Increasing the Production of Xenocoumacin 1 by Optimizing the Fermentation Process of Xenorhabdus Nematophila

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Research

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Abstract

Background: Xenocoumacin 1 (Xcn 1), a kind of water-soluble antibiotic discovered from the cell-free broth of *Xenorhabdus nematophila* YL001, has exhibited excellent activity against bacteria, oomycetes and fungi. However, the low yield limits the development and utilization of Xcn1. In order to increase the yield of Xcn1, the fermentation process was optimized in this study.

Results: Maltose and proteose peptone were identified as the best carbon and nitrogen sources that significantly affected Xcn1 production using a-factor-at-a-time approach. Response surface methodology was applied to optimize the medium constituents for Xcn1 production by *X. nematophila* YL001. Higher Xcn1-content (113.65 μg/mL) was obtained after optimizing medium components. The optimal levels of medium components were (g/L): proteose peptone 20.83, maltose 12.74, K_2HPO_4 3.77. Fermentation conditions, such as initial pH, inoculum size, temperature, rotation speed, liquid volume and the length of fermentation, were also investigated by using a-factor-at-a-time method to get a higher production of Xcn1. *X. nematophila* YL001 was able to produce higher Xcn1 (153.56 μg/mL) at 25°C, initial pH 7.0, inoculum size 10%, culture medium 75 mL in a 250 mL shake flash with an agitation rate of 150 rpm for 48h. Additionally, kinds, concentrations and adding time of the precursor were also investigated. *X. nematophila* YL001 was able to produce the highest Xcn1 (173.99 μg/mL) when the arginine was added to the broth with 3 mmol/L at the 12th hour. An overall 243.38% increase in Xcn1 content was obtained as compared with mean observed response at TSB medium.

Conclusions: To the best of our knowledge, there are no reports on optimization of fermentation process for Xcn1 production quantified by HPLC. The results show that nutrition, precursors and fermentation conditions had a highly influence on the production of Xcn1 by *X. nematophila* YL001. The optimized medium and fermentation conditions resulted in a 243.38% increase in Xcn1 production. This work will be helpful for the development of *X. nematophila* YL001 cultivation process for efficient Xcn1 production and lay a foundation for its industrial production.

Background

Microorganisms play an essential role in the discovery of new drugs and pesticides, since they are responsible for the majority of bioactive secondary metabolites [1–3]. Research and development of metabolites of microorganisms is one of primary and effective ways to develop novel pesticides. Xenocoumacin 1 (Xcn 1), a water-soluble antibiotic produced by *Xenorhabdus nematophila* belongs to dihydroisocoumarin derivative, is originally discovered in the fermentation broth of *Xenorhabdus* sp. Q1(ATCC 39497) [4]. Pharmacological studies on the Xcn1 showed that it has antibacterial and antifungal activity. There was high activity against *Escbericbia coli* ESS and *Cryptococcus neoformans* where MIC values are 0.5 μg/mL and 0.125 μg/mL, respectively [4]. Xcn1 also showed good biological activity on various oomycetes such as Xcn1 exhibits strong activity against five species of *Phytophthora*, with EC_{50} values ranging from 0.25 to 4.17 μg/mL [5]. In addition, Xcn1 efficiently controls the Phytophthora blight of pepper with a disease reduction of 99% at a concentration of 5 μg/mL assessed

on the third day after incubation of wound stem plants [6]. Therefore, Xcn1 has the potential to be developed as a new pesticide. However, the low yield and high cost of chemical synthesis Xcn1 limit its industrial production and application. Approximately 7–9 steps of chemical reaction are used to synthesize Xcn1 [7]. The low yield of Xcn1 in the broth of *X. nematophila* is also a serious limitation to its commercialization. The yield of Xcn1 of *X. nematophila* CB6 was 146μg/mL with 100mL B4 medium in 500mL flasks at 28°C and 180 rpm for 72h [8]. Yields of up to 300 μg/mL of Xcn1 was obtained from cultures of the *X. nematophila* All strain (ATCC 53200) in TSB medium at 28°C, pH 7.0, with sufficient agitation and aeration to maintain dissolved oxygen above 40% of saturation for 48h [4]. Xcn1, with concentration lower than 100μg/mL in TSB medium, was also separated from the fermentation broth of *X. nematophila* YL001 found in Yangling, China [9]. Therefore, it is necessary to improve the production of Xcn1 in the culture of *X. nematophila*.

Several strategies, improving the biosynthetic efficiency, such as tuning the expression of regulatory genes, increasing the supply of precursors, and over-expressing the biosynthesis and export genes, are used to increase antibiotic yields [10, 11]. On the one hand, precursors are crucial factors in achieving the production of the target natural products [12, 13], the production of antibiotics is increased by adding appropriate precursors. For instance, addition of valine, a biosynthetic precursors of glycopeptide antibiotic A40926, to minimal medium increased A40926 production [14]. Sodium decanoate is an effective precursor for synthesis of daptomycin from *Streptomyces roseosporus* NRRL11379 which is increased the daptomycin production remarkably [15]. On the other hand, promoter exchange experiments are used to induce higher levels of compounds production with biological activity [16, 17]. For example, exchange of the native promoter to the arabinose-inducible promoter is established in entomopathogenic bacteria to silence or activate gene clusters involved in natural product biosynthesis, which resulted in a tenfold increase in production for a number of compounds in *Photorhabdus luminescens* [18, 19]. In addition, understanding the underlying mechanism of gene expression regulation is necessary to improve the yield of known natural products [20]. The regulation of natural-product biosynthesis typically involves pleiotropic global regulators that either directly activate or repress biosynthetic genes or do so via cluster-situated activators or repressors [21]. Understanding the biochemical and molecular mechanisms of essential Xcn1 biosynthesis could aid in metabolic regulation for enhanced essential Xcn1 production. Arginine, leucine, urea and acetic acid are identified as precursors of Xcn1 biosynthesis in *X. nematophila* [22, 23]. Biosynthetic gene cluster of Xcn1 has been identified that the production of Xcn1 is promoted by *xcnA-L* genes and lessened by *xcnMN* genes [22]. In order to reduce the toxicity of Xcn1 to itself, *X. nematophila* splits Xcn1 by expressing *xcnM* and *xcnN* genes [24, 25]. The global regulator Lrp positively regulate the production of Xcn1 in *X. nematophila*, but LeuO regulates it negatively [26]. Induce-expressed or overexpressed *lrp* significantly increases the content of Xcn1, and not-expressed *leuo* controlling by non-inducible promoter also significantly increases the content of Xcn1 [26]. The global regulator Fliz up-regulates the expression of *xcnA-N* genes [27]. However, the molecular mechanisms of these global regulators on the regulation of Xcn1 biosynthesis are lacking. In addition, transcription regulator OmpR negatively regulates *xcnA-L* genes expression while positively regulates *xcnMN* expression, which led to a 3.3 fold yield of Xcn1 in *ΔompR* mutant [25]. Interestingly, transcription regulator CpxR not only regulates

the biosynthesis of Xcn1 with the same regulation model of OmpR, but also positively regulates *ompR* transcription [28, 29]. Nonetheless, it is unclear that how transcription regulator OmpR and CpxR regulate Xcn1 biosynthesis in molecular level. Differential regulation of OmpR to *xcnA-N* gene may be related to its binding position on the promoter. OmpR is a transcription inhibitor when it combines with the transcription initiation site, and active transcription when it combines with upstream activating sequence [30].

Yield of natural product is also improved by changing the medium composition such as carbon source, nitrogen source and inorganic ion or optimizing the fermentation conditions such as dissolved oxygen, pH, temperature and fermentation time [31–34]. The medium composition of *X. nematophila* YL001 and *X. stockiae* PB09 were optimized by response surface methodology, which enhancing the production of antibiotic and antifungal activity [35, 36]. With the optimized fermentation conditions, antibiotic yield of *X. nematophila* YL001 was increased compared with un-optimized, significantly [37]. Based on a two-stage pH control strategy, the effects of different pH on cell growth and antibiotic activity of *X. nematophila* YL00I were examined and it showed that the antibiotic production is significantly improved [38]. The antibacterial activity of *X. nematophila* D1 decreases significantly when cultured above 30ºC [33]. Adopting a three-stage oxygen supply control strategy to controlling the agitation speed and aeration rate, the dry cell weights and activity units of antibiotic are improved by 24.0% and 7.0% [39]. However, The accumulation of Xcn1 is toxic to the producing cells so the fermentation time also needs to be controlled [40]. Xcn1 production by the *X. nematophila* differs quantitatively also depending on the species of symbiotic bacteria [37]. Nevertheless, none of the above-mentioned studies reported the production of Xcn1 was increased accurately and quantitatively.

In this study, high performance liquid chromatography (HPLC) was used to detect the content of Xcn1 accurately. The effects of fermentation mediums, environmental factors and precursors on the yield of Xcn1 from the broth of *X. nematophila* YL001 was tested by a-factor-at-a-time method and response surface method (RSM).

Materials And Methods

Xcn1-producing microorganism

X. nematophila YL001 was isolated from its nematode symbiont, *Steinernema* sp. YL001, which was obtained from the soybean rhizosphere soil of Yangling, China (E: 107°59'-108°08'; N: 34°14'-34°20'; Soil type: Lou soil; average annual temperature: 12.9°C; average annual sunshine hours: 2163.8 h; average annual rainfall: 635.1 mm) [47, 48]. *X. nematophila* AN6, *X. nematophila* All, *X. nematophila* Mex were provided by Guangzhou Institute of Entomology.

Culture and fermentation

The entomopathogenic nematode symbiotic bacteria including *X. nematophila* have two phenotypic variants, called primary (I) and secondary (II). Only primary (I) exhibits considerable antimicrobial activity

[31, 49]. *X. nematophila* YL001, *X. nematophila* AN6, *X. nematophila* All and *X. nematophila* Mex were maintained in primary (I) and used throughout the study. To ensure the predominance of phase I population, refrozen cells were seeded on the surface of NA media supplemented with 0.04% triphenyltetrazolium chloride (w/v) and 0.025% bromothymol blue (w/v) (NBTA) and incubated at 28°C in the dark for 3 days [37, 39]. Fresh liquid culture was started from single blue colonies. Seed culture used for the fermentation passed two generations. A single colony of *X. nematophila* YL001 was cultured in a 250-ml flask containing 100 ml of fresh Luria-Bertani (LB) medium (1.0% Bacto Tryptone, 0.5% Yeast Extract, and 1.0% NaCl in water; pH 7.2) for 24 h at 28°C with shaking at 180 rpm. Then, 10mL of the seed culture of *X. nematophila* YL001 was transferred as a seed into a 250 mL flasks containing 100 mL of fermentation medium and the system was incubated for 48 h at 28°C with continuous agitation of 180 rpm. Methods of culture and fermentation were adjusted accordingly and used in all subsequent experiments.

Optimization of culture conditions and medium

Screening of basic medium

The one-factor-at-a-time method was used to select the original medium with the highest yield of Xcn1 from ten commonly used bacterial media. The fermentation medium included LB medium, Beef peptone yeast (BPY) medium (1.0% Peptone, 0.5% Beef Extract, 0.5% Yeast Extract, 0.5% Glucose, and 0.5% NaCl in water; pH 7.2), Trptic Soy Broth (TSB) medium (1.7% Bacto Tryptone, 0.3% Soya Peptone, 0.25% Glucose, 0.25% K2HPO4, and 0.5% NaCl in water; pH 7.2), BR medium (1.0% Beef Peptone, 0.3% Beef Extract, and 0.5% NaCl in water; pH 7.2), KB medium (0.5% Peptone, 0.3% Yeast Extract, and 0.25% Glucose in water; pH 7.2), NB medium (0.5% Beef Extract, 0.6% Peptone, and 1.5% NaCl in water; pH 7.2), NB + medium (0.3% Beef Extract, 0.5% Peptone, and 1% NaCl in water; pH 7.2), PP3 medium (2.0% Proteose Peptone in water; pH 7.2), PP3 + medium (2.0% Proteose Peptone and 1% NaCl in water; pH 7.2), YS medium (0.5% Yeast Extract, 0.05% $(NH_4)_2SO_4$, 0.05% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.5% NaCl in water; pH 7.2).

Selection of nutrient source

To evaluate the effect of carbon sources on the yield of Xcn1, various carbon sources such as glucose, fructose, maltose, starch, dextrin, lactose and sucrose were added. The nitrogen sources such as, soya peptone, bacto-tryptone, proteose peptone, beef peptone, yeast extract, potassium nitrate, urea and beef extract were added to screen nitrogen sources. To analyze the mineral sources, the inorganic salt such as, magnesium sulfate, sodium chloride, dipotassium hydrogen phosphate and sodium sulfate were added separately.

Experimental design and optimization by RSM

The fermentation medium was optimized by response surface methods. In order to enhance the production of Xcn1, central composite design (CCD) was used to optimize the concentrations of the variables such as carbon sources(X_1), nitrogen sources(X_2) and mineral sources(X_3). Variables were

analyzed at five different levels (-α, -1, 0, + 1, +α), for the selected three variables (maltose, proteose peptone and K_2HPO_4) 20 experimental trials has been performed. These 20 trials included six centre points, six axial points and eight factorial points. After which, the mean value of the response Y (the yield of Xcn1) was assayed in triplicate analysis. The CCD experiment was not only employed to analyze the interactions among the important three selected variables (proteose peptone, maltose and K_2HPO_4) but also to find their optimum levels.

Optimization of fermentation conditions

Six fermentation conditions, such as seed quantity, liquid loading, initial pH, rotating speed of shaking table, temperature and fermentation period, were optimized to determine the optimal fermentation conditions for improving the output of Xcn1 by one-factor-at-a-time method. Seed quantity was set at 4%, 6%, 8%, 10% and 12%. The initial pH was set at 5.0, 6.0, 7.0, 8.0 and 9.0. Liquid loading were set at 50, 75, 100, 125 and 150mL. The rotating speeds of the shaker were set at 50, 100, 150 and 200 rpm. Fermentation period were set at 24, 48, 72, 96 and 120 h. And temperatures of fermentation were set at 15, 20, 25, 30 and 35°C.

Optimization of precursors

To evaluate the effect of precursor on the yield of Xcn1, several precursors such as arginine, leucine, acetic acid and urea were added to the medium. The concentration of precursor substances in the medium was set at 2, 3 and 4 mmol/L to determine the optimal concentration. In order to determine the best adding time, precursors were added in different time, such as the 0th, 6th, 12th, 18th, 24th and 30th h, during fermentation.

Biomass content assessment

Biomass content was measured by optical density of the culture at 600 nm and biomass concentrations (DCW: g/L) were determined using a calibration curve [50]. The calibration curve was calculated using dilutions of a biomass suspension with known optical density. A fixed volume of dilutions was centrifuged at 8,000 rpm for 25 min (Himac CR 22G, Japan). The cells were washed and centrifuged repeatedly until the supernatant was clear. Then, cell pellets were freeze-dried and weighed. Thus, a relationship between biomass concentration (g/L) and optical density (600nm) can be determined.

Xcn1 contents analysis by HPLC

Previously purified Xcn1 was seen as the pure product to prepare the mother liquor with a concentration of 2000 μg/mL. Then, the mother liquor was dissolved with ultra-pure water and diluted it to 1000 μg/mL, 800 μg/mL, 600 μg/mL, 400μg/mL, 200 μg/mL and 100 μg/mL standard solution. Standard curve was drawn with Xcn1 concentration as abscissa and absorption peak area as ordinate. The linear regression equation was $y = 4.5173x + 18.658$, and the correlation coefficient is $R^2 = 0.9993$ (See Supplementary Figure S1, Additional File 1). In order to verify the accuracy of regression equation prediction, fermentation medium formula obtained by RSM was used to shake flask fermentation, repeated three times. The yield of Xcn1 was measured, and the average value of results was taken. The average value

was compared with the predicted result so that the accuracy of the model was verified. Nuclear magnetic resonance (NMR) spectrum and high resolution mass spectrum (HRMS) of Xcn1 was shown in the additional file (See Supplementary Figure S2-4, Additional File 1).

The broth of *X. nematophila* YL001 was centrifuged (8,000 rpm, 25 min, 4°C) to remove cells. The cell-free broth was added to the activated X-5 macroporous resin column (100cm×98cm, Nankai University Chemica Factory) for chromatographic separation. The macroporous resin column was eluted in five times of the volume with deionized-water, 50% methanol, 30% acetone with 0.01 mol/L HCl and 50% acetone with 0.02 mol/L HCl successively. The eluent of 30% acetone with 0.01 mol/L HCl was collected and concentrated to obtain the Xcn1 crude extract. Xcn1 crude extract was dissolved in 50% methanol and the fixed volume was 10 ml for detection by high performance liquid chromatography (HPLC). Purity of Xcn1 was determined using HPLC on a reversed-phase C_{18} column (Waters 2695 systems). Twenty microliters of sample were injected into a column (2.1×150mm), eluted with acetonitrile-0.1% trifluoroacetic acid (30:70) at a flow rate of 1 mL/min, monitored at 312nm, and the temperature of the column was 25°C. The high performance liquid chromatogram of experiment was shown in the additional file (See Supplementary Figure S5-17, Additional File 1).

Statistical analysis

Design expert 10 was used to analyze the variables. The 3D response graph and profile for predicted values and desirability level for factors were plotted using the same software. The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included the Fisher's F-test (overall model significance), its associated probability $P(F)$, correlation coefficient R, and determination coefficient R^2 that measures the goodness of fit of regression model. The analysis also included the t-value for the estimated coefficients and associated probabilities, $P(t)$. For each variable, the quadratic models were represented as contour plots. In addition, the fermentation conditions and precursors were analyzed using Origin 9.1 and Microsoft office Excel. Data were given from 3 duplications ± SE and data with different letters were significantly different at $P< 0.05$ by Duncan's multiple range test.

Results And Discussions

The yield differences of Xcn1 between strains of *X. neamtophlia*

The difference of Xcn1 production of different strains in TSB medium was shown in Figure 1 (a). The results showed that Xcn1 yield of *X. nematophila* All was the highest with 94.15 μg/mL. The Xcn1 yield of *X. nematophila* YL001 was 57.80 μg/mL in the middle level. *X. nematophila* AN6 and *X. nematophila* Mex showed low yield of Xcn1 with 25.60 μg/mL and 30.63 μg/mL, respectively. In this study, the Xcn1 production of *X. nematophila* All was significantly higher than that of *X. nematophila* Mex, *X. nematophila* AN6 and *X. nematophila* YL001 strains, which indicated that strain was an important factor of determining Xcn1 production.

Effect of different media on Xcn1 production

The results of cell growth and Xcn1 production of *X. nematophila* YL001 in different media were shown in Figure 1 (b-c). The results indicated that PP3 medium showed a maximum Xcn1 production (87.21 μg/mL) followed by BR medium (69.71 μg/mL) and KB medium (67.61 μg/mL). Lower Xcn1 production was observed in YS medium (11.12 μg/mL). The maximum cell growth in PP3 medium was found to be high when compared with other media. PP3 medium was selected as the original medium for the carbon and nitrogen sources selection studies because it was most suitable for cell growth and Xcn1 production of *X. nematophila* YL001. Medium played a decisive role in the growth and whole fermentation process of microorganisms [36, 37]. Previous studies have shown that PP3+ medium is the most suitable for the growth of *X. nematophila* YL001 [36]. However, in our study, PP3 medium is more suitable for the growth of *X. nematophila* YL001 than PP3+ medium. To some extent, the reason for this phenomenon is that the culture conditions are different. In addition, the yield of Xcn1 in PP3+ medium was significantly lower than that in PP3 medium. And our purpose was to improve the yield of Xcn1, so PP3 medium was selected as the original medium.

Effect of various nutrient sources on cell growth and Xcn1 production

Based on the original medium, single factor evaluation of inorganic salts (10 g/L), carbon source (5 g/L), and nitrogen source (20 g/L), were respectively conducted to explore the effect on cell growth and the production of Xcn1. Figure 2 (a) indicated that among the various nitrogen sources studied, the *X. nematophila* YL001 produced the maximum Xcn1 in proteose peptone (81.82 μg/mL), followed by tryptone (69.19 μg/mL) and yeast extract (63.34 μg/mL). The DCW in yeast extract (25.07 g/L) was found to be high when compared with other nitrogen sources. Among carbon source, maltose showed a maximum Xcn1 production (106.31 μg/mL) followed by fructose (101.67 μg/mL) and starch (98.57 μg/mL). Lower Xcn1 production was observed with the sucrose (37.06 μg/mL). The maximum DCW was found in starch (27.01 g/L) when compared with other carbon source (Figure 2 b). The effects of supplementation with various minerals were evaluated and were found to have potential impact on Xcn1 production. Among the various inorganic salt studied, the *X. nematophila* YL001 produced the maximum Xcn1 in K_2HPO_4 (112.30 μg/mL) and the minimum Xcn1 in NaCl (71.30 μg/mL). The DCW in Na_2SO_4 (25.47 g/L) was found to be high when compared with other inorganic salt (Figure 2 c).

The optimal concentrations of nitrogen, carbon and inorganic salts were determined by controlling their doses. Thus, proteose peptone (20 g/L), maltose (10 g/L) and K_2HPO_4 (5 g/L) were chosen as the source of nitrogen, carbon and inorganic salt for further experiments, respectively (Figure 2 d). By optimizing the nutrition of medium, the yield of Xcn1 was enhanced from 50.73 μg/mL to 117.99 μg/mL. Among the nitrogen sources, although yeast extract was the most suitable for the growth of *X. nematophila* YL001, proteose peptone was the most suitable for Xcn1 production. Fructose and starch were suitable for *X. nematophila* YL001 growth as carbon sources, but maltose was most suitable for Xcn1 production. In several inorganic salts, Na_2SO_4 and $MgSO_4$ were suitable for the growth of *X. nematophila* YL001, but K_2HPO_4 was the most suitable for the production of Xcn1. These results indicate that the growth of *X.*

nematophila YL001 is different from biosynthetic process of Xcn1. Previous studies showed that *X. nematophila* YL001 produced the most antibiotics when proteose peptone was used as nitrogen source [36]. When glucose and maltose were used as carbon source, *X. nematophila* YL001 produced a lot of antibiotics with concentration of 243.3 U/mL and 233.3 U/mL [36]. Going further, it was consistent with previous studies and also indicated that Xcn1 was the main active component of the antibiotic produced by *X. nematophila* YL001 [23, 41].

Optimization of medium constituents by response surface method

After determination of nitrogen sources, carbon sources and inorganic salts in the medium, the combined effects of concentration of medium constituents on Xcn1 production were further investigated. Table 1 showed experimental range and levels of the independent variables. The experimental results listed in Table 2 were analyzed using multiple regressions. The production of Xcn1 was found to be maximum at run 11 (113.65 μg/mL). The second-order polynomial model was proposed to evaluate the optimum levels of these selected variables and was shown in the following equation:

$$Y = -469.45715 + 33.78184X_1 + 31.64599X_2 + 43.41016X_3 - 0.53319X_1X_2 - 0.27222X_1X_3 - 0.72833X_2X_3 - 1.24623{X_1}^2 - 0.53066{X_2}^2 - 3.28788{X_3}^2$$

The optimum levels which were suitable for the maximum yield of Xcn1 were 1.274% maltose, 2.083% proteose peptone and 0.377% K_2HPO_4 in the media. In this study, the determination coefficient (R^2) confirmed the importance of statistical design, exhibiting minor experimental error and a fit regression equation. The R^2 of this designed CCD model was 0.9443. The F value of the model is 18.38, while the P value is less than 0.0001, indicating that the model has significance (Table 3). The saddle or elliptical nature of contour plot showed the significance of the good interactions between the respective variables. Figure 3 showed the contour plot and 3D response surface plot for the Xcn1 yield generated by the predicted CCD model. According to the model equation, we predicted that a maximum Xcn1 yield of 113.65 μg/mL could be achieved at 12.74 g/L maltose, 20.83 g/L proteose peptone and 3.77g/L K_2HPO_4. By HPLC, the content of Xcn1 was 117.99 μg/mL with the optimized medium, which was close to the predicted response. Compared with the output of Xcn1 of TSB medium (50.67 μg/mL), the output of Xcn1 of the optimized PP3 medium (113.65 μg/mL) increased by 112.65%.

Optimization of cultural conditions by a-factor-at-a-time method

Based on the above efforts, the fermentation conditions of *X. nematophila* YL001 was optimized further to improve the production of Xcn1. In order to get the most suitable conditions for the production of Xcn1, 6 kinds of fermentation conditions, such as the temperature, initial pH, inoculum size, rotation speed, liquid loading and fermentation time, were optimized for the fermentation. The production of Xcn1 was

assessed at different temperatures (15-35°C) and reached the maximum value of 119.87 μg/mL at 25°C (Figure 4 a). It showed that 25°C was the optimal fermentation temperature. Significantly, further increase in the temperature reduced the yield of Xcn1. Over different pH ranges (5-9) were assessed for Xcn1 production (Figure 4 b). Fermentation medium favoured pH 7 for maximum production of Xcn1 (125.72 μg/mL). Furthermore, an increase or decrease in the pH of the medium decreased the production of Xcn1. The yield of Xcn1 increased with the increased inoculum size but it decreased when inoculum size was over 10% (Figure 4 c). Thus, 10% was selected as the optimal inoculum size. The production of Xcn1 was assessed at different rotating speeds (50-200rpm) and reached the maximum value of 125 μg/mL at 100 rpm (Figure 4 d). There was no significant change in the yield of Xcn1 when the speed continued to increase. So 100 rpm was selected as the optimal rotating speed. The effect of medium loading on the production of Xcn1 was investigated by changing the volume of the medium. It showed that the optimal yield of Xcn1 was 153.56 μg/mL with 75mL medium in 250mL flasks (Figure 4 e). Length time of fermentation was also influenced toward the yield of Xcn1. The production of Xcn1 was assessed at different fermentation time (24-120h). There was no significant increase in the production of Xcn1 when the fermentation time increased (Figure 4 f). Consequently, 48h was selected as the optimal fermentation time.

Through the optimization of fermentation conditions, the yield of Xcn1 reached 153.56 μg/mL. Temperature and pH had significant influence on Xcn1 production of *X. nematophila* YL001, and high or low temperature and pH both led to a sharp decrease in Xcn1 production (Figure 5). Several other environmental conditions, such as inoculum size, rotation speed, liquid loading and fermentation time, could not make such a huge change in Xcn1 production. In this study, 7.0 was the most suitable pH for Xcn1 production. However, this was not consistent with the results obtained in previous studies. The weak alkaline pH environment was found to be beneficial for the production of Xcn1. And the production of Xcn1 were 2.49-fold higher at pH 8.5 relative to that at pH 7.0 [31]. To some extent, it may be due to the combined effects of various environmental conditions on Xcn1 production. Accordingly, more precise control of pH and temperature would help to increase the yield of Xcn1. In addition, pH7.0 was the most suitable for the growth of *X. nematophila* YL001 [38]. Thus, it needs to be further explored the relationship between the metabolism of growth of *X. nematophila* YL001 and the biosynthesis pathway of Xcn1.

Effect of precursor substances on Xcn1 production

Biosynthetic precursors, such as arginine, leucine, urea and acetic acid, affected biosynthesis of Xcn1 that natural product produced by *X. nematophila* YL001. Markedly, arginine influenced the production of Xcn1 and its optimal concentration was 3 mmol/L with the productivity of Xcn1 was 132.78 μg/mL (Figure 5). In order to determine the best occasion of adding, arginine (3 mmol/L) was added to the fermentation medium at the 0th, 6th, 12th, 18th, 24th, 30th h, during fermentation. The production of Xcn1 was assessed at 48h and reached the maximum value of 173.99 μg/mL with added arginine at the 12th h (Figure 5 e). Therefore, the 12th hour was selected as the optimal time to add arginine of 3 mmol/L to the fermentation medium. The yield of Xcn1 was increased to 173.99 μg/mL by adding precursors properly. Four precursors were involved in the biosynthesis of Xcn1 directly or indirectly. The

synthesis of arginine by microorganism needed urea to provide nitrogen. In the form of arginyl thioester, arginine was condensed with N-acyl-D-asparaginyl thioetser into the long chain of Xcn1 [23, 42]. This process occurred in the initial stage of Xcn1 long chain biosynthesis, hence arginine was the most helpful precursor for Xcn1 biosynthesis. This is consistent with the results of our study. Moreover, leucine and acetic acid were involved in the long chain synthesis of Xcn1, respectively, to form peptide bonds and extend the carbon chain [23, 42]. If precursors, such as arginine, leucine and acetic acid, were added in proper proportion at the right time, the production of Xcn1 would be further improved.

Previous studies evaluated the content of antibacterial active substances in fermentation broth of *X. nematophila* YL001 according to the size of bacteriostatic circle [36]. The above methods are not accurate enough, so we have adopted high performance liquid chromatography in this study. High performance liquid chromatography method was provided a more precise and accurate method for the Xcn1 detection and was proved to be a powerful tool for the optimization Xcn1 production by *X. nematophila* YL001. This method has broad prospects in follow-up study.

Microbial natural products are important sources for antibiotic discovery whether medicine or pesticides [43, 44]. However, the low yield limits the development and utilization of microbial natural products. In order to improve the output of antibiotic by microbes, biosynthesis metabolic regulation and fermentation process optimization had been researched [45, 46]. In an industrial point of view, to improve the production, various methods and techniques should be screened for Xcn1 production. We added some precursors of Xcn1 biosynthesis to the medium and optimized the fermentation process to increase the production of Xcn1 in this study. Furthermore, the fermentation of *X. nematophila* YL001 should be studied in pilot scale to further to optimize the fermentation conditions in the fermentation tank, so as to improve the output of Xcn1 and lay a foundation for its industrial production. With the use of macroporous resin X-5 in a fermentation setting, *X. nematophila* CB6 improved its ability to produce Xcn1 [40]. Perhaps, this method could be used to increase the production of Xcn1 in the fermentation broth of *X. nematophila* YL001.

Conclusion

In this study, the optimization of fermentation process was used to increase the production of Xcn1 in the fermentation broth of *X. nematophila* YL001. Medium, fermentation conditions and precursors were regulated and used to enhance the production of Xcn1. The result indicated that the production of Xcn1 on the optimal fermentation conditions reached 173.99 μg/mL, which was 3.43 times as much as that on the original conditions (TSB medium).

Declarations

Acknowledgements

Not applicable.

Authors' contributions

YW, JG and YH conceived and designed the research; YH, JG, SZ, and JH performed the experiments; YH, JG and ZY analyzed the data and wrote the paper; YH, YT and YW revised the article. All authors have read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional file.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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Tables

Table 1 Experimental range and levels of the independent variables

Variable	Factors (g/L)	Range and Levels				
		-1.682	-1	0	1	1.682
	Maltose	4.95	7	10	13	15.05
	Proteose peptone	9.91	14	20	26	30.09
	K_2HPO_4	2.48	3.5	5	6.5	7.52

Table 2 Central composite design for the production of Xcn1 by *X. nematophila* YL001

Run	Maltose(g/L)	Proteose peptone(g/L)	K_2HPO_4(g/L)	Xcn1 (μg/mL) Actual
1	10	9.91	5	34.79
2	10	20	5	108.28
3	10	30.09	5	75.16
4	7	26	3.5	99.16
5	13	26	6.5	41.45
6	7	14	6.5	56.66
7	10	20	5	103.94
8	10	20	7.52	62.52
9	10	20	5	103.42
10	10	20	5	105.41
11	10	20	2.48	113.65
12	7	26	6.5	77.84
13	10	20	5	104.72
14	13	14	6.5	63.56
15	15.05	20	5	53.32
16	13	26	3.5	72.57
17	7	14	3.5	56.66
18	13	14	3.5	63.56
19	4.95	20	5	101.25
20	10	20	5	110.23

Table 3 Analysis of variance for the production of Xcn1 using central composite design

Source	Sum of Squares	df	Mean Square	F Value	P Value
Model	11599.14	9	1288.79	18.38	<0.0001**
A- Maltose ()	1233.45	1	1233.45	17.59	0.0018
B- Proteose peptone ()	1027.77	1	1027.77	14.66	0.0033
C- K_2HPO_4 ()	1403.17	1	1403.17	20.01	0.0012
AB	736.90	1	736.90	10.51	0.0088
AC	12.00	1	12.00	0.17	0.6878
BC	343.74	1	343.74	4.90	0.0512
A^2	1812.96	1	1812.96	25.85	0.0005
B^2	5259.52	1	5259.52	75.00	<0.0001
C^2	788.68	1	788.68	11.25	0.0073
Residual	701.28	10	70.13		
Lack of Fit	665.31	5	133.06	18.49	0.0031**
Pure Error	35.98	5	7.20		
Cor Total	12300.42	19			

R^2= 94.43%, C.V. %=10.41

** Significant at 1% level.

Figures

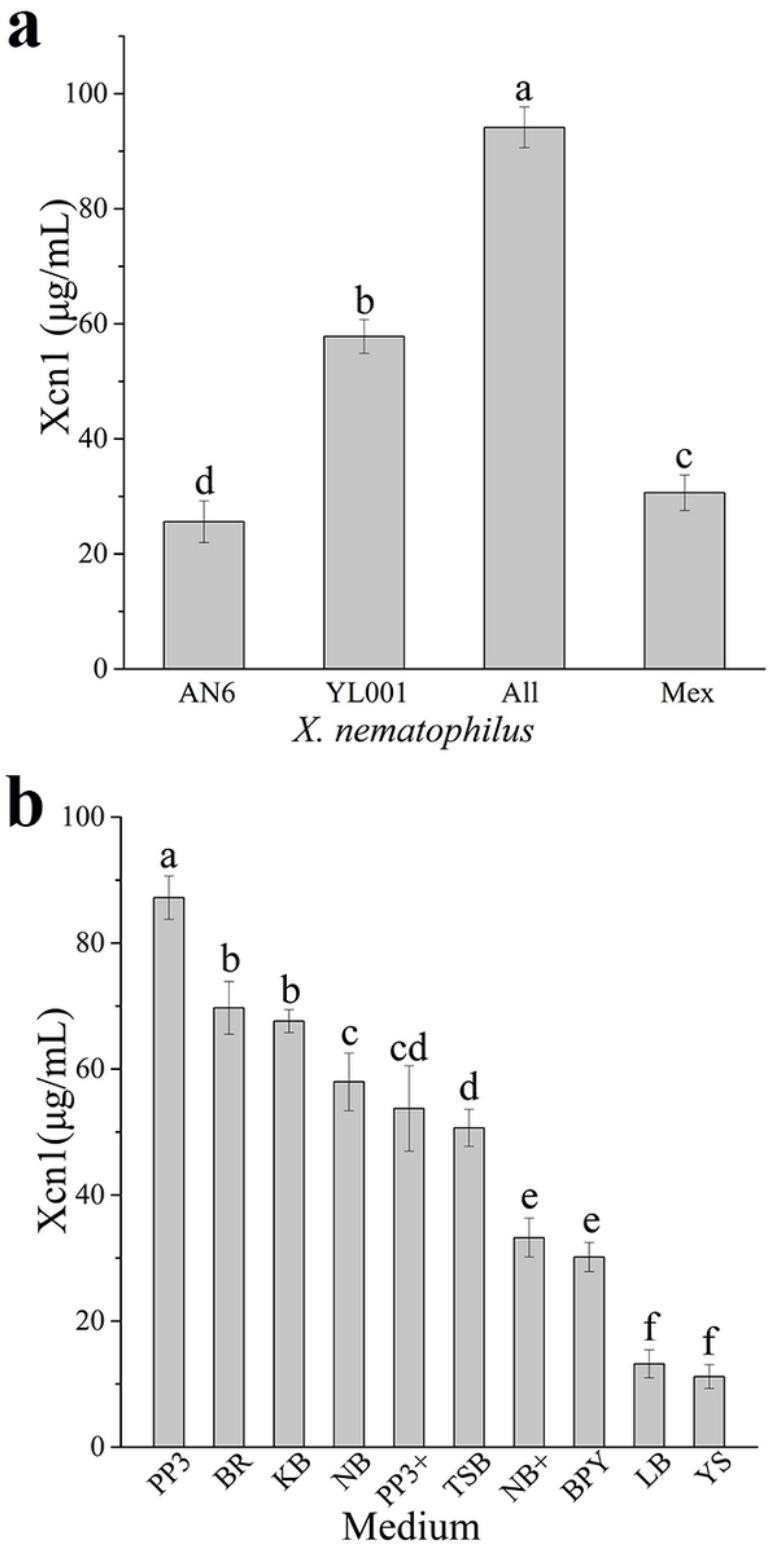

Figure 1

Effects of different medium and strains of Xenorhabdus nematophilus on cell growth and Xcn1 production. (a) Difference of Xcn1 production between strains of Xenorhabdus nematophilus. (b) Effects of different media on cell growth of X. nematophila YL001. (c) Effects of different media on Xcn1 production. The data represent the mean values of three independent replicates, and the error bars

represent the standard deviations. Data with different letters are significantly different at P□0.05 by Duncan's multiple range tests.

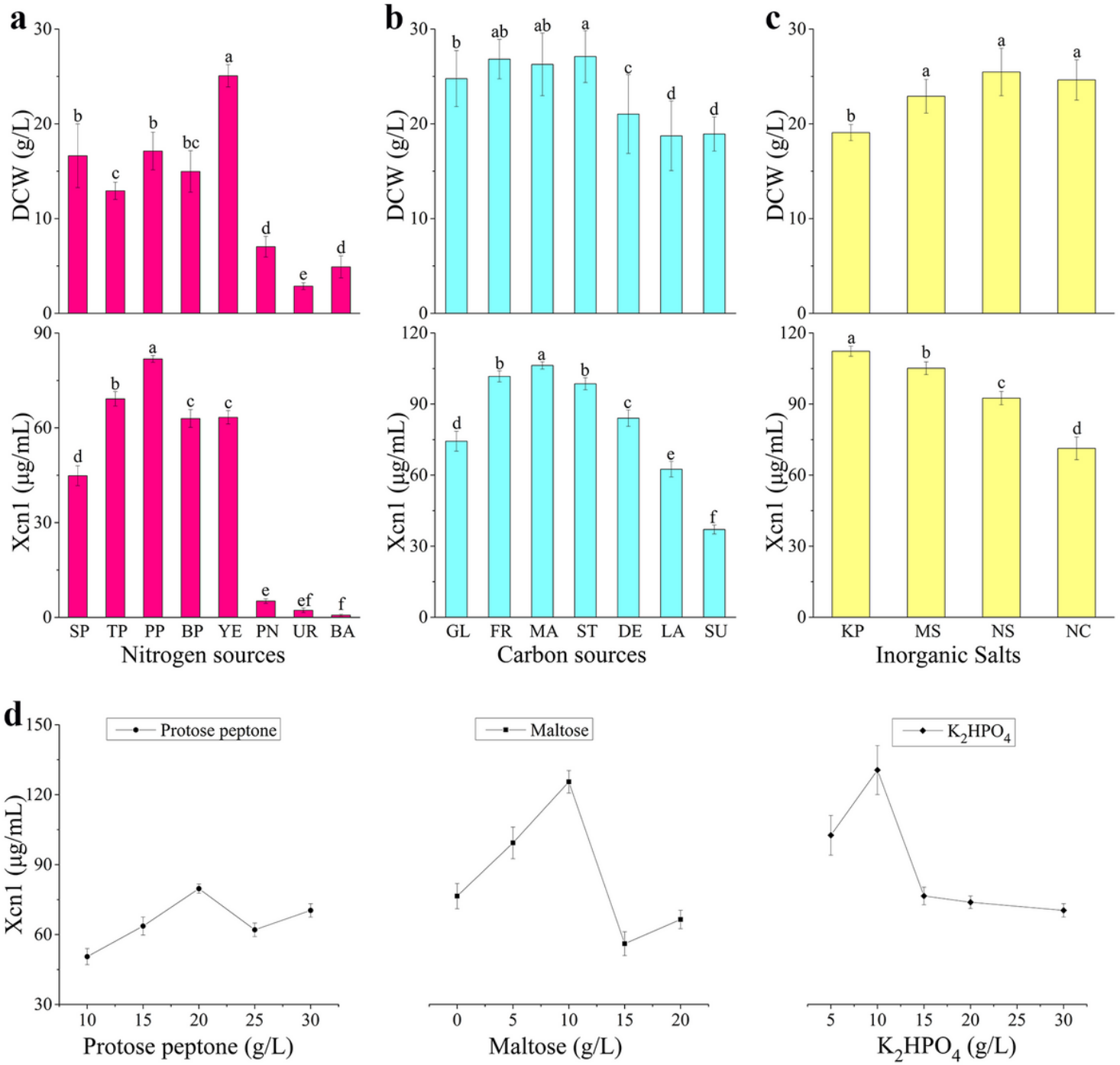

Figure 2

Effects of different nutrient sources on cell growth and Xcn1 production. (a) Effects of various nitrogen sources on cell growth and Xcn1 production. Abbreviation of nitrogen sources (SP: Soy peptone; TP: Tryptone; PP: Proteose peptone; BP: Beef peptone; YE: Yeast extract; PN: Potassium nitrate; UR: Urea; BA: Beef paste). (b) Effects of different carbon sources on cell growth and Xcn1 production. Abbreviation of carbon sources (GL: Glucose; FR: Fructose; MA: Maltose; ST: Starch; DE: Dextrin; LA: Lactose; SU: Sucrose). (c) Effects of various inorganic salts on cell growth and Xcn1 production. Abbreviation of

inorganic salts (KP: K2HPO4; MS: MgSO4; NS: Na2SO4; NC: NaCl). (d) Effects of various concentrations of proteose peptone, maltose and K2HPO4 on Xcn1 production. The data represent the mean values of three independent replicates, and the error bars represent the standard deviations. Data with different letters are significantly different at P□0.05 by Duncan's multiple range tests.

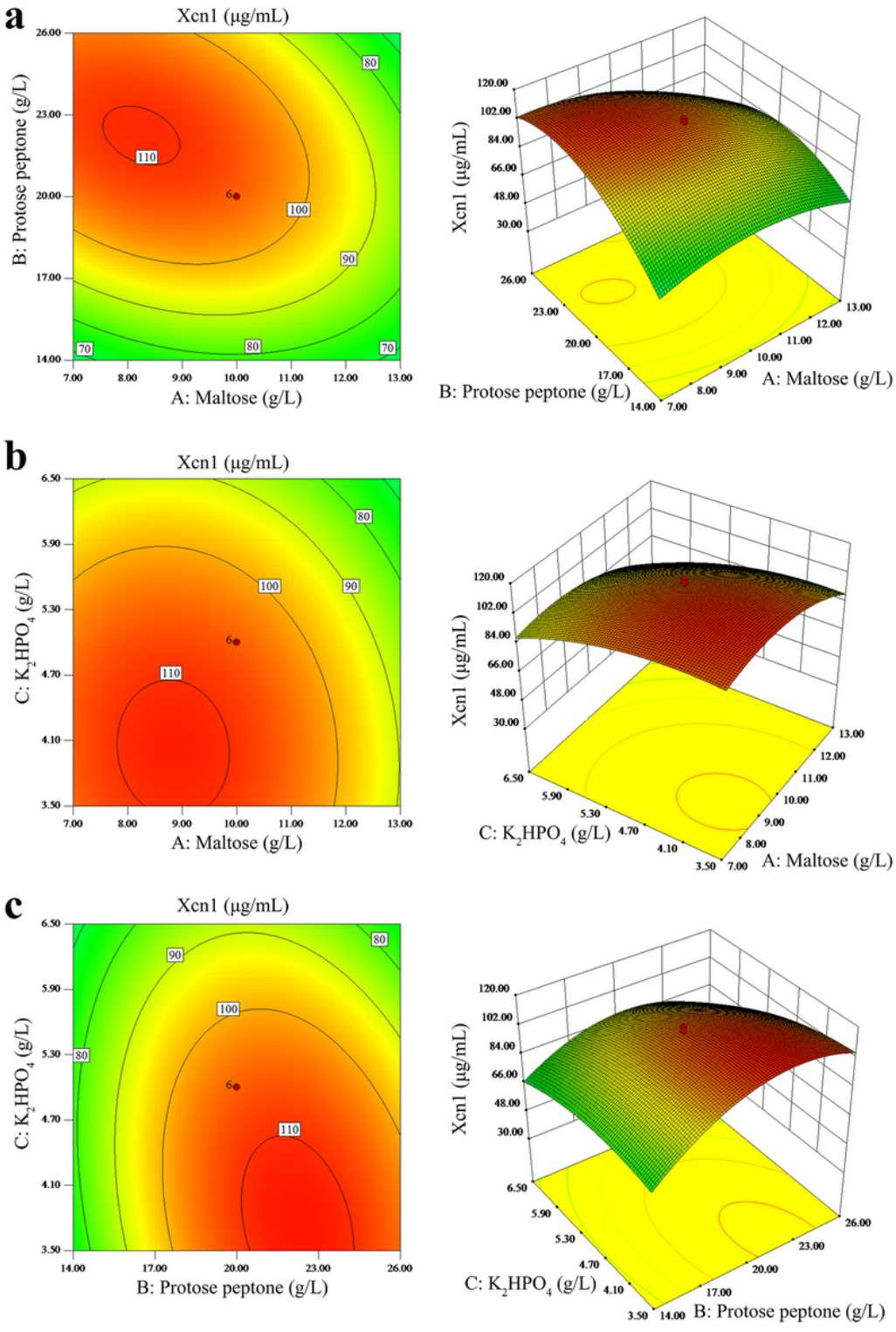

Figure 3

Response surface plot and contour plot. (a) The combined effects of maltose and proteose peptone on the Xcn1 production of X. nematophila YL001. (b) The combined effects of maltose and K2HPO4 on the Xcn1 production of X. nematophila YL001. (c) The combined effects of proteose peptone and K2HPO4 on the Xcn1 production of X. nematophila YL001.

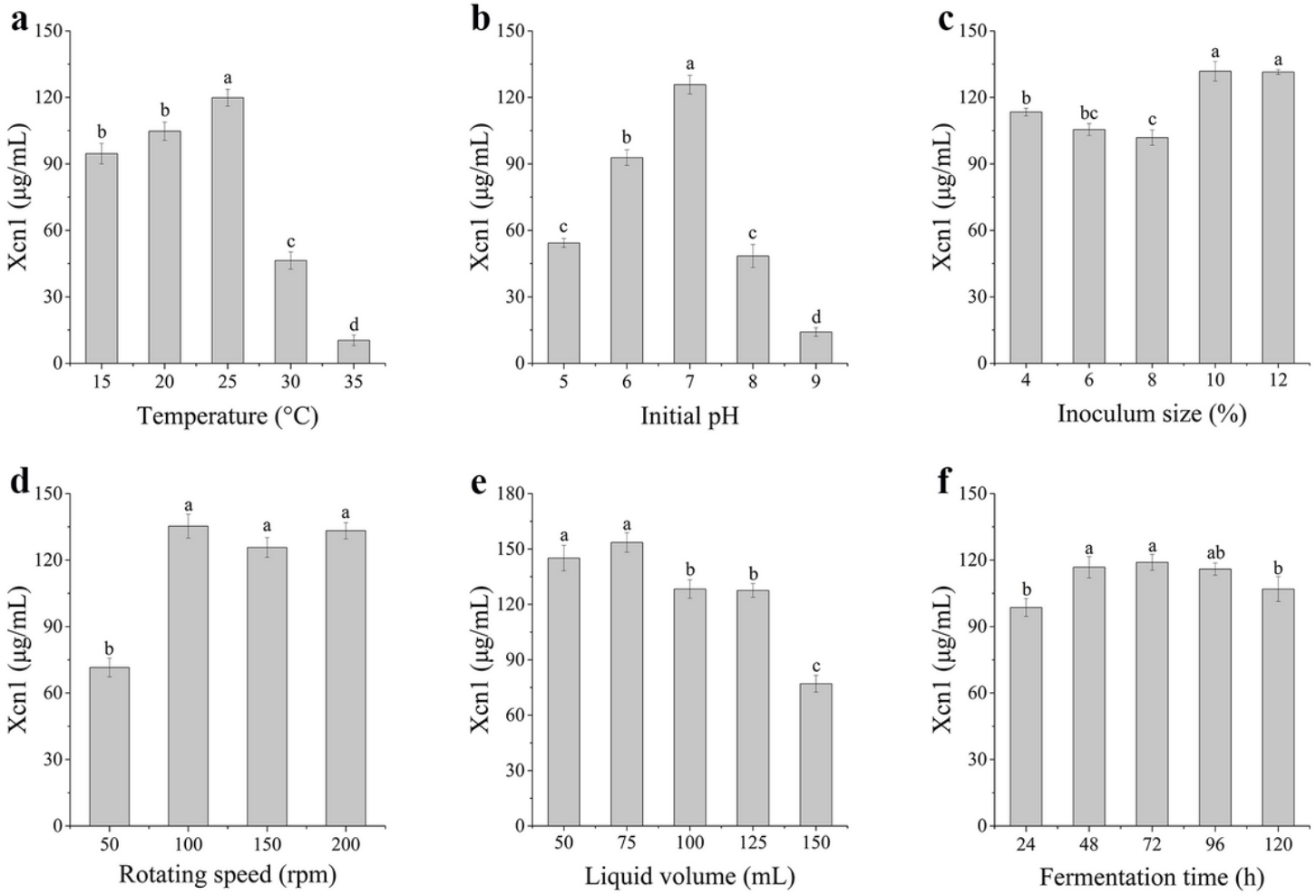

Figure 4

Effects of different fermentation conditions on the production of Xcn1. (a) Effects of various fermentation temperatures on Xcn1 production. (b) Effects of different initial pH of fermentation medium on Xcn1 production. (c) Effects of various inoculum size on Xcn1 production. (d) Effects of different rotating speed of shaker on Xcn1 production. (e) Effects of various volume of liquid medium on Xcn1 production. (f) Effects of different fermentation time on Xcn1 production. The data represent the mean values of three independent replicates, and the error bars represent the standard deviations. Data with different letters are significantly different at P□0.05 by Duncan's multiple range tests.

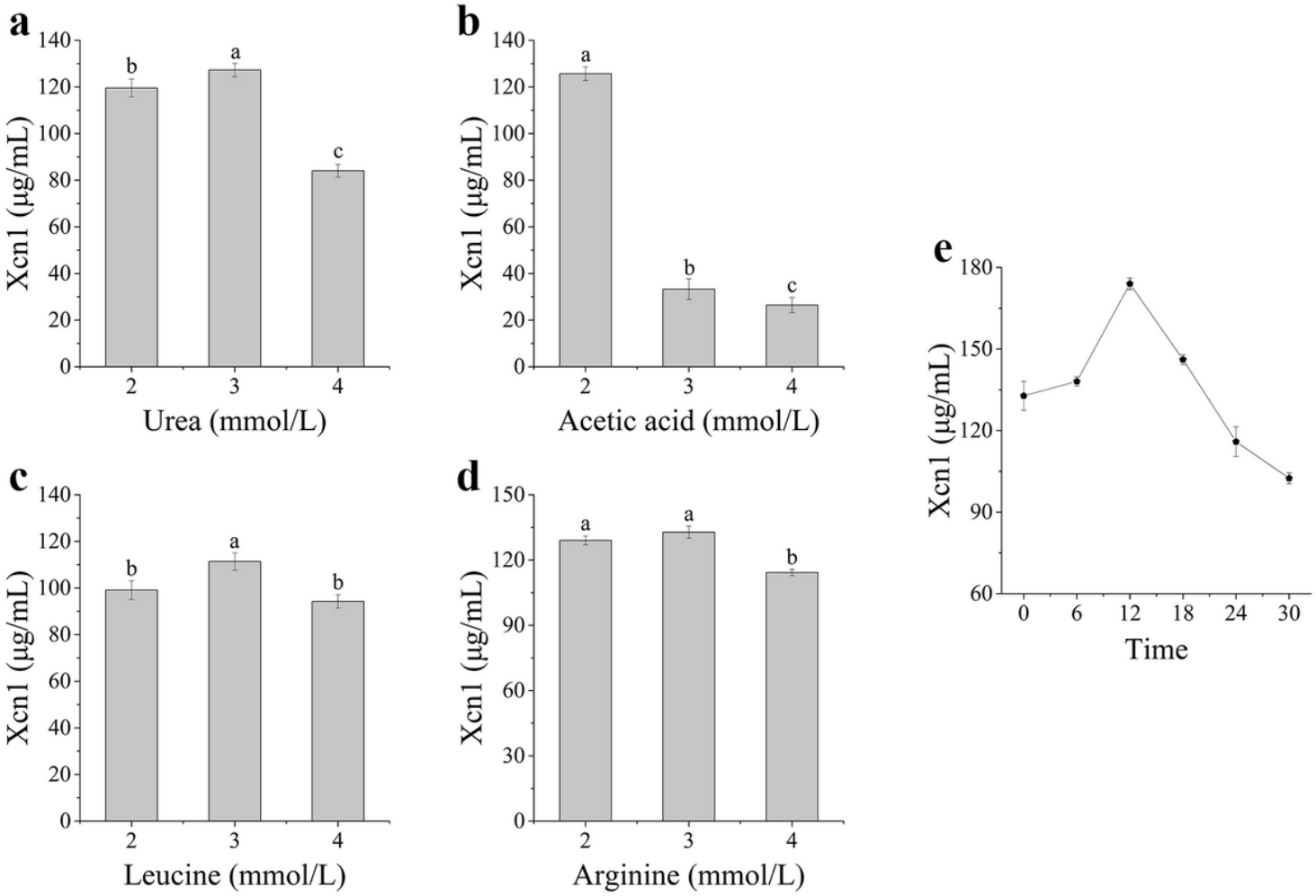

Figure 5

Effects of different precursor substances concentration and arginine adding time on Xcn1 production. (a) Effects of various concentration of urea on Xcn1 production. (b) Effects of different concentration of acetic acid on Xcn1 production. (c) Effects of various concentration of leucine on Xcn1 production. (d) Effects of different concentration of arginine on Xcn1 production. (e) Effects of adding time of arginine on Xcn1 production. The data represent the mean values of three independent replicates, and the error bars represent the standard deviations. Data with different letters are significantly different at P□0.05 by Duncan's multiple range tests.

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