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Tacrolimus - Strategies for Enhancing Tacrolimus Production at the Classical and Transcriptional Level

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Abstract: ***Tacrolimus (FK506) is a 23 membered polyketide macrolide, a potent immunosuppressant, and a useful secondary metabolite that exhibits 10 - 100 times more efficient than cyclosporine. It becomes an important therapeutic drug not only in transplantation but also used frequently in the treatment of severe atopic dermatitis, and secondary lymphedema. Biosynthesis of Tacrolimus is a multifaceted process involved in many essential pathways, but low productivity of tacrolimus fromStreptomyces sp. is a major concern regarding its increased global demand. To boost Tacrolimus production, many steps such as alteration in the biosynthetic pathway, strain improvements, optimizing culture conditions, and use of tacrolimus precursors have been adapted. This review focuses on different approaches at the nutritional and genetic engineering level to scale up the titer of tacrolimus.***

Keywords: Tacrolimus, FK506, secondary metabolite, *Streptomyces sp.*, immunosuppressant, transplantation, biosynthetic pathway

1. Introduction

In the field of medicine and surgery, organ transplantation is the key to save the lives of many patients affected with end - stage and irreversible organ damage and improving the quality of their life [1]. Somewhere between 1000 and 800 BC, transplantation was meticulously documented in the Charak Sanhita, written by Sushruta, known as the father of plastic surgery for his unparalleled efforts for successful nasal reconstruction [2]. Transplantation is a painstaking process where chances of three types of graft rejection such as hyper - acute, acute, and chronic rejection may occur [3]. Several types of research have been carried out to identify drugs that can reduce the chances of graft rejection. The remarkable discoveries of immunosuppressant molecules have paved the way for successful organ transplantation. In the beginning, the discovery of cyclosporine followed by tacrolimus (FK 506) (**Fig.1**) had made a significant contribution in the field of transplantation science [4]. Due to certain limitations of cyclosporine, which include hypertension and hyperlipidemia [5]. Tacrolimus was found to be 100 times more potent and efficacious than cyclosporine that was approved by the US Food and Drug Administration for liver transplantation in 1994 [6, 7]

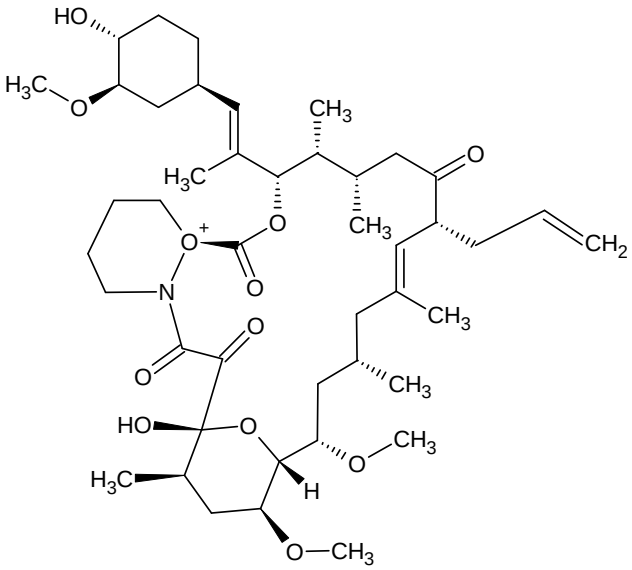

Figure 1: Structure of Tacrolimus FK506

The word tacrolimus originated from tsukubaensis, macrolide, and immunosuppressant [8]. Tacrolimus (FK 506) was isolated from a culture broth of *Streptomyces tsukubaensis*9993 in 1984 by Fujisawa Pharmaceutical at Tsukuba, Japan. It is a secondary metabolite and belongs to macrolides lactone antibiotics [9]. Tacrolimus suppresses immune cell functioning by acting as a calcineurin inhibitor. Calcineurin is a key protein required to allow transcription of Interleukin - 2 which results in T cell activation and proliferation. Tacrolimus binds to intracellular protein FKBP12 and forms a complex with other regulatory proteins that impede the phosphatase activity of calcineurin which inhibits the production of IL - 2 that restricts the proliferation of T cells, facilitate immunosuppressive activity, and finally prevent graft rejection [10]. Presently, tacrolimus reduces the chance of graft rejection in the transplantation of the liver, kidney, and heart. Other than transplantation, It has also been highlighted in the treatment of neuroprotective disorders [11]. Severe atopic dermatitis

[12]. and in the treatment of secondary lymphedema, caused by post - chemotherapy and radiation [13].

Howbeit, the treatment cost of transplantation after using Tacrolimus is quite high due to the high demand and low productivity of tacrolimus by tacrolimus producing strains [14]. To increase the productivity of tacrolimus, some of the studies have been suggested such as alteration in the biosynthetic pathway, strain improvements, optimizing culture conditions, and the use of tacrolimus precursors. Despite this, significant research has not been found for enhancing the ways of tacrolimus production and its cost - effective methods. This review updates on considerable efforts made in the past, and elucidates various strategies at the supplementation and genetic engineering level for enhancing tacrolimus production in order to meet increasing demands globally.

2. Metabolic regulation through supplementation of various compounds

Many practices have been attempted in the past for the addition of supplements or precursors in the respective culture medium of containing microorganisms to elevate the activity of secondary metabolites production. It has also been seen as a rational approach to increase the productivity of tacrolimus through the addition of precursor of tacrolimus biosynthetic pathways in the medium. These ideas came up when the enhancement of production was seen with respect to penicillin - producing fungus *Penicillin chrysogenum* NRRL 1951 by modulation in cysteine biosynthesis [15]. Even with the addition of methyl oleate in the culture medium of *Streptomyces clavuligerus* CKD 119, an increased yield of tacrolimus was noticed [16]. The use of soya oil as an additive had shown elevated production of tacrolimus in recent research [17]. Studies have shown increased tacrolimus production by the alteration of carbon and amino acid sources. It has been underlined in previous research that soya oil (30g/L) in combination with L - lysine (0.2 g/L) had significantly increased the production of tacrolimus by *Streptomyces sp*. MA6858 [18]. Further, the presence of amino acids such as lysine, leucine, proline threonine, and valine in the medium hasbeen shown to be an important factor for scaling up tacrolimus yield [19].

Piperdine and pyrimidine derivatives play an important factor in the production of secondary metabolites synthesis. In this context, Picolinic acid and pipecolic acid are the direct precursors for the biosynthesis of tacrolimus. The addition of Picolinic acid and pipecolic acid in the optimized medium of *Streptomyces tsubaensis* augmented the activity of tacrolimus several - fold. Pyrimidine derivatives such as nicotinamide and nicotinic acid also positively influenced the growth of *Streptomyces tsukubaensis* [20]. Hence, it is clearly evident that the presence of the precursor and cofactor is essentially needed in terms of enhancement of tacrolimus production and growth of *Streptomyces tsukubaensis.*

Association and well - regulated Citric acid pathways, Embeden - Meyerhof - Parnas (EMP), glycolysis, shikimate, and aminoacid metabolism play an essential role in tacrolimus biosynthesis. Precursors such as malonyl - COA, DHCHC, methylmalonyl – CoA, allymalonyl – CoA, methoxylmalonyl ACP synthesized in these pathways help for tacrolimus production [4]. Moreover, three - carbon compounds such as propylene glycol, propanol, or propionic acid promote the growth of *S. tsukubaensis* and eventually increase tacrolimus production [21].

Brazil nut oil containing unsaturated fatty acids, oleic, and linoleic acid was tested in scale - up of tacrolimus production. Exogenous feeding of Brazil nut oil into the culture medium of *S. tsukubaensis* remarkably boosted tacrolimus yield without affecting biomass [22]. Not only the addition of compound, precursor, or supplements into the medium but also feeding time also acts as an important factor for tacrolimus production due to diauxic growth of *S. tsukubaensis* [4]. In fact, tacrolimus Sequential adaptation (600 to 1600 mg) into the medium facilitated the highest reported tacrolimus production (972mg/l) by *Streptomyces sp. TST8 [*23]. Statistical experimental strategies such as using highly fractionate factorial design (Plakett - Burmann) followed by response surface method proved as one of the feasible techniques for screening variables for media optimization [23, 24] Analysis of Dextrine white, cotton seed meal (CSM), and polyethylene glycol (PEG) - 400 are proved to be the most significant variables through statistical analysis [25]. Optimized medium conditions for *S. tsukubaensis* by statistical approach have increased tacrolimus production 2.94 folds in comparison to basal media. Maximum production of Tacrolimus achieved 574 mg/l and 616mg/l at shake flask and 2.5 l bioreactor respectively [5].

In previous research, it had clearly marked that stress response induces more secondary metabolite production. Dimethyl sulfoxide (DMSO) and sodium thiosulfate are the most effective stressing compound for activating polyketide synthesis [26] and improve tacrolimusscale - up in *S. tsukubaensis NRRL 18488 [*27]. Therefore, optimum media composition is one of the important factors for the tacrolimus production at shake flask and fermenter level. Hence, it is further demonstrated that using different precursors, that influence polyketide biosynthesis and their feeding timing, as well as the right variables of the composition of media, are the most critical parameters for tacrolimus yield enhancer.

3. Engineering of the genetic makeup of Streptomyces Sp. for boosting tacrolimusproduction

Biosynthesis of tacrolimus is based on a hybrid system of polyketide 1 synthase and non - ribosomal peptide synthase (PKSI - NRPS) and regulated by a minimum of 19 genes of fkb cluster [28]. Short segment (*fkbQ, fkbN, fkbM, fkbD, fkbA, fkbP, fkbO, fkbB, fkbC, fkbL, fkbK, fkbJ, fkbI, fkbH, fkbG, allD, allR, allK and allA)* and extended segment (*fkbG, allM, allN, allP, allO, allS)* are locatedin the 5' region and the genes tcs6 - fkbRand /or tcs67 in the 3' region of the other species [29]. Some of these fkb gene clusters directly or indirectly play a unique role in the biosynthesis of intermediates or precursors involved in the tacrolimus production. The presence of genes such as *allM,*

allN, allP, allO, allS does not affect tacrolimus biosynthesis due to their low transcription level [30, 31]. However, overexpression or inactivation of other genes may have a significant effect on tacrolimus production. A detailed study on the transcriptome, proteome as well as metabolome level for the biosynthesis of polyketide has been carried out in the past globally [32, 33]. Overexpression of *fkbO, fkbL, fkbP*, fkbM, and fkdD genes in *S. tsukubaensis D852* showed promising results on tacrolimus biosynthesis especially in the formation of starting unit (4R, 5R) - 4, 5 - dihydroxycyclohex - 1 - enecarboxylic acid (DHCHC), pipecolate, and other important reactions [32].

The regulators fkbN, fkbR and allN regulate the fkb cluster. fkbN, fkbR and allN belong to the large regulatory protein of LuxR family [34], LysR [35], and AsnC family [36]respectively. After in - depth functional characterization of fkbN, fkbR and allN, it was found that fkbN and fkbR have shown a positive effect on tacrolimus production, while allN does not influence the biosynthesis of tacrolimus (Inactivation of fkbN and fkbR led to lack of tacrolimus production and decreased 20% yield respectively. Whilst overexpression of fkbN and fkbR markedly enhanced production of tacrolimus and increased the yield to 55% and 30% respectively [37]. FkbNis located on a short and extended segment of fkb cluster and FkbRis only present on the extended version, therefore inactivation of fkbN results in lack of production of tacrolimusas fkbN regulates the expression of the maximum number of genes located on fkb cluster [30].

Apart from this, genes involved in the synthesis of other intermediates in the biosynthetic pathway of tacrolimus were studied and found that inactivation of gdhA and ppc encoded to NADPH - dependent glutamate dehydrogenase and phosphoenolpyruvate carboxylase respectively boost the tacrolimus production [33]. Shikimate pathway and lysine arethe important factors required for the biosynthesis of tacrolimus with, *acoC and dapA* that play a crucial role. Hence the over expression of *aroC and dapA* stimulate the Tacrolimus production in *Streptomyces tsukubaensis* [38]. The review critically explores the roles of genes that may affect Tacrolimus production at the transcriptional level.

4. Conclusion and Future Aspects

Extensive research on tacrolimus and approval from FDA has opened a new way in the science of transplantation. However, its demand in the global market has increased day by day and the production cost has not matched its requirement. In order to resolve this problem, to enhance the production of tacrolimus, and to meet the global demand, an extensive strategy was used at the nutritional and transcriptional levels. At the nutritional level, altering the primary source of carbon and amino acid could enhance tacrolimus production [18]. Supplementation of oleate, soy oil, L - lysine, leucine, proline, threonine, valine, picolinic acid, pipecolic acid, brazil nut oil is some important practices to boost tacrolimus production. Stressing agents such as DMSO and sodium thiosulphate are also key players. At the transcription level, genes located on fkb cluster, outside of the cluster, and the regulatory proteins play a prominent role in scale - up tacrolimus production. FkbN is the essential target for increasing the activity of Tacrolimus [30]. Overexpression of fkbO, fkbL, fkbM, fkbP, FkbD [32] aroC, dapA [38] inactivation of gdhA, ppc [33] also have shown a major role in the enhancement of tacrolimus production. Integration of both nutritional aspect and transcriptional approach together may pave the way for the higher tacrolimus production. A gap between the two approaches should be filled and the use of a classical approach with metabolic engineering may be one of the excellent approaches to scale up tacrolimus production in near future.

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References

[1] J. M. Grinyó, Why is organ transplantation clinically important?, Cold Spring Harb Perspect Med.3 (2013). https: //doi. org/10.1101/cshperspect. a014985.
[2] M. C. Champaneria, A. D. Workman, S. C. Gupta, Sushruta: Father of plastic surgery, Ann Plast Surg.73 (2014) 2–7. https: //doi. org/10.1097/SAP.0b013e31827ae9f5.
[3] J. D. Ellett, G. Virella, K. D. Chavin, Transplantation immunology, Med Immunol Sixth Ed. (2007) 358–368. https: //doi. org/10.7326/0003 - 4819 - 76 - 5 - 854_3.
[4] C. Barreiro, M. Martínez - Castro, Trends in the biosynthesis and production of the immunosuppressant tacrolimus (FK506), Appl MicrobiolBiotechnol.98 (2014) 497–507. https: //doi. org/10.1007/s00253 - 013 - 5362 - 3.
[5] J. K. Patel, J. A. Kobashigawa, Cardiac transplant experience with cyclosporine, Transplant Proc.36 (2004) S323–S330. https: //doi. org/10.1016/j. transproceed.2004.01.039.
[6] T. KINO, T. GOTO, Discovery of FK - 506 and Update, Annals of the New York Academy of Sciences (1993) *68513–21*. doi: 10.1111/j.1749 - 6632.1993. tb35846. x.
[7] H. U. Meier - Kriesche, S. Li, R. W. G. Gruessner, J. J. Fung, R. T. Bustami, M. L. Barr, A. B. Leichtman, Immunosuppression: Evolution in practice and trends, 1994 - 2004, Am J Transplant.6 (2006) 1111–1131. https: //doi. org/10.1111/j.1600 - 6143.2006.01270. x.
[8] P. E. Wallemacq, R. Reding, FK506 (tacrolimus), a novel immunosuppressant in organ transplantation: Clinical, biomedical, and analytical aspects, Clin Chem.39 (1993) 2219–2228. https: //doi. org/10.1093/clinchem/39.11.2219.
[9] T. Kino, H. Hatanaka, M. Hashimoto, M. Nishiyama, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, H. Imanaka, (IFN - f) induced by antigens, J Antibiot.40 (1987) 1249–1255.
[10] G. Patel, T. P. Khobragade, S. R. Avaghade, M. D. Patil, S. H. Nile, G. Kai, U. C. Banerjee, Optimization

of media and culture conditions for the production of tacrolimus by Streptomyces tsukubaensis in shake flask and fermenter level, Biocatal Agric Biotechnol.29 (2020) 101803. https: //doi. org/10.1016/j. bcab.2020.101803.

[11] 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.

[12] W. W. Carr, Topical calcineurin inhibitors for atopic dermatitis: Review and treatment recommendations, Pediatr Drugs.15 (2013) 303–310. https: //doi. org/10.1007/s40272 - 013 - 0013 - 9.

[13] J. C. Gardenier, R. P. Kataru, G. E. Hespe, I. L. Savetsky, J. S. Torrisi, G. D. G. Nores, D. K. Jowhar, M. D. Nitti, R. C. Schofield, D. C. Carlow, B. J. Mehrara, Topical tacrolimus for the treatment of secondary lymphedema, Nat Commun.8 (2017). https: //doi. org/10.1038/ncomms14345.

[14] Patent WO 2007/039816 A2, PCT/IB2006/002788, Fermentation Process for the preparation of tacrolimus (2007).

[15] C. Barreiro, J. F. Martín, C. García - Estrada, Proteomics shows new faces for the old penicillin producer Penicillium chrysogenum, J Biomed Biotechnol.2012 (2012). https: //doi. org/10.1155/2012/105109.

[16] S. Mo, Y. H. Ban, J. W. Park, Y. J. Yoo, Y. J. Yoon, Enhanced FK506 production in Streptomyces clavuligerus CKD1119 by engineering the supply of methylmalonyl - CoA precursor, J Ind MicrobiolBiotechnol.36 (2009) 1473–1482. https: //doi. org/10.1007/s10295 - 009 - 0635 - 7.

[17] A. Mishra, S. Verma, Optimization of process parameters for tacrolimus (FK 506) production by new isolate of Streptomyces sp. using response surface methodology (RSM), J Biochem Technol.3 (2012) 419–425.

[18] B. P. Singh, B. K. Behera, Regulation of tacrolimus production by altering primary source of carbons and amino acids, Lett Appl Microbiol.49 (2009) 254–259. https: //doi. org/10.1111/j.1472 - 765X.2009.02652. x.

[19] Y. J. Yoon, C. Y. Choi, Nutrient effects on FK - 506, a new immunosuppressant, production by Streptomyces sp. in a defined medium, J Ferment Bioeng.83 (1997) 599–603. https: //doi. org/10.1016/S0922 - 338X (97) 81145 - 2.

[20] J. Turło, W. Gajzlerska, M. Klimaszewska, M. Król, M. Dawidowski, B. Gutkowska, Enhancement of tacrolimus productivity in Streptomyces tsukubaensis by the use of novel precursors for biosynthesis, Enzyme Microb Technol.51 (2012) 388–395. https: //doi. org/10.1016/j. enzmictec.2012.08.008.

[21] W. Gajzlerska, J. Kurkowiak, J. Turło, Use of three - carbon chain compounds as biosynthesis precursors to enhance tacrolimus production in Streptomyces tsukubaensis, N Biotechnol.32 (2015) 32–39. https: //doi. org/10.1016/j. nbt.2014.07.006.

[22] S. C. M. Silva, W. M. Ferrari, J. V. Moreira, T. T. Franco, M. A. Cremasco, Streptomyces tsukubaensis fermentation using Brazil nut oil to enhance tacrolimus production, J Appl Biotechnol Reports.6 (2019) 109–112. https: //doi. org/10.29252/JABR.06.03.05.

[23] S. Jung, S. Moon, K. Lee, Y. J. Park, S. Yoon, Y. J. Yoo, Strain development of Streptomyces sp. for tacrolimus production using sequential adaptation, J Ind MicrobiolBiotechnol.36 (2009) 1467–1471. https: //doi. org/10.1007/s10295 - 009 - 0634 - 8.

[24] D. Karamad, K. Khosravi - Darani, H. Hosseini, S. Tavasoli, A. W. Miller, Evaluation of Oxalobacterformigenes DSM 4420 biodegradation activity for high oxalate media content: An in vitro model, Biocatal Agric Biotechnol.22 (2019). https: //doi. org/10.1016/j. bcab.2019.101378.

[25] A. R. Tripathi, N. K. Singh, U. luhtra, Optimization of Nutrient Components for Tacrolimus Production by Streptomyces tsukubaensis using Plackett - Burman followed by Response Surface Methodology, IOSR J Pharm.4 (2014) 66–75. https: //doi. org/10.9790/3013 - 0407066075.

[26] G. Chen, G. Wang, X. Li, B. Waters, J. Davies, Enhanced Production of Microbial Metabolites in the Presence of Dimethyl Sulfoxide streptomycin (phenotypic suppression, for example) can be removed, and the pellet washed twice by resuspending in Morphological Observations Streptomyces glaucescenswa, J Antibiot (Tokyo).53 (2000) 1145–1153.

[27] Y. H. Ban, S. R. Park, Y. J. Yoon, The biosynthetic pathway of FK506 and its engineering: from past achievements to future prospects, J Ind MicrobiolBiotechnol.43 (2016) 389–400. https: //doi. org/10.1007/s10295 - 015 - 1677 - 7.

[28] M. Ordóñez - Robles, F. Santos - Beneit, J. F. Martín, Unraveling nutritional regulation of tacrolimus biosynthesis in Streptomyces tsukubaensis through omic approaches, Antibiotics.7 (2018) 1–19. https: //doi. org/10.3390/antibiotics7020039.

[29] D. Goranovič, M. Blažič, V. Magdevska, J. Horvat, E. Kuščer, T. Polak, J. Santos - Aberturas, M. Martínez - Castro, C. Barreiro, P. Mrak, G. Kopitar, G. Kosec, Š. Fujs, J. F. Martín, H. Petković, FK506 biosynthesis is regulated by two positive regulatory elements in Streptomyces tsukubaensis, BMC Microbiol.12 (2012). https: //doi. org/10.1186/1471 - 2180 - 12 - 238.

[30] Ordóñez - Robles, M., Rodríguez - García, A., & Martín, J. F. (2016). Target genes of the Streptomyces tsukubaensisFkbN regulator include most of the tacrolimus biosynthesis genes, a phosphopantetheinyl transferase and other PKS genes. Applied Microbiology and Biotechnology (2016), 100 (18), 8091–8103. doi: 10.1007/s00253 - 016 - 7696 - 0.

[31] J. S. Bauer, S. Fillinger, K. Förstner, A. Herbig, A. C. Jones, K. Flinspach, C. Sharma, H. Gross, K. Nieselt, A. K. Apel, dRNA - seq transcriptional profiling of the FK506 biosynthetic gene cluster in Streptomyces tsukubaensis NRRL18488 and general analysis of the transcriptome, RNA Biol.14 (2017) 1617–1626. https: //doi. org/10.1080/15476286.2017.1341020.

[32] D. Huang, M. Xia, S. Li, J. Wen, X. Jia, Enhancement of FK506 production by engineering secondary pathways of Streptomyces tsukubaensis and exogenous feeding strategies, J Ind MicrobiolBiotechnol.40

(2013) 1023–1037. https: //doi. org/10.1007/s10295 - 013 - 1301 - 7.

[33] D. Huang, S. Li, M. Xia, J. Wen, X. Jia, Genome - scale metabolic network guided engineering of Streptomyces tsukubaensis for FK506 production improvement, Microb Cell Fact.12 (2013) 1–18. https: //doi. org/10.1186/1475 - 2859 - 12 - 52.

[34] M. J. Bibb, Regulation of secondary metabolism in streptomycetes, https: //doi. org/10.1016/j. mib.2005.02.016.

[35] 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 MicrobiolBiotechnol.101 (2017) 4581–4592. https: //doi. org/10.1007/s00253 - 017 - 8242 - 4.

[36] R. Kolling, H. Lother, AsnC: An autogenously regulated activator of asparagine synthetase A transcription in Escherichia coli, J Bacteriol.164 (1985) 310–315. https: //doi. org/10.1128/jb.164.1.310 - 315.1985.

[37] S. J. Mo, Y. J. Yoo, Y. H. Ban, S. K. Lee, E. Kim, J. W. Suh, Y. J. Yoon, Roles of fkbN in positive regulation and tcs7 in negative regulation of FK506 biosynthesis in streptomyces sp. strain KCTC 11604BP, Appl Environ Microbiol.78 (2012) 2249–2255. https: //doi. org/10.1128/AEM.06766 - 11.

[38] C. Wang, J. Liu, H. Liu, S. Liang, J. Wen, Combining metabolomics and network analysis to improve tacrolimus production in Streptomyces tsukubaensis using different exogenous feedings, J Ind MicrobiolBiotechnol.44 (2017) 1527–1540. https: //doi. org/10.1007/s10295 - 017 - 1974 - 4.