Bioprocess and Biosystems Engineering (2018) 41:1743–1755
https://doi.org/10.1007/s00449-018-1997-x

RESEARCH PAPER

Enhancing candicidin biosynthesis by medium optimization and pH stepwise control strategy with process metabolomics analysis of *Streptomyces* ZYJ-6

Xiaoyun Liu[1] · Xiaojuan Sun[1] · Tong Wang[1] · Xin Zhang[2] · Xiwei Tian[1] · Yingping Zhuang[1] · Ju Chu[1]

Received: 14 May 2018 / Accepted: 2 August 2018 / Published online: 21 August 2018

Abstract
Candicidin is one of the most effective antimonilial agents. In order to enhance candicidin productivity, medium optimization and pH stepwise control strategy in process optimization were conducted by *Streptomyces* ZYJ-6. With the aid of Design Expert software and N/C/P-sources regulation, chemically defined medium fit for cell growth and candicidin biosynthesis was developed. Moreover, pH effects on cell growth and metabolism were investigated. The results indicated that the optimal pH for cell growth and candicidin biosynthesis were 6.8 and 7.8, respectively. The metabolomics analysis revealed that the pH stepwise control strategy (pH 6.8–7.8) combined the advantages of pH 6.8 and pH 7.8 and avoided precursor limitation in pH 6.8 and 7.8. Consequently, the pH stepwise control strategy played positive performance on cell growth and candicidin biosynthesis with the maximum titer of 5161 μg/mL. The titer of 5161 μg/mL was the highest level ever reported for candicidin production, which laid a solid foundation for industrial application. Additionally, pH stepwise control strategy was important reference for process optimization.

Keywords pH · Process optimization · Metabolomics · Candicidin · *Streptomyces*

Introduction

In 1965, about 50 polyenic antifungal antibiotics, which are the most effective antimonilial agents so far known, had been isolated and described [1]. This group includes four subgroups: tetraenes (pimaricin), pentaenes (lienomycin), hexaenes (dermostatin A) and heptaenes (candicidin). Among these, the most important polyenic antifungal antibiotics from actinomycetes belongs to the heptaenes which were also the most frequently produced polyenes [1]. The polyenic antifungal antibiotics, with a characteristic of three to eight conjugated double bonds, usually form transmembrane channels by interacting with sterols or ergosterol in the eukaryotic cell membrane, and then cause small molecules and ions leakage for cell death [2]. Because of sterols also existing in the cell membrane of human beings, candicidin is not used in clinical medicine at the present time in case. The heptaenes candicidin-producing strain *Streptomyces griseus* IMRU3570 was first isolated in 1948 from cow manure. Because of its high activity on *Candida albicans*, the antibiotic was named candicidin which was highly active upon yeasts and yeast-like fungi, but not upon filamentous fungi and bacteria [3].

Chen et al. [4] verified the candicidin and FR-008 are identical compounds in *Streptomyces griseus* IMRU3570 and *Streptomyces* FR-008, respectively. *Streptomyces* FR-008 was discovered in 1987 by Liang and Zhou [5]. They described the intraspecific protoplast fusion of two auxotrophic strains from *Streptomyces hygroscopicus* and found that different from their parental strains, FR-008 as well as other two fusants could produce novel antibiotics to kill *Saccharomyces cerevisiae*. FR-008 is a complex mixture of six components (FR-008-I to-VI) principally

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00449-018-1997-x) contains supplementary material, which is available to authorized users.

✉ Ju Chu
juchu@ecust.edu.cn

[1] State Key Laboratory of Bioreactor Engineering, School of Biochemical Engineering, East China University of Science and Technology, Room 413, Building 18, Shanghai 200237, People's Republic of China

[2] Shanghai Guojia Bioengineering Technology Research Center Co., Ltd., Shanghai 201403, People's Republic of China

differing from each other at C-3 and C-9. Combined inactivation of KR21 and DH18 from *Streptomyces* FR-008, a mutant (*Streptomyces* ZYJ-6) producing FR-008-III only was created. It has been reported that the antifungal activity of FR-008-III is superior to other components [6]. In this study, *Streptomyces* ZYJ-6 was selected as a potential host for FR-008-III/candicidin D production.

The synthesis of antibiotics by actinomycetes, specifically polyenic antifungal antibiotics, is of great scientific and economic importance. The onset (control and regulation) of antibiotics formation is still intriguing scientists both in academia and industry. Attempts had been made to improve the FR-008/candicidin production by *Streptomyces* FR-008 or *Streptomyces griseus* IMRU3570. Acker and Lechevalier [7] had explored nutritional requirements for the cell growth and candicidin production by *Streptomyces griseus* IMRU3570. They revealed that L-histidine and L-asparagine served best as single nitrogen sources, whereas mannose and glucose were superior carbon sources. Besides, the macroelements K, Mg, P, S and microelements Fe, Zn were also essential for the cell growth and candicidin production. The development of chemically defined medium has great significance in chassis microorganism culture [8], synthetic biology, rational production, separation and purification.

Unfortunately, there were few studies focusing on the process optimization of candicidin fermentation and its titer is still low. pH is one of the most important process parameters affecting cell growth and product formation [9]. In general, pH can affect nutrients availability and enzyme activity as a result of the charge change of cell membrane [10]. According to the previous reports, different pH conditions were adopted for the candicidin fermentation. With a soya peptone (SP)-glucose medium, the maximum candicidin production rate could be maintained and extended to a considerable time length by controlling the culture pH at 8.0 with a final yield up to 4 g/L [11]. Moreover, the candicidin yield was up to about 1.3 g/L under the condition of initial pH 7.2, and then pH was spontaneously kept at a constant level of 8.0 and greater than 8.0 at late phase in complex medium [12]. Additionally, properties of *p*-Aminobenzoic acid (PABA) synthase, the first enzyme for candicidin biosynthesis, were examined and the optimal pH in vitro were 9.0 [13].

Due to the important role of pH, pH shift and pH dual-stage strategies have been developed for enhancing secondary metabolites formation. Compared to pH-uncontrolled, pH-controlled, and two-stage pH-controlled conditions, the pH shift strategy effectively increased the CS103 (FR-008 derivative) yield and shortened the fermentation time from 120 to 96 h [2]. Additionally, Fei et al. [14] enhanced by 85.7% on acarbose production of *Streptomyces* M37 using a two-stage pH control strategy, compared to traditional batch fermentation without pH control. It was found that the alkaline pH shock could increase Val-A production by 27.43% in *Streptomyces hygroscopicus* [15].

This study has aimed to establish an efficient fermentation process technology to further enhance the efficiency of *Streptomyces* ZYJ-6 in producing FR-008-III/candicidin D (hereinafter referred as candicidin). First, the effects of medium components on the cell growth and candicidin production were investigated through statistical analysis. Second, based on the optimal medium in shake flask, the optimization of N/C/P-sources and pH control strategy was studied in 5 L bioreactor for further improvement of candicidin production. Simultaneously, the metabolomics profiling under different pH conditions was analyzed and discussed to elucidate the mechanism of pH affecting on candicidin biosynthesis. Finally, an efficient fermentation process with an integrated medium optimization and pH control strategy was developed.

Methods and materials

Microorganism and growth conditions

The *Streptomyces* ZYJ-6 was kindly donated by professor Delin You in Shanghai Jiao Tong University.

Aerobic cultivations of *Streptomyces* ZYJ-6 were started with glycerol stocks. 100 µL spores from glycerol stocks were incubated on the slant medium (2% agar, 2% mannitol, 2% soybean powder, pH 7.2) for 4-day cultivation at 30 °C. Spore suspension was harvested by washing the slant with 30 mL 0.9% NaCl solution. Subsequently, 10^7 spores per 100 mL was inoculated into the 100 mL medium (3% tryptone, 1% yeast extract, 10.3% sucrose, pH 7.2) in the 500 mL Erlenmeyer flask and grown 30 h at 30 °C and 220 rpm. Mycelia suspension of 1 mL was inoculated into 100 mL medium for shake flask fermentation in 500 mL Erlenmeyer flask and mycelia suspension of 300 mL was inoculated in 5 L bioreactor for further fermentation.

For shake flask fermentation, the primary medium was referenced to Ochi [16] (L^{-1}) and with some minor modifications: glucose 20 g, KH_2PO_4 0.5 g, glycine 5 g, $EDTANa_2$ 1.8 g, $MgSO_4{\cdot}7H_2O$ 20 g, $ZnSO_4{\cdot}7H_2O$ 35.7 mg, $CaCl_2$ 50 mg, $FeSO_4{\cdot}7H_2O$ 28.7 mg, $CuSO_4{\cdot}5H_2O$ 16 mg, $MnSO_4{\cdot}H_2O$ 9.1 mg, antifoam 0.3%, NaCl 5.0 g as an osmotic pressure regulator. Initial pH was adjusted to 7.2 by 4 M NaOH.

The 5-L bioreactor (Shanghai Guoqiang Bioengineering Equipment Co., Ltd. Shanghai, China) fermentation culture was carried out in 3 L working volume. The process parameters were as follows: the temperature was kept at 30 °C, the agitation was set at a constant speed rate of 400 rpm and the sterile airflow was kept at 1 vvm through a bottom sparger to keep overpressure of 0.05 MPa. The chemically defined

medium contained (L^{-1}): glucose 55 g, KH_2PO_4 1.5 g, $(NH_4)_2SO_4$ 1.8 g, $EDTANa_2$ 1.8 g, $MgSO_4{\cdot}7H_2O$ 8.6 g, $ZnSO_4{\cdot}7H_2O$ 35.7 mg, $CaCl_2$ 50 mg, $FeSO_4{\cdot}7H_2O$ 28.7 mg, $CuSO_4{\cdot}5H_2O$ 42 mg, $MnSO_4{\cdot}H_2O$ 9.1 mg, antifoam 0.3%, NaCl 9.0 g as an osmotic pressure regulator. The process pH was controlled by adding 10% ammonium hydroxide. The glucose concentration in feeding solution was 0.75 g (glucose)/g (deionized water). The feeding was started once the initial glucose was exhausted and then the residual glucose concentration was kept below 5 g/L. The glucose solution was sterilized separately at 110 °C for 40 min and the bioreactor with medium together was sterilized at 121 °C for 60 min.

Medium optimization in shake flask

Plackett–Burman (PB) design from Design Expert 10 was used for screening medium components with respect to their main effects but not to their interaction effects [17]. Each nutrient was tested at two concentrations (high and low) and these variables were screened with a 12-runs PB design shown in Table A1. The principle and steps were documented by Chen et al. [18].

Central composite design (CCD) from Design Expert 10 for three independent variables was used to obtain the combination of values and their interaction effects, which allows one to minimize the number of experiments required. The medium components selected for CCD were NaCl, $CuSO_4$ and $MgSO_4$.

N/C/P-sources regulation in bioreactor

N-source regulation was based on fixed C/N with shake flask but glycine was replaced by $(NH_4)_2SO_4$. P- and C-source were increased from 0.5 to 1.5 g/L and 20 to 55 g/L, respectively. pH was kept at 7.2 during the whole process.

Optimal pH in fermentation process

In 5 L bioreactor, all parameters were same but different pH values (6.8, 7.2, 7.5, 7.8 and 6.8–7.8) were set in fermentation processes. Since an immediate increase in pH might bring pH shock to cells, the pH was allowed to increase gradually from 44 to 48 h. In detail (Fig. 3e), pH 6.8 from 0 to 44 h, pH 7.0 from 44 to 45 h, pH 7.2 from 45 to 46 h, pH 7.4 from 46 to 47 h, pH 7.6 from 47 to 48 h, pH 7.8 from 48 h to the late phase ($t > 48$ h), respectively.

Sampling and pretreatment

Regular sampling (per 12 h) contained: 1 mL broth was centrifuged and the supernatant was determined for the residual glucose; 1 mL broth was taken out into 2 mL dimethylsulfoxide (DMSO) directly for the candicidin extraction; 3 mL × 3 were used for the dry cell weight (DCW).

Rapid sampling was set at 36 h, 46 h, 96 h and 156 h. 36 h was the time point in logarithmic phase; 46 h was in upcoming candicidin synthesis phase; 96 h was in plateau period and 156 h was in late stage. 1 mL broth was withdrawn into 8 mL isoamylol: base solution = 5:1 (v/v) and base solution was acetone: ethanol = 1:1 (mol/mol) by rapid sampling device within 0.2 s. The solution was centrifuged for 1 min at − 13 °C and 7500 rpm and the supernatant was discarded. The residual cell pellets with internal standard (IS) were extracted by 50% (v/v) methanol and then subjected to three cycles of freezing on liquid nitrogen for 3 min and thawing at − 30 °C on cryostat. The extracted solution was concentrated to 0.5 g for the detection of metabolites.

Analytical methods

The concentration of glucose was determined by a biosensor (SBA-40B, Shandong Science Academy, China). The candicidin was measured according to the procedures as described by Mao et al. [19]. Biomass accumulation was estimated by DCW analysis. Briefly, 3 mL of culture was deposited on pre-weighed filter paper and washed thrice with distilled water. The filter paper covered with the cells were dried to constant weight in a 70 °C oven for 36 h and allowed to cool in a desiccator. The filter paper was weighed and the biomass calculated.

Quantitative analysis of intracellular metabolites was performed by gas chromatograph mass spectrometer (GC-MS) with a 7890A GC coupling to a 5975C MSD single quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA). The determination conditions of the metabolites were referred to de Jonge et al. [20] with slight modification. Briefly, different column, HP5-5% Phenyl Methicone column (30 m × 250 μm internal diameter, 0.25 μm film thickness), was used. Sample of 1 μL was injected in splitless mode at 250 °C. During the analysis process, the flow rate of helium was kept at 1 mL/min. The GC column temperature gradient for organic acids (OA), phosphate sugars (PS) and sugar alcohols (SA): the initial temperature was set as 70 °C for 1 min and then increased with the speed of 10°C/min up to 300 °C and kept for 10 min. While for amino acids (AA) analysis was initially set as 100 °C for 1 min and then raised by a same speed of 10°C/min up to 300 °C and held at 300 °C for 10 min for cleaning. Electron ionization was operated with 70 eV and MS was operated in selected ion monitoring (SIM) mode as usually described. The temperature of transfer line, MS source and quadrupole were kept at 280 °C, 230 °C and 150 °C, respectively. Quantification of the metabolites was conducted by isotope dilution mass spectrometry (IDMS) [21].

The coenzymes and others were analyzed by ultra-high performance liquid chromatography-mass spectrometry tandem mass spectrometry (UHPLC-MS/MS) as described by Hong et al. [22].

Results and discussion

Medium optimization in shake flask

PB design was applied to screen the most significant medium components in candicidin production. The limits of variables and response of candicidin from different experimental trials were shown in Table A1. The analysis of variance (ANOVA) of PB model was described in Table A2. The p value was used as a tool to check the significance of each constituent. A low p value indicated a significant effect. The p value analysis shown that, among the tested variables, NaCl, $CuSO_4$ and $MgSO_4$ played significant roles in candicidin production ($p < 0.05$). Therefore, NaCl, $CuSO_4$ and $MgSO_4$ were selected for further optimization by CCD.

To examine the interaction effect of three significant components on candicidin production, a total of 20 experiments were performed (Table A3). The p values were also used to understand the significance of interaction pattern between the tested variables (Table A4). The F value of 4.06 and p value of 0.0164 implied the model was significant. According to these rules, NaCl and $MgSO_4$ were significant model terms; however, no interaction between them was found ($p > 0.1$). Through the CCD prediction and verification experiments, the optimal concentration of NaCl, $CuSO_4$ and $MgSO_4$ were 9.0 g/L, 42 mg/L and 8.6 g/L, respectively.

After optimization, the new medium recipe was more suitable for the candicidin production (Fig. 1). The glucose consumption and cell growth also displayed satisfactory performance, while broth pH would spontaneously increase from 7.2 to 8.0 and keep around 8.0 in the late period. In the following experiments, the optimized medium was adopted in 5 L bioreactor for further fermentation process optimization.

N/C/P-sources regulation in bioreactor

On the basis of the medium optimization in shake flask, the candicidin fermentation was scaled up to 5 L bioreactor. Nevertheless, the optimal medium in shake flask was not

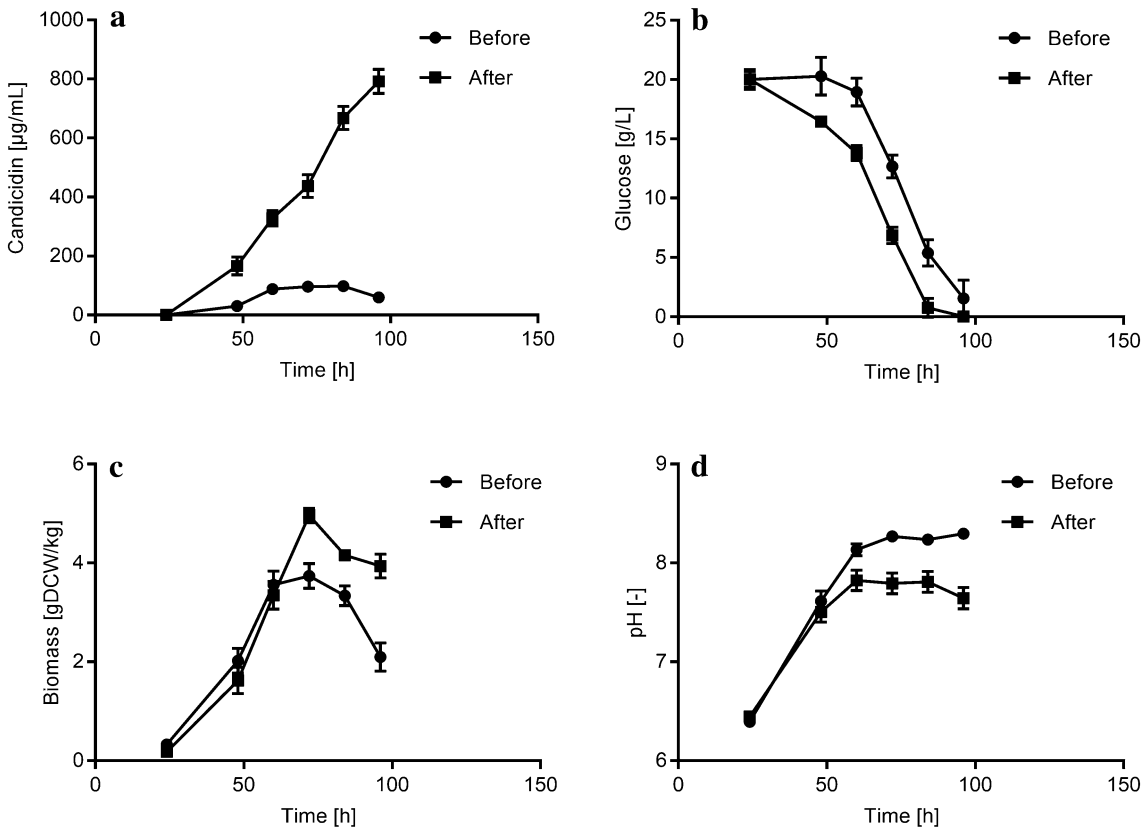

Fig. 1 Comparison of candicidin fermentation performances between before (solid circles) and after (solid square) the optimization of medium by *Streptomyces* ZYJ-6 in the shake flask culture. **a** Accumulation of candicidin with time; **b** consumption of glucose; **c** cell growth and **d** change of pH in the process. Each value represents the mean of three separate determinations with standard deviation

suitable for cultivation on the bioreactor scale, resulting in extremely low candicidin concentration in 5 L bioreactor (Fig. A1). Therefore, some process regulations were conducted to improve the fermentation performance on 5 L fermenter.

As mentioned above, pH would spontaneously increase from 7.2 to 8.0 and keep around 8.0 in shake flask. It was speculated that the glycine as an easily metabolic N and C source might be used primarily or utilized with glucose at same time, which led to pH increase in the whole shake flask fermentation process. In 5 L bioreactor, pH would be kept at constant 7.2; therefore, the glycine was replaced by $(NH_4)_2SO_4$ with fixed C/N ratio and the results are shown in Fig. 2. $(NH_4)_2SO_4$ was a physiological acid salt and its utilization would result in pH decrease so that pH could be kept in 7.2 by adding ammonium hydroxide. After the N-source regulation, candicidin production, glucose consumption, cell growth and oxygen uptake rate (OUR) level had a slight improvement compared with the initial fermentation, but still at relatively low level (Fig. 2). OUR is one of the fundamental physiological parameters of cell metabolism, which has close connection with the biosynthesis of products [23]. It is well known that the primary metabolism provides precursors for the secondary metabolism and thus enhances the primary metabolism is the pre-requisite for the improvement of secondary metabolism. However, the biomass was only around 5–6 g/kg in this case (Fig. 2c). The candicidin biosynthesis belongs to the secondary metabolism whereas the indispensable precursors PABA, malonyl-CoA and methylmalonyl-CoA are from the primary metabolism. Hence, in order to enhance the primary metabolism suitably and then improve the candicidin production, P-source regulation would be carried out afterwards.

The depletion of P-source appeared to trigger the onset of candicidin synthesis after a drastic reduction of the RNA synthesis rate [24]. Many literatures cast a veil over P-source in the candicidin biosynthesis and researchers always gave a conservative level of P-source. Martin and Demain [25] found phosphate concentrations above 1 mM decreased the incorporation rate of [^{14}C] propionate and [^{14}C] *p*-aminobenzoic acid into candicidin. Despite the above facts, sufficient supply of P-source was highly effective in enhancing the primary metabolism. Therefore, in medium components, level of KH_2PO_4 was aggrandized

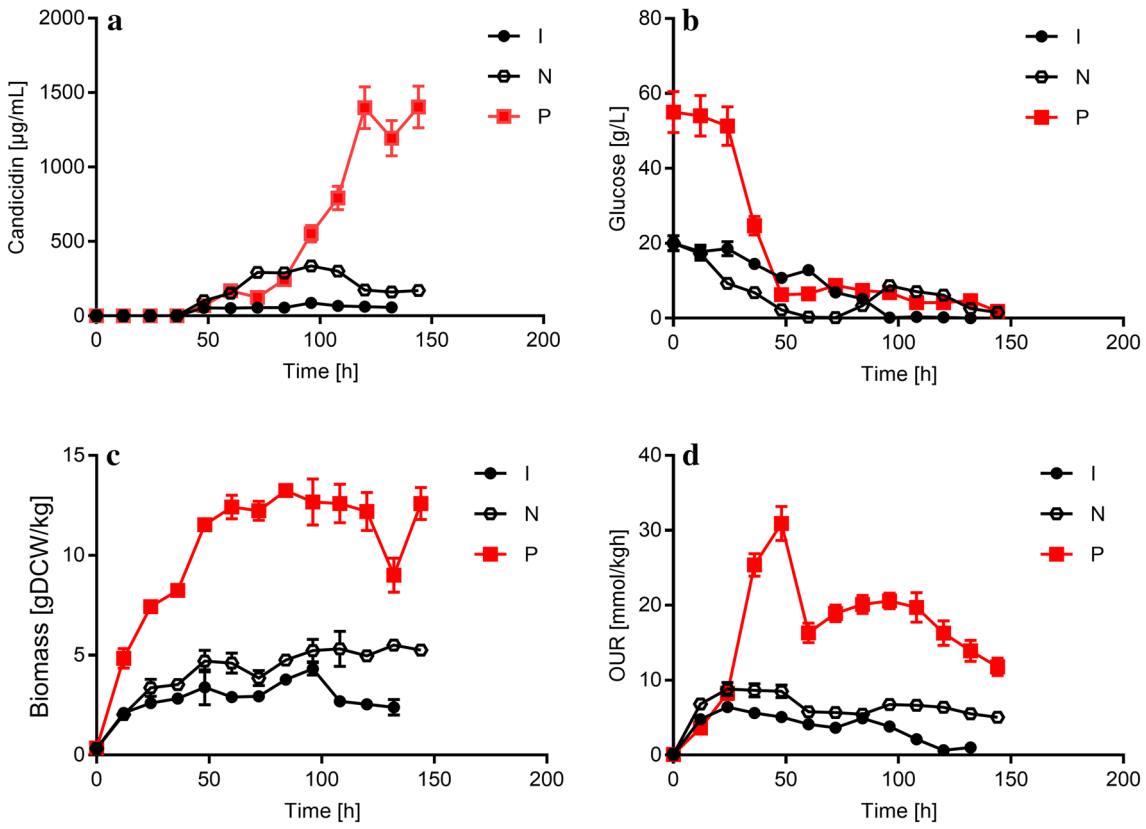

Fig. 2 Comparison of fermentation performances among initial medium (I, solid black circles), N-source regulation (N, hollow hexagon) and C/P-sources regulation (P, solid red square) by *Streptomyces* ZYJ-6 in 5 L bioreactor. **a** Accumulation of candicidin with time; **b** consumption of glucose; **c** cell growth and **d** change of OUR levels in the process. Each value represents the mean of three separate determinations with standard deviation

from 0.5 g/L (3.67 mM) to 1.5 g/L (11.02 mM) and with the overall consideration of nutrients balance, the glucose level was increased from 20 to 55 g/L with almost the same C/P ratio. Undoubtedly, pH was controlled by ammonium hydroxide so that the N-source was unlimited.

It was reported in Fig. 2 that P-source regulation displayed prominent performance. As expected, the maximum biomass concentration increased 3 times, compared to that in the initial fermentation and attained 12 gDCW/kg (Fig. 2c). The candicidin level had a qualitative leap and attained 1403 μg/mL (Fig. 2a), which was 3 times of 450 μg/mL cultured in shake flask with complex medium by *Streptomyces* ZYJ-6 and even higher than other mutants reported by Wang et al. [26]. The candicidin synthesis rate, q_P (7.08 mg/gh), was 3.5 times of N-source regulation (2.01 mg/gh) and 15.4 times of the initial fermentation (0.46 mg/gh, Table 1). Simultaneously, the process OUR profile also, to some extent, confirmed the P-source regulation results and its highest value attained 30.9 mmol/kg/h (Fig. 2d).

After the N-source regulation, controllable pH conditions were realized, which could enhance the cell growth and candicidin biosynthesis to some degree. Surprisingly, the candicidin synthesis rate and titer were further improved 3.5 and 8.2 times, respectively, with the C/P-sources regulation, compared to N-source regulation. Consequently, the titer of 1403 μg/mL was achieved, the highest level ever reported in *Streptomyces* ZYJ-6.

Table 1 *Streptomyces* ZYJ-6 fed-batch parameters at different conditions

Terms (-)	μ_{max} (/h)	q_S (g/gh)	q_P (mg/gh)	Yxp (mg/g)	Ysp (mg/g)
I (pH 7.2)	0.012	0.086	0.46	38.33	5.35
N (pH 7.2)	0.020	0.12	2.01	100.50	16.75
P (pH 7.2)	0.052	0.27	7.08	136.15	26.22
pH 6.8	0.065	0.19	4.63	71.23	24.37
pH 7.5	0.056	0.31	5.54	98.93	17.87
pH 7.8	0.051	0.28	11.64	228.24	41.57
pH 6.8–7.8	0.064	0.31	22.48	351.25	72.52

I initial medium from the optimization in shake flask, *N* N-source regulation, *P* P-source regulation, μ_{max} maximum specific growth rate, q_S glucose consumption rate and the unit is g glucose per g biomass per hour, q_P candicidin synthesis rate and the unit is mg candicidin per g biomass per hour, *Yxp* yield of candicidin based on biomass and calculated by q_P/μ and the unit is mg candicidin per g biomass, *Ysp* yield of candicidin base on glucose and calculated by q_P/q_S and the unit is mg candicidin per g glucose

Optimal pH in fermentation process

In N/C/P-sources regulation experiments, pH was kept at 7.2 by feeding 10% ammonium hydroxide as mentioned in literatures [8, 26]. It is well known that the optimal pH of *Streptomyces* is 6.5–8.0 and in the previous work, the pH range that could support candicidin production was 7.0–8.0 [3, 7, 11, 26]. To demonstrate the pH effect on the candicidin production, process pHs were maintained at 6.8, 7.2, 7.5 and 7.8, respectively (Fig. 3e) without changing of other operation parameters.

The time courses of candicidin production, residual glucose concentration, cell growth and OUR levels in the fed-batch culture of *Streptomyces* ZYJ-6 were shown in Fig. 3. When cells entered into the stationary phase, the candicidin began to be synthesized. As observed in Fig. 3, pH 7.8 was the most appropriate condition for the candicidin biosynthesis (Fig. 3a), but seemed to be not sutiable for the cell growth (Fig. 3c). In this study, glucose was used as a sole carbon source for the cell growth and candicidin production. Glucose concentration decreased sharply with the rapid cell growth at pH 6.8 (Fig. 3b). As observed in Fig. 3d, before the turning point (cell gorwth phase), OUR level at pH 6.8 performed the highest value, while after the turning point (cell stationary phase), pH 7.8 could maintian the relatively higher OUR than that under other conditions. The q_P of candicidin production for pHs at 6.8, 7.2, 7.5 and 7.8 were 4.63, 7.08, 5.54 and 11.64 mg/gh, respectively (Table 1). The μ_{max} for pHs at 6.8, 7.2, 7.5 and 7.8 were 0.065, 0.052, 0.056 and 0.051/h, respectively (Table 1). These results indicated that the optimal pH value for the cell growth was around 6.8, while the optimal pH value for the candicidin formation was around 7.8 by *Streptomyces* ZYJ-6.

On the basis of the above analyses, a stepwise pH control strategy (pH 6.8–7.8, Fig. 3e) was proposed for the further enhancement of candicidin production. As the OUR level at pH 6.8 had a downward tendency, the culture pH was increased gradually from 6.8 to 7.0 at 44 h, 7.0 to 7.2 at 45 h until 7.6 to 7.8 at 48 h (Fig. 3e). Phase I was defined as the condition to increase the cell growth by maintaining the pH at 6.8 from 0 to 44 h. Phase II was defined as the condition to accelerate the candicidin production by maintaining the pH at 7.8 from 48 h to the end of fermentation.

The results were summaried in Fig. 4. Under the pH 7.8, the candicidin concentration entered into plateau in the late phase ($t > 116$ h), whereas under pH 6.8–7.8 condition, the candicidin concentration exhibited a continuous increase. The maximum biomass reached eariler ($t = 72$ h) and followed by the significant decrease which was supposed to ascribe to the candicidin concentration entering into plateau at pH 7.8. As shown in Table 1, the candicidin titer and productivity increased up to 5161 μg/mL (Fig. 4a) and 22.48 mg/g h, respectively. The titer 5161 μg/mL was even

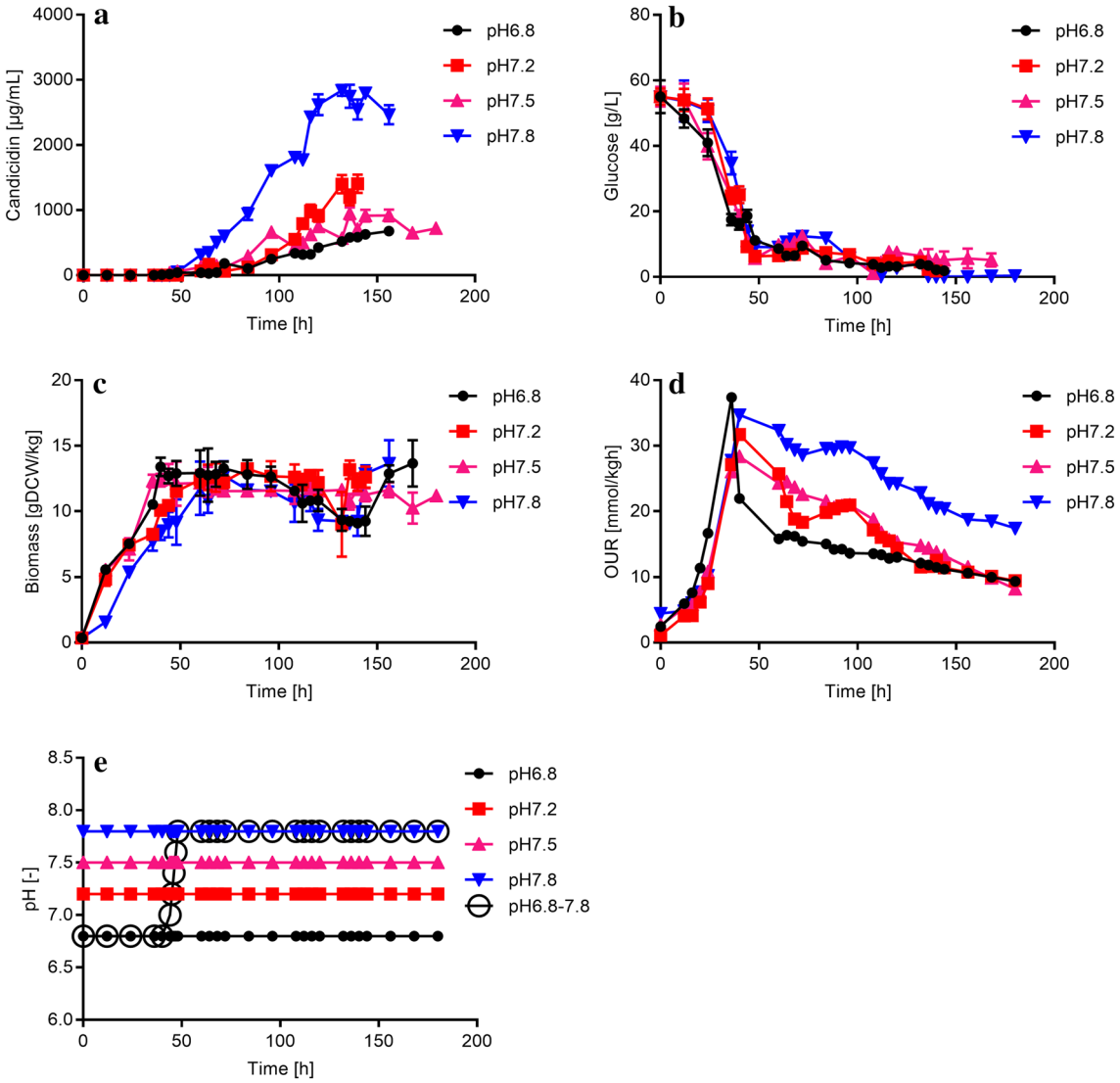

Fig. 3 Comparison of fermentation performances under different pH conditions, pH 6.8 (solid black circles), pH 7.2 (solid red square), pH 7.5 (solid pink triangle) and pH 7.8 (solid blue triangle) by *Streptomyces* ZYJ-6 in 5 L bioreactor. **a** Accumulation of candicidin with time; **b** consumption of glucose; **c**: cell growth and **d** change of OUR levels in the process. Each value represents the mean of three separate determinations with standard deviation. **e** Different pH conditions, pH 6.8–7.8 (hollow circles)

higher than 4 g/L, the highest candicidin level ever reported in the reference with *Streptomyces griseus* IMRU 3570 [11]. Regarding *Streptomyces* ZYJ-6, the candicidin concentration obtained in this study was even more than 10 times of 450 μg/mL in complex medium [26]. Productivity (q_P) is one of the key parameters to evaluate a bioprocess performance, its scale-up potential and commercial large-scale feasibility [27]. The q_P (22.48 mg/gh) under pH 6.8–7.8 was almost 2 times than pH 7.8 (11.64 mg/gh). Simultaneously the Yxp (yield based on biomass) and Ysp (yield based on glucose) were 1.5 and 1.7 times, respectively (Table 1).

All these results further confirmed that the optimal pH for *Streptomyces* ZYJ-6 growth was different from that for the candicidin production. On the other hand, through comparing the OUR levels, similar conclusions could be drawn that pH 6.8–7.8 control strategy was more sustainable than constant pH 7.8 strategy, and as in the pH 6.8–7.8 condition, OUR could keep at contant level during the whole late phase ($t > 60$ h), while it declined evidently under the constant pH 7.8 condtion. It was concluded that the stepwise pH control strategy improved the cell growth and candicidin productivities significantly comparing with the cultivations conducted at a fixed pH of 6.8 or 7.8.

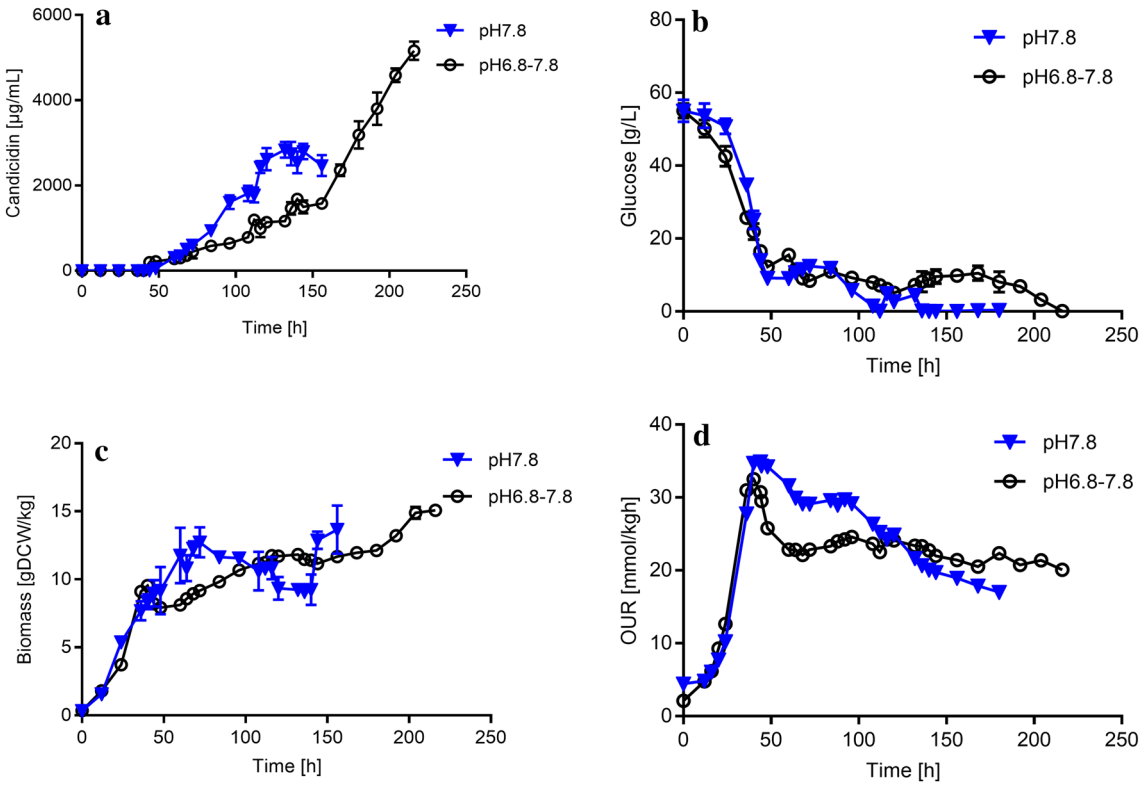

Fig. 4 Comparison of fermentation performances between pH 7.8 (solid blue triangle) and pH 6.8–7.8 (hollow circles) by *Streptomyces* ZYJ-6 in 5 L bioreactor. **a** Accumulation of candicidin with time; **b** consumption of glucose; **c** cell growth and **d** change of OUR levels in the process. Each value represents the mean of three separate determinations with standard deviation

Metabolomics analysis under different pH conditions

Compared to fixed pHs, the stepwise pH control strategy could vastly improve the candicidin production. It is assumed that the expression of candicidin polyketide synthase genes is constant in all of these growth conditions. Thus, it was reasonable to speculate that different pH strategies caused changes on intracellular metabolite levels, which further led to significant difference on final candicidin titers. To confirm this hypothesis, intracellular metabolites from pH 6.8, pH 7.8 and pH 6.8–7.8 at 4 cultivation time points (36, 46, 96 and 156 h) were obtained and analyzed by a combination of GC-IDMS and UHPLC-MS/IDMS. Almost 40 metabolites were identified and quantified, including precursors of candicidin, amino acids, intermediates of center carbon metabolism and others (Table 2).

First, the data structure and quality were assessed by principal component analysis (PCA, Fig. 5a). The four time points from pH 6.8 (red) were separated clearly from other conditions. While although time points from pH 7.8 (green) and pH 6.8–7.8 (blue) possessed a little intersection, most parts were separated clearly as well. It was indicated that intracellular metabolites data were suitable to monitor the culture processes. Second, to further identify which metabolites were closely associated with the candicidin production, the variable importance of the projection (VIP) was generated by partial least squares (PLS) analysis. A higher VIP score suggested that the metabolites contributed more significantly to candicidin biosynthesis. A total of 16 identified metabolites were selected (VIP score > 1) for further investigation (Fig. 5b). They were malonyl-CoA, PABA, propionyl-CoA, citrate, alanine, asparagine, glycine, threonine, proline, fumarate, malate, ADP, α-ketoglutarate, arabitol, serine and methylmalonyl-CoA. Most of them belong to precursors, amino acids and tricarboxylic acid cycle (TCA) intermediates, which would be discussed in detail. Third, heatmap combined with the cluster analysis reconfirmed the above conclusion (Fig. 5c). Most precursors were assigned to one subgroup with candicidin. Subsequently, metabolites highly correlated with candicidin biosynthesis were classified into different categories and were mapped onto metabolic pathways (Fig. 6).

Among precursors, malonyl-CoA gained the highest VIP score of 2.15 in the detected metabolites, which implied the most important role in candicidin biosynthesis. PABA and propionyl-CoA were ranked second and third, respectively. Methylmalonyl-CoA had the lowest VIP score of 1.02, but in

Table 2 Intracellular metabolites concentration in groups with pH 6.8, pH 7.8 and pH 6.8–7.8 at different timepoints: 36, 46, 96 and 156 h

Metabolites	36 h			46 h			96 h			156 h		
	pH 6.8	pH 7.8	pH 6.8–7.8	pH 6.8	pH 7.8	pH 6.8–7.8	pH 6.8	pH 7.8	pH 6.8–7.8	pH 6.8	pH 7.8	pH 6.8–7.8
Precursors												
Shikimate	< 0.1	0.1 ± 0	0.7 ± 0.1	0.3 ± 0	0.6 ± 0.2	0.4 ± 0.1	0.5 ± 0	0.2 ± 0	0.2 ± 0	0.7 ± 0.1	0.9 ± 0	0.1 ± 0
PABA	1.5 ± 0.4	2.6 ± 0.3	1.6 ± 0.2	1.1 ± 0.1	4 ± 0.5	7.3 ± 0.4	1.8 ± 0.1	2.8 ± 0.2	6.4 ± 0.4	1.8 ± 0.1	2.8 ± 0.3	6.4 ± 0.4
CoA	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.2 ± 0	0.8 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.1 ± 0	0.2 ± 0
MalonylCoA	6.3 ± 1.9	8.7 ± 1.7	5.9 ± 0.2	5.6 ± 1.2	6.8 ± 2.3	6.5 ± 3.6	1.4 ± 0.3	1.4 ± 0.2	4.9 ± 1.1	0.7 ± 0.1	0.6 ± 0.1	9.8 ± 6.4
MethylmalonylCoA	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	1.6 ± 0.1	2 ± 0.5	0.4 ± 0.1	0.2 ± 0	3.1 ± 0.7	< 0.1	0.1 ± 0	2.4 ± 0.4
AcetylCoA	2.4 ± 0.1	2.6 ± 0.6	3.3 ± 0.5	1.7 ± 0.5	1.4 ± 0.2	1.4 ± 0.3	0.5 ± 0.1	0.7 ± 0.1	1.5 ± 0.3	0.1 ± 0	0.3 ± 0	1.8 ± 0.3
PropionylCoA	0.3 ± 0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.1 ± 0	0.1 ± 0	1 ± 0.2	< 0.1	< 0.1	0.6 ± 0.1
Amino acids metabolism												
Alanine	18.1 ± 1.8	94 ± 19.9	25.9 ± 1.4	14.4 ± 1	179.8 ± 9.9	72.5 ± 13.5	25.3 ± 5.9	209.9 ± 22.3	51.2 ± 3.7	11.1 ± 2	165.9 ± 33.2	169.9 ± 24.3
Glycine	1.2 ± 0.1	3.3 ± 0.6	2 ± 0.1	1.2 ± 0.1	9.4 ± 0.7	3.4 ± 0.6	2.1 ± 0.4	5.4 ± 0.4	2.6 ± 0.2	0.8 ± 0.2	6.1 ± 0.6	4.2 ± 0.6
Valine	1.9 ± 0.1	2.4 ± 0.4	5.7 ± 0.3	1.9 ± 0.2	7.5 ± 0.5	7.8 ± 1.6	2.7 ± 0.6	14.6 ± 1.3	6.3 ± 0.5	0.9 ± 0.1	11.2 ± 2.4	6.2 ± 0.9
Leucine	1.7 ± 0.3	2.7 ± 0.3	5.5 ± 0.2	2.1 ± 0.1	5.8 ± 0.5	6.5 ± 1.3	3.3 ± 0.9	8.7 ± 1.4	5.4 ± 0.2	1.2 ± 0.1	6.5 ± 1.3	3.6 ± 0.5
Isoleucine	0.4 ± 0.1	0.9 ± 0.1	1.5 ± 0.1	0.6 ± 0	2.4 ± 0.2	1.7 ± 0.5	0.7 ± 0.1	3.3 ± 0.4	1.3 ± 0.2	0.3 ± 0.1	2.3 ± 0.4	1.7 ± 0.2
Proline	0.7 ± 0.2	0.9 ± 0.2	0.9 ± 0	0.5 ± 0	1.9 ± 0.1	2 ± 0.4	1.1 ± 0.3	2.6 ± 0.4	2.1 ± 0.2	0.4 ± 0.1	2.4 ± 0.5	1.8 ± 0.3
Methionine	0.6 ± 0.1	0.8 ± 0.1	1.2 ± 0.2	0.6 ± 0.1	1 ± 0.1	1.2 ± 0	1.2 ± 0.4	1.5 ± 0.3	1.1 ± 0	0.8 ± 0.1	1.1 ± 0.2	0.8 ± 0.2
Serine	1.5 ± 0.1	3.6 ± 0.7	2.6 ± 0.1	1.2 ± 0.1	7.3 ± 0.5	4 ± 0.8	1.8 ± 0.4	6.4 ± 0.6	2.8 ± 0.2	0.8 ± 0.2	5.3 ± 1.1	3.8 ± 0.6
Threonine	1.8 ± 0.1	3.1 ± 0.6	3.7 ± 0.1	1.4 ± 0.1	8.4 ± 0.5	4.3 ± 0.8	2.1 ± 0.5	14.4 ± 1.6	3.4 ± 0.2	0.8 ± 0.2	13.4 ± 0.1	10.1 ± 1.5
Phenylalanine	1.1 ± 0	1.9 ± 0.4	2.1 ± 0.2	1.3 ± 0.3	3.8 ± 0.2	3.4 ± 0.6	9.3 ± 2.5	3.5 ± 0.4	4.9 ± 0.2	4.3 ± 0.4	5.4 ± 0.8	4.2 ± 0.6
Aspartate	3.7 ± 0.3	8.5 ± 1.5	7.7 ± 1.2	3.4 ± 0.1	12.6 ± 0.6	12.5 ± 2.5	6 ± 1.5	7.7 ± 1.3	7.2 ± 0.2	3.5 ± 0.8	4 ± 1.3	5.8 ± 1
Glutamate	111.7 ± 15.5	168.5 ± 37.1	163.3 ± 8.5	49.1 ± 3.7	230 ± 11.8	215.9 ± 36.6	121 ± 36.1	96.7 ± 11.5	154.8 ± 18.2	36.1 ± 6.8	65.1 ± 16.4	139.3 ± 23
Ornithine	0.6 ± 0	2 ± 0.4	1.2 ± 0.1	0.4 ± 0.1	1.5 ± 0.1	1.7 ± 0.3	0.8 ± 0.2	0.9 ± 0.2	1 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
Asparagine	1 ± 0.3	1.7 ± 0.1	1.5 ± 0.1	0.5 ± 0	3.2 ± 0.2	2 ± 0.6	1.5 ± 0.4	3.6 ± 0.4	0.9 ± 0.1	0.6 ± 0.2	4.7 ± 1	3.6 ± 0.4
Glutamine	14.6 ± 1.7	20.9 ± 4.9	51.4 ± 6	5.9 ± 0.5	36.8 ± 1.6	32.2 ± 5.8	15.6 ± 4.3	17.1 ± 4.6	18.1 ± 0.7	4.3 ± 1.1	12.7 ± 3.1	25.2 ± 4.4
Tyrosine	0.4 ± 0	0.7 ± 0.2	1 ± 0	0.5 ± 0	1.5 ± 0.1	0.1 ± 0	0.9 ± 0.1	1.8 ± 0.4	1.7 ± 0.3	0.6 ± 0.1	1 ± 0.1	0.5 ± 0.1
Center carbon metabolism												
Pyruvate	0.7 ± 0.1	0.7 ± 0.1	0.9 ± 0	1.4 ± 0.1	1 ± 0.1	0.4 ± 0.1	1.5 ± 0.2	0.8 ± 0	0.6 ± 0	0.8 ± 0.1	0.6 ± 0	0.6 ± 0
Succinate	0.5 ± 0.1	2.4 ± 0.6	0.5 ± 0.1	0.6 ± 0.1	1.1 ± 0.1	1.6 ± 0.3	0.8 ± 0.2	0.5 ± 0	0.6 ± 0	0.2 ± 0.1	0.4 ± 0.1	0.6 ± 0.1
Fumarate	0.3 ± 0	0.5 ± 0.2	0.2 ± 0	0.2 ± 0	0.3 ± 0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0
Malate	0.6 ± 0	0.5 ± 0.1	0.6 ± 0	0.2 ± 0	0.4 ± 0	0.6 ± 0.1	0.5 ± 0.1	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.3 ± 0
α-ketoglutarate	0.3 ± 0	0.3 ± 0.1	0.5 ± 0	0.1 ± 0	0.4 ± 0	0.4 ± 0.1	0.2 ± 0	0.2 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
Ribose 5-phosphate	1.6 ± 0.1	1.5 ± 0.3	1.6 ± 0.3	0.9 ± 0	2.9 ± 0.2	2.5 ± 0.5	5.7 ± 1.2	0.9 ± 0.2	1.3 ± 0.1	3.8 ± 0.9	1.1 ± 0.2	0.7 ± 0
Glyceraldehyde 3-phosphate	1.4 ± 0.4	7.2 ± 1.8	3.5 ± 0.3	3.3 ± 0.8	5.4 ± 0.7	9.1 ± 0.8	7.1 ± 0.2	7.6 ± 1.5	4.8 ± 1.7	8.3 ± 1.2	8.4 ± 1	4.8 ± 1.2
6-Phospho-gluconate	1.3 ± 0.4	0.6 ± 0.1	1 ± 0.1	0.6 ± 0.1	2.4 ± 0.3	0.7 ± 0.1	0.6 ± 0.1	3.6 ± 0.1	0.8 ± 0	5.1 ± 0.8	3 ± 0.4	1.1 ± 0.1

Table 2 (continued)

Metabolites	36 h			46 h			96 h			156 h		
	pH 6.8	pH 7.8	pH 6.8–7.8	pH 6.8	pH 7.8	pH 6.8–7.8	pH 6.8	pH 7.8	pH 6.8–7.8	pH 6.8	pH 7.8	pH 6.8–7.8
3-Phosphoglycerate	1.7 ± 0.3	4.2 ± 0.5	2.9 ± 0.6	1.8 ± 0.1	1.3 ± 0.4	2.1 ± 0.1	< 0.1	1.4 ± 0.2	2.9 ± 0.4	2.4 ± 0.1	2.7 ± 0.2	2.4 ± 0.3
Citrate	0.5 ± 0	1.4 ± 0.3	0.7 ± 0.2	0.8 ± 0.1	1.8 ± 0.3	1.7 ± 0.4	4.7 ± 0.4	0.7 ± 0.1	3.6 ± 0.5	5.7 ± 0.1	2.3 ± 0	4.7 ± 0.1
Fructose 6-phosphate	1.6 ± 0.1	1.7 ± 0.6	1.5 ± 0.3	1.3 ± 0	2.7 ± 0.2	2.7 ± 0.5	6.3 ± 0.5	2.3 ± 0.4	2.8 ± 0.1	4.6 ± 0.5	3.2 ± 0.5	2.2 ± 0.3
Glucose 6-phosphate	5.8 ± 0.4	6.2 ± 1.3	6.4 ± 0.9	5 ± 0.3	1.7 ± 0.8	7.4 ± 2.1	8.1 ± 2.8	9.4 ± 1.4	12.5 ± 0.7	2.2 ± 3.4	12.2 ± 1.8	9.4 ± 1.6
Others												
Erythritol	1 ± 0.2	0.7 ± 0.1	1.1 ± 0	1 ± 0	0.9 ± 0.1	1.7 ± 0.3	3.3 ± 0.5	1.3 ± 0.1	1.9 ± 0.1	3.1 ± 0.4	1.9 ± 0.2	1.7 ± 0.2
Xylitol	0.8 ± 0	0.2 ± 0	0.4 ± 0.1	0.1 ± 0	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.1 ± 0	0.2 ± 0	0.3 ± 0.1	0.2 ± 0	0.3 ± 0
Arabitol	1.2 ± 0.2	0.9 ± 0.3	1.6 ± 0	0.9 ± 0	2 ± 0.6	1 ± 0.1	1.1 ± 0.3	3.4 ± 0.2	0.7 ± 0.2	0.3 ± 0.1	1.7 ± 0	0.2 ± 0
ADP	0.3 ± 0.1	0.1 ± 0	0.8 ± 0.1	< 0.1	0.1 ± 0	0.2 ± 0	< 0.1	0.3 ± 0	0.2 ± 0	0.2 ± 0	< 0.1	< 0.1
ATP	0.6 ± 0.1	2.3 ± 0.4	3.7 ± 0.9	0.1 ± 0	1.3 ± 0.1	0.3 ± 0	1.6 ± 0.1	0.1 ± 0	0.7 ± 0	0.3 ± 0.1	0.2 ± 0	0.7 ± 0.1

The values shown here represent the means of three independent experiments and the error bars represent standard deviations of three values. The concentration of the metabolites was given in μmol/gDCW

same cluster group with candicidin (Fig. 5c). Malonyl-CoA, PABA and methylmalonyl-CoA were the direct precursors for the candicidin biosynthesis (Fig. 6). At pH 6.8 and pH 7.8, malonyl-CoA consumed quickly and ranged from 6.3 to 8.7 μmol/gDCW at 36 h to 0.7 and 0.6 μmol/gDCW at 156 h (Table 2). While at pH 6.8–7.8, malonyl-CoA concentration was always maintained high level from 5.9 μmol/gDCW at 36 h to 9.8 μmol/gDCW at 156 h. The results indicated that malonyl-CoA was abundant at pH 6.8–7.8 but deficient at pH 6.8 or pH 7.8. PABA, the starting unit of candicidin biosynthesis, was produced and converted from shikimate metabolism (Fig. 6). PABA was always at lower level in pH 6.8 (1.1–1.8 μmol/gDCW, Table 2). Thus, it could be reasonably assumed that there might be something inhibited the intracellular flux toward PABA at pH 6.8. Except for malonyl-CoA and PABA, methylmalonyl-CoA was also the direct precursors for candicidin biosynthesis and propionyl-CoA was the direct precursor of methylmalonyl-CoA (Fig. 6). The remarkable deficiency of methylmalonyl-CoA and propionyl-CoA was observed during the major candicidin biosynthesis phase ($t > 96$ h, < 0.1 μmol/gDCW, Table 2) which was verified that precursors were insufficient both at pH 6.8 and pH 7.8.

Amino acid metabolism, supporting the protein synthesis and cell growth, was documented as an important source of polyketide precursors [28]. There were 6 amino acids, which were closely associated with candicidin biosynthesis by PLS i.e., alanine [VIP score = 1.71], asparagine [VIP score = 1.61], glycine [VIP score = 1.47], threonine [VIP score = 1.41], proline [VIP score = 1.40] and serine [VIP score = 1.06] (Fig. 5b). Most strikingly, strong differences in the intracellular level of amino acids was observed under different pH conditions. The above mentioned amino acids had significantly lower pools in pH 6.8 and declined to lowest level at 156 h. While, in pH 7.8, the 6 amino acids were at higher level than other conditions at 46 h (onset time of candicidin biosynthesis) and increased continually until 96 h (except for glycine and serine) and the level of these amino acids at 96 h was dropped till 156 h. In contrast, the levels of 6 amino acids decreased at 96 h and increased again at 156 h in pH 6.8–7.8. These results might be the reason why candicidin biosynthesis entered into plateau in pH 7.8 but could keep rapid synthesis rate (22.48 mg/gh, Table 1) after 156 h in pH 6.8–7.8. Moreover, alanine, glycine, serine and threonine could convert to acetyl-CoA via pyruvate. Acetyl-CoA was the direct and important precursor of malonyl-CoA (Fig. 6). Asparagine and threonine were the indirect precursors of methylmalonyl-CoA (Fig. 6). Presumably, this was the reason for deficiency of malonyl-CoA and methylmalonyl-CoA after 96 h in pH 7.8.

Central carbon metabolism supplied fundamental precursors and energy for the secondary metabolism and amino acid metabolism. Based on the suggestions of PLS, citrate

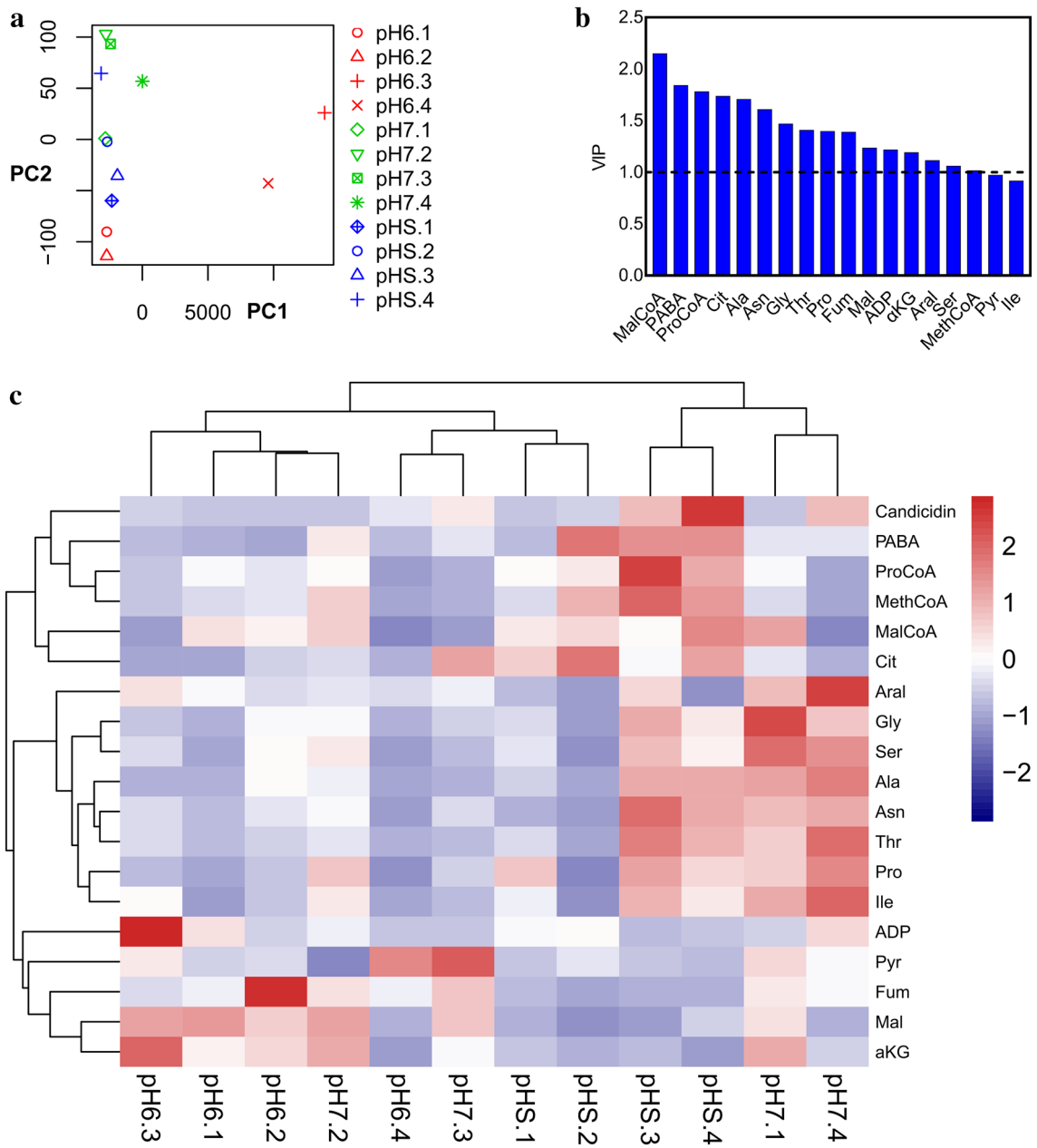

Fig. 5 PCA, PLS and Cluster analysis of intracellular metabolites from pH 6.8, pH 7.8 and pH 6.8–7.8. The samples were withdrawn from the cultivations at 36, 46, 96, and 156 h. **a** PCA-derived score plots. **b** PLS-derived the variable importance of the projection (VIP) histogram. **c** Cluster-derived heatmap. pH *x.y: x* was pH condition and 6 means pH 6.8; 7 means pH 7.8; S denotes pH 6.8–7.8, y was the sampling time point and 1–4 means first (36 h) to forth (156 h) sampling points. e.g. pH 6.1 was the first sampling point (36 h) in pH 6.8, pH 7.2 was the second sampling point (46 h) in pH 7.8 and pH S.3 was the third sampling point (96 h) in pH 6.8–7.8. MalCoA, malonyl-CoA; PABA, para aminobenzoic acid; ProCoA, propionyl-CoA; Cit, citrate; Ala, alanine; Asn, asparagine; Gly, glycine; Thr, threonine; Pro, proline; Fum, fumarate; Mal, malate; αKG, α-ketoglutarate; Aral, arabitol; Ser, serine; MethCoA, methylmalmonyl-CoA; Pyr, pyruvate

(VIP score = 1.74), fumarate (VIP score = 1.39), malate (VIP score = 1.23) and α-ketoglutarate (VIP score = 1.19) (Fig. 5b) implied a close association with candicidin biosynthesis. As described in Table 2, no significant difference was found in above metabolite concentration among different pH conditions. The facts revealed that although the TCA was important in supplement of precursors and energy, it was not the bottleneck of candicidin biosynthesis under different pH conditions.

These results proved that the concentration of TCA metabolites had little differences; nevertheless, the differentiation would appear in amino acids metabolism and

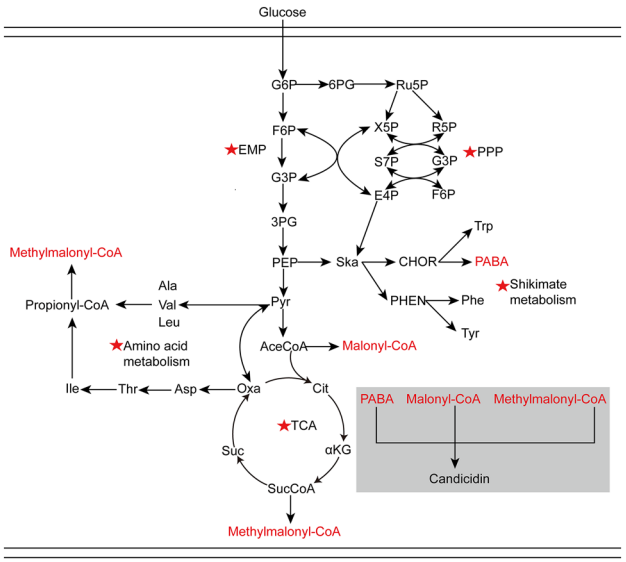

Fig. 6 Schematic representation of the proposed metabolic pathways closely associated with the candicidin biosynthesis in *Streptomyces* ZYJ-6. The key pathways are marked by red pentacles. EMP, Embden-Meyerhof-Parnas pathway; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; G3P, glyceraldehyde 3-phosphate; 3PG, 3-phospho-glycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; AceCoA, acetyl-CoA; Cit, citrate; αKG, α-ketoglutarate; SucCoA, succinyl-CoA; Suc, succinate; Oxa, oxaloacetate; Asp, aspartate; Thr, threonine; Ile, isoleucine; Ala, alanine; Val, valine; Leu, leucine; 6PG, 6-phospho-gluconate; Ru5P, ribulose 5-phosphate; X5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; Ska, shikimic acid; CHOR, chorismate; Trp, tryptophan; PABA, para aminobenzoic acid; PHEN, prephenic acid; Phe, phenylalanine; Tyr, tyrosine

precursors supplement, which led to different candicidin titers. Specifically, at pH 6.8, low levels of amino acids were insufficient to PABA, malonyl-CoA and methylmalonyl-CoA in whole candicidin biosynthesis process; at pH 7.8, amino acid concentration started to drop from 96 to 156 h resulting in the deficiency of malonyl-CoA and methylmalonyl-CoA in late phase of candicidin biosynthesis and causing candicidin biosynthesis into a plateau; at pH 6.8–7.8, due to amino acids increased continually after 96 h, abundant precursors PABA, malonyl-CoA and methylmalonyl-CoA were guaranteed to the rapid and continuous candicidin biosynthesis. To sum up, the stepwise pH control strategy (pH 6.8–7.8) was favorable to supply abundant precursors for the candicidin biosynthesis.

Conclusion

Based on the medium optimization in shake flask, chemically defined medium was developed for the cell growth and candicidin production. However, the medium could not be applied to bioreactor directly. By the N/C/P-sources regulation, the candicidin titer reached to 1403 μg/mL; through the optimization of cultivation pH, the titer attained to 2839 μg/mL in pH 7.8; benefit from the optimization of pH, pH stepwise strategy was proposed and unprecedented titer 5161 μg/mL was achieved, which was the highest level ever reported for candicidin production and availability for further industrial application. Metabolomics analysis revealed the pH stepwise control strategy could supply more abundant precursors than fixed pHs and this strategy provides important clue for process optimization of other secondary metabolites.

Acknowledgements We thank Prof. Dr. Delin You (Shanghai Jiao Tong University) for supplying *Streptomyces* ZYJ-6. This work was financially supported by a grant from the Major State Basic Research Development Program of China (973 Program, no. 2012CB721000G), NWO-MoST Joint Program (2013DFG32630) and national key special Program 2017YFF 0204600.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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