

## Effects of *cpxR* on the growth characteristics and antibiotic production of *Xenorhabdus nematophila*

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### Summary

**CpxR is a global response regulator that negatively influences the antimicrobial activities of *Xenorhabdus nematophila*. Herein, the wildtype and  $\Delta cpxR$  mutant of *X. nematophila* were cultured in a 5-l and 70-l bioreactor. The kinetic analysis showed that  $\Delta cpxR$  significantly increased the cell biomass and antibiotic**

**activity. The maximum dry cell weight (DCW) and antibiotic activity of  $\Delta cpxR$  were  $20.77 \pm 1.56 \text{ g L}^{-1}$  and  $492.0 \pm 31.2 \text{ U ml}^{-1}$  and increased by 17.28 and 97.33% compared to the wildtype respectively. Xenocoumacin 1 (Xcn1), a major antimicrobial compound, was increased 3.07-fold, but nematophin was decreased by 48.7%. In 70-l bioreactor, DCW was increased by 18.97%, while antibiotic activity and Xcn1 were decreased by 27.71% and 11.0% compared to that in 5-l bioreactor respectively. Notably, pH had remarkable effects on the cell biomass and antibiotic activity of  $\Delta cpxR$ , where  $\Delta cpxR$  was sensitive to alkaline pH conditions. The optimal cell growth and antibiotic activity of  $\Delta cpxR$  occurred at pH 7.0, while Xcn1 was increased 5.45- and 3.87-fold relative to that at pH 5.5 and 8.5 respectively. These findings confirmed that  $\Delta cpxR$  considerably increased the biomass of *X. nematophila* at a late stage of fermentation. In addition,  $\Delta cpxR$  significantly promoted the biosynthesis of Xcns but decreased the production of nematophin.**

### Abbreviations

$\bar{\mu}$	average specific cell growth rate ( $\text{h}^{-1}$ )
$\bar{q}_p$	average specific antibiotic production rate ( $\text{U mg}^{-1} \text{ h}^{-1}$ )
$\bar{q}_s$	average specific glucose consumption rate ( $\text{h}^{-1}$ )
DCW	dry cell weight ( $\text{g L}^{-1}$ )
DO	dissolved oxygen (%)
$P_{\text{max}}$	maximal antibiotic activity ( $\text{U ml}^{-1}$ )
$P_p$	antibiotic productivity ( $\text{U ml}^{-1} \text{ h}^{-1}$ )
$P_x$	cell production rate ( $\text{g L}^{-1} \text{ h}^{-1}$ )
$q_p$	specific antibiotic production rate ( $\text{U mg}^{-1} \text{ h}^{-1}$ )
$q_s$	specific glucose consumption rate ( $\text{h}^{-1}$ )
$X_{\text{max}}$	maximal cell density ( $\text{g L}^{-1}$ )
$Y_{p/S}$	antibiotic yield of glucose ( $\text{U g}^{-1}$ )
$Y_{p/X}$	antibiotic yield on the cell ( $\text{U g}^{-1}$ )
$Y_{x/S}$	cell yield on glucose ( $\text{g g}^{-1}$ )
$\mu_{\text{max}}$	maximal specific cell growth rate ( $\text{h}^{-1}$ )
$\mu$	specific cell growth rate ( $\text{h}^{-1}$ )

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[Correction added on 8 February 2019, after first online publication: the presentation of the authors' names have been corrected in this version].

## Introduction

*Xenorhabdus nematophila* is a unique Gram-negative bacterium that develops a mutualistic association with an infective dauer juvenile (IJ) insect-pathogenic nematode in the genus of *Steinernema* (Thomas and Poinar, 1979). Recent research has revealed *X. nematophila* to be a rich source for natural products with potent biological or pharmaceutical activities (Challinor and Bode, 2015; Bozhüyük *et al.*, 2016; Tobias *et al.*, 2017). In *X. nematophila* ATCC 19061, 7.5% of the genome encoding proteins is involved in secondary metabolism; however, the majority of these encoded molecules are cryptic (Chaston *et al.*, 2011). Until now, *X. nematophila* has been known to produce several secondary metabolites, including indole derivatives (Paul *et al.*, 1981; Sundar and Chang, 1993; Li *et al.*, 1995), nematophin (Li *et al.*, 1997), insecticidal proteins (Morgan *et al.*, 2001; Sergeant *et al.*, 2003; Yang *et al.*, 2009; Sheets *et al.*, 2011; Hinchliffe, 2010), benzylideneacetone (Ji *et al.*, 2004), xenocoumacins (Xcns) (McInerney *et al.*, 1991) and the major class of non-ribosomal produced secondary metabolites (Crawford *et al.*, 2011). Due to structural diversity, these metabolites display a wide range of bioactivities with potential interest for pharmaceutical, agricultural and chemical applications. These naturally occurring antibiotics and their derivatives may provide beneficial leads in the future research and manufacturing of agrochemicals.

Nematophin is a 3-indoleethyl 3'-methyl-2'-oxopentamide consisting of an N-terminal  $\alpha$ -keto acid and a C-terminal tryptamine (TRA), first isolated from strain *X. nematophila* BC1. It displays strong antifungal and antibacterial activities (Li *et al.*, 1997; Kennedy *et al.*, 2000). A monomodular non-ribosomal peptide synthetase (NRPS), RdpD, is responsible for the biosynthesis of nematophin (Cai *et al.*, 2017). Xenocoumacins, including Xcn1 and Xcn2, are the principal antimicrobial compounds secreted by *X. nematophila*. Xcn1 exhibits marked antimicrobial activity against Gram-positive bacteria and fungal strains, while Xcn 2 shows substantially reduced bioactivity (McInerney *et al.*, 1991; Huang *et al.*, 2005, 2006; Yang *et al.*, 2011; Zhou *et al.*, 2017). Meanwhile, it has been demonstrated that fourteen genes (*xcnA-xcnN*) are involved in the synthesis of Xcns in *X. nematophila*. The genes *xcnA-L* are responsible for Xcn1 synthesis, whereas the genes *xcnM* and *xcnN* control the conversion of Xcn1 into Xcn2 (Park *et al.*, 2009). A prominent increase in Xcn1 along with a 20-fold reduced cell viability following attenuated Xcn 2 production suggests that the conversion of Xcn 1 to Xcn 2 is a resistance mechanism to circumvent self-toxicity (Park *et al.*, 2009).

The rapid bacterial adaptation ability to changing environmental conditions is crucial for their growth and

pathogenicity. Reports have shown that the growth of *X. nematophila* occurs in the nematode vesicle, a stressful and nutrient-deficient environment. Nevertheless, *X. nematophila* is speculated to encounter a relatively nutrient-rich environment, when released from nematode vesicles to insect haemolymph (Goodrich-Blair and Clarke, 2007; Herbert and Goodrich-Blair, 2007). In response to nutrient upshift, the bacteria grow rapidly and produce several metabolites to overcoming the insect immune system (Forst and Nealson, 1996) and preventing the growth of various bacterial and fungal competitors (Akhurst, 1982; Chen *et al.*, 1994). Several reports confirmed that the antimicrobial activity of *X. nematophila* varies according to the environment conditions (Chen *et al.*, 1996; Wang *et al.*, 2008a,b, 2010, 2011). However, it remains unclear how *X. nematophila* recognizes these changes or how these signals are associated with the antimicrobial activity. Increasing evidence indicates that two two-component systems (CpxRA and EnvZ/OmpR) are involved in responding to both of the nematode and insect environments (Park and Forst, 2006; Herbert *et al.*, 2007; Tran and Goodrich-Blair, 2009; Tran and Goodrich-Blair, 2009). As a sensor histidine kinase, CpxA and EnvZ phosphorylate its cognate response regulator CpxR and OmpR in response to change in pH and osmolality as well as other stimuli and regulate key genes essential for establishing interactions with each of these hosts.

As a global regulator, OmpR also represses antibiotic production, in  $\Delta ompR$  mutant strain, Xcn1 level and expression of *xcnA-L* were increased, while Xcn2 level and *xcnMN* expression were decreased (Park and Forst, 2006; Park *et al.*, 2009). Interestingly, CpxR also exhibits a similar regulatory mechanism for Xcns biosynthesis as OmpR. In  $\Delta cpxR$  mutant strain, the levels of *xcnA-L* expression were amplified, while *xcnMN* expression was diminished (Tang, 2015). However, to date, there is no report concerning the effects of CpxR on the growth characteristics and antibiotics biosynthesis potential of *X. nematophila*. Therefore, the present study investigated the effects of CpxR on the antibiotics production and growth of *X. nematophila*. The kinetics analysis of batch fermentation of the wildtype and  $\Delta cpxR$  mutant of *X. nematophila* YL001 was carried out in a 5-l laboratory-scale bioreactor. Specifically, we tested whether CpxR influenced the production of nematophin and Xcns.

## Results

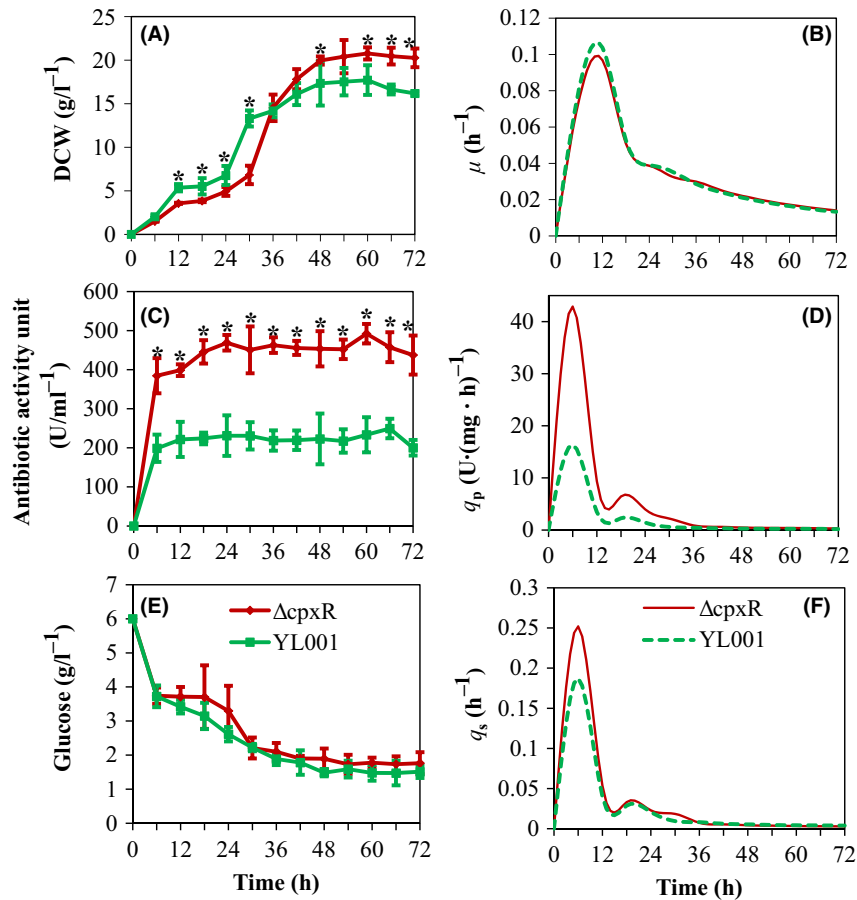
### *Effects of cpxR disruption on cell growth and antibiotics production*

The batch fermentation kinetics of the wildtype and  $\Delta cpxR$  of *X. nematophila* YL001 were studied in a 5-l bioreactor. Evidently, a distinct exponential and stationary

phase was observed from the bacterial growth curve (Fig. 1A). During 0–36 h of the bioprocess, the DCW of the wildtype strain was higher than that of  $\Delta cpxR$ ; however, after 36 h, the DCW of  $\Delta cpxR$  was found to be higher in comparison with the wildtype strain. Due to the rapid growth of wildtype compared with  $\Delta cpxR$  at earlier fermentation stage, DO concentration and pH decreased rapidly (Fig. 2). The maximum DCW of the wildtype and  $\Delta cpxR$  showed an optimal value of  $17.71 \pm 1.35$  and  $20.77 \pm 1.56 \text{ g L}^{-1}$ , respectively, when fermentation proceeded to the stationary phase, where the DO concentration was 17.8 and 3.5% respectively (Figs 1A and 2). However, the maximum specific cell growth rate ( $\mu_{\max}$ ) of  $\Delta cpxR$  was  $0.099 \pm 0.007 \text{ h}^{-1}$  and found to be lower compared with the wildtype strain (Fig. 1B). The cell yields on glucose ( $Y_{X/S}$ ) and cell production rate ( $P_X$ ) of  $\Delta cpxR$  were higher than that of the wildtype strain

(Table 1). It is obvious that  $\Delta cpxR$  mutation favoured the cell production. In consonance with diminishing cell biomass of  $\Delta cpxR$  during 0–36 h, the consumption of glucose was declined.

During the bioprocess,  $\Delta cpxR$  showed a significantly higher ( $p < 0.05$ ,  $t = 43.33$ ,  $d_f = 10$ ) antibiotic activity (Fig. 1C). The maximum antibiotic activity ( $492.0 \pm 31.2 \text{ U ml}^{-1}$ ) of  $\Delta cpxR$ , which was increased by 97.33% compared with the wildtype ( $249.33 \pm 15.23 \text{ U ml}^{-1}$ ), was achieved at 60 h. Profiles of the specific antibiotic production rate ( $q_p$ ) of the wildtype strain and  $\Delta cpxR$  showed identical trends. The time duration to reaching the maximum  $q_p$  was similar, whereas the declining rate was found to be different after  $q_p$  reached the maximum (Fig. 1D). The maximum  $q_p$  of the wildtype and  $\Delta cpxR$  was  $16.35 \pm 1.23$  and  $42.87 \pm 3.67 \text{ U mg}^{-1} \text{ h}^{-1}$  respectively. During the bioprocess, the average specific antibiotic biosynthesis rate of



**Fig. 1.** Time profiles of wildtype and  $\Delta cpxR$  mutant of *Xenorhabdus nematophila* YL001 during cultivation in the 5-l bioreactor.

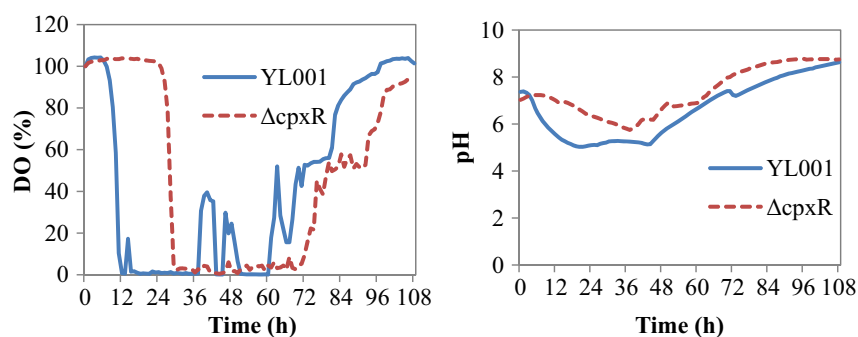
A. Cell growth  
B. Specific cell growth rate ( $\mu$ ).

C. Antibiotic activity.

D. Specific antibiotic production rate ( $q_p$ ).

E. Glucose.

F. Specific glucose consumption rate ( $q_s$ ). Error bars indicate standard deviations from three independent experimental measurements. Asterisk symbols denote significant difference at  $P = 0.05$  at different time between wildtype and  $\Delta cpxR$  mutant of *Xenorhabdus nematophila* YL001.



**Fig. 2.** The variations in dissolved oxygen concentration and pH of wildtype and  $\Delta cpxR$  mutant of *Xenorhabdus nematophila* YL001 during cultivation in the 5-l bioreactor.

**Table 1.** Batch fermentation parameters of wildtype and  $\Delta cpxR$  of *Xenorhabdus nematophila* YL001 at pH 7.0 in 5- and 70-l bioreactor.

Parameters	5 L		70 L
	YL001	$\Delta cpxR$	$\Delta cpxR$
$X_{\max}$ (g L <sup>-1</sup> )	17.71 ± 1.35	20.77 ± 1.56 <sup>a</sup>	24.71 ± 2.31 <sup>b</sup>
$P_{\max}$ (U ml <sup>-1</sup> )	249.33 ± 15.23	492.0 ± 31.20 <sup>a</sup>	381.07 ± 15.74 <sup>b</sup>
$P_X$ (g L <sup>-1</sup> h <sup>-1</sup> )	0.30 ± 0.05	0.35 ± 0.03	0.41 ± 0.06 <sup>b</sup>
$P_P$ (U ml <sup>-1</sup> h <sup>-1</sup> )	3.78 ± 0.28	8.20 ± 1.20 <sup>a</sup>	9.07 ± 2.53
$\mu$ (h <sup>-1</sup> )	0.107 ± 0.02	0.099 ± 0.007	0.035 ± 0.003 <sup>b</sup>
$\bar{\mu}$ (h <sup>-1</sup> )	0.037 ± 0.003	0.036 ± 0.004	0.021 ± 0.002 <sup>b</sup>
$q_p$ (U mg <sup>-1</sup> h <sup>-1</sup> )	16.35 ± 1.23	42.87 ± 3.67 <sup>a</sup>	6.410 ± 0.89 <sup>b</sup>
$\bar{q}_p$ (U mg <sup>-1</sup> h <sup>-1</sup> )	2.29 ± 0.26	6.04 ± 1.25 <sup>a</sup>	1.348 ± 0.26 <sup>b</sup>
$Y_{X/S}$ (g g <sup>-1</sup> )	2.82 ± 0.08	3.05 ± 0.55 <sup>a</sup>	5.626 ± 1.06 <sup>b</sup>
$Y_{P/S}$ (U g <sup>-1</sup> )	55.05 ± 6.21	116.52 ± 9.35 <sup>a</sup>	87.32 ± 12.3
$Y_{P/X}$ (U g <sup>-1</sup> )	14.99 ± 1.36	23.69 ± 0.89 <sup>a</sup>	16.44 ± 1.42 <sup>b</sup>
$q_s$ (h <sup>-1</sup> )	0.187 ± 0.06	0.252 ± 0.03 <sup>a</sup>	0.132 ± 0.002 <sup>b</sup>
$\bar{q}_s$	0.028 ± 0.004	0.036 ± 0.002 <sup>a</sup>	0.024 ± 0.003 <sup>b</sup>

Data are presented as a mean ± standard deviation from three replicated experiments.

**a.** Followed by the data in the column indicates significant differences of  $\Delta cpxR$  and YL001 in 5-l bioreactor at  $P = 0.05$ .

**b.** Followed by the data in the column indicates significant differences of  $\Delta cpxR$  between fermentation in 5- and 70-l bioreactor at  $P = 0.05$ .

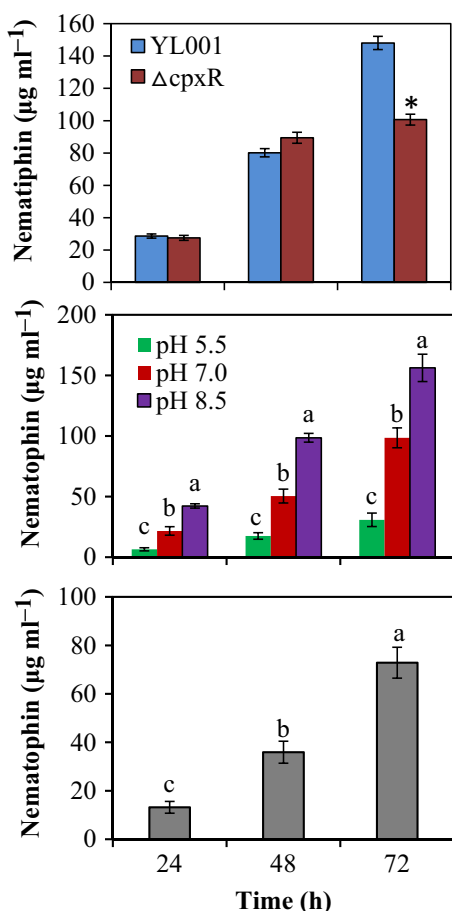
the wildtype and  $\Delta cpxR$  was  $6.04 \pm 1.25$  and  $2.29 \pm 0.26$  U mg<sup>-1</sup> h<sup>-1</sup> respectively. Similarly, the optimum antibiotic yield on glucose ( $Y_{P/S}$ ), the antibiotic productivity ( $P_P$ ) and the optimum antibiotic yield on cell ( $Y_{P/X}$ ) of  $\Delta cpxR$ , which were  $116.52 \pm 9.35$  U g<sup>-1</sup>,  $8.20 \pm 1.20$  U ml<sup>-1</sup> h<sup>-1</sup> and  $23.69 \pm 0.89$  U g<sup>-1</sup>, respectively, were higher than that of the wildtype strain (Table 1). It can be concluded that  $\Delta cpxR$  could significantly increase the antibiotic activity of *X. nematophila*.

Based on our findings that  $\Delta cpxR$  mutant exhibited higher antibiotic activity against *B. subtilis*, we also investigated the variations of nematophin and Xcns. At 24 and 48 h of the bioprocesses,  $\Delta cpxR$  had no obvious ( $p > 0.05$ ,  $t = 1.22$ ,  $d_f = 4$ , 24 h;  $t = -2.20$ ,  $d_f = 4$ , 48 h) effect on nematophin production, but at 72 h, the levels of nematophin in  $\Delta cpxR$  were significantly decreased by 48.7% compared with the wildtype strain (Figs 3A and S1). In addition,  $Y_{P/S}$ ,  $P_P$ ,  $Y_{P/X}$  and  $q_p$  of nematophin in  $\Delta cpxR$  were decreased by 41.12, 32.04, 45.68 and 45.67%, respectively, compared with the wildtype strain

(Table 2). However, Xcn1 in  $\Delta cpxR$  was 6.41-fold greater in contrast with the native strain (Fig. S2). These results indicate that CpxR positively regulates the production of nematophin while negatively regulates Xcns synthesis.

The rapid growth and increase in biomass of the wildtype at an early phase of fermentation resulted in the rapid decrease in DO concentration compared with  $\Delta cpxR$  (Fig. 2). Correspondingly, glucose uptake of the wildtype was faster than  $\Delta cpxR$  (Fig. 1E). At the late stage of fermentation, an increase in biomass of  $\Delta cpxR$  led to decreased DO concentration, but the uptake of glucose between two strains had no significant difference (Fig. 1E). At the end of the fermentation, the glucose concentration in the broth of the strains was 1.51 and 1.76 g L<sup>-1</sup>, and the total glucose consumption was 74.8 and 70.67%. Profiles of the specific glucose consumption rate ( $q_s$ ) of the wildtype and  $\Delta cpxR$  displayed comparable trends. The time duration of attaining the maximum  $q_s$  was identical, but a different decaying trend





**Fig. 3.** The concentration of nematophin produced by wildtype and  $\Delta cpxR$  mutant of *Xenorhabdus nematophila* YL001. A. The wildtype and  $\Delta cpxR$  mutant were cultured at pH 7.0 in 5-l bioreactor. B.  $\Delta cpxR$  mutant was cultured at pH 5.5, 7.0 and 8.5 in 5-l bioreactor. C.  $\Delta cpxR$  mutant was cultivated at pH 7.0 in 70-l bioreactor. Error bars indicate standard deviations from three independent samples. Different lowercase letters above the bar indicate significant differences at  $P = 0.05$ . Asterisk symbols denote significant difference at  $P = 0.05$ .

was observed after  $q_s$  reached the maximum (Fig. 1F). Notably, the maximum  $q_s$  and the average specific glucose consumption rate of the wildtype and  $\Delta cpxR$  showed a significant difference (Table 1).

#### Effects of pH on the cell growth and antibiotics production of $\Delta cpxR$

At different initial pH, cell growth was observed to be considerably changed with the pH operation applied (Fig. 4A). At pH 8.5, the presence of an obvious lag phase was characterized by a higher DO concentration at an early phase of fermentation (Fig. 5). During 0–42 h of the fermentation, the DCW was reduced at pH 8.5 in

comparison with other pH conditions. After 48 h, the DCW was observed to be higher than that at pH 5.5 and lower than that at pH 7.0. The highest DCW of each original pH displayed an optimum value of  $20.44 \pm 2.65 \text{ g L}^{-1}$  at pH 7.0, which was reduced by 31.5% and 21.14% at pH 5.5 and 8.5 respectively. The maximum specific cell growth rate ( $\mu_{max}$ ) at pH 5.5, 7.0 and 8.5 was  $0.065 \pm 0.013$ ,  $0.065 \pm 0.009$  and  $0.054 \pm 0.011 \text{ h}^{-1}$ , and the average specific cell growth rate during the bioprocess was  $0.030 \pm 0.01$ ,  $0.030 \pm 0.005$  and  $0.024 \pm 0.007 \text{ h}^{-1}$  respectively (Fig. 4B, Table 3). Likewise, the optimal cell yield on glucose ( $Y_{X/S}$ ) and the maximum cell productivity ( $P_X$ ), that is, which were  $4.03 \pm 1.25 \text{ g g}^{-1}$  and  $0.357 \pm 0.005 \text{ g L}^{-1} \text{ h}^{-1}$  at pH 7.0, were higher than that at other pH conditions (Table 3). The optimum original pH for maximal cell proliferation of  $\Delta cpxR$  was found to be pH 7.0.

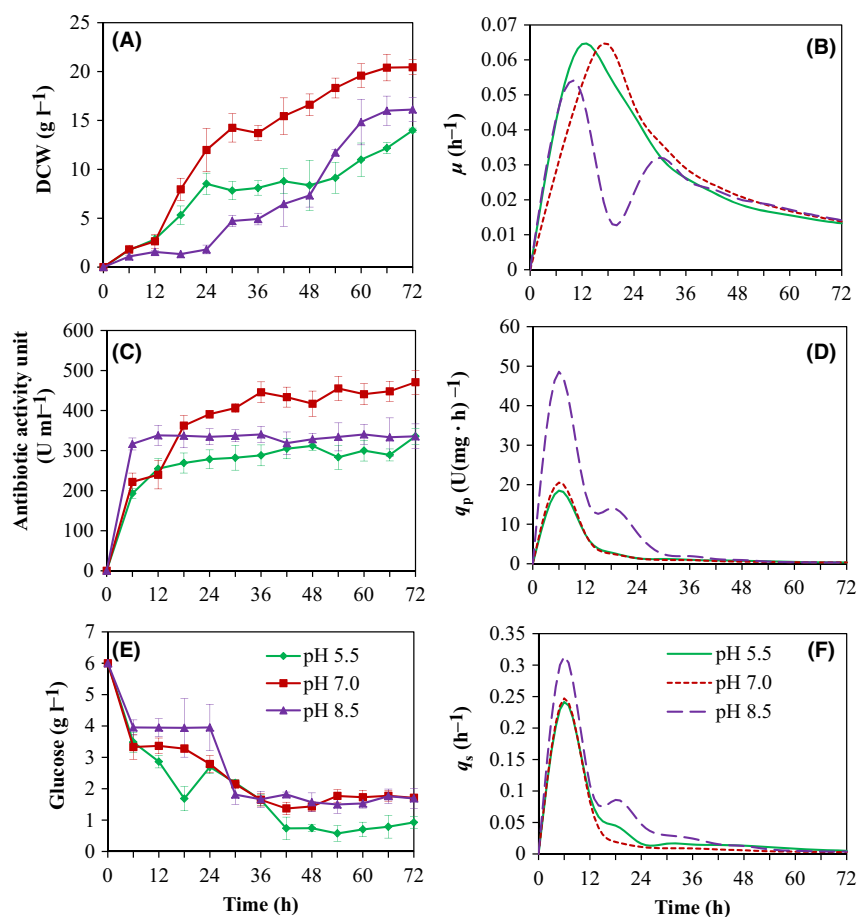
The influence of different pH on the antibiotic production potential of  $\Delta cpxR$  is portrayed in Fig. 4C. A higher antimicrobial activity was observed at pH 8.5 than that at pH 5.5 and 7.0 during the first 0–18 h. However, after 24 h of bioprocess, the antibiotic activity was significantly increased at neutral pH (pH 7.0) in contrast with pH 5.5 and 8.5. The maximum antibiotic activity ( $470.71 \pm 15.67 \text{ U ml}^{-1}$ ), which was 28.68% and 27.67% higher compared with pH 5.5 and 8.5, was obtained at pH 7.0, where the maximum DCW was obtained. Profiles of the specific antibiotic production rate ( $q_p$ ) exhibited similar trends at varying pH, and  $q_p$  reached the maximum value at 6 h of the bioprocesses. The highest  $q_p$  was achieved at pH 8.5 and reduced by 61.97% and 57.71% at pH 5.5 and 7.0 respectively (Fig. 4D, Table 3). Consistent with the  $q_p$  of  $\Delta cpxR$  at different pH, during the bioprocess, the  $\bar{q}_p$  of  $\Delta cpxR$  at pH 8.5 was also significantly higher than that at pH 5.5 and 8.5 (Table 3). Similarly, the highest antibiotic yield on glucose ( $Y_{P/S}$ ) and the antibiotic productivity ( $P_P$ ) achieved at pH 7.0 were  $92.79 \pm 2.58 \text{ U g}^{-1}$  and  $6.54 \pm 0.58 \text{ U ml}^{-1} \text{ h}^{-1}$  respectively. Nevertheless, the maximum antibiotic yield on the cell ( $Y_{P/X}$ ) was  $24.00 \pm 1.09 \text{ U g}^{-1}$  at pH 5.5 (Table 3). The findings inferred that among the different pH investigated, pH 7.0 was found to be promising for antibiotic biosynthesis due to the elevated cell growth at this pH.

The influence of varying pH on the biosynthesis of nematophin and Xcns was also determined. A noticeable effect ( $p < 0.05$ ) of different pH was observed in Xcns biosynthesis. At pH 7.0, Xcn1 production was 5.45- and 3.87-fold higher than that at pH 5.5 and 8.5 respectively (Table 4, Figs S3 and S4). In addition, pH also had a significant effect on nematophin production (Fig. S5). In comparison with pH 5.5 and 7.0, the nematophin concentration was recorded to be significantly higher at pH

**Table 2.** Parameters of the batch fermentation of nematophin produced by *Xenorhabdus nematophila* YL001 wildtype and  $\Delta cpxR$  at different pH in 5- and 70-l bioreactor.

Parameters	5 L			70 L	
	YL001	$\Delta cpxR$		$\Delta cpxR$	
	pH 7.0	pH 5.5	pH 7.0	pH 8.5	pH 7.0
Nematophin ( $\mu\text{g ml}^{-1}$ )	148.06 $\pm$ 5.61 a	42.30 $\pm$ 3.67 c	100.67 $\pm$ 3.38 b	156.2 $\pm$ 3.67 a	72.85 $\pm$ 2.58*
$P_P$ ( $\mu\text{g ml}^{-1} \text{h}^{-1}$ )	2.06 $\pm$ 0.35 a	0.59 $\pm$ 0.12 c	1.40 $\pm$ 0.26 b	2.17 $\pm$ 0.18 a	1.01 $\pm$ 0.03*
$q_p$ ( $\mu\text{g mg}^{-1} \text{h}^{-1}$ )	0.127 $\pm$ 0.015 a	0.042 $\pm$ .011 c	0.069 $\pm$ .0012 b	0.135 $\pm$ .005 a	0.042 $\pm$ .012*
$Y_{P/S}$ ( $\mu\text{g g}^{-1}$ )	32.98 $\pm$ 2.58 b	9.86 $\pm$ 1.67 d	19.42 $\pm$ 2.31c	36.26 $\pm$ 0.58 a	16.70 $\pm$ 0.37*
$Y_{P/X}$ ( $\mu\text{g g}^{-1}$ )	9.15 $\pm$ 1.32 a	3.02 $\pm$ 0.85 c	4.97 $\pm$ 0.78 b	9.69 $\pm$ 1.35 a	3.04 $\pm$ 0.79*

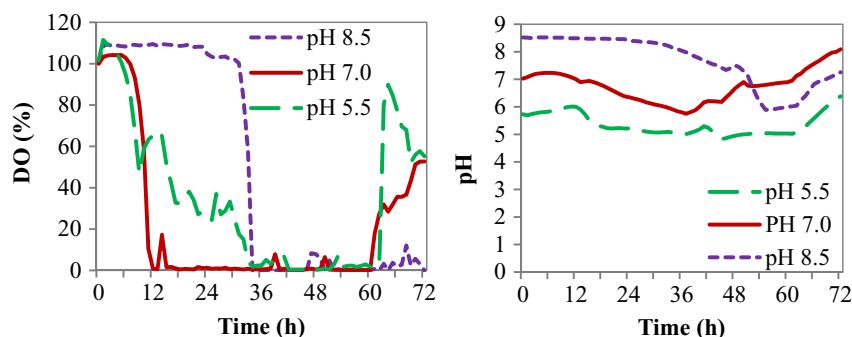
Data are presented as a mean  $\pm$  standard deviation from three replicated experiments. Different lowercase letters followed by the data in each row indicate significant differences at  $P = 0.05$ . '\*' followed by the data in the column indicates significant differences of  $\Delta cpxR$  fermentation in pH 7.0 between in 5- and 70-l bioreactor at  $P = 0.05$ .

**Fig. 4.** Time profiles of  $\Delta cpxR$  mutant of *Xenorhabdus nematophila* YL001 at different pH during cultivation in the 5-l bioreactor.

- A. Cell growth.
- B. Specific cell growth rate ( $\mu$ ).
- C. Antibiotic activity.
- D. Specific antibiotic production rate ( $q_p$ ).
- E. Glucose.
- F. Specific glucose consumption rate ( $q_s$ ).

8.5. At 72 h, the concentration of nematophin under pH 8.5 was  $156.25 \pm 3.67 \mu\text{g ml}^{-1}$ , 1.59- and 3.69-fold greater than that at pH 7.0 and 5.5. Likewise, the optimal

$Y_{P/S}$ ,  $P_P$ ,  $Y_{P/X}$  and  $q_p$  of nematophin at pH 8.5 were 86.71, 58.39, 101.04 and 101.49% higher compared with that at pH 7.0 (Fig. 3B, Table 2).



**Fig. 5.** The variations in dissolved oxygen concentration and pH of  $\Delta cpxR$  mutant of *Xenorhabdus nematophila* YL001 during cultivation at different pH in the 5-l bioreactor.

**Table 3.** Parameters of the batch fermentation of  $\Delta cpxR$  at different pH in the 5-l bioreactor.

Parameters	$\Delta cpxR$		
	pH 5.5	pH 7.0	pH 8.5
$X_{max}$ (g L <sup>-1</sup> )	13.99 ± 1.38 c	20.44 ± 2.65 a	16.12 ± 0.57 b
$P_{max}$ (U ml <sup>-1</sup> )	335.71 ± 25.73 b	470.71 ± 15.67 a	340.48 ± 20.36 b
$P_X$ (g L <sup>-1</sup> h <sup>-1</sup> )	0.231 ± 0.03 b	0.357 ± 0.005 a	0.166 ± 0.02 b
$P_P$ (U ml <sup>-1</sup> h <sup>-1</sup> )	4.66 ± 1.13 b	6.54 ± 0.58 a	5.67 ± 1.67 ab
$\mu$ (h <sup>-1</sup> )	0.065 ± 0.013 a	0.065 ± 0.009 a	0.054 ± 0.011 ab
$\bar{\mu}$ (h <sup>-1</sup> )	0.030 ± 0.01 a	0.030 ± 0.005 a	0.024 ± 0.007 b
$q_p$ (U mg <sup>-1</sup> h <sup>-1</sup> )	18.495 ± 2.56 c	20.540 ± 1.68 b	48.569 ± 4.331 a
$\bar{q}_p$ (U mg <sup>-1</sup> h <sup>-1</sup> )	3.098 ± 0.556 b	3.192 ± 0.796 b	8.392 ± 1.875 a
$Y_{X/S}$ (g g <sup>-1</sup> )	3.26 ± 0.34 b	4.03 ± 1.25 a	3.74 ± 1.13 ab
$Y_{P/S}$ (U g <sup>-1</sup> )	78.28 ± 5.30 b	92.79 ± 2.58 a	76.18 ± 6.13 b
$Y_{P/X}$ (U g <sup>-1</sup> )	24.00 ± 1.09 a	23.03 ± 3.24 a	22.95 ± 0.67 a
$q_s$ (h <sup>-1</sup> )	0.241 ± 0.052 b	0.247 ± 0.007 b	0.314 ± 0.021 a
$\bar{q}_s$ (h <sup>-1</sup> )	0.042 ± 0.006 a	0.036 ± 0.013 a	0.057 ± 0.015 a

Data are presented as a mean ± standard deviation from three replicated experiments. Different lowercase letters followed by the data in each row indicate significant differences at  $P = 0.05$ .

**Table 4.** The relative amount of xenocoumactins (Xcn1 and Xcn2) in  $\Delta cpxR$  of *Xenorhabdus nematophila* YL001 at different pH

pH	Relative amount of Xcns <sup>a</sup>	
	Xcn1	Xcn2
5.5	1 c	1 b
7.0	5.45 a	1.06 b
8.5	1.41 b	2.4 a

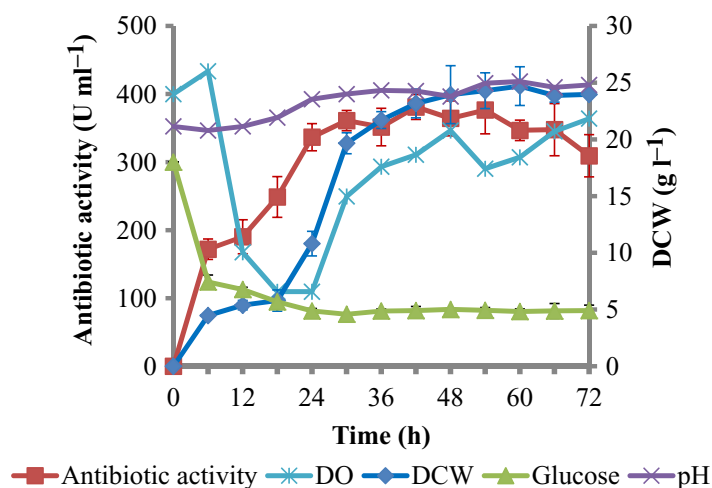
a. The peak area of extracted ion chromatogram (EIC) of Xcns at different pH/the peak area of extracted ion chromatogram (EIC) of Xcns at pH 5.5, the peak area was calibrated by its OD<sub>600</sub> value, and the relative amount of Xcns at pH 5.5 was referred to 1. Different lowercase letters followed by the data of Xcn1 and Xcn2 at different pH indicate significant differences at  $P = 0.05$ .

The rapid growth and rise in biomass at an early phase of fermentation at pH 7.0 resulted in the rapid decrease of DO concentration compared with that at pH 5.5 and 8.5 (Fig. 5). Consistently, glucose uptake at pH 7.0 was faster than that at pH 5.5 and 8.5 (Fig. 4E). Similarly, at the late stage of fermentation, a higher

biomass at pH 7.0 resulted in the increased uptake of glucose. During 0–42 h of the fermentation, the total glucose consumption at pH 5.5, 7.0 and 8.5 was 76.0, 87.67 and 69.67% respectively. At the end of the fermentation, the glucose concentration was 1.71, 0.93 and 1.69 g L<sup>-1</sup> at pH 5.5, 7.0 and 8.5 respectively. However, the highest  $q_s$  ( $0.314 \pm 0.021$  h<sup>-1</sup>) was obtained at pH 8.5 and decreased by 30.29% and 27.13% at pH 5.5 and 7.0 respectively (Fig. 4D). The average specific glucose consumption rate during the bioprocess at pH 5.5, 7.0 and 8.5 was  $0.042 \pm 0.006$ ,  $0.036 \pm 0.013$  and  $0.057 \pm 0.015$  h<sup>-1</sup> respectively (Table 3).

*Batch fermentation characteristics of  $\Delta cpxR$  mutation in the 70-l bioreactor*

Based on the 5-l bioreactor-based fermentation results, the batch fermentation kinetics of  $\Delta cpxR$  mutation was carried out in the 70-l bioreactor. The growth profile displayed a distinctive exponential and stationary phase, and antibiotic production occurred throughout the



**Fig. 6.** Time-course data of batch fermentation of  $\Delta cpxR$  mutant of *Xenorhabdus nematophila* YL001 in 70-l bioreactor with the initial pH of 7.0, the agitation speed of 150 rpm and the aeration rate of 40.0 L min<sup>-1</sup>.

bioprocess (Fig. 6). The maximum DCW and antibiotic activity ( $P_{max}$ ) in 70-l bioreactor were  $24.71 \pm 2.31$  g L<sup>-1</sup> and  $381.07 \pm 15.74$  U ml<sup>-1</sup>, which was increased by 18.97%, and decreased by 27.71% compared to that in 5-l bioreactor respectively. The concentration of nematophin ( $72.85 \pm 2.58$   $\mu$ g ml<sup>-1</sup>) produced by  $\Delta cpxR$  at 72 h was decreased by 26.06% compared to the 5-l bioreactor.  $Y_{P/S}$ ,  $P_P$ ,  $Y_{P/X}$  and  $q_p$  of nematophin in 70-l bioreactor were decreased by 14.01, 26.28, 36.93 and 37.31% compared with that in the 5-l bioreactor (Fig. 3C, Table 2). Xcn1 of  $\Delta cpxR$  was decreased by 11.0% relative to that in the 5-l bioreactor (data not shown).

## Discussion

In contrast to the wildtype strain,  $\Delta cpxR$  mutant exhibited a prolonged lag phase, and lower DCW at early- and mid-stage. These findings were consistent with the previous report, in which *cpxR* inactivation results in a prolonged lag phase when cultivated in either LB culture or haemolymph (Herbert *et al.*, 2007). At the late stage of fermentation, the DCW of  $\Delta cpxR$  was enhanced relative to the wildtype, whereas, in the previous study, the cell biomass between a *cpxR* deletion mutant and wildtype of *X. nematophila* had no significant difference (Herbert *et al.*, 2007). The difference in growth profiles between the parent strain and  $\Delta cpxR$  derivative may be associated with the rapid adaptability to fluctuating environmental perturbations. CpxRA could sense the changing environment and regulate the growth and metabolisms to adapt the environment of insect haemolymph (Herbert *et al.*, 2007; Tran and Goodrich-Blair, 2009; Herbert *et al.*, 2009). However,  $\Delta cpxR$  mutant was defective in adapting

to this nutrient source and displayed a protracted lag phase. This may be due to the lack of metabolism adaptability of  $\Delta cpxR1$  mutant to the nutrients available in haemolymph but is more prone to insect antimicrobial activities (Herbert *et al.*, 2007). Previously, it has been found that the  $\Delta cpxR1$  exhibited a prolonged lag phase during *in-vitro* growth in haemolymph (Herbert *et al.*, 2007), a finding consistent with the idea that it is defective in acclimatizing to this nutrient source.

Generally, antibiotic biosynthesis is induced at high cell density and is regulated, in most of the cases by quorum sensing (Romero *et al.*, 2011). In this study, in all cases, the nematophin biosynthesis could be improved by raising the concentration of antibiotic-producing cells (Fig. 3). Li *et al.* (1997) and Park *et al.* (2009) reported comparable outcomes that the concentration of nematophin and Xcns increased by increasing the bacterial cell density and attained the maximal level at the stationary phase. However, in  $\Delta cpxR$  mutant, the level of Xcn1 was increased significantly while nematophin was decreased during the stationary phase, where the DCW of  $\Delta cpxR$  was higher. Our findings confirmed that quorum sensing is not an essential process to regulate the antimicrobial biosynthesis (Singh and Forst, 2016), and global regulators particularly govern the biosynthesis of small molecules in *X. nematophila* (Park *et al.*, 2009; Engel *et al.*, 2017). Therefore, identifying and engineering of these regulators can provide a noteworthy approach for the discovery of new secondary metabolites and increased production of useful molecules.

In this study, the  $\Delta cpxR$  strain exhibited an increase in antibiotic activity. This suggested that the biosynthetic pathway of secondary metabolites was induced in  $\Delta cpxR$ . However, although the production of Xcn1 in



$\Delta cpxR$  was 3.07-fold higher, nematophin production was significantly decreased. Meanwhile, we did not rule out the possibilities that the *cpxR* gene disruption could induce the production of previously cryptic metabolites, and the biological activities of these induced cryptic metabolites need to be further validated by purification and characterization. Taken together, the improved antibiotic activity of  $\Delta cpxR$  might be attributed to the mutual effect of higher Xcn1 or the cryptic antibiotics produced in  $\Delta cpxR$  strain.

In CpxRA signal transduction system, CpxA senses a variety of stresses and changes in external conditions, including pH, phosphorylates CpxR, and activates this response regulator to regulate the expression of its regulon (Herbert *et al.*, 2007). Previously, we have demonstrated the significant influence of different pH on the growth profile and antibiotic biosynthesis titre of *X. nematophila* YL001 (Wang *et al.*, 2011). However, it is uncertain whether CpxA can sense the pH changing and affect the growth and antibiotic production of *cpxR*-inactivated mutant. Our study indicated that pH had a marked consequence of the growth and production of antibiotic by  $\Delta cpxR$  strain. The cell biomass, the maximum DCW and antibiotic production of  $\Delta cpxR$  strain all were varied at different pH conditions. However, there was no significant difference in cell biomass of the wildtype at different pH conditions (Wang *et al.*, 2011). The growth difference between the wildtype and  $\Delta cpxR$  strain might be related to the hypersensitivity of  $\Delta cpxR$  strain to alkaline conditions. Danese and Silhavy (1998) found that the *cpxR* null mutant of *E. coli* was the most hypersensitive to alkaline condition, and showed marked growth defects at an extreme alkaline pH. Also, the antibiotic production of  $\Delta cpxR$  strain had a significant difference between pH 5.5, 7.0 and 8.5 (Table 1, Fig. S3). Our previous study revealed that antibiotic activity and the relative amount of Xcn1 of wildtype at pH 8.5 were higher relative to that at pH 5.5 and pH 7.0 (Guo, 2015). Moreover, in *X. nematophila*, CpxR and OmpR negatively regulate the production of Xcn1 (Park *et al.*, 2009; Tang, 2015). Although CpxR can be activated by alkaline pH, it works as a CpxA-dependent response (Siryaporn and Goulian, 2008; Wolfe *et al.*, 2008). At pH 8.5, the expression level of *cpxR* and *ompR* of wildtype of *X. nematophila* YL001 was significantly lower than that at pH 5.5 and 7.0 (Guo, 2015). Therefore, the Xcn1 levels of the wildtype were increased at pH 8.5. However, the *ompR* expression level of  $\Delta cpxR$  strain at pH 8.5 was significantly greater than that at pH 5.5 and 7.0 (Tang, 2015). Thus, at pH 7.0 the  $\Delta cpxR$  strain exhibited the higher levels of Xcn1 and antibiotic activity. The ability of  $\Delta cpxR$  strain to sense the changes in pH, and regulating the growth and antibiotic production might be related to the cross-talk

between a histidine kinase and a non-cognate response regulator of different two-component signalling systems. Siryaporn and Goulian (2008) detected the cross-talk between the histidine kinase CpxA and non-cognate response regulator OmpR in *E. coli*. Hence, we speculated that OmpR of  $\Delta cpxR$  strain could be triggered by alkaline pH conditions.

Based on the scale-up method of a constant mass transfer coefficient, *X. nematophila* YL001 was successfully scaled-up from shaking flask to a 5-l bioreactor, and further to a 70-l bioreactor (Wang, 2004). In this study, according to the fermentation condition of *X. nematophila* YL001 in a 70-l bioreactor,  $\Delta cpxR$  strain was cultured in a 70-l bioreactor.  $\Delta cpxR$  strain exhibited the similar kinetics profiles, compared with fermentation in the 5-l bioreactor. However, the profiles of DO concentration of  $\Delta cpxR$  strain in 5- and 70-l bioreactor had a significant difference ( $p < 0.05$ ,  $t = 5.491$ ,  $d_f = 16$ ). In the 70-l bioreactor, the DO concentration was above 20% during the whole bioprocess but was maintained to around zero level in a growth phase in the 5-l bioreactor. The fermentation condition in 70-l bioreactor could ensure the sufficient supply of oxygen in the fermentation process that is favourable to both the cell growth and metabolic activity. So, the maximum DCW of  $\Delta cpxR$  strain in the 70-l bioreactor was increased by 18.97%, but the maximum antibiotic activity was decreased by 27.71%, compared to that in the 5-l bioreactor. These findings were consistent with the results of scale-up of *X. nematophila* YL001 from a 5-l to the 70-l bioreactor. These showed that the fermentation conditions used in the 70-l bioreactor were beneficial for cell growth, but not favourable to the biosynthesis of antibiotics. Thus, the optimum fermentation condition for antibiotic production by  $\Delta cpxR$  strain will be further investigated.

These findings confirmed that  $\Delta cpxR$  mutation significantly increased the biomass of *X. nematophila* at a late stage of fermentation, and promoted the production of Xcns, but decreased the production of nematophin. In addition, pH had remarkable effects on the bacterial cell growth and antimicrobial activity of  $\Delta cpxR$  mutant. Thus, this work provides an efficient way to obtain massive antimicrobial production of Xcns or nematophin in *X. nematophila*.

## Experimental procedures

### Microorganism

The *X. nematophila* wildtype strain was isolated from the soil in Yangling, China. The *cpxR*-null mutant strain ( $\Delta cpxR$ ) derived from YL001 by the allelic exchange that contains a kanamycin (Km) cassette instead of the *cpxR* gene was used (Tang, 2015). Phase I variant of *X.*

*nematophila* preserved in glycerol at  $-70^{\circ}\text{C}$  was used as seed inoculum for cultures. NBTA NA medium supplemented with triphenyl tetrazolium chloride ( $0.040\text{ g L}^{-1}$ ) and bromothymol blue ( $0.025\text{ g L}^{-1}$ ) was employed to test the phase variant of *X. nematophila*.

#### Preparation of inoculum

A loopful of phase I of *X. nematophila* YL001 wildtype and  $\Delta\text{cpxR}$  was inoculated into 250-ml Erlenmeyer flasks comprising 100 ml sterilized NB (NA without agar) medium with the final pH of 7.2. The flasks were then incubated in dark at  $28^{\circ}\text{C}$  in a temperature-controlled rotary shaker at an agitation speed of 150 rpm for 16 to 24 h.

#### Batch fermentation characteristics in the laboratory-scale bioreactor

Fermentation of  $\Delta\text{cpxR}$  of *X. nematophila* YL001 was investigated in batch fermentation using a 5-l bioreactor (Eastbio, China). The pH, temperature, DO and agitation speed were monitored online by a computer-coupled system. The medium containing ( $\text{g L}^{-1}$ ) glucose 6.13, peptone 21.29,  $\text{KH}_2\text{PO}_4$  0.86,  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  1.50,  $\text{Na}_2\text{SO}_4$  1.72,  $\text{K}_2\text{HPO}_4$  1.11 and  $(\text{NH}_4)_2\text{SO}_4$  2.46 was adjusted to pH 7.2 by the addition 1 M HCl or 1 M NaOH. The influence of varying pH on the growth pattern and antibiotic production of  $\Delta\text{cpxR}$  was also evaluated in batch fermentation using the same bioreactor. Details have been shown in the Supporting Information.

#### Batch fermentation process in 70-l bioreactor

Fermentation of  $\Delta\text{cpxR}$  was also carried out in a 70-l bioreactor (Eastbio, China) with a working volume of 50 L. The bioreactor was equipped with two six-blade disc turbine impeller. The pH, temperature, DO and agitation speed were monitored online. The fermentation medium, pH, temperature and inoculum level used were identical with the 5-l bioreactor. Throughout the fermentation bioprocessing, the temperature levels were regulated automatically. The bioreactors were operated under well-controlled conditions as mentioned above for 5-l bioreactor for 72 h.

#### Analytical methods

The method for the determination of DCW ( $\text{g L}^{-1}$ ), the specific cell growth rate ( $\mu$ ), the glucose concentration and the antibiotic activity assay was shown in Supporting Information. All of the results were averaged and presented as the mean  $\pm$  standard deviation from triplicate experiments. Student's t-test using SPSS 18.0 (SPSS, Chicago, IL, USA) statistically analysed the data.

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#### Conflict of interest

The authors have no conflict of interest.

#### Author Contributions

Y.H.W. proposed and supervised the project. X.Z. designed the experiments. S.Q.G., Z.Y.W., B.L.L., J.T.G., X.L.F. and Q.T. conducted the experiments. S.Q.G., M.B., X.L.F. and Y.H.W. analysed the data and drafted the manuscript. All authors read and approved the final manuscript.

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