Deacetoxycephalosporin C synthase (expandase): Research progress and application potential

Xiaofan Niu, Jian Zhang, Xianli Xue, Depei Wang, Lin Wang, Qiang Gao

Abstract

Cephalosporins play an indispensable role against bacterial infections. Deacetoxycephalosporin C synthase (DAOCS), also called expandase, is a key enzyme in cephalosporin biosynthesis that epoxides penicillin to form the hexavalent thiazide ring of cephalosporin. DAOCS in fungus Acremonium chrysogenum was identified as a bifunctional enzyme with both ring expansion and hydroxylation, whereas two separate enzymes in bacteria catalyze these two reactions. In this review, we briefly summarize its source and function, improvement of the conversion rate of penicillin to deacetoxycephalosporin C through enzyme modification, crystallography features, the prediction of the active site, and application perspective.

1. Introduction

Penicillin came into people’s sight in 1928 and save millions of peoples’ lives from bacterial infections in the later period. However, beneath its ever supreme antibacterial glory, penicillin also encounters the severe development of bacterial resistance due to its massive use, furthermore, penicillin itself has the disadvantages of narrow antibacterial spectrum and easy to cause anaphylactic shock. At this expecting the severe development of bacterial resistance due to its massive use, beneath its ever supreme antibacterial glory, penicillin also encounters peoples’ sight in 1928 and save millions of peoples’ lives from bacterial infections in the later period. However, beneath its ever supreme antibacterial glory, penicillin also encounters the severe development of bacterial resistance due to its massive use, furthermore, penicillin itself has the disadvantages of narrow antibacterial spectrum and easy to cause anaphylactic shock. At this expecting time, a new kind of antibiotics came into being—cephalosporin. In 1945, Italian scientists discovered a spectral antibiotic with strong inhibition against both Gram-positive and Gram-negative bacteria when doing a drug study on the offshore sewage of Sardinia, Italy. In 1955, the first cephalosporin antibiotic, cephalosporin C, was isolated and identified, and is rapidly expanding as an antibacterial chemotherapy drug in a family of cephalosporins, which was widely prepared and used in clinic. In 1976, Kohsaka and Demain first reported the ring expansion reaction of penicillin N to deacetylcephalosporin (DAOCS) in the cell-free reaction system of Cephalosporium aeremnonium [1]. In 1983, the DAOCS in C. aeremnonium was firstly purified by Kupta et al. [2]. In 1994, the cephalosporin sales volume exceeded penicillin, and the number of its varieties ranked the first among all kinds of antibiotics.

β-Lactam antibiotics have been widely used to treat various bacterial infections for over half a century. This kind of antibiotics has the advantages of strong bactericidal activity, low toxicity, broad indications and good clinical efficacy [3]. To date, penicillin and cephalosporin are the most characteristic β-lactam antibiotics. Both act as inhibitors of bacterial peptidoglycan biosynthesis, the advantage of cephalosporin over penicillin lies in their resistance to penicillin β-lactamase. Although cephalosporin has overcome some of the drawbacks of penicillin, there are still some problems to be solved in its production process.

At present, a third of cephalosporins are synthesized from penicillin in industry [4], the industrial production of cephalosporin includes chemical synthesis and potential enzymatic synthesis. The chemical synthesis process is complicated and accompanied with the production of by-products. Enzymatic synthesis has more obvious advantages and is an effective method for the production of cephalosporin. The key enzyme in enzymatic synthesis is deacetoxycephalosporin C synthase (DAOCS) (EC 1.14.20.1), but its natural substrate, penicillin N, is not easily obtained artificially. Penicillin G is the earliest clinical antibiotic due to its strong bactericidal force, easy industrial availability, low toxicity, and environmentally friendly [5]. Therefore, in order to facilitate the acquisition of cephalosporin, the research greatly focuses on
improving the conversion rate of penicillin G to DAOCS.

2. The origin and function of DAOCS

After decades of efforts by many researchers, 9 DAOCS genes have been cloned and identified. To date, the bifunctional DAOCS/deacetylcephalosporin C synthase (DACS) has been isolated from 8 bacterial strains (Amycolatopsis lactamurans, Pochonia chlamydosporia, Streptomyces clavuligerus, Streptomyces chartreusis, Streptomyces ambiguofaciens, Streptomyces jumonjinensis, Nocardia lactamurans, Lysobacter lactamgenus) (Table 1). Here, A. chrysogenum DAOCS and S. clavuligerus DAOCS/DACS are under overview as typical examples.

Cephalosporin biosynthesis is mediated by DAOCS, which is mainly responsible for the ring expansion of the five-member thiazole ring of the penicillin nucleus to form the six-member dihydrothiazide ring of the cephalosporin nucleus, namely the ring expansion reaction, which is also a rate-limiting step in the process of cephalosporin biosynthesis. There is no doubt that DAOCS is the bridge between penicillin and cephalosporin [11].

In both fungi and bacteria, the key enzymes for the biosynthesis of penicillin and cephalosporin are isopenicillin N synthase (IPNS) and DAOCS, respectively [12]. First, three amino acids, L-α-aminoadipic acid, L-cysteine and L-valine, were condensed to form LLD-ACV (d-ι-α-aminoadipyl-D-cysteinyl-D-valine) tripeptide under the catalysis of non-ribosomal peptide LLD-ACV synthase [13]. IPNS catalyzes the cyclic production of isopenicillin N from ACV tripeptide, which is then isomerized to penicillin N by isopenicillin N isomerase. DAOCS catalyzes the five-member thiazole of penicillin N to oxidative cyclization to form a six-member dihydrothiazide ring of cephalosporin, which will generate DAOC. Next, DAOC hydroxylation leads to deacytlation of cephalosporin C (DAC), which is formed by DAC acetylation [14]. The following Fig. 1 depicts this specific process of cephalosporin biosynthesis.

In the above-mentioned cephalosporin biosynthesis, penicillin ring expansion reaction and DAOC hydroxylation reaction are slightly different between fungi and bacteria. In this review, the discussion of DAOCS from S. clavuligerus (scDAOCS) and A. chrysogenum (acDAOCS) was evaluated as examples. In A. chrysogenum, the dual-functional enzyme DAOCS/DACS encoded by cef gene not only catalyzes the expansion of penicillin N to produce DAOC, but also catalyzes the hydroxylation of DAOC to produce DAC [16]. However, in the case of S. clavuligerus, the expansion ring of penicillin N towards DAOC and the hydroxylation of DAOC are independently catalyzed by DAOCS encoded by cefB gene and DACs encoded by cefB gene [17]. Although all the known DAOCS exhibits highly specificity of the natural penicillin N substrate, but their substrate spectra are quite narrow. Since penicillin G is a cheap industrial antibiotics but with much low conversion rate to DAOCS, therefore, the directional modification of DAOCS to improve the conversion rate of other penicillin is extremely important.

3. DAOCS as an Iron(II) and 2-oxoglutarate dependent enzyme

DAOCS belongs to the family of non-heme iron (II) and 2-oxoglutarate (2OG or α-ketoglutarate) dependent oxygenases [18]. Most of the enzymes in this family use iron (IV) and 2 oxygenated [19] intermediates to initiate different oxidative transformations [20] and play an indispensable role in biochemical reactions [21]. DAOCS forms a ternary complex with α-ketoglutarate and substrate, which then react with oxygen to form ferriyl intermediates, initiating the following series of reactions [22].

In the study of DAOCS, reductants other than α-ketoglutarate acid and Fe$^{2+}$ were found to be required for its activity [23], such as vitamin C, molecular oxygen, ATP and DTT (dithiothreitol) [24] as cofactors to participate in the reaction of cephalosporin generated by penicillin expansion [25]. However, Cu$^{2+}$, Co$^{2+}$ and Zn$^{2+}$ have a stronger inhibitory effect on DAOCS [26]. Besides, para-hydroxymercurate and 5,5′-dithiobis (2-nitrobenzoic acid) also inactivate the native scDAOCS. Not only the cofactors affect enzyme activity, but also the order in which cofactors and substrates are added [27]. DAOCS exists in solution as a balanced mixture of monomers and oligomers, whereas DTT addition partially reverses the aggregation.

4. Effect of modification on DAOCS

Since penicillin N is a natural substrate for DAOCS, the modification of DAOCS to improve the conversion rate of other kinds of penicillin is extremely important [28]. But, penicillin N is at a disadvantage compared to other penicillin [29] in terms of economic cost and clinical use. So, in order to improve the conversion rate of DAOCS from penicillin, many related researches have been conducted by changing the enzyme reaction conditions, or by modifying the DAOCS. For example, single mutation, double mutation and combined mutation were carried out on the enzyme, and the mutants that could significantly improve the enzyme activity and the conversion rate of penicillin were screened out. Next, we discuss the modification of DAOCS on its activity and conversion rate.

Based on the study of the crystal structure of DAOCS and the scientiffic deduction of the experimental results, it is shown that the C-terminal arm of DAOCS plays an important role in assisting the transformation of penicillin substrate, but the specific mechanism remains to be explored. According to the current studies, there are several conjectures as follows: C-terminal may be helpful to introduce the substrate into the active site [30], C-terminal amino acid residues are related to the activity of DAOCS [31], modification of the C-terminal might make the DAOCS more receptive to its unnatural penicillin substrates (penicillin G and penicillin V) [32]. By combining the existing theories with the experimental results, we can purposefully modify the C-terminal to achieve the desired effect.

4.1. Effect of single mutation on DAOCS

4.1.1. Effect of directional modification of acDAOCS

It has been reported that A. chrysogenum DAOCS/DACS (acDAOCS/ DACS) residue N305 has a significant effect on enzyme activity, and C-terminal residue M306 has substrate selectivity and catalytic specificity [33]. By establishing the DAOCS/DACS model, it was observed that the side chain of C-terminal residue R307 extended from the substrate binding region, while the side chains of N305, M306 and R308 pointed to the substrate. The results of a series of experiments proved this conjecture.

Therefore, the residues N305, R307 and R308 of acDAOCS/DACS located near its C-terminal were respectively replaced by leucine in some experiments. The results showed that the transformation ability of N305L and R308L mutants to penicillin analogue was significantly improved. R308 was identified as a residue with significant influence on the activity of acDAOCS/DACS [34]. The kinetic data demonstrated that

| Microorganism                  | GenBank ID   | Peptide length |
|--------------------------------|-------------|---------------|
| Amycolatopsis lactamurans [6]  | Q03047      | 314 aa        |
| Acremonium chrysogenum (A. chrysogenum) [7] | P11935 | 332 aa        |
| P. chlamydosporia              | XP018144307 | 313 aa        |
| N. lactamurans                 | Z13974      | 314 aa        |
| L. lactangus YK90 [8]          | CA639984    | 319 aa        |
| S. clavuligerus NRRL 3585 [9]  | P16548      | 311 aa        |
| S. jumonjinensis NRRL 5741     | AF317908    | 311 aa        |
| S. chartreusis 102813 [10]     | AV318743    | 311 aa        |
| S. ambifaciens 293A4 [10]      | AV318742    | 311 aa        |
R308L and N305L had better affinity for the substrate penicillin G. After the initial attempts, more mutations were made at the R308 site. Among the numerous R308 mutants, the relative specificity of the transformation of various penicillin analogues was studied, and the specific activity of R308L, R308V, R308I and R308T against penicillin G was increased by 3.46–7.62 fold [35]. R308L and R308I revealed the most meaningful improvement in the conversion of penicillin G at about 520% and 760%, respectively, and showed the widest substrate specificity and improved catalytic activity, capable of converting all penicillin analogues in the tests. These experimental results suggest that R308 occupies the strategic entry and exit channels in DAOCS.

### 4.1.2. Effect of modified scDAOCS

In the construction of mutants of scDAOCS, residues not directly participated in substrate recognition were selected to construct several mutants, such as Q126M, T213V, S261M, S261A and Y184A, all of which exhibited increased activity to penicillin G [36]. In the single round of random mutation, the kcat/Km values of G79E, V275I, C281Y, N304K, I305L and I305M were significantly increased, indicating that the enzyme activity was improved [37]. Q126, T213 and S261 were also identified as the sites with significant effects on enzyme activity [38]. scDAOCS contains 7 cysteine residues, and attempts have been made to mutate 3 cysteine residues at 100, 155 and 197 sites of DAOCS to alanine residues [39]. However, the results were not very satisfactory, in which the property of C100A mutant was similar to that of wild-type enzymes, furthermore, the activities of C155A and C197A mutants on both penicillin N and penicillin G were not improved but decreased.

Similarly, in *S. clavuligerus*, studies have been devoted to directional modification of hydrophilic residues N301, Y302, N304, R306 and R307 near the end of scDAOCS C-terminal, which were replaced by a hydrophobic leucine residue, respectively. Calculation and analysis indicated that N304 was strategically located in the catalytic chamber of scDAOCS [40], it is important to attract different substrates into the center. In the bioassay of penicillin analogue transformation, it is found that the enzyme activities of both mutants N304L and R306L significantly enhanced the conversion rate of penicillin substrate by 145–400% as shown in Fig. 1.

**Fig. 1.** The biosynthesis process of cephalosporin [15].
4.2. Effect of iterative combined mutagenesis on scDAOCS

C-terminal in DAOCS remains unclear. penicillin analogues through C-terminal mutation or the combination of improve the enzyme activity of scDAOCS and the transformation of penicillin analogues. These results suggest that it is reasonable to further which is used to guide the combined mutagenesis route. First, the tar

crystal structure indicates that in a trimer unit, the C-terminal of a molecule is cyclically inserted into the active site of its neighboring molecule. This arrangement impedes the production of the crystalloasubstrate complex [54]. This also explains the rationality of constructing a series of C-terminal modified DAOCs mutants.

6. The application prospect of DAOCs

As described above, the key enzyme DAOCs plays an indispensable role as the rate-limiting step in cephalosporin biosynthesis. A number of attempts, such as random or targeted mutation and genetic engineering, have been continuously carried out to enhance DAOCs activity for higher DAOC production.

Due to the weak antibacterial activity of cephalosporin C, the clinical important cephalosporins are manufactured by ligation of various side chains to the 7-amino residue of the intermediate, 7- aminodeacetoxycephalosporanic acid (7-ADCA). Currently in industrial prac

tice, 7-ADCA is prepared using chemical expansion of penicillin V or penicillin G. This process has many disadvantages, such as strict reaction condition, high cost and serious environmental pollution, etc. To overcome these disadvantages, enzymatic synthesis using DAOCs came into human’s sight. However, native DAOCs only recognize penicillin N rather than penicillin G as substrate, the effective modification of native DAOCs for penicillin G is imperative with modern molecular biology and synthetic biology. For example, Lin et al. enhanced the yield of pheny lactoyl-7-ADCA (the precursor of 7-ADCA) from penicillin G by 11-fold using engineered Escherichia coli as efficient whole cell catalyst by reconstitution of TCA cycle with DAOCs.

In another case, avermectin (AVM) is a kind of 16-membered ring macrolide fermented by Streptomyces avermitilis. As a world-famous anthelmintic and pesticide antibiotics, AVM was awarded 2015 Nobel Prize in Physiology or Medicine. However, the AVM yield was very low in the fermentation. Based on decades of related efforts in China, the 5 M strategy (Mine, Model, Manipulation, Measure and Manufacture) was proposed by Zhang et al. [55]. With the aid of synthetic biology principle and technique, intelligent AVM production has been achieved with the titer increase by ~1000-fold and the sale price decreased by ~40-fold, which led China to be the only producer of raw AVM product in the world since late 2015. Therefore, this successful example can draw lessons from for improving DAOCs substrate spectrum and activity,
7-ADCA and cephalosporin production. On the other hand, the success in modification might greatly facilitate efficient and new cephalosporin development as well as for other kinds of antibiotics.

7. Summary

To date, the research progress in DOACS has greatly enhanced our understanding for cephalosporin biosynthesis and potential manufacture application. Since new DAOCS might be still isolated, identified and purified from various strains, we should have been using scientific theoretical knowledge and the latest technology to continuously improve its quality to meet our production needs. Future studies on DAOCS should try to improve the conversion rate of DAOCS to various penicillin substrates. If DAOCS mutants can be constructed to significantly increase the conversion rate, it will mean more efficient and clean production of cephalosporin. At the same time, the catalytic active site of DAOCS should be further explored through the study of its structure, the mechanism of its C-terminal influence on the activity of DAOCS, and the directed modification of DAOCS will finally realize the efficient intelligent bio-manufacture of cephalosporin in industry to contribute to our life and medical care.

Credit author statement

Xiaofan Niu: Writing – original draft, Methodology, Jian Zhang: Data curation, Validation, Xianli Xue: Resources, Depei Wang: Funding acquisition; Lin Wang: Visualization, Qiang Gao: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. All authors read and agreed to the publication of the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgment

This work was supported by the Key Project of Natural Science Foundation of Tianjin Municipal Science and Technology Bureau of China (20JCZDJC00140), National Basic Research Program (973 Program) of China (2013CB734004), National Natural Science Foundation of China (31700775, 31471725, 31902193, 61603273), Key Technology R&D Project of Shandong Province of China (Fostering Talent Project) (2016GRC201).

References

[1] Kohsaka M, Demain AL. Conversion of penicillin N to cephalosporin(s) by cell-free extracts of Cephalosporium acremonium. Biochem Biophys Res Commun 1975;70(2):465–73.
[2] Kupka J, Shen Y-Q, Wolfe S, Demain AL. Studies on the ring-cyclization and ring-expansion enzymes of beta-lactam biosynthesis in Cephalosporum acremonium. Can J Microbiol 1983;29(5):488–96.
[3] Thykaer J, Nielsen J. Metabolic engineering of beta-lactam production. Metab Eng 2003;5(1):56–69.
[4] Lloyd MD, Lipcomb SJ, Hewitt KS, Hengsru GMH, Baldwin JE, Schofield CJ. Controlling the substrate selectivity of deacetoxycephalosporin/ deacetylccephalosporin C synthase. J Biol Chem 2004;279(15):15420–6.
[5] Adrio JL, Hintermann GA, Demain AL, Piret JM. Construction of hybrid bacterial deacetoxycephalosporin C synthases (expandases) by in vivo homologous recombination. Enzyme Microb Technol 2002;31(7):932–40.
[6] Coque J, Martin LF, Liras P. Characterization and expression in Streptomyces lividus of cad and cef genes from Nocardia lactamdurans: the organization of the cefamycin gene cluster differs from that in Streptomyces clavuligerus. Mol Gen Genet 1993;236(3):453–8.
[7] Samson SM, Dotzlaw J, Sliz ML, Becker GW, Van Frank RM, Veal LE, Yeh WK, Miller JR, Queener SW, Ingolia TD. Cloning and expression of the fungal expandase/hydroxylase gene involved in cephalosporin biosynthesis. Nat Biotechnol 1987;5:1207–14.
[8] Kimura H, Iwaz M, Sumino Y. Molecular analysis of the gene cluster involved in cephalosporin biosynthesis from Lysobacter lachamgenus YK90. Appl Microbiol Biotechnol 1996;44(5):589–96.
[9] Koivacevíc S, Weigel BJ, Tobin MB, Ingolia TD, Miller JR. Cloning, characterization, and expression in E. coli of the Streptomyces clavuligerus gene encoding a deacetoxy/deacetylcephalosporin C synthase. J Bacteriol 1989;171(2):764–66.
[10] Hung S-J, Yang Y-B, Deng C-H, Wei C-L, Liaw S-H, Tsai Y-C. Family shuffling of expandase genes to enhance substrate specificity for penicillin G. Appl Environ Microbiol 2004;70(10):6257–63.
[11] Chen H, Han H, Xu G. Progress in modification and its prospects of application. China Biotechnol 2000;20(1):27–36 (in Chinese).
[12] Yeh WK. Evolving enzyme technology for pharmaceutical applications: case studies. J Ind Microbiol Biotechnol 1997;19(5–6):134–42.
[13] Martínez JF, Ullán RV, García-Estrada C. Regulation and compartmentalization of beta-lactam biosynthesis. Microb Biotechnol 2010;3(3):285–99.
[14] Chin HS, Sim J, Seh KL, Sim IS. Deacetylccephalosporin C synthane isoyrnes exhibit diverse catalytic activity and substrate specificity. FEMS Microbiol Lett 2003;218(2):251–7.
[15] Oster LM, van Scheltinga ACT, Vlegeld K, Hose AM, Renninger KN, Newland JM. High-level semi-synthetic production of the potent antimarial artemisinin. Nature 2013;496(7446):528–32.
[16] Marquet S, Hausinger RP. Catalytic mechanisms of Fe(II)- and 2-oxoglutarate-dependent oxygenases. J Biol Chem 2015;290(34):20702–11.
[17] Solomon EJ, Light KM, Liu LV, Smrc M, Wong SD. Geometric and electric structure contributions to function in non-heme iron enzymes. Acc Chem Res 2013;46(11):2725–39.
[18] Adrio JL, Cho H, Piret JM, Demain AL. Inactivation of deacetoxycephalosporin C synthase in extracts of Streptomyces clavuligerus during bioconversion of penicillin G to deacetylccephalosporin G. Biochem Biophys Res Commun 1995;218(1):497–501.
[19] Cho H, Adrio JL, Luengo JM, Wolfe S, Ocran S, Hintermann G, Piret JM, Demain AL. Elucidation of conditions allowing conversion of penicillin G and other penicillins to deacetylccephalosporin C by resting cells and extracts of Streptomyces clavuligerus NPI. Proc Natl Acad Sci U S A 1998;95(19):11544–8.
[20] Lee H-J, Lloyd MD, Scholli CJ, Vlegeld K. The effect of cysteine mutations on recombinant deacetoxycephalosporin C synthase from S. clavuligerus. Biochem Biophys Res Commun 2000;267(1):482–8.
[21] Corries J, Martin LF, Castro JM, Liras P. Purification and characterization of a 2-oxoglutarate-linked ATP-independent deacetoxycephalosporin C synthase of Streptomyces lactamdurans. J Gen Microbiol 1987;133(11):3165–74.
[22] Wu L, Fan K, Ji J, Yang K. Evaluation of penicillin expandase mutants and complex substrate inhibition characteristics at high concentrations of penicillin G. Proc Natl Acad Sci U S A 2012;109(43):29887–9.
[23] Gao Q, Demain A. Improvement in the bioconversion of penicillin G to deacetylcephalosporin C G by elimination of agitation and addition of deacne. Appl Microbiol Biotechnol 2001;57(4):511–3.
[24] Liao MD, Lee H-J, Harolds K, Zhang ZH, Baldwin JE, Schofield CJ, Churchman N, Garner CD, Harra T, van Scheltinga ACT, Vlegeld K, Vklund DJAC, Hlad J, Anderson I, Danielsson A, Ibhikhabari R. Studies on the active site of deacetoxycephalosporin C synthase. J Mol Biol 1999;287(5):943–60.
[25] Vlegeld K, van Scheltinga ACT, Lloyd MD, Hara T, Ramaswamy S, Perrasik A, Thompson A, Lee H-J, Baldwin JE, Schofield CJ, Hlad J, Anderson I. Structure of a cephalosporin synthase. Nature 1998;394(6695):805–9.
[26] Lee H-J, Schofield CJ, Vlegeld K. Active site mutations of recombinant deacetoxycephalosporin C synthase. Biochem Biophys Res Commun 2002;292(1):66–70.
[27] Hu Y, Zhu B. Study on genetic engineering of Acremonium chrysogenum, the cephalosporin C-producer. Synth Syst Biotechnol 2016;10(3):143–9.
[28] Wu X-B, Fan K-Q, Wang Q-H, Yang K-Q. C-termius mutations of Acremonium chrysogenum deacetoxy/deacetylcephalosporin C synthase with improved activity toward penicillin analogs. FEMS Microbiol Lett 2005;246(1):110–13.
[29] Xie X, Tian Y, Ji J, Wu WB, Fan K-Q, Yang K-Q. Saturation mutagenesis of Acremonium chrysogenum deacetoxy/deacetylcephalosporin C synthase R308 site
confirms its role in controlling substrate specificity. Biotechnol Lett 2011;33(4): 805–12.

[36] Mo H, Wu X, Zhou X, Liu Y. Study on site-directed mutagenesis of Streptomyces clavuligerus deacetoxycephalosporin C synthase. J Jinan Univ (Nat Sci Med Ed) 2006;27(2):176–83 (in Chinese).

[37] Wei C-L, Yang Y-B, Wang W-C, Liu W-C, Hsu J-S, Tsai Y-C. Engineering Streptomyces clavuligerus deacetoxycephalosporin C synthase for optimal ring expansion activity toward penicillin G. Appl Environ Microbiol 2003;69(4): 2306–12.

[38] Ji J, Tian X, Fan K, Yang K. New strategy of site-directed mutagenesis identifies new sites to improve Streptomyces clavuligerus deacetoxycephalosporin C synthase activity toward penicillin G. Appl Microbiol Biotechnol 2012;93(6):2395–401.

[39] Ji J. Research progress of directional modification of penicillin DAOCS of Streptomyces clavuligerus. Jiangsu Agr Sci 2013;41(7):13–15 (in Chinese).

[40] Chin HS, Sim J, Sim TS. Mutation of N304 to leucine in Streptomyces clavuligerus deacetoxycephalosporin C synthase creates an enzyme with increased penicillin analogue conversion. Biochem Biophys Res Commun 2001;287(2):507–13.

[41] Chin HS, Goo KS, Sim TS. A complete library of amino acid alterations at N304 in Streptomyces clavuligerus deacetoxycephalosporin C synthase elucidates the basis for enhanced penicillin analogue conversion. Appl Environ Microbiol 2004;70(1): 607–9.

[42] Chin HS, Sim TS. C-terminus modification of Streptomyces clavuligerus deacetoxycephalosporin C synthase improves catalysis with an expanded substrate specificity. Biochem Biophys Res Commun 2002;295(1):55–61.

[43] Gao Q, Demain AL. Improvement in the resting-cell bioconversion of penicillin G to deacetoxycephalosporin G by addition of catalase. Lett Appl Microbiol 2002;34(4): 290–2.

[44] Ji J, Fan K, Tian X, Zhang X, Zhang Y, Yang K. Iterative combinatorial mutagenesis as an effective strategy for generation of deacetoxycephalosporin C synthase with improved activity toward penicillin G. Appl Environ Microbiol 2012;78(21): 7809–12.

[45] Reetz MT, Praad S, Carballeira JD, Gamulya Y, Bocola M. Iterative saturation mutagenesis accelerates laboratory evolution of enzyme stereoselectivity: rigorous comparison with traditional methods. J Am Chem Soc 2010;132(26):9144–52.

[46] Reetz MT, Carballeira JD. Iterative saturation mutagenesis (ISM) for rapid directed evolution of functional enzymes. Nat Protoc 2007;2(4):891–903.

[47] Gao Q, Piret JM, Adrio JL, Demain AL. Performance of a recombinant strain of Streptomyces lividans for bioconversion of penicillin G to deacetoxycephalosporin G. J Ind Microbiol Biotechnol 2003;30(3):190–4.

[48] Sim J, Sim TS. Mutational evidence supporting the involvement of tripartite residues His183, Asp185, and His243 in Streptomyces clavuligerus deacetoxycephalosporin C synthase for catalysis. Biosci Biotechnol Biochem 2014; 64(4):828–32.

[49] Sim J, Wong E, Chin HS, Sim TS. Conserved structural modules and bonding networks in isopenicillin N synthase related non-haem iron-dependent oxygenases and oxidases. J Mol Catal B: Enzym 2003;23(1):17–27.

[50] Chin HS, Goh KW, Teo KC, Chan MY, Lee SW, Ong LGA. Predicting the catalytic sites of Streptomyces clavuligerus deacetylcephalosporin c synthase and clavamine synthase 2. Afr J Microbiol Res 2011;5(21):3357–66.

[51] Stok JE, Baldwin JE. Development of enzyme-linked immunosorbent assays for the detection of deacetoxycephalosporin C and isopenicillin N synthase activity. Anal Chim Acta 2006;577(2):153–62.

[52] Baldwin JE, Crabbe MJC. A spectrophotometric assay for deacetoxycephalosporin C synthase. FEBS Lett 1987;214(2):357–61.

[53] Valegård K, van Scheltinga ACT, Dubus A, Ranghino G, Öster LM, Hajdu J, Andersson I. The structural basis of cephalosporin formation in a mononuclear ferrous enzyme. Nat Struct Mol Biol 2004;11(1):95–101.

[54] Lee H-J, Lloyd MD, Clifton IJ, Baldwin JE, Schofield CJ. Kinetic and crystallographic studies on deacetoxycephalosporin C synthase (DAOCS). J Mol Biol 2001;308(3):937–48.

[55] Gao Q, Tan G-Y, Xia X, Zhang L. Learn from microbial intelligence for avermectins overproduction. Curr Opin Biotechnol 2017;48:251–7.