STRATEGIES FOR ENHANCING PRODUCTIVITY OF ASCOMYCIN - A REVIEW

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I. ABSTRACT:

Ascomycin is a polyketide macrolide antibiotic and potent immunosuppressant due to calcium-dependent serine/threonine protein phosphatase inhibitor. It was firstly isolated from *Streptomyces hygroscopicus* var *ascomyceticus* and is known as an important therapeutic drug to prevent graft rejection and frequently used in many therapeutic domains. Biosynthesis of ascomycin is a multifaceted and complex process involving many essential pathways. Its low productivity from *Streptomyces hygroscopicus* is a major concern. To increase ascomycin production, many steps such as alteration in the biosynthetic pathway have been adapted so far. This review is majorly focusing on different approaches at the media optimization, statistical approach as well as genetic engineering level to scale up the titer of ascomycin.

Keywords: Ascomycin, *Streptomyces hygroscopicus.* Immunosuppressant, Calcineurin Inhibitor, Transplantation, Fermentation.

II. INTRODUCTION:

Many people are living a quality life even after transplantation surgery. Organ transplantation was one of its remarkable discoveries to save many lives after irreversible organ damage. This was not possible without the discovery of many immunosuppressive drugs which play a crucial role in reducing the chances of graft rejection and successful organ transplantation. [1]. In this segment, Ascomycin (FK 520) is one of the effective drugs in organ transplantation and exhibits diverse roles in pharmacological and biological activities such as anticonvulsive effect and neuroprotectant [2], antimalarial [3], antifungal [4], immunosuppressive [5]. Due to the complex structure, still many things are unclear about it. The structure was shown in Figure 1.

Several microorganisms have been shown an important role in producing primary and secondary metabolites. One of its groups' Actinomycetes produces different kinds of secondary metabolites. *Streptomyces,* which come under the largest genus of actinobacteria, have greater potential to produce multifunctional active pharmaceutical ingredients and biologically active substances in several therapeutic categories [6]. This was shown in figure 2. *Streptomyces hygroscopicus subsp. ascomyceticus* is used in the industrial production of ascomycin. Tadashi *et. al.,* 1966 described an antifungal agent known as ascomycin, which is produced by cultivating a strain of *Streptomyces hygroscopicus* var.*ascomyceticus* under submerged aerobic conditions [7].

There are many analogs of ascomycin. *S. tsukubaensis* was the first to synthesize tacrolimus (FK506), an ascomycin analog [8, 9], in 1984 [10]. Another macrolactam termed rapamycin (sirolimus) was identified in an antifungal screening method of fermentation broths [11, 12], it is also an immunosuppressant, antifungal, anticancer, and anti-aging medication [11]. They bind to the abundant intracellular binding protein FKBP12 with a high affinity (FK506-binding protein; also called macrophilin [12]) and suppress the production of Th1 (interferon- and IL-2) and Th2 (IL-4 and IL-10) cytokines. Ascomycin is also widely used as a starting material in the synthesis of Pimecrolimus [13].

Due to the structural complexity of ascomycin, its biosynthesis is completely reliant on microbial synthesis at the moment; as a result, researchers are concentrating their efforts on microbial synthesis. The strain *S. hygroscopicus var. ascomyceticus* is well studied for the production of ascomycin, and previous scientific reports concluded that the wild strain produced ascomycin in a very small amount; thus, various yield improvement techniques were used, proving that there is a large scope of such techniques for yield improvement. This review focus on various yield improving strategies of Ascomycin.

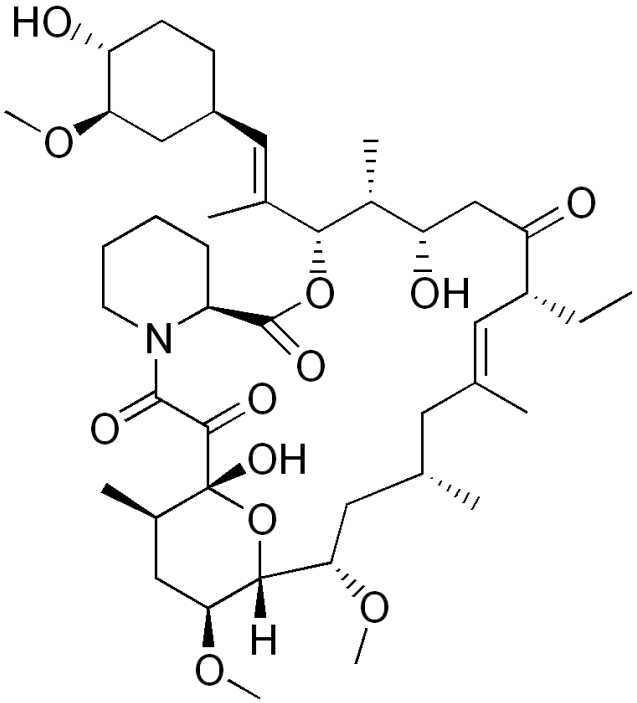

Fig. 1 Structure of Ascomycin

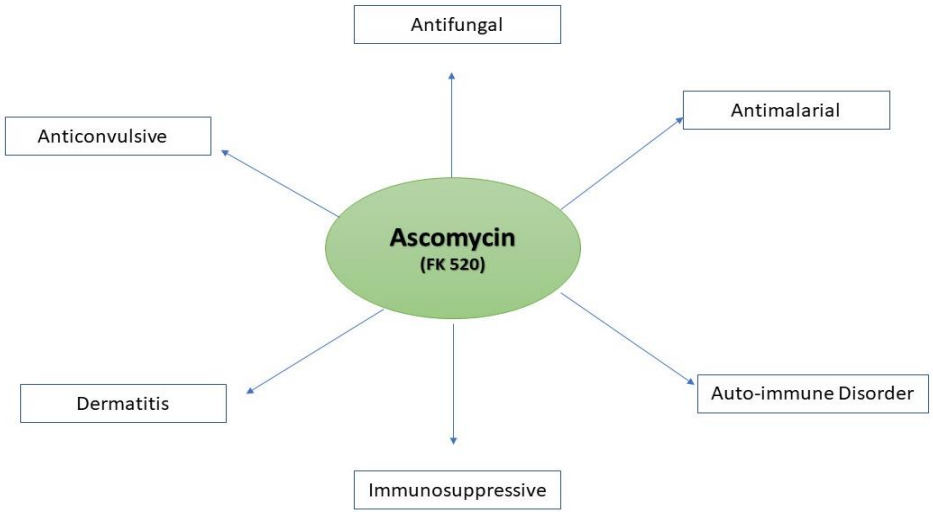

Fig.2 Role of ascomycin in the different therapeutic segments.

III. OPTIMIZATION OF CRITICAL PARAMETERS AT SHAKE FLASK AND FERMENTATION LEVEL:

A critical step in the production of bioactive metabolites specially ascomycin is the study of culture growth and the screening of shake flask and fermentation media and its various parameters. Several studies have been done in this regard. Interactions between microorganisms and growth media have long been recognized as important in determining the rate of metabolite production and yield. Microorganisms grow on or in nutrient-rich growth media. Culture enrichment is necessary for proper microorganism growth. Carbon, nitrogen, minerals,' salt for the osmotic balance of culture,' and water are all required for microbial enrichment. The production of specific metabolites in high titers may be possible if proper control and regulation are maintained at various levels, such as via extracellular nutrient transport and metabolism, precursor formation, and so on [14]. In my previous studies, P3 medium was used which contains Dextrose, dextrin white, and glycerol were used as carbon sources in P3, whereas soya-flour and soya-peptone were used as nitrogen sources supplemented with lysine. Because lysine is an essential precursor in the ascomycin pathway, supplementing it had a favorable effect on ascomycin titer. As a result, media outlets advocated soya flour, which is high in lysine. Corn starch, malto-dextrin, glycerol, and maltose were used as carbon sources in the P8 medium, whereas soya-flour and dry yeast were used as nitrogen sources. The high concentration of amino acids in soya flour, a rich source of lysine, and dry yeast aided cell development, boosting the titer of ascomycin in the P8 medium. The three-stage strategy was found to have a greater influence on ascomycin titer maximization. The impact of physical parameters (pH, PMV percent, and microscope) on productivity was studied at various intervals. P3 and P8 mediums were chosen for additional studies based on the results obtained. On the 11th day of culture in P8 production media, S2 seed that had been cultivated for 44 hours produced a better yield. The second set of trials yielded a maximum ascomycin concentration of 0.5 mg/g, whereas the first set yielded a concentration of 0.2 mg/g. [15]. some researchers investigated ascomycin production under various cultivation conditions. *S. hygroscopicus no. KK317* was cultivated aerobically for 12 hours under submerged fermentation, supplemented with aqueous carbohydrate solution and nitrogenous fertilizers. After extraction, a flocculent white precipitate of pure ascomycin with a concentration of 200 mg/L was obtained [6].

In another study, Preparation of the spore stocks of *S. hygroscopicus* ATCC 14891, Reeves *et.al.* used SY medium for the production of ascomycin and its analogs in tryptic soy broth with 50 mM TES buffer, pH 7, and 1% glucose (TSBGM) [16]. Qi *et al.* used *S. hygroscopicus* var. *ascomyceticus* FS35 as a starting strain for obtaining a mutant strain *S. hygroscopicus* SA68 from a combination of femtosecond laser irradiation and shikimic acid enduring screening which resulted in 270 mg/L ascomycin. The seed and production medium. Seed medium consists of 10 g/L soluble starch, 30 g/L glucose, 6 g/L peptones, 6 g/L yeast powder, and 2 g/L CaCO3. and production medium contains

24 g/L soluble starch, 40 g/L dextrin, 5.0 g/L peptone, 7.0 g/L yeast powder, 2.0 g/L corn steep liquor, 11 mL/L soybean oil, 0.5 g/L K2HPO43H2O, 1.5 g/L (NH4)2SO4, 1.0 g/L MgSO47H2O, and 1.0 g/L CaCO3 were prepared in an unbaffled shake flask [7]. Song et al. investigated *Streptomyces hygroscopicus var. ascomyceticus* FS35 for ascomycin synthesis in the fermentation medium [17]. In a separate study, Yu et al. developed a high-yield S. hygroscopicus SFK-36 strain by ARTP-induced mutagenesis of the original *S. hygroscopicus* ATCC 14891 strain in seed media containing 8.0 g/L corn steep liquor, 10.0 g/L glucose, 3.0 g/L cottonseed meal, and 1.0 g/L KH2PO4 with pH 7.0 and production media of 20.0 g/L soluble starch, 40.0 g/L dextrin, 5.0 g/L yeast powder, 5.0 g/L peptone, 5.0 g/L corn steep liquor, 1.0 g/L K2HPO43H2O, 1.5 g/L (NH4),0.5 g/L MnSO4H2O, 1 g/L MgSO47H2O, 1 g/L CaCO3, and 1 g/L soybean oil with 6.5 pH [8]. The mutant *S. hygroscopicus* SFK-36 was able to produce 495.3 mg/L of ascomycin after ARTP-induced mutagenesis of the original strain, but the production medium was further adjusted by RSM to increase the output up to 1466.3 mg/L of ascomycin [8]. In the improved medium, SFK-36 showed a higher rate of carbon utilization. This research concluded that using a mix of standard mutagenesis breeding and medium adjustment to boost ascomycin production is a viable option. In addition to those various strategies were reported during medium design and optimization to improve the efficiency of the production medium for primary and secondary metabolites. OFAT (change one factor at a time) and multiple factors in media statistical techniques such as Plakett Burman design, Taguchi design, Central composite design, Box Behnken design, Response surface methodology (RSM) [18].

Other parameters such as foaming are also critical parameters for ascomycin production. One of the research groups has been discovered a link between ascomycin and foam formation. In the batch fermentation process of *Streptomyces hygroscopicus* FS-35 in a 7.5 L bioreactor, foaming diminishes the working volume and inhibits the synthesis of macrolide immunosuppressive ascomycin [19]. When *S. hygroscopicus var. ascomyceticus*

ATCC 14891 was cultured for 180–240 hours at 24–32 degrees Celsius with agitation speeds of 2.0–4.0 m/s, ascomycin production was enhanced to 400 mg/L [20].

IV. ENHANCING PRODUCTION AT GENE LEVEL AND THROUGH ADVANCED METHODOLOGY:

Because of the structural complexity of ascomycin, its biosynthesis is completely dependent on microbial synthesis; thus, researchers are focusing their direction on microbial synthesis. Even all efforts made by traditional methods, still yield ascomycin production still limited therefore understanding the biosynthesis mechanism of ascomycin has been greatly aided by genetic engineering [17, 21].

The group of researchers investigated the overall biosynthesis of ascomycin in *S. hygroscopicus var. ascomyceticus* (ATCC 14891) and discovered four gene clusters (*fkbB, fkbC, fkbA, fkbP*) for the biosynthesis of unusual polyketide extender units [22]. In another study, it was demonstrated that pentose phosphate biosynthesis and aromatic amino acid played a crucial role for the precursor molecules involved in ascomycin biosynthesis which results in enhancing ascomycin production. Therefore, genes from the pentose phosphate (zwf gene, encoding glucose-6-phosphate dehydrogenase) and shikimate (aroA gene, encoding 3-deoxy-7-phosphoheptulonate synthase) pathways have been selected as potential ascomycin production target genes for further enhancement of ascomycin production [23]. Other studies suggested that the ascomycin biosynthetic gene cluster contained seven co-transcription units, including *fkbW, fkbU, fkbR1/R2, fkbE/F/G, fkbB/C/L/K/J/I/H, fkbO/P/A/D/M*, and *fkbS/Q/N*, which were responsible for ascomycin synthesis with yield enhancement [8]. One of the studies exhibited role of pathway-specific positive regulators for increasing ascomycin production. Overexpression of *fkbN also* enhanced the ascomycin yield 1800mg/L. They also reported that the inactivation of *fkbN* greatly decreased ascomycin production [24]. There is still a need to explore the biosynthetic pathways of Ascomysin.

Apart from this, the use of a femtosecond laser to boost FK520 synthesis in *S. hygroscopicus* appeared to be a promising method. *Streptomyces hygroscopicus var. ascomyceticus* NT2-11, an N-methyl-N-nitro-N-nitrosoguanidine (NTG)-induced strain derived from *S. hygroscopicus*, was used for the first time to improve the ascomycin (FK520) yield (ATCC14891). FS35 has a 45 percent higher FK520 production capacity than its parent strain, NT2-11. The FK520 fermentation titer of FS35 achieved 300 mg/L under ideal fermentation conditions

by use of a femtosecond laser [21]. Another approach was to combine 13C-labeling experiments with in silico pathway analysis could be a potential strategy to boost ascomycin production. The metabolic network model of *S. hygroscopicus* SA68 was calibrated using 13C-labeling studies. Using the EFMA method, prospective target genes for increasing ascomycin synthesis were discovered. With the use of the model, a high-yield strain TD-Pyc-FkbO was created, with ascomycin output reaching 610 mg/L, an increase of 84.8 percent over the parent strain SA68 [25]. According to Wang et al., the ethylmalonyl-CoA pathway's two targets, *hcd, and ccr* were chosen for overexpression, and the experimental results showed that ascomycin production improved more in both single- and double-overexpression strains. These findings indicated that increasing ethylmalonyl-CoA supply could boost ascomycin output effectively [26]. The deletion and overexpression of the target genes *fkbE and fkbF*, respectively, were used to explore the involvement of these genes in ascomycin synthesis. Ascomycin synthesis increased by 45.6 percent when *fkbE* was overexpressed. To boost ascomycin production, even more, researchers created the *fkbR1 and fkbE* combinatorial overexpression strain OfkbRE, which raised ascomycin yield by 69.9% to 536.7 mg/L when compared to the parent strain. These findings revealed a useful method for increasing ascomycin synthesis by modifying *FkbR1* and its target gene [17].

To boost ascomycin synthesis, researchers used a combination of adsorbent resin HP20 addition and metabolic profile studies. These findings showed that combining HP20 addition with metabolic profiling data might be used to successfully improvement of ascomycin production. The ascomycin production was eventually increased to 460 mg/L, representing a 53.3 percent increase over the original condition by this process [4]. The original *S. hygroscopicus* ATCC 14891 strain was treated with atmospheric and room temperature plasma to develop a stable high-producing *S. hygroscopicus* SFK-36 strain that produced 495.3 mg/L ascomycin, a 32.5 percent increase in ascomycin over the ATCC 14891 strain. Then, using response surface methods, the fermentation medium was adjusted to boost ascomycin production even more. In fast culture, the ascomycin yield reached 1466.3 mg/L in the optimal medium, which contained 81.0 g/L soluble starch, 57.4 g/L peanut meal, and 15.8 g/L soybean oil. A significant amount of yield increased with this [8].

In addition to this, there are some more ways to further enhance ascomycin production. Such as genome shuffling [27] and further changes in transcription and translational level [28].

V. CONCLUSION:

Ascomycin has piqued the interest of researchers and pharmaceutical businesses due to its key pharmacological properties and market potential. To date, some efforts have been done to improve ascomycin yield. Medium optimization is still one of the most closely studied phenomena that occur before large-scale metabolite production [29], and it comes with a slew of obstacles. previously media optimization was done using traditional approaches, which were costly, time-intensive, and involved numerous experiments with varying degrees of accuracy. Nonetheless, with the introduction of current mathematical/statistical tools, media optimization has grown more lively, effective, efficient, cost-effective, and stable in its output. The best fermentation conditions (e.g., pH, temperature, agitation speed, etc.) and medium components (e.g., carbon, nitrogen, etc.) must be discovered and adjusted when constructing a production medium. Additionally, maximum product concentration might be reached by adjusting the aforementioned parameters [30, 31].

One of the better aspects for selecting target genes and pathway-specific positive regulators for boosting ascomycin synthesis is genetic engineering. Genes from the pentose phosphate and shikimate pathways, for example, should be investigated further to improve ascomycin synthesis

The combination of dynamic fermentation characteristics, enzyme activities and metabolites levels in related pathways, and gene expression levels can provide deep insights into the unearthing complete biosynthesis pathways of ascomycin in order to maximize the production which can fulfill the global market demand of ascomycin.

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VII. REFERENCES:

[1] U. Luthra, A. Tomar, Tacrolimus - Strategies for Enhancing Tacrolimus Production at the Classical and Transcriptional Level, 10 (2021) 233–237. https://doi.org/10.21275/SR211005095325.

[2] G. Sierra-Paredes, G. Sierra-Marcuño, Ascomycin and FK506: Pharmacology and Therapeutic Potential as Anticonvulsants and Neuroprotectants, CNS Drug Rev. 14 (2008) 36–46. https://doi.org/10.1111/j.1527-3458.2008.00036.x.

[3] P. Monaghan, M. Fardis, W.P. Revill, A. Bell, Antimalarial effects of macrolactones related to FK520 (ascomycin) are independent of the immunosuppressive properties of the compounds, J Infect Dis. 191 (2005) 1342–1349. https://doi.org/10.1086/428454.

[4] H. Qi, S. Zhao, H. Fu, J. Wen, X. Jia, Enhancement of ascomycin production in *Streptomyces hygroscopicus var. ascomyceticus* by combining resin HP20 addition and metabolic profiling analysis, J Ind Microbiol Biotechnol. 41 (2014) 1365–1374. https://doi.org/10.1007/s10295-014-1473-9.

[5] B.F.J. Dumont, M.J. Staruch, S.L. Koprak, J.J. Siekierka, C.S. Lin, R. Harrison, T. Sewe, V.M. Kindt, U.T. Beattie, M. Wyvratt, N.H. Sigal, Matthew Wyvratt,S and Nolan H. Sigal*, 176 (1992).

[6] Tadashi A., Nogata M., Nakano, K. Ascomycin and Process for its production, US 3,244,592 (1966).

[7] H. Qi, S. Zhao, J. Wen, Y. Chen, X. Jia, Analysis of ascomycin production enhanced by shikimic acid resistance and addition in Streptomyces hygroscopicus var. ascomyceticus, Biochem. Eng. J. 82 (2014) 124–133. https://doi.org/10.1016/j.bej.2013.11.006.

[8] Z. Yu, X. Shen, Y. Wu, S. Yang, D. Ju, S. Chen, Enhancement of ascomycin production via a combination of atmospheric and room temperature plasma mutagenesis in Streptomyces hygroscopicus and medium optimization, AMB Express. 9 (2019). https://doi.org/10.1186/s13568-019-0749-x.

[9] K. Wu, L. Chung, W.P. Revill, L. Katz, C.D. Reeves, The FK520 gene cluster of Streptomyces hygroscopicus var. ascomyceticus (ATCC 14891) contains genes for biosynthesis of unusual polyketide extender units, Gene. 251 (2000) 81–90. https://doi.org/10.1016/S0378-1119(00)00171-2.

[10] M.A.R.C. Bulusu, K. Baumann, A. Stuetz, Chemistry of the immunomodulatory macrolide ascomycin and related analogues., 2011. https://doi.org/10.1007/978-3-7091-0748-5_2.

[11] A. Kudelski, S.N. Sehgal, A new antifungal antibiotic taxonomy of the producing streptomycete and isolation of the active principle , Department of Microbiology , Ayerst Research Laboratories , Montreal , Quebec ., Canada (Received for publication June 17 , 1975.

[12] T. Okuda, T. Hatano, K. Yazaki, Praecoxin B, C, D and E, novel ellagitannins from stachyurus praecox, Chem. Pharm. Bull. 31 (1983) 333–336. https://doi.org/10.1248/cpb.31.333.

[13] Kumar, P., Sharma, S. K., Malviya, H. K., Balasubramaniam, R. C., Dalal, A., An improved fermentation process for preparing Ascomycin, WO 2007/029082, 2007.

[14] Rokem, J. S., Lantz, A. E., Nielsen, J., Systems biology of antibiotic production by microorganisms. Nat. Prod. Rep. 24: 1262–1287, 2007.

[15] U. Luthra, H. Kumar, A. Tripathi, S. Talele, V. Gupte, A. Biotechnology, Accepted Manuscript, (2019).

[16] C.D. Reeves, L.M. Chung, Y. Liu, Q. Xue, J.R. Carney, W. Peter Revill, L. Katz, A new substrate specificity for acyl transferase domains of the ascomycin polyketide synthase in Streptomyces hygroscopicus, J. Biol. Chem. 277 (2002) 9155–9159. https://doi.org/10.1074/jbc.M111915200.

[17] K. Song, L. Wei, J. Liu, J. Wang, H. Qi, J. Wen, Engineering of the LysR family transcriptional regulator FkbR1 and its target gene to improve ascomycin production, Appl. Microbiol. Biotechnol. 101 (2017) 4581–4592. https://doi.org/10.1007/s00253-017-8242-4.

[18] V. Singh, S. Haque, R. Niwas, A. Srivastava, M. Pasupuleti, C.K.M. Tripathi, Strategies for fermentation medium optimization: An in-depth review, Front. Microbiol. 7 (2017). https://doi.org/10.3389/fmicb.2016.02087.

[19] X. Xin, H. Qi, J. Wen, X. Jia, Y. Chen, Reduction of foaming and enhancement of ascomycin production in rational Streptomyces hygroscopicus fermentation, Chinese J. Chem. Eng. 23 (2015) 1178–1182. https://doi.org/10.1016/j.cjche.2014.04.006.

[20] K. Parveen, S.S. Kumar, M.H. Kumar, an improved fermentation process for preparing ascomycin, WO 2007/029082, 2007.

[21] H.S. Qi, X. Xin, S.S. Li, J.P. Wen, Y.L. Chen, X.Q. Jia, Higher-level production of ascomycin (FK520) by Streptomyces hygroscopicus var. ascomyceticus irradiated by femtosecond laser, Biotechnol. Bioprocess Eng. 17 (2012) 770–779. https://doi.org/10.1007/s12257-012-0114-2.

[22] K. Wu, L. Chung, W.P. Revill, L. Katz, C.D. Reeves, The FK520 gene cluster of Streptomyces hygroscopicus var. ascomyceticus (ATCC 14891) contains genes for biosynthesis of unusual polyketide extender units, Gene. 251 (2000) 81–90. https://doi.org/10.1016/S0378-1119(00)00171-2.

[23] A. Jacobi, M. Braeutigam, V. Mahler, E. Schultz, M. Hertl, Pimecrolimus 1% cream in the treatment of facial psoriasis: A 16-week open-label study, Dermatology. 216 (2008) 133–136. https://doi.org/10.1159/000111510.

[24] B. Wang, J. Sui, B. Yu, C. Yuan, L. Guo, A.M.A. El-aty, ur na l P of, Carbohydr. Polym. (2020) 117314. https://doi.org/10.1016/j.carbpol.2020.117314.

[25] H. Qi, M. Lv, K. Song, J. Wen, Integration of parallel 13C-labeling experiments and in silico pathway analysis for enhanced production of ascomycin, Biotechnol. Bioeng. 114 (2017) 1036–1044. https://doi.org/10.1002/bit.26223.

[26] J. Wang, C. Wang, K. Song, J. Wen, Metabolic network model guided engineering ethylmalonyl-CoA pathway to improve ascomycin production in Streptomyces hygroscopicus var. ascomyceticus, Microb. Cell Fact. 16 (2017) 1–13. https://doi.org/10.1186/s12934-017-0787-5.

[27] W. Du, D. Huang, M. Xia, J. Wen, M. Huang, Improved FK506 production by the precursors and product-tolerant mutant of Streptomyces tsukubaensis based on genome shuffling and dynamic fed-batch strategies, J. Ind. Microbiol. Biotechnol. 41 (2014) 1131–1143. https://doi.org/10.1007/s10295-014-1450-3.

[28] B. Bednarz, M. Kotowska, K.J. Pawlik, Multi-level regulation of coelimycin synthesis in Streptomyces coelicolor A3(2), Appl. Microbiol. Biotechnol. 103 (2019) 6423–6434. https://doi.org/10.1007/s00253-019-09975-w.

[29] K. Sambyal, R.V. Singh, Bioprocess and genetic engineering aspects of ascomycin production: a review, J. Genet. Eng. Biotechnol. 18 (2020). https://doi.org/10.1186/s43141-020-00092-0.

[30] M. Gupte, P. Kulkarni, A study of antifungal antibiotic production by Thermomonospora sp MTCC 3340 using full factorial design, J. Chem. Technol. Biotechnol. 78 (2003) 605–610. https://doi.org/10.1002/jctb.818.

[31] E. Franco-Lara, H. Link, D. Weuster-Botz, Evaluation of artificial neural networks for modelling and optimization of medium composition with a genetic algorithm, Process Biochem. 41 (2006) 2200–2206. https://doi.org/10.1016/j.procbio.2006.06.024.