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Alkaline pH shock enhanced production of validamycin A in fermentation of *Streptomyces hygroscopicus*

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ABSTRACT

Validamycin A (Val-A) is produced by *Streptomyces* as a secondary metabolite with wide agricultural applications of controlling rice sheath blight, false smut and damping-off diseases. The effect of alkaline pH shock on enhancing Val-A production and its mechanism were investigated. A higher yield of Val-A was achieved by NaOH shock once or several times together with faster protein synthesis and sugar consumption and alkaline pH shock can increase Val-A production by 27.43%. Transcription of genes related to amino acid metabolism, carbon metabolism and electron respiratory chain was significantly up-regulated, accompanied by the substantial increase of respiratory activity and glutamate concentration. Val-A production was promoted by a series of complex mechanisms and made a response to pH stress signal, which led to the enhancement of glutamate metabolism and respiration activity. The obtained information will facilitate future studies for antibiotic yield improvement and the deep revealment of molecular mechanism.

1. Introduction

The antifungal antibiotic validamycin A (Val-A), produced by the fermentation of the industrial strain *Streptomyces hygroscopicus* 5008, has been widely used as a prime control agent against rice sheath blight, false smut and damping-off diseases in East Asia. Much attention has been paid to the improvement of the Val-A yield but seems to do little to increase the productivity. pH is a comprehensive reflection of microbial metabolism, thus affecting the fermentation process and cell growth. pH can affect the direction of metabolism, and the optimal pH of growth and synthesis of products in different stages are different. The same microorganism may produce different metabolites in various environmental pH conditions. For instance, the main production of sucrose fermented by *Aspergillus niger* at pH 2-3 was citric acid, but when pH changed to 7, it was mainly oxalic acid (Lu et al., 2016). Therefore, in the process of fermentation, using the appropriate strategy to control pH can get the desired products.

Fermentation pH also has a great influence on the fermentation of *Streptomyces,* and some of them have specific response measures. The synthesis of different antibiotics requires different pH, and even the same antibiotic at different culture stages requires different pH to get the highest production. According to Chan's research (Chan et al., 2015), a two-stage pH control strategy by shifting the culture pH from 5.5 to 5.8 after 112 h of cultivation was proposed to improve the production of enduracidin and the productivity increased by 51.2%. For another example, ε-poly-L-lysine production was improved by 36.6% at most through seed stage development based on in-situ pH monitoring (Sun et al., 2015; Xu et al., 2015).

Environmental stress has been a crucial approach for the promotion of secondary metabolites in *Streptomyces*, and the environmental signal can improve the yield of secondary metabolites by starting the complex transduction system (Li et al., 2016). *Streptomyces* bacteria, based on different types of environmental factors, have many unique signal transduction mechanisms. In *Streptomyces coelicolor* A3(2), pH shock induced overexpression of regulatory and biosynthetic genes for actinorhodin production (Kim et al., 2007, 2008). Additionally, in *Streptomyces* sp. CK4412, expression of a putative acid-shock-induced gene SCO7832 stimulated tautomycetin production via pathway-specific regulatory genes' overexpression (Park et al., 2009). Besides, two-component regulatory system is also a very important way of regulation. It has been reported that two-component system DraR/DraK was involved in the regulation of antibiotic biosynthesis in *Streptomyces coelicolor* (Yeo et al., 2013). In *Streptomyces albulus* M-Z18, physiological responses to acidic pH for high ε-poly-L-lysine production were

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studied, and the genes related to transcription regulation, stress response protein, translocator, cell wall and cell membrane, secondary metabolite production, DNA and RNA metabolism, as well as ribosome subunit played major roles in the acidic pH response (Ren et al., 2015). However, there is little information about the effects of pH shock on Val-A production and gene expression in biosynthetic pathways, which are of great value to improve antibiotic fermentation and reduce the costs. Until recently, we have firstly employed alkaline pH shock to enhance Val-A production, and the preferable pH for pH shock was determined primarily at about 8.0 (Zhou et al., 2016).

This work aimed to investigate the effects of pH shock on the fermentation process of Val-A and to reveal the relationship between pH and the yield of Val-A. Based on the changing rules of production of Val-A synthesized by *S. hygroscopicus* 5008 in different pH shock conditions and the latest genomic research progress, the impact of pH on gene expression and its rules on physiological levels were studied to explore the mechanism that pH shock improved the yield of Val-A. First, pH was selected as an environmental stress to improve Val-A production in terms of metabolic activity analyses, and an efficient fermentation strategy with pH shock was constructed. Furthermore, the effects of pH shock on gene expression and cell microenvironment were successively explored. Finally, the mechanism of pH shock on Val-A production was comprehensively discussed. To the best of our knowledge, this is the first attempt to adopt an environmental stress on Val-A fermentation. It is also a good reference for the production of antibiotics by other actinomycetes.

2. Materials and methods

2.1. Microorganism and fermentation conditions

The used strain *Streptomyces hygroscopicus* 5008 (CGMCC 4.1026), a Val-A producer in industry, was preserved in China General Microbiological Culture Collection Center.

Sporulation agar medium contained the following: soybean meal 20 g/L, maltose 20 g/L, and agar 20 g/L. The medium was adjusted to pH 7.0 by using 2 mol/L NaOH, and sterilized in an autoclave for 20 min at 121 °C. Spores were collected and suspended in 20% (v/v) glycerol after cultivation for eight days at 37 °C and stored at −80 °C for future use.

Seed medium consisted of the following: corn powder 30 g/L, soybean meal 22 g/L, yeast extract 10 g/L, NaCl 2 g/L, and KH_2PO_4 0.8 g/L. Precultivation was performed in 250 mL shake flasks containing 50 mL seed medium at 37 °C and 220 rpm on a rotary shaker after inoculation with 50 μL of spore suspension (1.5×10^7 cfu/mL).

For the Val-A production, fermentation medium comprised the following: corn powder 100 g/L, soybean meal 25 g/L, yeast extract 5 g/L, NaCl 1 g/L, and KH_2PO_4 1.5 g/L. 5 mL of seed culture was inoculated to 250 mL flasks containing 50 mL of fermentation medium and fermented for 5 days at 37 °C and 220 rpm (Zhou, et al., 2012; Zhou and Zhong, 2015).

2.2. pH shock strategy for Val-A production

2.2.1. Different alkaline solution for pH shock

When the fermentation lasted for 24 h, the pH of the fermentation broth in different treatment groups was adjusted from natural pH 6.4 to pH 8.0 by adding 2 mol/L NaOH, 2 mol/L KOH, and 2 mol/L ammonia, respectively.

2.2.2. Different time points for pH shock

The fermentation of six different treatment groups lasted for 4 h, 8 h, 12 h, 16 h, 20 h and 24 h respectively and then the pH of the fermentation broth was adjusted to pH 8.0 by adding 2 mol/L NaOH for further culture.

2.2.3. Different treatment times

Three groups were as follows:

Once pH shock treatment: when the fermentation lasted for 20 h, the pH of the fermentation broth was adjusted to pH 8.0 by adding 2 mol/L NaOH.

Twice pH shock treatment: when the fermentation lasted for 20 h and 44 h, the pH of the fermentation broth was adjusted to pH 8.0 with 2 mol/L NaOH.

Thrice pH shock treatment: when the fermentation lasted for 20 h, 44 h and 68 h, the pH of the fermentation broth was adjusted to pH 8.0 with 2 mol/L NaOH.

2.3. Analyses of cell weight, residual carbon source and Val-A productivity

2 mL samples of fermentation broth were taken every day for the analysis of cell weight, residual carbon source and Val-A productivity. The samples were centrifuged at 12,000*g* for 5 min. The 0.5 mL of supernatant was extracted by the same volume of chloroform and then filtered by 0.22-μm hydrophilic filter. The filtered sample with Val-A was analyzed by high-performance liquid chromatography (HPLC) method (Iwasa et al., 1971). The normal dry cell weight method was not feasible as corn powder and soybean powder in seed medium were insoluble, so standard Bradford method was used to measure the intracellular protein to reflect the growth of the cell (Kieser et al., 2000). The total residual sugar as carbon source in medium was determined by standard phenol-sulfuric acid method (Liao et al., 2009).

2.4. Extracellular and intracellular pH measurements

Extracellular pH (pH_{ex}, the medium pH) was measured by the pH microelectrode meter (Unisense, Denmark).

Intracellular pH (pH_i) was measured with the pH sensitive dye 2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Beyotime Institute of Biotechnology, Jiangsu, China). A dual-excitation ratio method by means of multiscan spectrum (SpectraMax M5, CA, USA) was used for this study; excitation wavelengths were 440 nm and 488 nm, while emission wavelength was 535 nm. An *in vivo* calibration was performed with the ionophore nigericin (Sigma, Lezennes, France) and a set of high K^+ phosphate buffer of determined pH ranging from 6.5 to 8.5 (Corvini et al., 2000).

2.5. DNA microarray experiments and microarray data analysis

Total RNA was isolated and purified by RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, and the quality and quantity were checked using the Agilent Bioanalyzer 2100 system (Agilent, CA, USA). Total RNA was amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color. The Cy3-labeled cRNA was purified by RNeasy Mini Kit. Hybridization was performed in Hybridization Oven for 17 h. After hybridization, the slides were washed in Gene Expression Wash Buffer Kit, and the microarrays were scanned by Agilent Microarray Scanner G2565CA.

Using Feature Extraction Software 10.7, acquisition and quantification of array images were performed to normalize raw data with Quantile algorithm. Normalized expression ratios were calculated for each gene and tested for significance with the criteria $|\text{fold change}| > 2.0$ and $p < .05$. The change value with the lowest p value in a statistical analysis (t-test) was employed as the most reliable one. To represent the variation in triplicate measurements for each culture condition with one technical replicate, the coefficient of variation (CV) was estimated. Among the samples, at least 98% of the genome yielded detectable transcripts, and the average coefficient of variation did not exceed 0.15, as recommended by Agilent for the quality control.