Chapter 1

Genetic Engineering of *Acremonium chrysogenum*, the Cephalosporin C Producer

Youjia Hu

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/55471

1. Introduction

Acremonium chrysogenum, belongs to Filamentous fungi, is an important industrial microorganism. One of its metabolites, cephalosporin C (CPC), during fermentation is the major resource for production of 7-amino cephalosporanic acid (7-ACA), an important intermediate for the manufacture of many first-line anti-infectious cephalosporin-antibiotics, in industry.

Cephalosporins belong to the family of beta-lactam antibiotics. Comparing the first-discovered penicillin, cephalosporins have obvious advantages since they are more stable to penicillinase and are more effective to many penicillin-resistant strains. The incidence of adverse effects for cephalosporins is also lower than that for penicillins and other anti-infectious agents. Thus, cephalosporins are among the most-widely used anti-infectious drugs clinically. In China, the research on cephalosporins started from the 1960s, and cefoxitin was first developed in 1970. In the past 30 years, cephalosporin-antibiotics are one the most developed medicines on the domestic market. They accounts for more than 40% of the anti-infectious drug market share.

As the major resource for manufacturing 7-ACA, the production and cost of CPC is of the utmost importance in the cephalosporin-antibiotics market. The Ministry of Science and Technology of China has listed the fermentation of CPC as the major scientific and technical project in the past 30 years due to the continuous demand of strain improvement for the CPC-producing *Acremonium chrysogenum*.

Because of the limitation of traditional techniques on strain improvement for *A. chrysogenum*, along with the ubiquitous applications of molecular biology, genetic engineering has become a powerful tool to manipulate the antibiotic producing strain and to obtain a high-yielding mutant strain. This paper will summarize the most recent developments on genetic manipulation of *A. chrysogenum*.

2. Biosynthesis of CPC

The industrialization of CPC fermentation has been established tens of years ago with the breakthrough in key technologies including fermentation yield, fermentation regulation and preparation and purification. Nevertheless, there has been a lot of publications, recently on the improvement of CPC-producing strain by traditional methods, such as UV [1] or NTG [2]mutagenesis, and optimization of fermentation process [3], as well. However, most of the latest strain breeding techniques are at the molecular level, and the most important approach has been the research on the biosynthesis of the target metabolite.

The biosynthesis of CPC during the fermentation of *A. chrysogenum* has been well investigated. There are two gene clusters on the chromosome that are involved in the biosynthesis of CPC. The "early" cluster consists of *pcbAB-pcbC* and *cefD1-cefD2*. The *pcbAB-pcbC* encode two enzymes responsible for the first two steps in CPC biosynthesis [4]. While the *cefD1-cefD2* encode proteins that epimerize isopenicillin N (IPN) to penicillin N [5]. The "late" cluster consists of *cefEF* and *cefG* genes, which encode enzymes responsible for the last two steps [6].

The biosynthesis pathway of CPC is illustrated in figure 1. The ACV synthase, encoded by the *pcbAB* gene, condenses 3 precursors L-α-aminoadipic acid, L-cysteine, L-valine to the ACV tripeptide. The ACV is then cyclized into IPN by IPN synthase encoded by *pcbC* gene. The step from IPN to penicillin N is catalyzed by a two-component epimerization system encoded by *cefD1-cefD2*. The *cefEF* encodes a unique bi-functional enzyme, deacetyloxy-cephalosporin C (DAOC) synthase-hydroxylase which successively transforms penicillin N into DAOC and deacetyl-cephalosporin C (DAC). The last step in CPC biosynthesis is catalyzed by a DAC-acetyltransferase (DAC-AT) which is encoded by *cefG*. The crystal structure of DAC-AT has been published [7]. It has been shown that DAC-AT belongs to α/β hydrolase family according to the formation of DAC-enzyme complex [7]. Among these, *pcbAB*, *cefEF* and *cefG* were considered as the rate-limiting steps in CPC biosynthesis [8].

In recent years, some other regulatory proteins, which have been found to be important in CPC biosynthesis, as well as their coding genes have been discovered. For example, Ac*veA*, a homologue of *veA* from *Aspergillus*, regulates the transcription of all 6 major CPC biosynthesis genes including *pcbAB*, *pcbC*, *cefD1*, *cefD2*, *cefEF* and *cefG*. Disruption of Ac*veA* leads to a dramatic reduction of CPC yield.

A *cefP* gene located in the early cluster of CPC biosynthesis cluster has just been characterized. This gene encodes a transmembrane protein anchored in a peroxisome. It regulates the epimerization of IPN to penicillin N catalyzed by CefD1-CefD2 two-component enzyme complex in peroxisome. The *cefP* disruptant accumulated IPN and lost CPC production [10]. To compensate for the disruption of *cefP*, both *cefP* and *cefR* need to be introduced simultaneously. The CefR is the repressor of CefT, and stimulates the transcription of *cefEF*. A mutant *A. chrysogenum* without *cefR* showed delayed transcription of *cefEF* and accumulation of penicillin N resulted in reduction of CPC yield [11].

A *cefM* gene was also found downstream of *cefD1*. Disruption of *cefM* accumulates penicillin N with no CPC production at all [12]. It is suggested that CefM may be involved in the

http://dx.doi.org/10.5772/55471

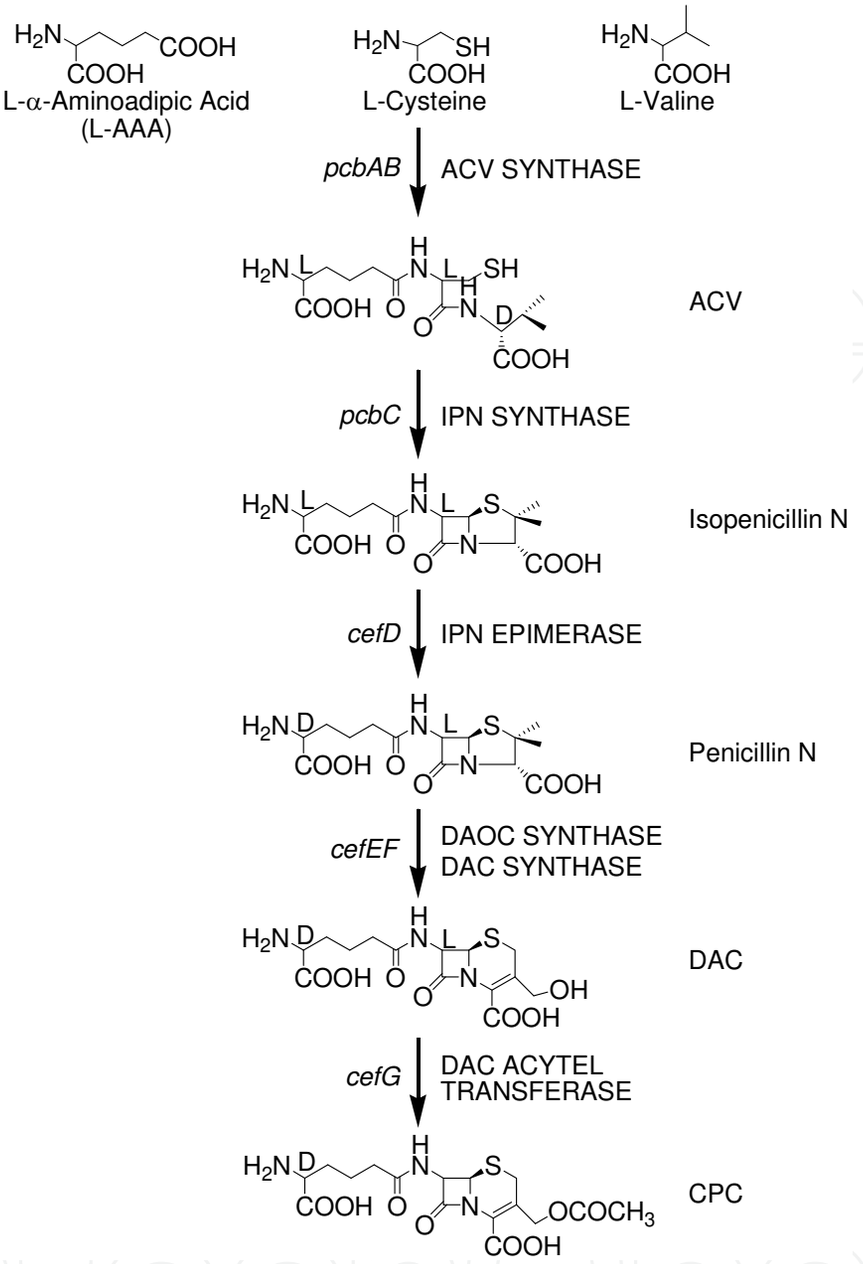

Figure 1. The biosynthesis pathway of CPC

translocation of penicillin N from the peroxisome to the cytoplasm. Without *cefM*, cells are unable to transport penicillin N which gets epimerized in peroxisome into cytoplasm, from where CPC is synthesized.

3. Techniques for molecular breeding

Acremonium chrysogenum belongs to the family of Filamentous fungi. The techniques for genetic breeding are somehow difficult to manipulate due to its complicated structure of the cell wall

and the special life cycle. Our laboratory has started the molecular breeding of *A. chrysogenum* at a relatively early stage based on some published results from host, transformation, homologuous recombination and selectable marker of *A. chrysogenum* [13, 14].

To introduce exogenous DNA into *A. chrysogenum*, a traditional PEG-mediated protoplast transformation method is commonly used [15]. Since we are focusing on high-yield, or industrial strains, which usually have a stronger restriction-modification system than type strain, the traditional transformation method is not efficient enough for foreign gene introduction.

Agrobacterium tumefaciens mediated transformation has been widely used in plant genetic engineering, and in some of the Filamentous fungi including *Penicillium chrysogenum* and *Aspergillus nidulans* as well [17]. We have developed an adapted *A. tumefaciens* mediated transformation protocol for *A. chrysogenum*, which has a higher transformation efficiency than the PEG- mediated method [17], and more importantantly, this protocol can also be applied in *A. chrysogenum* high-yield strain. This is the first report of *A. tumefaciens* mediated *A. chrysogenum* transformation in the world.

Considering the significant improvement after introduction of *vgb*, VHb protein coding gene, we use error-prone PCR together with DNA shuffling to artificially evolve the *vgb* gene *in vitro*. After primary and secondary screening, a higher active mutant protein was obtained. *E. coli* bearing this mutant VHb produce 50% more biomass than its counterpart bearing the original VHb under limited oxygen environment [18].

A lot of basic research was done to facilitate the genomic DNA extraction [19] and endogenous promoter capture [20] from the chromosome of *A. chrysogenum*. A notable progress is the cloning of *pcbAB-pcbC* bi-directional promoter from the chromosome of *A. chrysogenum* [21]. This allows for the convenient manipulation of *A. chrysogenum* by introduction of multiple genes.

The last step in CPC biosynthesis, DAC transformed into CPC catalyzed by DAC acetyltransferase, was further investigated, as many reports have demonstrated that this is the rate-limiting step while DAC acetyltransferase coding gene, *cefG* has a low transcription rate *in vivo*. Our study showed that recombinant expressed DAC acetyltransferase can transform DAC into CPC *in vitro* in the presence of acetyl CoA [22]. The enzymological and kinetic study of the recombinant DAC acetyltransferase help us better understand the catalytic mechanism of the enzyme and make it possible to improve its enzymatic activity *in vivo* [23].

4. Molecular breeding of *Acremonium chrysogenum*

Among the three rate-limiting enzymes, PcbAB is relatively difficult to manipulate due to its larger coding gene. Thus, researchers focus on *cefEF* and *cefG* for molecular breeding of *A. chrysogenum*. Besides, extra copy numbers of *cefT* could increase the yield of CPC in the mutant *A. chrysogenum* [24]. And, overexpression of *cefP* and *cefR* in *A. chrysogenum* can decrease the accumulation of penicillin N and promote the yield of CPC by about 50% [11].

http://dx.doi.org/10.5772/55471

The fermentation process of *A. chrysogenum* is an extreme oxygen-consumption procedure. All the rate-limiting enzymes are oxygen-requiring enzymes. The *Vitreoscilla* Hemoglubin (VHb) is very attractive since it is capable of oxygen transmission in oxygen-limiting environments. A recombinant strain bearing VHb can significantly improve the usage of oxygen during the fermentation process and increase the product yield, which has been proven in *Aspergillus* [25]. Introduction of *vgb*, the coding gene for VHb, into *A. chrysogenum* can also maintain a higher specific growth rate and specific production rate resulting in a 4-5 fold higher yield of the mutant strain [26]. Actually, there are many industrial *A. chrysogenum* strains that express a recombinant *vgb*.

The earliest report on genetic modification for *A. chrysogenum* was published in 1989, when researchers from Eli Lilly Co. introduced an extra copy of *cefEF-cefG* fragment into *A. chrysogenum* which resulted in a 15%-40% higher producing mutant strain [27]. This was the first evidence that molecular breeding could be a powerful tool in strain improvement of *A. chrysogenum*.

Although controlled by the same bi-directional promoter, the transcription levels of *cefEF* and *cefG* showed a huge difference as shown by RT-PCR. The transcription of *cefG* is much lower than that of *cefEF*. This leads to the accumulation of DAC in the metabolites since they can not be efficiently transformed into CPC. As a matter of fact, CPC/DAC ratio is a quality control parameter in the industrial production of CPC fermentation. Thus, the introduction of extra copy numbers of *cefG* produced an engineering strain whose CPC yield is 3 folds higher than the parental strain [28].

There is another report on the introduction of *cefT* into *A. chrysogenum*,where the resulting mutant doubled the CPC yield [29]. This could be attributed to the enhancement of CefT, the efflux pump protein, so that the feedback inhibition *in vivo* triggered by the fermentation product was attenuated, resulting in a higher product yield.

Using molecular breeding technology, some CPC derivatives can be directly produced by engineering *A. chrysogenum* fermentation. For example, by disruption of *cefEF* and introduction of *cefE* originating from *Streptomyces clavuligerus*, a novel DAOC producing strain was obtained, which if followed by two enzymatic transformations, the industrially important 7-ADCA can be produced [30]. By introduction of the coding genes simultaneously into *A. chrysogenum* for the two enzymes used the industrial production of 7-ACA by immobilized enzymatic transformation, the engineering strain can produce 7-ACA by fermentation [31].

Besides the introduction of exogenous genes, disruption and/or silencing of the endogenous genes is also a common strategy for genetic breeding of a certain strain. The recently developed RNA interference (RNAi) technique can be used as an alternative to silence the transcription of target genes instead of homologous recombination. RNAi in *A. chrysogenum* was first published in 2007 [32]. The latest report was silencing of *pcbC* gene in *Penicillium chrysogenum* and *cefEF* gene in *A. chrysogenum* by RNAi [33]. These reports demonstrated the feasibility of RNAi technique in Filamentous fungi.

There is another interesting research for the molecular breeding of *A. chrysogenum* in a different idea. As we mentioned before, CefD1-CefD2 is a two-component enzyme complex that

transforms IPN into penicillin N by an epimerization system located in the peroxisome. *cefD1-cefD2* block mutant lacking this epimerization system accumulated a large amount of IPN to more than 650μg/mL, almost the total relative CPC yield. With this mutant, the unstable IPN, which has never been purified before, could be now be purified by several steps using chromatography [34]. Characterization of its half-life and stability under a variety conditions can greatly help in the investigation of IPN.

It is worth noting that all of the above genetic breeding reports were on the background of an *A. chrysogenum* type strain C10, whose CPC yield is only 1 mg/mL, far less than the industrial production level. Although some good achievements were obtained in improvement of *A. chrysogenum* fermentation and modification of metabolic products, those achievements are still far away from application in industry.

5. Industrialization research on molecular breeding of *A. chrysogenum*

Our research is focused on the molecular breeding of *A. chrysogenum* high-yield and/or industrial strains. We introduced different combinations of *cefG*/*cefEF*/*cefT*/*vgb* genes into CPC high-producing strain and found that an extra copy of *cefG* has a significant positive effect on CPC fermentation level. Since random integration occurring in *A. chrysogenum*, different transformants with *cefG* introduction showed different elevated levels, with some at 100%. An extra copy of *vgb* gene also displayed a significant improvement up to 30% more of the CPC yield. Meanwhile, introduction of *cefEF* and *cefT* has no obvious effect on CPC production in the high-yield strain [35]. This revealed the apparent discrepancy between the genetic background of the type strain and the high-yield strain, and also suggested that endogenous *cefEF* and *cefT* may already achieve high bioactivity after several rounds of mutagenesis breeding that a high-yield strain usually undertaken.

We then applied this achievement to a CPC industrial strain. Although we didn't obtain a mutant that doubled the CPC yield, we did obtain an engineering strain whose CPC yield was increased by 20%, which has a promising industrialized potential.

We also tried the RNAi technique in the high-yield strain. A plasmid vector containing *cefG* double strain RNA transcription unit was constructed and transformed into high-yield *A. chrysogenum*. The *cefG* transcription level in the transformants was measured by quantitative RT-PCR. Two mutant strains were found to have a decreasing *cefG* transcription level of up to 80%. Their CPC yield was also found to decrease by 34.6% and 28.8%, respectively [36]. This result demonstrated the feasibility of RNAi application in high-yield *A. chrysogenum* and possible, industrial strain. Moreover, this is important for metabolic pathway reconstitution and novel CPC derivatives fermentation in *A. chrysogenum*.

The fermentation product of *A. chrysogenum*, CPC, is the major resource for industrial manufacturing of 7-ACA, the important intermediate of a large variety of cephalosporins antibiotics. A common producing route of 7-ACA is the chemical semi-biosynthesis. To date, the more environmental-friendly biotransformation has been widely used in industry. Although two

step transformation dominates in the market [37], research on one step transformation from CPC to 7-ACA is still hot. However, the substrate specificity of CPC acylase still remains unsolved [38].

Whether two-step or one-step, fermentation of CPC is the prerequisite followed by enzymatic biotransformation *in vitro*. We are thinking of introducing CPC acylase gene into *A. chrysogenum* to construct the engineering strain that can produce 7-ACA directly by fermentation, a breakthrough in the production of 7-ACA.

A CPC acylase gene was designed according to the codon bias of *A. chrysogenum* and introduced into an industrial strain. Our result showed that this CPC acylase was expressed in *A. chrysogenum* with bioactivity. The recombinant acylase can transform the original product CPC into 7-ACA *in vivo*, makes the engineering strain capable of direct fermentation of 7-ACA. Based on enzymological profiles of CPC acylase *in vitro*, we performed a preliminary optimization of medium composition and culture condition and the CPC yield was increased significantly with as least 30% of the CPC fermented being transformed into 7-ACA [40]. We believe this *in vivo* conversion can be more effective if a more powerful transcription cassette and more copy number can be introduced, with the incorporation of traditional breeding technology, and finally, bring this technique to industry.

6. Perspectives

As a novel tool for strain improvement, genome shuffling is of widespread concern in the field of industrial microbiology since it was first reported [41]. This has been applied in Bacteria and Streptomyces, and the yield of a lot of metabolites got a substantial increase by genome shuffling. However, genome shuffling in Filamentous fungi is rare, maybe due to the undeveloped genetic manipulation system. In 2009, the cellulase production in *Penicillin decumbens* was reported to be increased by 40% with the help of genome shuffling [42]. But this achievement resulted largely in primary metabolites. As we all know, the regulation of secondary metabolites, as well as the genetic manipulation of *A. chrysogenum* is much more complicated. Since the exogenous genes were randomly integrated in the chromosome of *A. chrysogenum*, we suggest that genome shuffling can effectively improve the fermentation of the strains based on the established genetic techniques in our laboratory.

The biosynthesis of CPC in *A. chrysogenum* has been investigated thoroughly. However, the mechanism of its regulation as well as the biosynthesis of precursors in primary metabolism is still unclear [43]. The full sequence of *A. chrysogenum* is yet to be completed, although there are more than 10 species belonging to the Filamentous fungi that have already been sequenced [44]. To better understand the genetic basis of *A. chrysogenum*, we realize that comparative proteomics could be used to study the molecular breeding without the genomic sequence of *A. chrysogenum*. By identifying those different expressed proteins during CPC fermentation, fermentation may be proposed based on the popular theory of metabolic engineering and system biology [45].

Besides its use in studying the mechanism of disease development, the application of comparative proteomics in antibiotic-producing microorganisms also showed promise. For instance, 345 different proteins were identified as critical during the conversion from primary to secondary metabolism in *Streptomyces coelicolor* [46]. Another example is research on *Penicillin chrysogenum* where 950 proteins involved in precursor biosynthesis, stress response and pentose phosphate pathway were found to be related to the fermentation yield in 3 penicillin-producing strains [47].

Thus, we believe that the molecular breeding of *A. chrysogenum* should consist of genome shuffling, optimization of secondary metabolism, improvement of precursor biosynthesis and energy metabolism as well. Although there are still big effects need to be put in the basic and practical research of *A. chrysogenum*, the molecular bred engineering strains will play an important role in the industrial production of CPC and its derivatives.

Author details

Youjia Hu

Address all correspondence to: bebydou@hotmail.com

Shanghai Institute of Pharmaceutical Industry, Department of Biopharmaceuticals, Shanghai, China

References

[1] Ellaiah P, Adinarayana K, Chand GM, Subramanyam GS, Srinivasulu B. Strain improvement studies for cephalosporin C production by *Cephalosporium acremonium. Pharmazie*, 2002, 57(7): 489–490.

[2] Ellaiah P, Kumar JP, Saisha V, Sumitra JJ, Vaishali P. Strain improvement studies on production of cephalosporin C from *Acremonium chrysogenum* ATCC 48272. *Hindustan Antibiot Bull*, 2003, 45-46(1-4): 11–5.

[3] Lee MS, Lim JS, Kim CH, Oh KK, Yang DR, Kim SW. Enhancement of cephalosporin C production by cultivation of *Cephalosporium acremonium* M25 using a mixture of inocula. *Lett Appl Microbiol*, 2001. 32(6): 402–406.

[4] Gutierrez S, Diez B, Montenegro E, Martin JF. Characterization of the *Cephalosporium acremonium pcbAB* gene encoding alpha-aminoadipyl-cysteinyl-valine synthetase, a large multidomain peptide synthetase: linkage to the *pcbC* gene as a cluster of early cephalosporin biosynthetic genes and evidence of multiple functional domains. *J Bacteriol*, 1991, 173(7): 2354–2365.

[5] Martin JF, Ullan RV, Casqueiro J. Novel genes involved in cephalosporin biosynthesis: the three-component isopenicillin N epimerase system. *Adv Biochem Eng Biotechnol*, 2004, 88: 91–109.

[6] Gutierrez S, Velasco J, Fernandez FJ, Martin JF. The *cefG* gene of *Cephalosporium acremonium* is linked to the *cefEF* gene and encodes a deacetylcephalosporin C acetyltransferase closely related to homoserine O-acetyltransferase. *J Bacteriol*, 1992, 174(9): 3056–3064.

[7] Lejon S, Ellis J, Valegard K. The last step in cephalosporin C formation revealed: crystal structures of deacetylcephalosporin C acetyltransferase from *Acremonium chrysogenum* in complexes with reaction intermediates. *J Mol Biol*, 2008, 377(3): 935–944.

[8] Brakhage AA, Thon M, Sprote P, Scharf DH, Al-Abdallah Q, Wolke SM, Hortschansky P. Aspects on evolution of fungal beta-lactam biosynthesis gene clusters and recruitment of trans-acting factors. *Phytochemistry*, 2009, 70(15-16): 1801–1811.

[9] Dreyer J, Eichhorn H, Friedlin E, Kurnsteiner H, Kuck U. A homologue of the *Aspergillus velvet* gene regulates both cephalosporin C biosynthesis and hyphal fragmentation in *Acremonium chrysogenum. Appl Environ Microbiol*, 2007, 73(10): 3412–3422.

[10] Ullan RV, Teijeira F, Guerra SM, Vaca I, Martin JF. Characterization of a novel peroxisome membrane protein essential for conversion of isopenicillin N into cephalosporin C. *Biochem J*, 2010, 432(2): 227–236.

[11] Teijeira F, Ullan RV, Fernandez-Lafuente R, Martin JF. CefR modulates transporters of beta-lactam intermediates preventing the loss of penicillins to the broth and increases cephalosporin production in *Acremonium chrysogenum. Metab Eng*, 2011, 13(5): 532-543.

[12] Teijeira F, Ullan RV, Guerra SM, Garcia-Estrada C, Vaca I, Martin JF. The transporter CefM involved in translocation of biosynthetic intermediates is essential for cephalosporin production. *Biochem J*, 2009, 418(1): 113–124.

[13] Kuck U, Hoff B. New tools for the genetic manipulation of filamentous fungi. *Appl Microbiol Biotechnol*, 2010, 86(1): 51–62.

[14] Meyer, V. Genetic engineering of filamentous fungi--progress, obstacles and future trends. *Biotechnol Adv*, 2008, 26(2): 177–185.

[15] Skatrud PL, Queener SW, Carr LG, Fisher DL. Efficient integrative transformation of *Cephalosporium acremonium. Curr Genet*, 1987, 12(5): 337–348.

[16] Groot MJ, Bundock P, Hooykaas PJ, Beijersbergen AG. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nat Biotechnol*. 1998, 16: 839-842

[17] Xu W, Zhu C, Zhu B. An efficient and stable method for the transformation of heterogeneous genes into *Cephalosporium acremonium* mediated by *Agrobacterium tumefaciens*. *J Microbiol Biotechnol*, 2005, 15(4): 683–688.

[18] Yuan N, Hu Y, Zhu C, Zhu B. DNA shuffling of *Vitreoscilla* Hemoglobin. *China Biotechnology*, 2006, 26(11):14-19.

[19] Xu W, Zhu C, Zhu B, Yao X. A new method for isolation of chromosomal DNA from filamentous fungus *Cephalosporium acremonium*. *Journal of Shenyang Pharmaceutical University*. 2004, 21(3): 226–229.

[20] Zhang P, Zhu C, Zhu B, Zhao W. A convenient method to select DNA fragments of *Cephalosporium acremonium* with promoter function. *Microbiology*. 2004, 31(3): 97–100.

[21] Zhang P, Zhu C, Zhu B. Cloning of bidirectional *pcbAB-pcbC* promoter region from *Cephalosporium acremonium* and its application. *Acta Microbiologica Sinica.* 2004. 44(2): 255–257.

[22] Chen D, Yuan N, Hu Y, Zhu C, Zhao W, Zhu B. Cloning, expression and activity analysis of DAC-acetyltransferase gene from *Acremonium chrysogenum*. *Chinese Journal of Antibiotics*, 2006. 31(7): 395–399.

[23] Chen D, Hu Y, Zhu C, Zhu B. Optimization on soluble expression of a recombinant DAC-acetyltransferase from *Acremonium chrysogenum* and its enzyme kinetics. *Chinese Journal of Pharmaceuticals*, 2007, 38(9): 625–628.

[24] Nijland JG, Kovalchuk A, van den Berg MA, Bovenberg RA, Driessen AJ. Expression of the transporter encoded by the *cefT* gene of *Acremonium chrysogenum* increases cephalosporin production in *Penicillium chrysogenum. Fungal Genet Biol*, 2008, 45(10): 1415–1421.

[25] Lin YH, Li YF, Huang MC, Tsai YC. Intracellular expression of *Vitreoscilla* hemoglobin in *Aspergillus terreus* to alleviate the effect of a short break in aeration during culture. *Biotechnol Lett*, 2004, 26(13): 1067–1072.

[26] DeModena JA, Gutierrez S, Velasco J, Fernandez FJ, Fachini RA, Galazzo JL, Hughes DE, Martin JF. The production of cephalosporin C by *Acremonium chrysogenum* is improved by the intracellular expression of a bacterial hemoglobin. *Bio/Technology*, 1993. 11(8): 926–929.

[27] Skatrud P, Tietz A, Ingolia T, Cantwell C, Fisher D, Chapman J, Queener S. Use of recombinant DNA to improve production of cephalosporin C by *Cephalosporium acremonium*. *Bio/Technology*, 1989, 7(5): 477–485.

[28] Gutierrez S, Velasco J, Marcos AT, Fernandez FJ, Fierro F, Barredo JL, Diez B, Martin JF. Expression of the *cefG* gene is limiting for cephalosporin biosynthesis in *Acremonium chrysogenum. Appl Microbiol Biotechnol*, 1997, 48(5): 606–614.

[29] Ullan RV, Liu G, Casqueiro J, Gutierrez S, Banuelos O, Martin JF. The *cefT* gene of *Acremonium chrysogenum* C10 encodes a putative multidrug efflux pump protein that

significantly increases cephalosporin C production. *Mol Genet Genomics*, 2002, 267(5): 673–683.

[30] Velasco J, Luis Adrio J, Angel Moreno M, Diez B, Soler G, Barredo JL. Environmentally safe production of 7-aminodeacetoxycephalosporanic acid (7-ADCA) using recombinant strains of *Acremonium chrysogenum. Nat Biotechnol*, 2000, 18(8): 857–861.

[31] Isogai T, Fukagawa M, Aramori I, Iwami M, Kojo H, Ono T, Ueda Y, Kohsaka M, Imanaka H. Construction of a 7-aminocephalosporanic acid (7ACA) biosynthetic operon and direct production of 7ACA in *Acremonium chrysogenum. Biotechnology (N Y)*, 1991, 9(2): 188–191.

[32] Janus D, Hoff B, Hofmann E, Kuck U. An efficient fungal RNA-silencing system using the DsRed reporter gene. *Appl Environ Microbiol*, 2007, 73(3): 962–970.

[33] Ullan RV, Godio RP, Teijeira F, Vaca I, Garcia-Estrada C, Feltrer R, Kosalkova K, Martin JF. RNA-silencing in *Penicillium chrysogenum* and *Acremonium chrysogenum*: validation studies using beta-lactam genes expression. *J Microbiol Methods*, 2008, 75(2): 209–218.

[34] Vaca I, Casqueiro J, Ullan RV, Rumbero A, Chavez R, Martin JF. A preparative method for the purification of isopenicillin N from genetically blocked *Acremonium chrysogenum* strain TD189: studies on the degradation kenetics and storage conditions. *J Antibiot*, 2011, 64(6): 447-451.

[35] Liu Y, Gong G, Xie L, Yuan N, Zhu C, Zhu B, Hu Y. Improvement of cephalosporin C production by recombinant DNA integration in *Acremonium chrysogenum. Mol Biotechnol*, 2010, 44(2): 101–109.

[36] Gong G, Liu Y, Hu Y, Zhu C, Zhu B. Down-regulation of *cefG* gene transcription in an industrial strain of *Acremonium chrysogenum* by RNA interference. *Biotechnology Bulletin*. 2010, 10: 193–197.

[37] Conlon HD, Baqai J, Baker K, Shen YQ, Wong BL, Noiles R, Rausch CW. Two-step immobilized enzyme conversion of cephalosporin C to 7-aminocephalosporanic acid. *Biotechnol Bioeng*, 1995. 46(6): 510–513.

[38] Sonawane VC. Enzymatic modifications of cephalosporins by cephalosporin acylase and other enzymes. *Crit Rev Biotechnol*, 2006, 26(2): 95–120.

[39] Liu Y, Gong G, Hu Y, Zhu C, Zhu B. Expression of cephalosporin C acylase in *Acremonium chrysogenum*. *Chinese Journal of Pharmaceuticals*, 2009, 40(12): 902-906.

[40] Liu Y, Gong G, Zhu C, Zhu B, Hu Y. Environmentally safe production of 7-ACA by recombinant *Acremonium chrysogenum. Curr Microbiol*, 2010, 61(6): 609–614.

[41] Zhang YX, Perry K, Vinci VA, Powell K, Stemmer WP, del Cardayre SB. Genome shuffling leads to rapid phenotypic improvement in bacteria. *Nature*, 2002, 415(6872): 644–646.

[42] Cheng Y, Song X, Qin Y, Qu Y. Genome shuffling improves production of cellulase by *Penicillium decumbens* JU-A10. *J Appl Microbiol*, 2009, 107(6): 1837–1846.

[43] Schmitt EK, Hoff B, Kuck U. Regulation of cephalosporin biosynthesis. *Adv Biochem Eng Biotechnol*, 2004, 88: 1–43.

[44] Jones MG. The first filamentous fungal genome sequences: *Aspergillus* leads the way for essential everyday resources or dusty museum specimens? *Microbiology*, 2007, 153(Pt 1): 1–6.

[45] Thykaer J, Nielsen J. Metabolic engineering of beta-lactam production. *Metab Eng*, 2003, 5(1): 56–69.

[46] Manteca A, Sanchez J, Jung HR, Schwammle V, Jensen ON. Quantitative proteomics analysis of *Streptomyces coelicolor* development demonstrates that onset of secondary metabolism coincides with hypha differentiation. *Mol Cell Proteomics*, 2010, 9(7): 1423–1436.

[47] Jami MS, Barreiro C, Garcia-Estrada C, Martin JF. Proteome analysis of the penicillin producer *Penicillium chrysogenum*: characterization of protein changes during the industrial strain improvement. *Mol Cell Proteomics*, 2010, 9(6): 1182–1198.