

Article

Improving the Yield of Xenocoumacin 1 by P_{BAD} Promoter Replacement in *Xenorhabdus nematophila* CB6

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Abstract: Xenocoumacin 1 (Xcn1), which is produced by *Xenorhabdus nematophila* CB6, exhibits strong inhibition activity against plant pathogens, especially fungi and oomycetes. Therefore, it has attracted interest in developing it into a novel biofungicide applicable for plant protection. However, its low yield with concomitant high cost during the fermentation process limits its widespread application. In this study, we replaced the native promoter of *xcnA* with the arabinose-inducible *araBAD* promoter (P_{BAD}), a well-known and widely used promoter for expressing heterologous genes, to evaluate its effects on Xcn1 yield and antimicrobial activity. Compared with wildtype strain, the fermentation yield of Xcn1 was improved from 68.5 mg/L to 249.7 mg/L (3.6-fold) and 234.9 mg/L (3.4-fold) at 0.5% and 1.0% L-arabinose concentration, respectively. We further explored the transcription level of the biosynthesis related genes of Xcn1 and found that their upregulation resulted in the yield improvement of Xcn1. Moreover, the antimicrobial activity of Xcn1 against *Bacillus subtilis* and *Phytophthora capsici* was determined by agar diffusion plate and growth inhibition assay, as expected, it was also found to be enhanced. The promoter-replacement strategy utilized here improves the yield of Xcn1 efficiently, which provides a basis for the industrial production of Xcn1.

Keywords: *X. nematophila*; xenocoumacin 1; P_{BAD} promoter; antimicrobial activity



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1. Introduction

Xenorhabdus nematophila, forming a mutual association with the specific soil-dwelling nematode *Steinernema carpocapsae*, is a type of Gram-negative bacteria of the Morganellaceae family [1–5]. This type of bacteria colonizes in a specialized intestinal receptacle of their cognate nematode partners when the nematodes are in the infective juvenile (IJ) form. As an entomopathogenic complex, they can invade an insect's body cavity (hemocoel) [2,6–8]. After entering the hemocoel, the nematode releases *X. nematophila*; then, the bacteria secrete different compounds, including immunosuppressive compounds and toxins, to overcome the host immune system and kill it [9–14]. With the quick proliferation of *X. nematophila*, they also secrete a large amount of exoenzymes for degrading insect tissue, and both *X. nematophila* and the digested macromolecules derived from the insect host could be utilized by the nematode as nutrient resources for proliferation [15–21]. Finally, the nematodes develop into the IJ stage, and, together with *X. nematophila*, they emerge from the insect cadaver and search for a new insect host [3,4,6,22].

It is well-known that *X. nematophila* can produce numerous secondary metabolites during its life cycle, and many of them exhibit antimicrobial activity, including indole derivatives [20,23,24], xenocoumacins (Xcns) [1,4,25–30], peptides [31–33], benzylineacetone [34], and nematophin [18,23,35,36]. These metabolites not only have diverse chemical

structures but also great application potential in the medical and agricultural fields due to their remarkable biological activity. Xenocoumacin (Xcns), mainly composed of Xcn1 and Xcn2, are the major antimicrobial compounds identified from *X. nematophila* [37,38]. It has been proved that a 39 kb cluster of 14 genes (*xcnA-xcnN*), which include two nonribosomal peptide synthetases (NRPSs) and three polyketide synthases (PKSs), was in charge of the biosynthesis of Xcns [4]. Among them, genes *xcnA-L* are responsible for the biosynthesis of Xcn1, and the conversion of Xcn1 into Xcn2 is catalyzed by the enzymes encoded by *xcnM* and *xcnN* in a concerted manner [4].

Xcn1 exhibits a strong antifungal activity against *Alternaria alternata*, *Botrytis cinerea*, *Rhizoctonia solani*, and *Phytophthora* species, etc. [26,36,39,40]. Xcn2, however, shows significantly decreased bioactivities [26,37,40]. *Phytophthora infestans* is one of the most important plant pathogens, which can cause potato late blight disease, the most devastating disease during potato production [26]. It is reported that Xcn1 exhibited strong inhibition effects against the mycelial growth of *P. infestans*, reaching 100% inhibition at 1.5 µg/mL. Moreover, it also effectively inhibited other species of *Phytophthora*, with EC₅₀ values below 4.2 µg/mL [26,39,40]. Therefore, Xcn1 has great potential to be used as a new biofungicide for plant protection. However, the low fermentation yield leading to high cost hampers the practical application of Xcn1 in the agricultural field.

While it is a challenging task, different strategies have been employed to improve the production of Xcn1. Yang et al. tried to improve the yield of Xcn1 by physical and chemical mutagenesis methods, the classical strategy for strain improvement, and several positive mutants were obtained, with inhibition activity enhanced in the range of 60–70% [41,42]. Fermentation optimization is another traditional strategy for increasing the titer of microbial secondary metabolites. The effects of medium components, parameters of fermentation process, such as initial pH, inoculum size, temperature, speed of rotation, etc., and feeding precursor on the yield of Xcn1 were systematically investigated, the highest yield reaching 173.99 µg/mL under the optimal fermentation conditions [35,43–45]. In situ separation technology (ISPR), which could remove Xcn1 from the fermentation broth, therefore preventing the conversion of Xcn1 to Xcn2, was reported to improve the yield of Xcn1 from 42.5 to 73.8 µg/mL in three days [46]. Furthermore, regulators, particularly the global regulators, related to the production of Xcn1 were studied. It was established that the global regulators OmpR, LeuO, and CpxR were negative regulators of Xcn1, negatively regulating the transcription of *xcnA-L*, while Lrp and FliZ positively regulated the production of Xcn1 [4,28,47].

As described above, despite multiple efforts were attempted to increase the yield of Xcn1, it still cannot meet the requirement of industrial production. Therefore, new strategies for engineering the production strain, which might complement previous endeavors, should be attempted. Replacement of the natural promoter of a gene cluster of interest with a strong constitutive promoter or an inducible promoter whose regulation mechanism is well understood could be an effective strategy to improve the yield of the aimed secondary metabolite [48,49]. It has been reported that the well-known *P*_{BAD} promoter was used successfully to improve the production of secondary metabolites in the entomopathogenic bacteria *Photobacterium luminescens* and *Xenorhabdus doucetiae* [49]. Hence, we speculate that replacement of the original promoter of *xcnA* with the *P*_{BAD} promoter could be an useful strategy to improve the yield of Xcn1. Therefore, in this study, we constructed a ΔP_{BAD} -*xcnA* mutant based on *X. nematophila* CB6 and studied its effects on Xcn1 production and antimicrobial activity.

2. Materials and Methods

2.1. Bacterial Strains, Media, and Growth Conditions

X. nematophila CB6 was isolated from the entomopathogenic nematode *Steinernema* sp. screened from a soil sample in Beijing, China. Details of the strains and plasmids used in this study are provided in Table 1. *X. nematophila* CB6 and *E. coli* were cultured with Luria–Bertani (LB) broth (Bacto tryptone 10 g/L, yeast extract 5 g/L, and NaCl 10 g/L) or

LB agar media (15 g/L agar). *X. nematophila* CB6 was cultured at 28 °C, and *E. coli* was grown at 37 °C. Unless noted otherwise, strains were initially grown overnight in 3 mL of LB medium, then inoculated into 100 mL of fresh LB medium in a 250 mL conical flask and incubated for the desired period. When screening the *X. nematophila* CB6 mutants of single crossover homologous recombination, ampicillin and gentamicin were added to the medium with a final concentration of 100 and 50 µg/mL, respectively. An LB agar plate (no NaCl) containing 50 µg/mL of kanamycin and 5% sucrose was applied for screening the second crossover homologous recombination mutants. *Bacillus subtilis* was grown at 37 °C on an LB medium, and *Phytophthora capsici* was grown at 25 °C on potato dextrose agar (PDA).

Table 1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Relevant Genotype, Phenotype, or Characteristic(s)	Reference or Source
Strains		
<i>X. nematophila</i> CB6 <i>P_{BAD}-xcnA</i>	Wildtype, phase I variant; Amp ^r CB6Δ <i>P_{BAD}-xcnA</i> ::Km ^r	Laboratory stock This study
<i>E. coli</i>		
DH5α	F-φ80d <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>end</i> A1 <i>recA1</i> <i>hsdR17</i> (r _k ⁻ , m _k ⁺) <i>supE44</i> λ- <i>thi-1</i> <i>gyrA96</i> <i>relA1</i> <i>phoA</i>	TransGen Biotech
S17-1 λpir	<i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsdR</i> -M+. RP4-2Tc::Mu Km::Tn7 in the chromosome	Laboratory stock
Plasmids		
pBAD30	Expression vector; Amp ^r , <i>ori</i> p15A, <i>ori</i> F1	Laboratory stock
pYBA-1132	Expression vector; Km ^r , <i>ori</i> pBR322	Laboratory stock
pJQ200SK	Suicide vector; Gm ^r , Suc ^s , SacB, <i>ori</i> p15A, <i>ori</i> T	Laboratory stock
pJQP _{BAD} - <i>xcnA</i>	Plasmid pJQ200SK carrying 859 bp upstream homology arm, 1075 bp kanamycin resistant cassette, 1245 bp <i>P_{BAD}</i> promoter fragment and 853 bp downstream homology arm	This study

Note: Amp^r, ampicillin resistance; Km^r, kanamycin resistance; Gm^r, gentamicin resistance; Suc^s, Sucrose sensitive.

2.2. Construction of Δ*P_{BAD}-xcnA* Mutant Strain

To construct the Δ*P_{BAD}-xcnA* mutant strain by homologous recombination, upstream (859 bp) and downstream (853 bp, beginning with the start codon ATG of *xcnA* gene) fragments were amplified from CB6 chromosomal DNA with primer pairs XcnA-5DT/XcnA-P1 and XcnA-3DT/XcnA-P2 using FastPfu fly DNA polymerase (TransGen Biotech, China) according to the manufacturer's instructions. The PCR fragment containing a kanamycin-resistant cassette (1075 bp) was amplified from pYBA-1132 using primer pairs Km-F/Km-R. The *P_{BAD}* promoter fragment was amplified using primer pairs *P_{BAD}*-F/*P_{BAD}*-R from pBAD30 plasmid. pJQ200SK plasmid was linearized with infusion-F and infusion-R primers. All these fragments were connected by a seamless cloning and assembly kit (Beijing Zoman Biotechnology Co., Ltd, Beijing, China) following the user manual. The resulting plasmid pJQP_{BAD}-*xcnA* was transformed into *E. coli* S17-1 λpir and transferred into wild type *X. nematophila* CB6 strain by conjugation. The detailed steps are as follows: *E. coli* S17-1 λpir and *X. nematophila* CB6 were cultured in an LB medium at 37 °C and 28 °C, respectively. When liquid cultures were grown to OD₆₀₀ 0.6–0.8, 1 mL cultures were harvested and washed twice using fresh LB medium. The cells were resuspended in 50 µL LB. The *E. coli* S17-1 λpir and *X. nematophila* were mixed and spotted onto LB agar plate without antibiotics. The plate was grown at 28 °C for 18 h. Then, the bacterial colony was resuspended in the liquid LB medium and spread on an LB agar plate containing ampicillin and gentamicin. The single crossover homologous recombination bacteria were screened by selecting colonies and confirmed by PCR [4]. Thereafter, we cultured the colonies for 12 h in LB (without NaCl) and spread them on an LB plate containing 50 µg/mL of kanamycin and 5% sucrose (no NaCl) [50]. Sucrose resistant colonies were picked into LB

supplemented with kanamycin and incubated at 28 °C. The replacement generated via the second crossover was confirmed by sensitivity to gentamicin as well by PCR [50].

2.3. Monitoring the Growth of $\Delta P_{BAD}\text{-}xcnA$ Mutant Strain

Wildtype CB6 and $\Delta P_{BAD}\text{-}xcnA$ strains were grown overnight in an LB medium at 28 °C by shaking at 200 rpm. Then, the cells were transferred to a fresh LB medium with an initial OD₆₀₀ value reaching 0.05. Thereafter, L-arabinose (0.5–2%) was added at 8 h, and the cultivation was continued for 48 h. For measuring cell density, the optical density of wildtype CB6 and $\Delta P_{BAD}\text{-}xcnA$ strains at 600 nm [OD₆₀₀] was monitored at predetermined time points. Each experiment was performed in triplicate.

2.4. Analysis of the Yield of Xcns

Analysis of the yield of Xcns was performed according to a previously reported method [4,25,26,46] by high-performance liquid chromatography (HPLC). The equal volume of MeOH was added into the cell-free fermentation broth for HPLC analysis. The HPLC analysis condition was used according to previously described methods [4,46]. The yields of Xcn1 and Xcn2 were calculated based on comparison with standards we obtained previously [25,39].

2.5. Quantitative Real-Time PCR (qRT-PCR)

The total ribonucleic acid (RNA) of the wildtype CB6 strain and the $\Delta P_{BAD}\text{-}xcnA$ mutant strain was extracted using a bacterial RNA extraction kit (TransGen Biotech, ER501-01). RNA reverse transcription to cDNA was performed using One-Step gDNA Removal and a cDNA Synthesis SuperMix kit (TransGen Biotech, AE311-04). qRT-PCR was carried out using SYBR Green qPCR SuperMix (GenStar, A304-10) according to the manufacturer's instructions. The reaction was performed as follows: 95 °C denaturation step for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. The *recA* gene was chosen as the internal reference gene to normalize other genes' expressions [4,46]. The relative transcript levels were determined using the $2^{-\Delta\Delta C_T}$ method with three independent experiments [51]. All the primers for qRT-PCR are listed in Table 2.

Table 2. Primers used in this study.

Primer	Sequence (5'→3')	Use
XcnA-5DT	CGGTATCGATGTTATTGCTGTTTGTATTGTG	Mutant construction
XcnA-P1	CATAGGCTCCTTAAGTTGGTGCCATAATAATAG	Mutant construction
Km-F	ACCAACTTAAGGAGCCTATGGAAACTGGGA	Mutant construction
Km-R	CAGACAATTGTCAGAAGAAGCTCGTCAAGAAG	Mutant construction
P_{BAD} -F	GTTCTTCTGACAATTGCTGATTTCGTTACC	Mutant construction
P_{BAD} -R	TCTTCTTCATATGCTAGCCTCCTGTTAGCCCA	Mutant construction
XcnA-P2	AGGCTAGCATATGAAGAAGACGATTTTGTAGTTG	Mutant construction
XcnA-3DT	ATTCGATATCAAGCCCCATTACCATTCTCAA	Mutant construction
XcnA-Pro-TF	CAGGTATAGTAAATTAATAGGGG	Mutant construction
XcnA-Pro-TR	GTTTCTCTTCACTACTCCAACG	Mutant construction
infusion-F	CAGCAATAACATCGATACCGTCCGACCTCG	Mutant construction
infusion-R	AATGGGGCTTGATATCGAATTCCTGCAGCC	Mutant construction
xcnA-Fwd	GCATAGAGCCTCGTGAATTAGAG	qPCR analysis
xcnA-Rev	GATATGTTCTCTCACCCGGGG	qPCR analysis
xcnF-Fwd	GCTGGCGTGATCAAATCTCTTC	qPCR analysis
xcnF-Rev	CAGGCAATTGACTTTATTCCCATC	qPCR analysis
xcnG-Fwd	CTGTTGTGGTGATGGCGAATG	qPCR analysis
xcnG-Rev	GAAAACAGGGAAAGGAAGGCAC	qPCR analysis
xcnM-Fwd	CGTATTGATGTTCAATGGAGTTGATG	qPCR analysis
xcnM-Rev	ATGATCTTGAATATGCTCAGCCAAC	qPCR analysis
recA-Fwd	GCTGAAATTCTATGCGTCTGTCC	qPCR analysis
recA-Rev	CTGTTTGAATGGTGCTGCAACTTTG	qPCR analysis

2.6. Antimicrobial Activity Assay

To assess the antimicrobial activity of the wildtype *X. nematophila* CB6 and the $\Delta P_{BAD}\text{-}xcnA$ mutant, we performed an agar diffusion plate assay and a growth inhibition assay against *Bacillus subtilis* and *Phytophthora capsici*, which were sensitive to Xcn1 in our previous studies [26,46,52]. Concerning the agar diffusion plate assay, sample wells were prepared on a two-layer agar diffusion plate, in which 20 mL of medium containing 0.75% agar and $10^7\text{--}10^8$ cells of *B. subtilis* were layered above 10 mL of 2% sterilized agar. Then the tested sample was added into the wells, and the plates were cultured at 28 °C for 24 h to measure the diameter of the inhibition zone. To test the antifungal activity of *X. nematophila*, the *P. capsici* strain that is sensitive to Xcn1 was chosen as a model fungus. Briefly, the tested sample was mixed with a PDA medium with a final concentration of 0.5 $\mu\text{g/mL}$ of Xcn1 and then poured onto a Petri dish. The mycelia PDA block was cut from the edges of a five-day colony of *P. capsici* and then was placed at the center of the plate for culturing at 25 °C. After seven days, the diameter of *P. capsici* was measured, and the growth inhibition rate was calculated using the following equation: growth inhibition rate (%) = $100 \times [(\text{Diameter of control fungal colony} - \text{Diameter of test fungal colony}) / \text{Diameter of control fungal colony}]$ [53]. Each treatment was repeated three times.

3. Results

3.1. Construction of $\Delta P_{BAD}\text{-}xcnA$ Strain

The yield of Xcn1 was related with the transcription level of its biosynthetic gene cluster, thus, we speculated that promoting the transcription level of Xcn1 biosynthetic gene cluster by replacement of the original promoter of *xcnA* with the P_{BAD} promoter could boost the yield of Xcn1. To test this hypothesis, we first performed a homologous recombination experiment to construct the $\Delta P_{BAD}\text{-}xcnA$ mutant. Then the resulted mutants were confirmed by PCR and sequencing using external primers XcnA-Pro-TF and XcnA-Pro-TR (Figure 1). The sizes of the PCR product were consistent with the expected results, which were 2300 bp and 4193 bp when using the genomes of wildtype and mutant strains as templates, respectively. Furthermore, the sequencing results were identical with the aimed replacement. Therefore, it proved that the *xcnA* original promoter in *X. nematophila* CB6 was successfully replaced with the P_{BAD} promoter, correspondingly, the *xcnA* original promoter was knocked out from *X. nematophila* CB6.

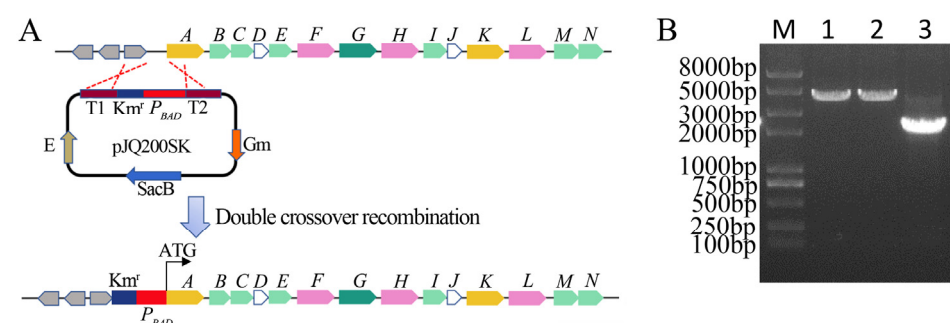


Figure 1. Construction of the $\Delta P_{BAD}\text{-}xcnA$ mutant strain by homologous recombination. (A) The process for changing the promoter of *X. nematophila* CB6. (B) Identification of the $\Delta P_{BAD}\text{-}xcnA$ mutant with external primers XcnA-Pro-TF/XcnA-Pro-TR, Lane M, DNA molecular marker (Direct-load StarMarker D2000 Plus II); Lane1, 2, $\Delta P_{BAD}\text{-}xcnA$ mutant, 4193 bp; Lane 3, wildtype, 2300 bp.

3.2. Growth Profile of $\Delta P_{BAD}\text{-}xcnA$ Strain

Once obtained the mutant, we were rather curious whether the genetic manipulation performed in the CB6 strain could affect its growth, which might subsequently influence the yield of Xcn1. In the whole fermentation process, the $\Delta P_{BAD}\text{-}xcnA$ mutant displayed a similar growth curve with the wildtype CB6 strain. In addition, the production of Xcn1 in the $\Delta P_{BAD}\text{-}xcnA$ mutant strain must be coupled with the addition of arabinose utilized for activating the inducible promoter P_{BAD} . So, it was also necessary to assess the influence of

arabinose on the growth of $\Delta P_{BAD}\text{-}xcnA$ mutant strain. When 0.5% L-arabinose was added to the LB medium, the growth curve of the $\Delta P_{BAD}\text{-}xcnA$ mutant was decreased relative to the wildtype CB6 strain. With the increase in the added L-arabinose, a greater impact on the growth was observed (Figure 2).

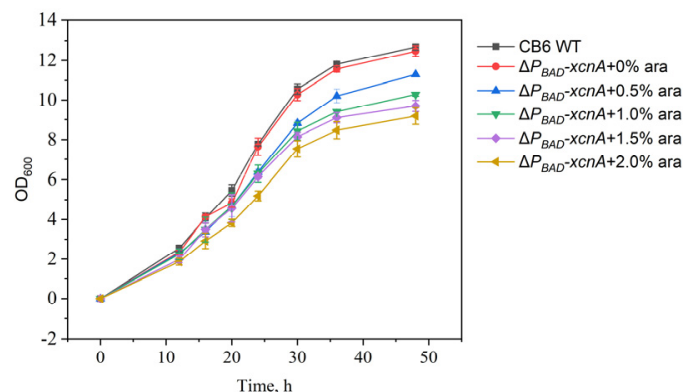


Figure 2. Growth curves of $\Delta P_{BAD}\text{-}xcnA$ mutant strain, adding different concentrations of L-arabinose and wildtype CB6 strain.

3.3. Effects of P_{BAD} Promoter Replacement on the Yield of $Xcn1$

As a type of tight control promoters, P_{BAD} in the $\Delta P_{BAD}\text{-}xcnA$ mutant could function as a master switch for modulating $Xcn1$ and $Xcn2$ production. Indeed, $Xcns$ cannot be detected in the fermentation broth of $\Delta P_{BAD}\text{-}xcnA$ mutant without the addition of L-arabinose (Figure 3). After adding arabinose, a significant amount of $Xcns$ was detected by HPLC. The $Xcn1$ yields, however, did not improve with the increase in L-arabinose concentration, and it kept a similar level between 0.5 and 1.5% L-arabinose in the culture media. When the L-arabinose concentration reached 0.5% and 1%, relative to the wildtype strain the fermentation yield of $Xcn1$ of the $\Delta P_{BAD}\text{-}xcnA$ mutant at 48 h was improved from 68.5 mg/L to 249.7 mg/L (3.6-fold) and 234.9 mg/L (3.4-fold), respectively (Figure 3). When 2% L-arabinose was added to the medium, the yield of $Xcn1$ reduced significantly, although it was still higher than the wildtype CB6 strain. The yield of $Xcn2$, however, was the highest, about 4.4-fold more than the wildtype CB6 strain, in the medium containing 2% L-arabinose. The total yield of $Xcns$, namely the sum of $Xcn1$ and $Xcn2$, was also investigated, and as shown in Figure 3, relative to the WT CB6 strain remarkably higher yields were found in the $\Delta P_{BAD}\text{-}xcnA$ mutants induced by L-arabinose of different concentrations.

3.4. Transcriptional Analysis of the Xcn Genes

To evaluate the xcn genes' transcription levels of the $\Delta P_{BAD}\text{-}xcnA$ mutant in different L-arabinose concentrations, qRT-PCR was employed to monitor the four critical genes ($xcnA$, $xcnF$, $xcnG$, and $xcnM$) during the fermentation process (Figure 4). Consistent with our expectations, induced by L-arabinose, all of the four genes in the $\Delta P_{BAD}\text{-}xcnA$ mutant showed higher transcription levels relative to the wildtype CB6 strain at both 24 h and 36 h. It is worth noting that the expression levels of $xcnA$, encoding the important NRPS enzyme responsible for $Xcn1$ biosynthesis, in the $\Delta P_{BAD}\text{-}xcnA$ mutant were enhanced 7-fold, 9-fold, 8-fold, and 6-fold at 36 h with addition of 0.5%, 1.0%, 1.5%, and 2% L-arabinose, respectively. Another crucial gene $xcnF$, encoding a PKS enzyme involved in $Xcn1$ biosynthesis, was also highly upregulated with 2.7-fold, 3.2-fold, 2.3-fold, and 2.9-fold increase at 0.5%, 1.0%, 1.5%, and 2% L-arabinose, respectively. The transcription levels of $xcnG$, encoding a specific peptidase that can cleave the inactive prexenoscoumactins into the active compound $Xcn1$, were increased at 36 h by 2.4-fold, 3.0-fold, 2.0-fold, and 1.9-fold, respectively. The over-transcription of $xcnA$, $xcnF$, and $xcnG$, involved in $Xcn1$ biosynthesis, was in concert with the observed higher $Xcn1$ yield produced by the $\Delta P_{BAD}\text{-}xcnA$ mutant. The transcription level of the $xcnM$ gene was increased by 2.5-fold, 2.3-fold, 2.0-fold, and

2.2-fold at 36 h with the addition of 0.5%, 1.0%, 1.5%, and 2% L-arabinose, respectively. It led to a higher conversion rate from Xcn1 to Xcn2, therefore, increased Xcn2 yield.

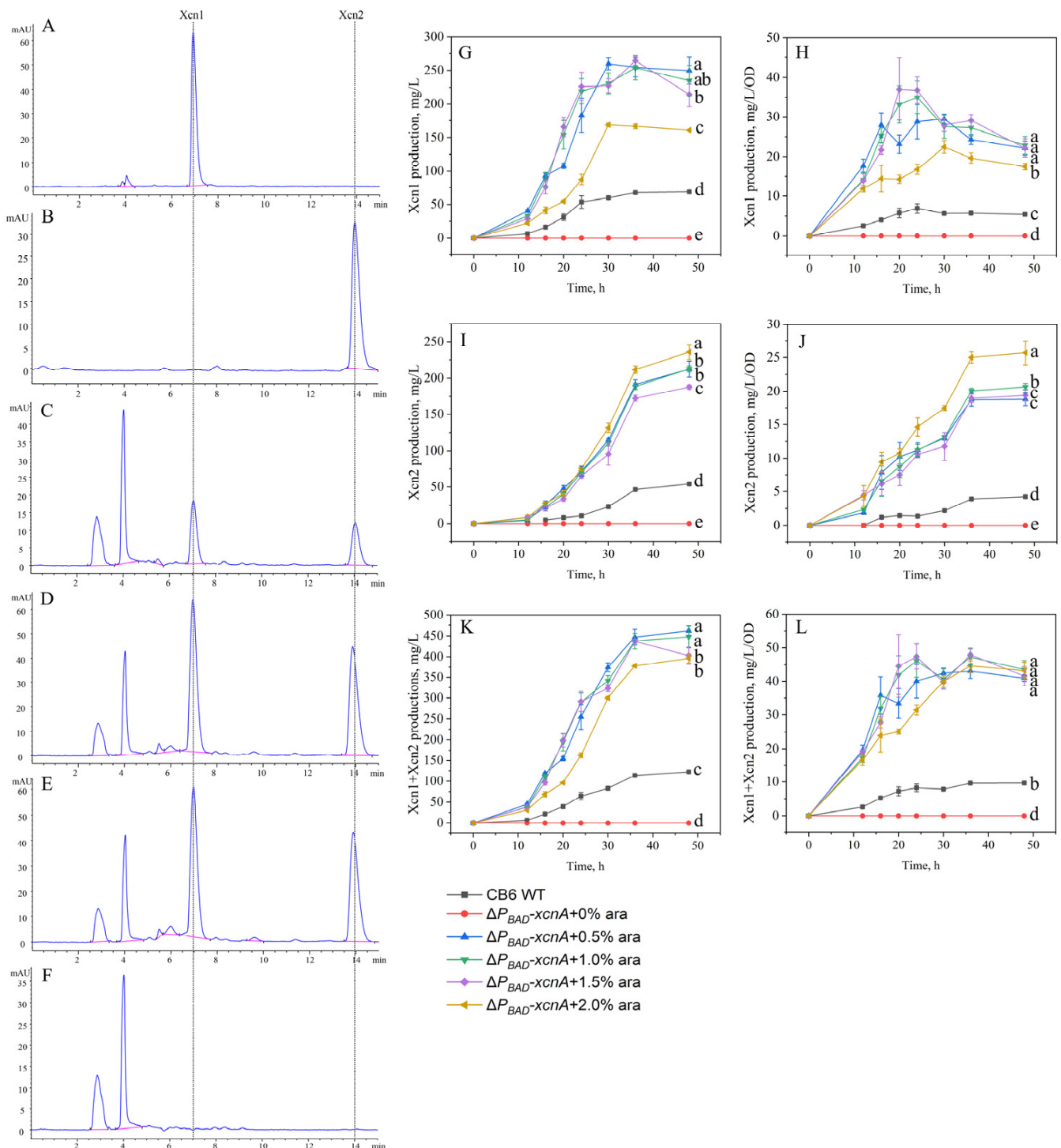


Figure 3. HPLC chromatogram of fermentation samples at 48 h and the dynamic curve of Xcn1 and Xcn2 detected by HPLC during the fermentation process. (A) Xcn1 standard (100 $\mu\text{g}/\text{mL}$), (B) Xcn2 standard (100 $\mu\text{g}/\text{mL}$), (C) fermentation sample of WT CB6, (D) fermentation sample of $\Delta P_{BAD}\text{-}xcnA$ mutant induced by 0.5% L-arabinose, (E) fermentation sample of $\Delta P_{BAD}\text{-}xcnA$ mutant induced by 2% L-arabinose, (F) fermentation sample of uninduced $\Delta P_{BAD}\text{-}xcnA$ mutant, (G) the dynamic curve of Xcn1, (H) the dynamic curve of Xcn1 per OD, (I) the dynamic curve of Xcn2, (J) the dynamic curve of Xcn2 per OD, (K) the dynamic curve of Xcn1+Xcn2, and (L) the dynamic curve of Xcn1+Xcn2 per OD. Error bars represent the standard deviation of three independent replicates. Data were subjected to the Duncan ANOVA test, curves labelled with different lowercase letters at 48 h were significantly different at $p \leq 0.05$.

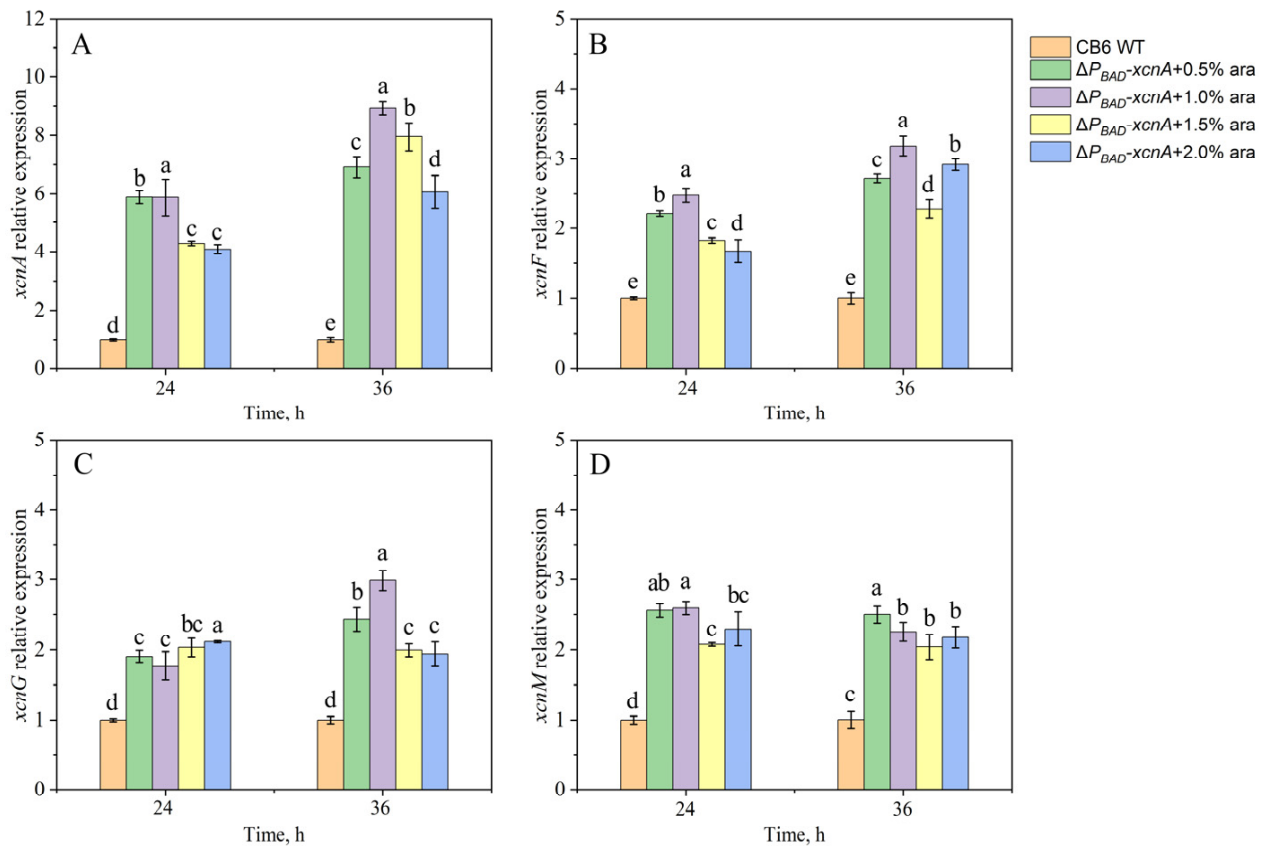


Figure 4. (A) *xcnA* relative expression (B) *xcnF* relative expression (C) *xcnG* relative expression (D) *xcnM* relative expression. Relative transcription levels of the biosynthesis and conversion related genes of Xcn1 (*xcnA*, *xcnF*, *xcnG*, and *xcnM*) were measured by qRT-PCR; error bars represent the standard deviation of three independent replicates. Data were subjected to the Duncan ANOVA test, columns labelled with different lowercase letters were significantly different at $p \leq 0.05$.

3.5. Antimicrobial Activity of $\Delta P_{BAD}\text{-}xcnA$ Mutant Strain

We utilized an agar diffusion plate assay and growth inhibition assay against *B. subtilis* and *P. capsici*, respectively, to test whether the higher yield of Xcn1 gave a corresponding greater antimicrobial activity. As expected, in comparison with the wildtype CB6 strain, the fermentation broth of $\Delta P_{BAD}\text{-}xcnA$ mutant exhibited higher antimicrobial activity, the zone of inhibition against *B. subtilis* significantly increased from 11.83 mm to 16.34 mm (Figure 5A, Table 3). Similarly, better inhibition activity against *P. capsici* was detected for the fermentation broth of the $\Delta P_{BAD}\text{-}xcnA$ mutant strain as well. As shown in Figure 5B and Table 3, similar inhibition effects were achieved using the fermentation broth of the $\Delta P_{BAD}\text{-}xcnA$ mutant strain and the wildtype CB6 strain in 500-fold and 137-fold dilution, the diameter of *P. capsici* being 18.50 mm and 17.64 mm, (Figure 5B), and corresponding inhibition rates being 77.80% and 78.80% (Table 3), respectively. The results indicated that the antimicrobial effects of the $\Delta P_{BAD}\text{-}xcnA$ mutant were significantly improved due to the enhanced yield of Xcn1 and possible contribution from increased Xcn2.

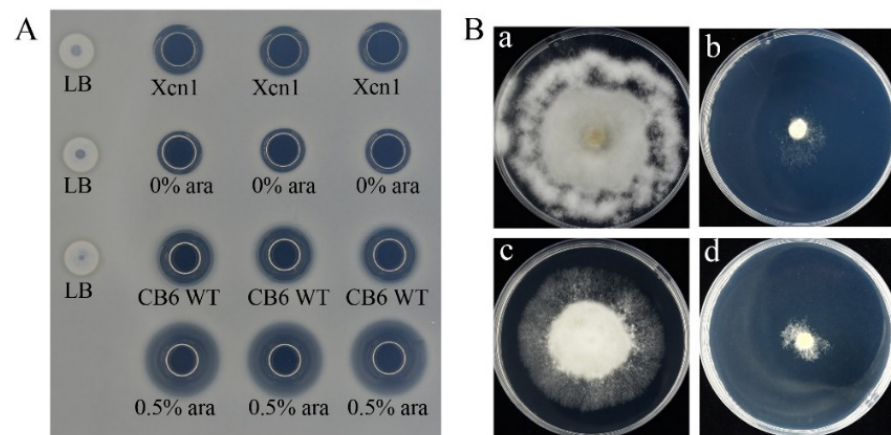


Figure 5. The antimicrobial activity of wild type CB6 strain and $\Delta P_{BAD-xcnA}$ mutant strain on *B. subtilis* and *P. capsici*. (A) Antibacterial activity was studied by agar diffusion plate assay using *B. subtilis* as the indicator. Xcn1 represents 50 µg/mL Xcn1, CB6 WT represents the cell-free fermentation broth of wild type strain, 0% ara and 0.5% ara represent the cell-free fermentation broth of uninduced and induced $\Delta P_{BAD-xcnA}$ mutant, respectively. (B) Antifungal activity of $\Delta P_{BAD-xcnA}$ mutant was assessed by growth inhibitory assay using *P. capsici* as the indicator. (a) control, (b) 137-fold dilution of the fermentation broth of wild type CB6 strain mixed with PDA medium with a final concentration of 0.5 µg/mL of Xcn1, (c) 137-fold dilution of the fermentation broth of uninduced $\Delta P_{BAD-xcnA}$ mutant mixed with PDA medium, and (d) 500-fold dilution of the fermentation broth of $\Delta P_{BAD-xcnA}$ mutant induced by 0.5% L-arabnose mixed with PDA medium with a final concentration 0.5 µg/mL of Xcn1.

Table 3. Inhibitory effect of the cell-free fermentation broth against *B. subtilis* and *P. capsici*.

Samples	Inhibition Zone Against <i>B. subtilis</i> (mm)	Inhibition Rate of <i>P. capsici</i> (%) *
50 µg/mL Xcn1	11.30 ± 0.26 c	ND
CB6 wildtype	11.83 ± 0.29 b	78.80 ± 0.92 a
$\Delta P_{BAD-xcnA}$ + 0% ara	9.67 ± 0.28 d	24.80 ± 1.39 b
$\Delta P_{BAD-xcnA}$ + 0.5% ara	16.34 ± 0.58 a	77.80 ± 1.59 a

* To detect inhibition rate, there was 137-fold dilution of the fermentation broth of wildtype CB6 and $\Delta P_{BAD-xcnA}$ + 0% ara and 500-fold dilution of the fermentation broth of $\Delta P_{BAD-xcnA}$ +0.5% ara. ND: not done. Numerical values were mean ± SD by three replicates. Means were separated using Duncan's multiple range test. Different lowercase letters were significantly different at $p \leq 0.05$ for the same columns.

4. Discussion

Xcn1 has great potential to be applied in the agricultural field to protect crops against the infection of many pathogens due to its wide range of antimicrobial activity [25,26,28,39,40]. However, the present fermentation yield of Xcn1 is still low, which hampers its large-scale production. Numerous well-known strategies have been reported to promote the yield of secondary metabolites in microorganisms, for example, increasing the precursor supply, deleting the competing pathways, engineering the crucial enzymes of the biosynthetic pathway, engineering regulatory networks, and controlling the transcription of the biosynthetic gene cluster [49,54–64]. In our research, we found that the biosynthetic gene cluster of Xcn1 remained at a low transcriptional level during the fermentation process (unpublished data), so we supposed that a promoter replacement strategy could be effective to increase its transcription, thereby improving the yield of Xcn1.

The P_{BAD} promoter, which possesses the advantage to accurately control the transcription level of target genes with varying concentrations of inducer, has been used extensively for modulation of gene expression [49,65,66]. It can play an important role in natural products isolation and structure elucidation through strictly regulating overproduction (with

inducer) or loss (without inducer) of the desired compound in fermentation process [49]. This strategy is also widely applicable to activate gene clusters that are “silent” under common conditions and will enrich our knowledge of natural products and their biological functions [49].

Previous research had reported that the mutant strains generated by genetic manipulation were often accompanied by the changes in phenotype and physiology, for instance morphology, metabolites, growth, etc. [4,8,28,64]. Here, the growth curves of the $\Delta P_{BAD}\text{-}xcnA$ mutant strain and wildtype CB6 strain in LB were similar when no L-arabinose was added, indicating the genetic manipulation caused no obvious influence on its growth. When we added L-arabinose to the LB broth, the P_{BAD} promoter was activated, thus, the production of Xcn1 increased remarkably. These results are in agreement with previous research where Bode et al. applied arabinose-inducible promoters in entomopathogenic bacteria to overproduce the desired natural products for their structure elucidation [49]. As Xcn2 was converted from Xcn1, we also paid attention to the production changes in Xcn2. Obviously, the yield of Xcn2 was also increased, coupled with the increased yield of Xcn1. Correspondingly, relative to the wildtype strain, the higher transcription level of *xcnA*, *xcnF*, *xcnG*, and *xcnM* were observed in the $\Delta P_{BAD}\text{-}xcnA$ mutant strain with the induction of L-arabinose, which revealed the underlying mechanism for the increased yield of Xcn1 and Xcn2. However, the induction strength for the P_{BAD} promoter could be increased within a certain range of L-arabinose concentration, and once exceeded, the transcription levels of target genes might be reduced [67]. Indeed, 2% L-arabinose resulted in lower transcription levels of *xcnA*, *xcnF*, and *xcnG* at 36 h compared with 0.5–1% L-arabinose. As we observed in the growth curves, a high concentration of L-arabinose inhibited the growth of the mutant strain, this is another reason responsible for the decreased yield of Xcn1 in the fermentation medium containing 2% L-arabinose. Moreover, a large amount of Xcn2, which was converted from Xcn1 mediated by both the XcnM and XcnN enzymes, was found in the fermentation broth. So, in future research, the addition of an adsorber resin to the culture during the fermentation of the $\Delta P_{BAD}\text{-}xcnA$ strain may be a practicable way to in situ collect the produced Xcn1 to reduce its conversion [46].

In previous research [4,26,37], Xcn1 was regarded as the main antimicrobial compound in *X. nematophila*. In other words, the higher concentration of Xcn1 corresponds to higher antimicrobial activity. In this study, we improved the yield of Xcn1 remarkably in the $\Delta P_{BAD}\text{-}xcnA$ mutant, so more obvious antimicrobial activity should be identified in the corresponding fermentation broth. As anticipated, higher inhibition activity against model strains *B. subtilis* and *P. capsici* was detected. The study presented here provides an effective strategy to engineer the CB6 strain to increase the yield of Xcn1; together with other complementary means, for instance, increasing the precursor supply, deleting the competing pathways, optimizing the fermentation process, controlling the regulators, and enhancing the resistance of the CB6 strain to Xcn1 et al., the greater improvement of the yield of Xcn1 could be realized in the near future, paving the way for its industrial production.

5. Conclusions

In summary, we achieved the remarkable improvement of the yield of Xcn1 by promoter replacement strategy, the fermentation yield of Xcn1 in the $\Delta P_{BAD}\text{-}xcnA$ mutant strain was improved from 68.5 mg/L to 249.7 mg/L helped by the induction of 0.5% L-arabinose, and it was accompanied with increased antimicrobial activity. Further study revealed that upregulation of the biosynthesis related genes of Xcn1 contributed to the yield improvement. We hope that the promoter replacement strategy employed here could be used as a reference for other *Xenorhabdus/Photorhabdus* species, which might promote the commercial development of more nature products from nematode associated bacteria.

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