Journal of Biotechnology 142 (2009) 271–274

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Short Communication

Effect of fermentation temperature on validamycin A production by *Streptomyces hygroscopicus* 5008

Yueqiao Liao[a], Zhen-Hua Wei[b], Linquan Bai[a], Zixin Deng[a], Jian-Jiang Zhong[a,*]

[a] *Key Laboratory of Microbial Metabolism, Ministry of Education, School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, 800 Dong-Chuan Road, Shanghai 200240, China*
[b] *State Key Laboratory of Bioreactor Engineering, School of Biotechnology, East China University of Science and Technology, Shanghai 200237, China*

ARTICLE INFO

Article history:
Received 21 November 2008
Received in revised form 8 April 2009
Accepted 25 April 2009

Keywords:
Validamycin production
Fermentation temperature
Streptomyces hygroscopicus
Gene expression
Enzyme activity

ABSTRACT

Validamycin A (VAL-A), produced by *Streptomyces hygroscopicus*, is an important anti-fungal agro-antibiotic. In this work, the effect of fermentation temperature on VAL-A biosynthesis by *S. hygroscopicus* 5008 was investigated between 28 °C and 42 °C, and an interesting threshold of temperature for VAL-A biosynthesis was found between 35 °C and 37 °C. At a relatively higher temperature, a much higher VAL-A productivity was obtained together with faster protein synthesis and sugar consumption. Transcriptional analysis of samples from early, middle and late stages of fermentation at various temperatures demonstrated that three operons, *valABC, valKLMN* and *valG*, for all eight necessary structure genes, were dramatically promoted when temperature reached the threshold. Activities of both glucose-6-phosphate dehydrogenase (G6PDH) of pentose-phosphate pathway and ValG of VAL-A biosynthesis were also enhanced at a higher cultivation temperature. The interesting temperature effect with a 2 °C threshold shift from 35 °C to 37 °C on the antibiotic biosynthesis was understood to be related to the gene transcriptional levels and key enzyme activities.

1. Introduction

Validamycin A (VAL-A), also called as jinggangmycin in China, is an anti-fungal aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* var. *limoneus* (Iwasa et al., 1970) and *S. hygroscopicus* var. *jinggangensis* 5008 (*S. hygroscopicus* 5008, hereafter) (Shen, 1988). Because of its high level of effectiveness as a fungicide as well as its lack of threat to human and animal health, VAL-A has been one of the most important and widely used plant protection reagents for the treatment of rice sheath blight disease in East Asia.

Cultivation temperature generally affects metabolite biosynthesis. However, there is still lack of information on structure genes expression and enzyme activity during fermentation of secondary metabolite production by industrially important species, and no detailed in-depth studies on the impact of temperature on VAL-A production were reported.

Recently the biosynthetic mechanism of VAL-A was gradually revealed, and a whole gene cluster responsible for its biosynthesis in *S. hygroscopicus* 5008 was cloned (Yu et al., 2005) and eight genes located in three operons, *valABC, valKLMN* and *valG*, were identified (Bai et al., 2006). The *valG* codes for a glycosyltransferase responsible for the final step of VAL-A biosynthesis modifying the validoxylamine structure with a glucose moiety to yield VAL-A (Bai et al., 2006).

In this work, the effect of temperature on VAL-A fermentation kinetics was investigated including the gene transcriptional level of three operons possessing all eight necessary genes required for the biosynthesis of VAL-A during the fermentation process. The activities of enzymes, representing abundance of precursor and efficiency of the last step of the VAL-A biosynthesis pathway, were also studied to gain insight into the mechanism of temperature effects on antibiotic production.

2. Material and methods

2.1. Sampling and fermentation analysis

Spores of *S. hygroscopicus* 5008 were stored, activated and cultivated as reported (Yu et al., 2005). Six duplicates were carried out for each temperature condition. To determine the intracellular protein concentration, mycelia were centrifuged, washed and then distributed to two Eppendorf tubes, with lysozyme added to one of the tubes to lysis the cells as reported (Kieser et al., 2000). Standard Bradford method was used to determine the protein released from the mycelia. The total residual sugar was determined by standard phenol-concentrated sulfuric acid method (Hodge and Hofreiter, 1962) and VAL-A production by HPLC (Yu et al., 2005).

* Corresponding author. Tel.: +86 21 34206968; fax: +86 21 34204831.
E-mail address: jjzhong@sjtu.edu.cn (J.-J. Zhong).

0168-1656/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2009.04.015

Table 1
Sequence of primer pairs for Quantitative RT-PCR.

Name	Primer sequence
valABC-F	ACGACTACACCACGCAGGAC
valAABC-R	ATGACGCACAGGTGGAACTC
valKLMN-F	CAGGTGGCTGCCTTGATG
valKLMN-R	CGGGAAGGTCTGTGCTGA
valG-F	CATCCCATGTGCCACTGCT
valG-R	CGACTCGGTATCCAAGGTCT
16sRNA-F	TATTGGGCGTAAAGAGC
16sRNA-R	CACCGCTACACCAGGAA

2.2. RNA and quantitative RT-PCR assay

The transcription levels of *valABC*, *valKLMN* and *valG* genes were determined by quantitative RT-PCR carried out on an Eppendorf Mastercycler Realplex^2 with One Step PrimeScript^{TM} RT-PCR Kit (Takara) according to manufacturer's procedure. Control reactions were set by adding RNA without reverse transcription instead of cDNA. After pre-denature at 95 °C for 5 min, amplification occurred in two steps: 15 s at 95 °C for denature and 30 s at 60 °C for both annealing and extension for 40 cycles. The sequences of primer pairs are listed in Table 1.

2.3. Determination of G6PDH and ValG enzyme activities

Activity of glucose-6-phosphate dehydrogenase (G6PDH) was analyzed as reported (Deutsch, 1983). The ValG activity assay was done as follows. The reaction mixture (100 μl) with 25 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 20 mM NH_4Cl, 15 mM UDP-glucose, 10 mM validoxylamine A, and 2 μl of crude enzyme solution was incubated for 3 h at 30 °C, 35 °C, 37 °C, and 42 °C, respectively, in keeping with their fermentation temperature. Subsequently, 100 μl of chloroform was added to stop the reaction and the protein was removed. Enzyme activity was determined by measuring the formation of VAL-A transformed from validoxylamine A by HPLC. Protein concentration of the crude enzyme solution was determined by standard Bradford method.

3. Results and discussion

3.1. Fermentation profiles under various cultivation temperatures

Due to the insoluble residues of corn and soybean powder in the liquid medium, total intracellular protein was determined to represent the growth of *S. hygroscopicus* 5008 instead of cell dry weight. The effect of fermentation temperature on the total intracellular protein was significant (Fig. 1b). At the early stage, the intracellular protein accumulation rate was faster at a higher fermentation temperature. However, at a lower temperature, a higher protein concentration was achieved at the middle stage of fermentation. At the late stage of fermentation, the protein concentration at a high fermentation temperature rapidly decreased, and the inhibitory effect of a high concentration of VAL-A on cell growth and a more rapid decrease of intracellular protein was confirmed in our Supplementary experiments (data not shown).

As shown in Fig. 1d, VAL-A was rapidly accumulated during the early and middle stages of fermentation at relatively higher fer-

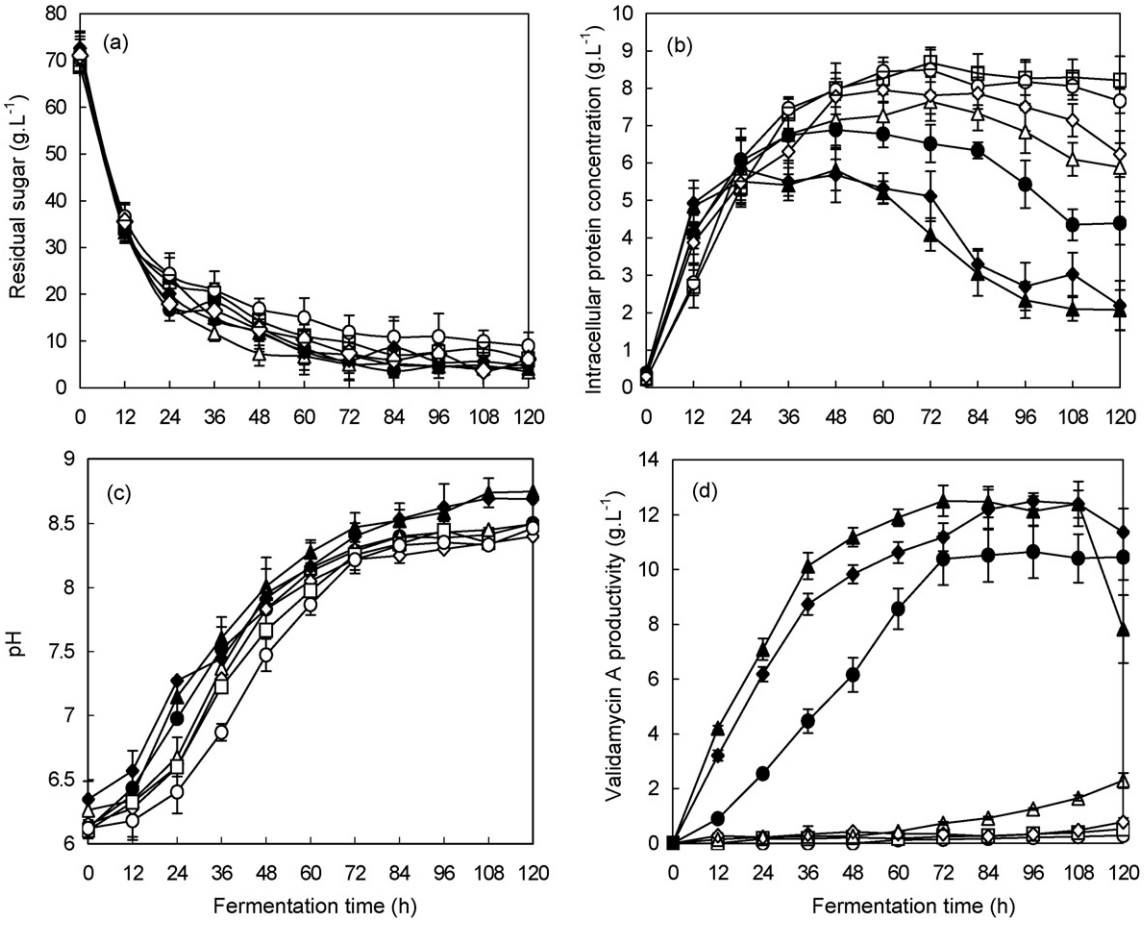

Fig. 1. Fermentation profile at various temperatures of 28 °C (○), 30 °C (□), 33 °C (□), 35 °C (□), 37 °C (●), 40 °C (□) and 42°C (▲), respectively. (a) Total residual sugar during fermentation; (b) concentration of intracellular protein; (c) variation of pH; (d) validamycin A production.

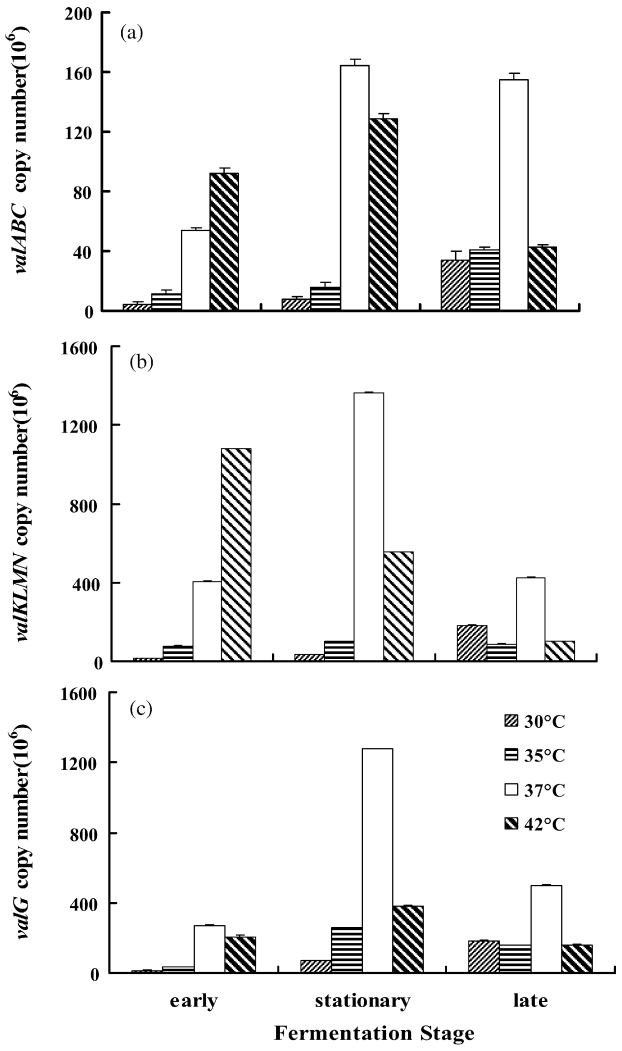

Fig. 2. Effect of fermentation temperature on the transcription level (copy number/μL) of genes *valABC* (a), *valKLMN* (b) and *valG* (c) of *S. hygroscopicus* 5008 cells at 30 °C, 35 °C, 37 °C and 42 °C, respectively. Each data point represents the mean ± S.D. from three independent samples.

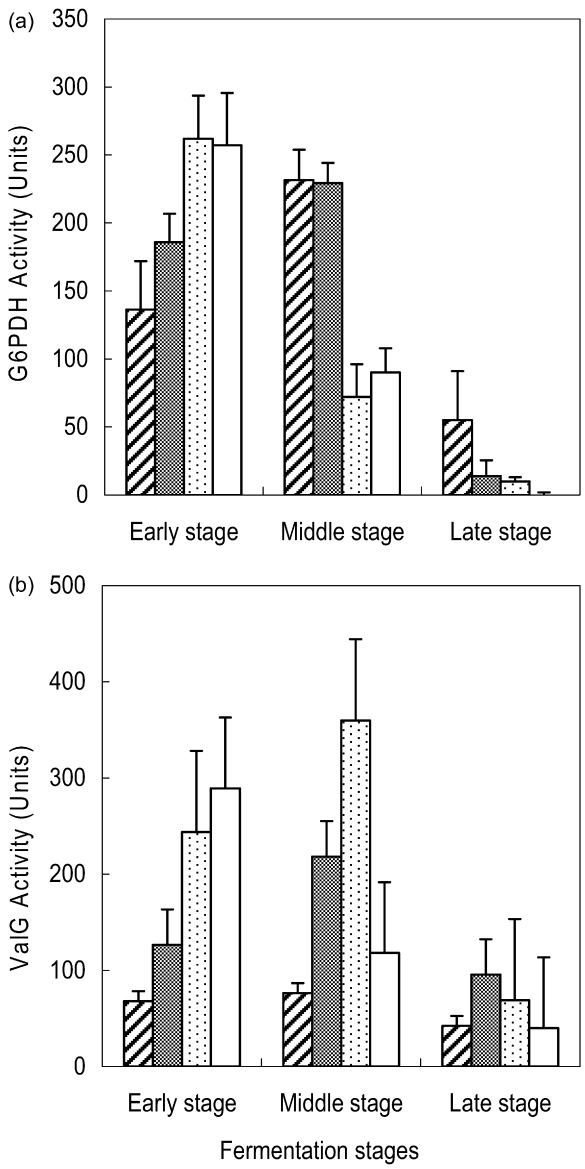

Fig. 3. Effect of fermentation temperature on activities of crude enzymes extracted from mycelia, which were collected from early, middle, and late stages of fermentation. Temperature: 30 °C (slash bar), 35 °C (dark bar), 37 °C (dot bar) and 42 °C (blank bar). (a) G6PDH activity; (b) valG glycosyltransferase activity.

mentation temperatures (37 °C, 40 °C and 42 °C), while the VAL-A production at lower temperatures (28 °C, 30 °C, 33 °C and 35 °C) was very low. Surprisingly, a significant difference of validamycin A production was observed by increasing just 2 °C from 35 °C to 37 °C, which was concluded to be a temperature threshold. When the temperature reached the threshold, certain cell signaling might be triggered that will cascade into gene expression, and ultimately result in a significant increase in the antibiotic biosynthesis.

At higher fermentation temperatures, the final residual sugar was lower with a slightly faster consumption rate in the early stage (Fig. 1a). Fermentation temperature also impacted pH. As temperature increased, the pH increased more rapidly during the early fermentation stage (Fig. 1c).

3.2. Response of gene transcription to fermentation temperature

For RNA and quantitative RT-PCR assay, mycelia from 10 ml of culture broth fermented at 30 °C, 35 °C, 37 °C and 42 °C were collected at the early stage (16 h), middle stage (48 h) and late stage of fermentation (120 h), respectively. Total RNA samples were extracted by Trizol (Sigma, USA) following the manufacturer's procedure and treated with DNase I (Promega) to remove the residual genomic DNA. After that, RNA samples were reverse-transcribed with M-MLV system (Promega) following the manufacturer's instructions for quantitative real-time PCR (qRT-PCR) assay. As shown in Fig. 2, as temperature increased, the transcriptional levels of all three operons *valABC*, *valKLMN* and *valG* were significantly affected. At the early and middle stages of fermentation, as temperature increased from 35 °C to 37 °C, the transcriptional levels of *valABC*, *valKLMN* and *valG* were dramatically promoted, which was consistent with the rapid increase of VAL-A production between 35 °C and 37 °C. The transcriptional levels of *valABC* and *valKLMN* at 42 °C were higher than those at lower temperatures, which was in agreement with the highest productivity (production rate) at 42 °C. The results showed that fermentation temperature had a significant effect on the transcription of genes responsible for VAL-A synthesis.

3.3. Effect of temperature on key enzyme activities

Temperature can play a regulatory role not only at transcriptional levels but also at post-transcriptional levels (Mitobe et al., 2008). G6PDH, the key enzyme in the regulation center of the pentose-phosphate pathway (PPP), provides the validamycin A

biosynthesis with the precursor sedoheptulose 7-phosphate (S7P). As shown in Fig. 3a, at the early stage of fermentation, a much more vigorous G6PDH activity for samples at 37 °C and 40 °C was observed compared to that from 30 °C to 35 °C. The high G6PDH activity suggested a high PPP metabolism rate providing the VAL-A biosynthetic pathway with plenty of S7P. The enzyme activity at each temperature generally dropped upon entering the middle stage of fermentation, and almost no G6PDH activity was detected at 42 °C after 5 days.

To examine the temperature effect on the enzyme level of VAL-A pathway, the enzyme activity of the final step of the VAL-A biosynthesis was monitored. As shown in Fig. 3b, the valG activity consistently increased along with the increase of temperature at the early stage of fermentation. At the middle stage of fermentation, the respective valG activity at 30 °C, 35 °C and 37 °C continued to increase, while its activity at 42 °C decreased. At the end of fermentation, the enzyme activity was low in all cases. ValG activity was higher at 42 °C than 37 °C at 16 h, so it had a high VAL-A production rate (higher slope level of VAL-A production) at 42 °C in the early stage. In the middle and late stages, VAL-A production rate at 42 °C was lower than that at 37 °C, where valG gene copy number and ValG activity for the former case were also lower than the latter. The higher VAL-A concentrations in the middle and late stages at 42 °C were caused by the accumulation in the early stage.

In conclusion, the results of this work indicated that a relatively high fermentation temperature brought about a vigorous PPP metabolism, a higher protein synthesis rate and a rapid increase of transcription level and consequently a high VAL-A productivity.

Acknowledgements

Financial support from the National High Technology R&D Program (863 project # 2006AA10A202) of the Ministry of Science & Technology of China, National Basic Research Program of China (973 Program, No. 2009CB118906), National Natural Science Foundation of China (No. 30821005), and the Shanghai Leading Academic Discipline Project (project # B203) was appreciated. We also thank Ms. Antonia Palkovic of Plant Department of UC Davis (USA) for her linguistic advice.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2009.04.015.

References

Bai, L.Q., Li, L., Xu, H., Minagawa, K., Yu, Y., Zhang, Y., Zhou, X., Deng, Z.X., 2006. Functional analysis of the validamycin biosynthetic gene cluster and engineered production of validoxylamine A. Chem. Biol. 13, 387–397.

Deutsch, J., 1983. Glucose-6-phosphate dehydrogenase. In: Methods of Enzymatic Analysis, 3rd ed. Verlag Chemie, pp. 191–197.

Hodge, J.E., Hofreiter, B.T., 1962. Determination of reducing sugars and carbohydrates. In: Whistler, R.L., Wolfrom, M.L. (Eds.), Methods in Carbohydrate Chemistry, vol. 1. Academic Press, New York, pp. 380–394.

Iwasa, T., Yamamoto, H., Shibata, M., 1970. Studies on validamycins, new antibiotics.II. Production and biological properties of validamycin A and B. J. Antibiot. (Tokyo) 24, 107–113.

Kieser, T., Bibb, M.J., Chater, K.F., Butter, M.J., Hopwood, D.A., 2000. Practical Streptomyces Genetics: A Laboratory Manual. John Innes Foundation, Norwich, United Kingdom, 342–453.

Mitobe, J., Morita-Ishihara, T., Ishihama, A., Watanabe, H., 2008. Involvement of RNA-binding Protein Hfq in the post-transcriptional regulation of invE gene expression in *Shigella sonnei*. J. Biol. Chem. 283, 5738–5747.

Shen, Y.C., 1988. Research and development of agro-antibiotic, validamycin. Chin. J. Antibiot. 6, 58–61.

Yu, Y., Bai, L.Q., Minagawa, K., Jian, X., Li, L., Li, J., Chen, S., Deng, Z.X., 2005. Gene cluster responsible for validamycin biosynthesis in *Streptomyces hygroscopicus* subsp. *jinggangensis* 5008. Appl. Environ. Microbiol. 71, 5066–5076.