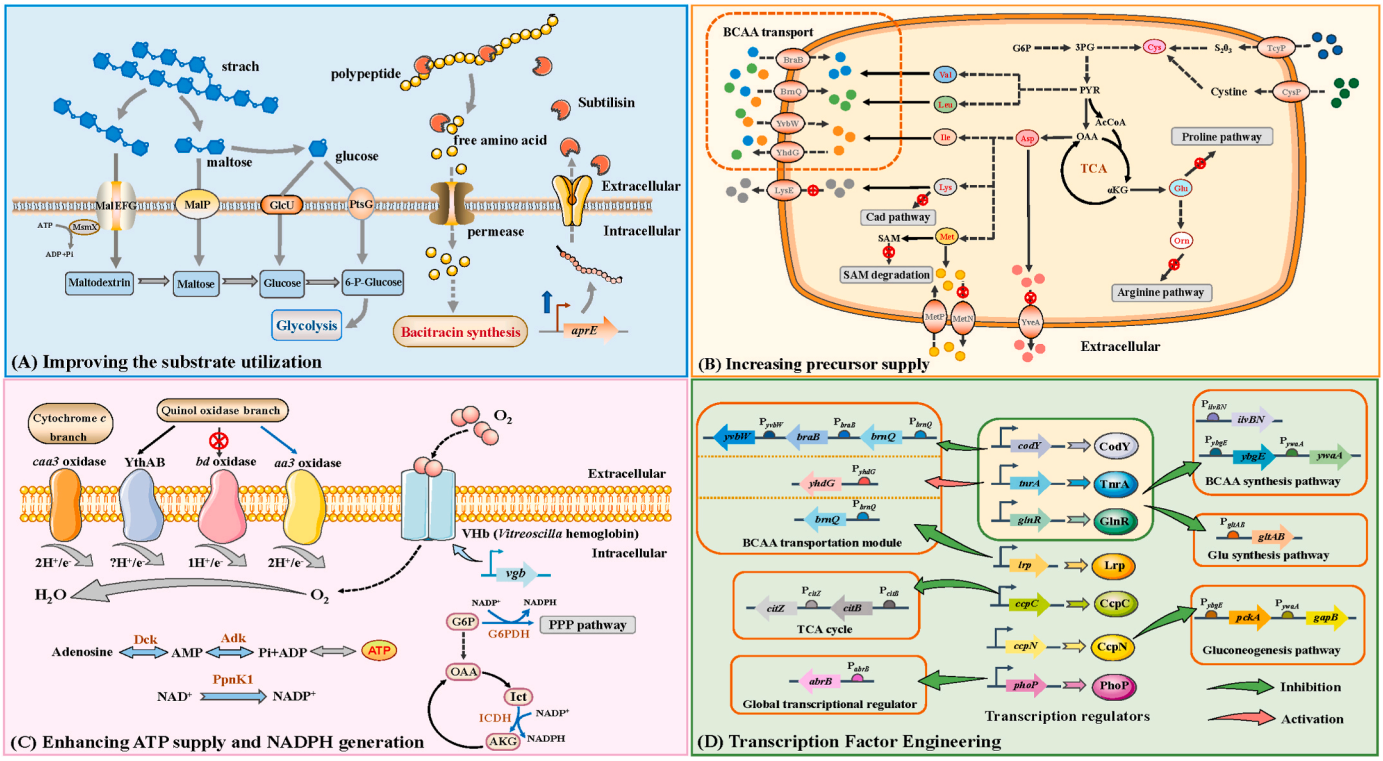

Fig. 2. Metabolic engineering tactics for improving bacitracin production in *B. licheniformis*. (A) Improving the substrate utilization. MalEFG, maltose/maltodextrin ABC transporter; MalP, maltose-specific phosphotransferase system IICB component; GlcU, glucose permease; PtsG, glucose-specific phosphotransferase system IICB; Subtilisin, serine alkaline protease encoded by *aprE*. (B) Increasing precursor supply. G6P, glucose 6-phosphate; 3 PG, 3-phospho-glycerate; PYR, pyruvate; OAA, oxaloacetate; a-KG, alpha-ketoglutaric acid; TCA, tricarboxylic acid cycle. (C) Enhancing ATP supply and NADPH generation. Dck, adenosine kinase; Adk, adenylate kinase; PpnK1, ATP-NAD kinase; VHb, *Vitreoscilla* hemoglobin. G6PDH, glucose-6-phosphate dehydrogenase; ICDH, isocitrate dehydrogenase. (D) Transcription Factor Engineering. Four BCAA permease genes, *ywbW*, *braB*, *brnQ* and *yhdG*; *citZ*, citrate synthase gene; *citB*, aconitate hydratase gene; *ilvBN*, acetolactate synthase gene; Two BCAA aminotransferase genes, *ybgE* and *ywaA*; *gltAB*, glutamate synthase gene; *pckA*, phosphoenolpyruvate carboxykinase gene; *gapB*, glyceraldehyde-3-phosphate dehydrogenase gene. Deletion of genes and blocking of pathways are indicated by "⊗".

improve economic prospects, the diaminopimelate dehydrogenase pathway from *C. glutamicum* was introduced into *B. licheniformis* to increase L-Lys yield. Subsequently, the L-Lys supply was further increased by weakening the Lys degradation pathway and engineering Lys transporters, and bacitracin yield was increased by 28.95% [56]. In *B. licheniformis*, L-Orn is synthesized from L-glutamate via four enzymatic steps, which are regulated by arginine repressor ArgR. Three strategies have been conducted to improve L-Orn synthesis, including disruption of metabolic pathway from glutamate to proline, removal of inhibitor ArgR, and overexpression of NAD kinase, which resulted in a 71.40% increase of L-Orn concentration and a 16.50% increase of bacitracin yield, respectively [57]. Furthermore, L-Cys supply was strengthened by increasing the expression of L-serine acetyltransferase gene *cysE* and L-Cys synthase gene *cysK*, as well as thiosulfate/sulfate intracellular transporter CysP, thereby alleviating oxidative stress and facilitating bacitracin synthesis simultaneously [58]. SAM is an essential methyl donor that participates in the biosynthesis of various antibiotics, such as avermectin, erythromycin, and lincomycin [59–61]. Through modification of SAM synthesis and degradation pathways, and engineering Met transporters, bacitracin yield was increased by 28.97% [51]. To increase the supply of branched-chain amino acids (BCAAs), feedback-resistant IlvBN^{fbr} and LeuA^{fbr} were simultaneously introduced into *B. licheniformis* DW2, with the overexpression of two importers (BraB and YvbW) and an aminotransferase YbgE, which led to a 31.05% increase of bacitracin yield [62]. In addition, BCAA permease YhdG was identified as a L-Ile exporter in *B. licheniformis*, in contrast to the previous results obtained in *B. subtilis* [63]. Moreover, deletion of *yhdG* has been found to increase the intracellular L-Ile concentration by 6.27 times and bacitracin yield by 11% [64]. These findings demonstrated that strengthening the supplies of precursor amino acids is an efficient strategy for bacitracin overproduction (Fig. 2B). However, how to balance the metabolic flux distribution of synthesis module of each precursor amino acid remains a challenge to be resolved.

3.3. Strategies for improving ATP supply and NADPH generation

As the essential energy resource for metabolic reactions, ATP plays a critical role in the production of various metabolites [65]. In addition, ATP functions as the substrate, product, activator, or inhibitor in diverse metabolic pathways, thus rationally engineering ATP supply is considered as an effective strategy for achieving high-level yield of target product [66]. To evaluate whether manipulating ATP supply is beneficial for bacitracin synthesis (Fig. 2C), Zhang et al. have blocked the high energy-intensive cytochrome *bd* oxidase branch and overexpressed adenosine kinase Dck in *B. licheniformis*, and bacitracin yield and ATP content were increased by 21.66% and 49.32%, respectively [67]. In addition, TCA cycle was strengthened by overexpression of citrate synthase CitZ and isocitrate dehydrogenase Icd, and the engineered strain demonstrated a high yield of bacitracin in shaker flask [68]. Moreover, increasing the dissolved oxygen content augments ATP supply and facilitates antibiotic production [69]. In particular, Wu et al. have increased the availability of oxygen via heterologous expression of *Vitreoscilla* hemoglobin VHb, thereby increasing bacitracin A titer by 126.67% under the oxygen-restricted conditions [16].

NADPH is a critical cofactor in microbial anabolism, particularly in the synthesis of amino acids [70–72]. In general, three main pathways, transhydrogenase reactions, TCA cycle, and pentose phosphate pathway, are involved in NADPH generation. Bacitracin is a polypeptide antibiotic composed of 11 types of amino acids, and most of which are produced by NADPH-dependent pathways. Thus, the supply of NADPH is considered as a crucial factor in bacitracin production (Fig. 2C). Zhu et al. observed that overexpression of glucose-6-phosphate dehydrogenase Zwf led to a 61.24% increase of NADPH concentration, as well as a 12.43% increase of bacitracin yield in *B. licheniformis* [73]. Alternatively, NADPH availability was enhanced by strengthening the expression of NAD kinase PpnK1, thus further elevating bacitracin production [57]. In addition, with the in-depth understanding of protein structure and development of rational design tools, the cofactor specificity of NADPH-dependent enzymes have been converted to NADH-dependent, and further applied in the overproduction of L-Lys, L-Val, L-Glu, which strategy may also benefit bacitracin biosynthesis [74–76].

3.4. Engineering transcription factors to increase bacitracin biosynthesis

On the basis of an in-depth understanding of microbial metabolic pathways, the efficiency of target metabolite synthesis has been improved through direct up- or down-regulation of metabolic pathways [77]. However, the complicated metabolic networks in microorganisms usually pose challenges in achieving the desired aims. For example, overexpression of one or several genes may lead to the excessive accumulation of intermediate metabolites, whereas down-regulation of genes can cause growth retardation or metabolic disorders [78]. Thus, transcription factor engineering, a powerful tool regulating multiple key genes globally and dynamically, has been developed to construct the optimal microbial chassis for target metabolite synthesis [79,80].

Transcription factors are sequence-specific proteins that usually contain the DNA-binding and regulatory domains, and can simultaneously regulate the expression of multiple genes at the transcriptional level by interacting with specific sequences in promoter regions [81]. In recent years, several transcription factors have been demonstrated to play critical roles in bacitracin synthesis in *B. licheniformis* (Fig. 2D). For example, leucine-responsive regulatory protein Lrp, which is known to alter complex cellular metabolic pathways in bacteria and archaea, has been identified as a repressor of BCAA transporter BrnQ, and bacitracin yield was increased by 14.71% in the *lrp* deletion strain [82,83]. CcpA, CcpC, and CcpN are three major carbon metabolism regulators involved in regulating carbon acquisition, TCA cycle, and gluconeogenesis pathway, respectively [84–86]. Disruption of *ccpC* and *ccpN* increased the levels of ATP and NADPH, and further facilitated bacitracin synthesis. In contrast, deleting *ccpA* significantly decreased bacitracin yield [87]. Furthermore, three nitrogen metabolism TFs, GlnR, CodY, and TnrA, have been demonstrated to serve as the efficient machinery for control of intracellular BCAA concentrations through regulating the expression of key genes (*ilvA*, *ilvBHC-leuABCD*, *ilvD*, *ybgE*, and *ywaA*) in BCAA synthetic pathways [88–90]. To strengthen the supply of BCAAs, while avoiding triggering nitrogen catabolite repression by the presence of excessive BCAAs, extra copies of genes *glnR*, *codY*, and *tnrA* were integrated into the genome of *B. licheniformis*, thus increasing bacitracin yields by 12.98%, 14.17%, and 16.20%, respectively [87]. In addition, the mediation of phosphorus metabolism by TCS PhoR–PhoP has been demonstrated to manipulate antibiotic synthesis in many antibiotic-producing bacteria [91]. In *B. licheniformis*, *phoP* deletion was confirmed as an effective strategy for enhanced production of bacitracin. In addition, transcriptional level of *abrB* was decreased by 37.13% in a *phoP*-deficient strain, however, the results of electrophoretic mobility shift assay demonstrated that regulator PhoP does not interact directly with the promoter regions of *abrB*, and the underlying mechanism is currently unclear [87].

4. Optimizing fermentation process for bacitracin production

To meet commercial production demands and decrease fermentation costs, several agricultural by-products have been used as the raw materials for industrial biochemical production, such as soybean meal, cottonseed meal, and rapeseed meal [92]. These raw materials are rich in amino acids, vitamins, and inorganic salts, and they are typical nitrogen resources used by microorganisms for antibiotics production [93]. A combination of 7% soybean meal with 2% rapeseed cake has been confirmed as an economical and optimal formula for bacitracin production, bacitracin yield was increased by 12.40% in a 50 L fermentor [53](Fig. 3). In addition, lactose supplementation was proven to be better than glucose supplementation in maintaining bacitracin

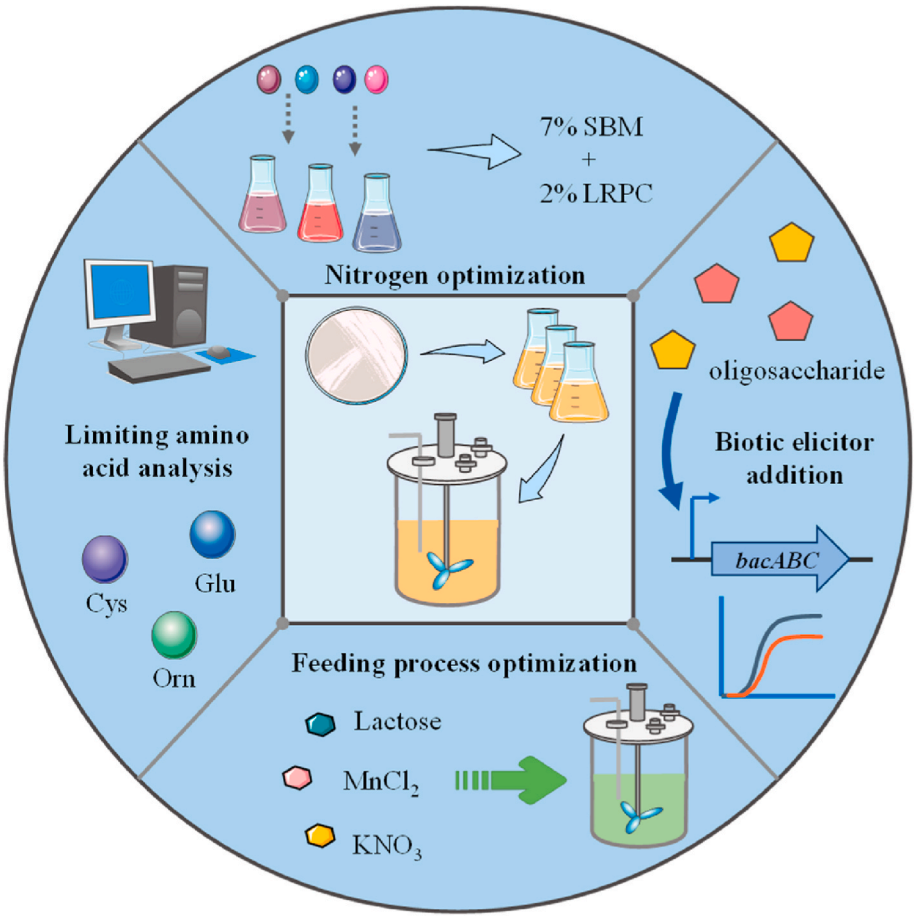

Fig. 3. Fermentation process optimization for bacitracin production. SBM, soybean meal; LprC, low protein rapeseed cake.

synthesis, during the middle and late stages of fed-batch fermentation in a 10 L bioreactor [94](Fig. 3).

During the large-scale bacitracin fermentation process, the factors that may limit cell growth or bacitracin synthesis are supposed to be identified (Fig. 3). In particular, the dissolved oxygen content decreases to zero in the early period, thus potentially resulting in the insufficient energy supply, and further restricts bacitracin synthesis. In general, the presence of a nitrate-reducing system can cause *Bacillus* to grow actively under oxygen-limited conditions [95]. Chen et al. demonstrated that exogenous addition of 514.39 mg/L KNO_3 and 45.35 mg/L Orn increased bacitracin yield by 10.80% in a 10 L bioreactor [54]. Moreover, the NADH oxidation efficiency is closely associated with ATP supply, a critical factor during microbial fermentation [96]. Supplementation with 0.30 mg/L $MnCl_2$ could increase acetoin reductase activity and NADH oxidation efficiency, which benefited cell growth and bacitracin synthesis [97]. Generally, oligosaccharides are biotic elicitors for the syntheses of antibiotics and enzymes. According to previous research, combined addition of oligoguluronate and mannan oligosaccharide positively affected the transcriptional level of bacitracin biosynthesis genes *bacABC*, thereby leading to a 36.50% increase of bacitracin A production [98].

5. Concluding remarks

Bacitracin is a promising peptide antibiotic with many ideal features for applications in animal feed additives and veterinary medicine. Because of its great robustness, high suitability for modification, and non-pathogenicity, *B. licheniformis* provides an ideal bacitracin-producing platform, and has been applied in industrial production for more than 20 years [99–101]. Herein, we reviewed the regulation of bacitracin biosynthesis, and proposed the access, pull, drive, and regulate tactics for designing excellent microbial cell factories for bacitracin production. In addition, several potential limiting factors, such as DO, nitrate concentration, and NADH oxidation efficiency, that influence cell growth and bacitracin synthesis during the large-scale fermentation process have also been identified. Nonetheless, several challenges must be addressed to facilitate the expansion of bacitracin industry.

In recent years, owing to the effects of COVID-19 pandemic, the costs of raw materials and logistics have gradually increased worldwide. Soybean meal is an important nitrogen source for microbial fermentation, and it is also the most extensively used plant-based protein raw material in feed industry [102,103]. Therefore, the growing demand for soybean meal in fermentation industry may restrict the development of animal husbandry. In addition, the complex composition of soybean meal is not conductive to subsequent analytical process and bacitracin extraction. Thus, a complete synthetic medium may be a superior choice for industrialized production of bacitracin, and the precursor amino acid synthetic pathways need to be enhanced in *B. licheniformis*.

During the large-scale bacitracin fermentation process, short branched-chain fatty acids (SBCFAs) and biogenic amines (BAs) generated from amino acid catabolism cause strong odor pollution, which severely affect people's physical and mental health [104,105]. Therefore, the synthesis pathways of SBCFAs and BAs need to be weakened or blocked in *B. licheniformis*. BCFAs are carboxylic acids with four to six carbon atoms that produced by BCAAs catabolism [106]. The expression of *bkd* operon has been confirmed as the limiting factor for SBCFAs synthesis [107]. By manipulating the native promoter of *bkd*operon, SBCFAs concentration is expected to be controlled at a low level. In addition, putrescine, cadaverine and spermidine are the main BAs that produced by decarboxylation of amino acids in *B. licheniformis* [108,

109]. Thus, ornithine decarboxylase SpeF, lysine decarboxylase YaaO, and spermidine synthetase SpeE are supposed to be blocked for the reduction of BAs during bacitracin synthesis.

Bacitracin A is the most bioactive component of bacitracin, in which two L-Ile molecules are presented at positions 5 and 8, and this difference depends on the substrate preferences of bacitracin synthases BacA and BacC [11,18]. NRPSs are multi-module enzymes that assemble abundant peptides with high structural and functional diversity [110]. A typical NRPS module usually contains the adenylation, peptidyl carrier protein, and condensation domains, which synergistically activate and incorporate amino acid monomers into an elongating peptide chain [111]. The modular NRPS structure provides the possibility of artificially designing nonribosomal peptide assembly lines for new peptidyl backbone synthesis [112,113]. Thus, rational design of substrate-selective adenylation domains can enhance the preferences of BacA and BacC for L-Ile, and potentially increases the proportion of bacitracin A during bacitracin production.

Author's contributions

J Zhu: Methodology, Investigation, Data curation, Software, Writing - original draft. **S Wang**: Investigation, Data curation, Software. **C Wang**: Data curation. **Z Wang**: Methodology, Investigation, Data curation. **G Luo**: Methodology, Investigation, Data curation. **J Li**: Writing - review & editing. **Y Zhan**: Writing - review & editing. **D Cai**: Investigation, Data curation, Writing - review & editing. **S Chen**: 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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