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STRATEGIES AND APPROACHES FOR ANTIBIOTIC YIELD IMPROVEMENT IN ACTINOMYCETES

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ABSTRACT

Actinomycetes represent a valuable source of commercially significant antibiotics. However, the yields of antibiotics in wild Actinomycetes usually could not meet the commercial needs and so antibiotics yield improvement is a constant requirement in the drug industry. Various methods and strategies such as traditional strain breeding methods and fermentation optimization methods have been tried and some new ideas such as mixed culture and quorum sensing along with empirical approaches are still on the way to gain this ultimate goal. This review will focus on the strategies and approaches for Actinomycetes antibiotics augment.

KEYWORDS: Actinomycetes, antibiotics, strain breeding, fermentation optimization.

INTRODUCTION

Antibiotics as secondary metabolites produced by plants and microbials are a prolific source of clinically valuable natural products such as anti-pathogenic bacteria/fungi, anticancer and antiviral compounds. Actinomycetes is the largest antibiotic-producing genus in the microbial world and about 70% current pharmaceutical antibiotics were produced by Actinomycetes. However, as the increase ofpathogenic microbials antibiotic resistance, new antibiotics are in great demand. Actinomycetes still play crucial role because a great deal of structurally novel compounds with prominent antibiotics potential were discovered from this genus. Nevertheless, the yields of will-be antibiotics in wild strains is usually too low. In order for such natural compounds to become commercial medicine, improvement of production

process is necessary since titers produced by wild strains could never compete with the meets of antibiotic industry. Several strategies and methods were developed for secondary metabolite yield improvement in actinomycetes hitherto. Strain breedingand fermentation process optimizations are chief strategies for overproduction of bioactive secondary metabolites. Several approaches were further included in each strategies (Tab 1). In this article, the merits and shortcomings of the various approaches were reviewed to give readers comparatively comprehensive ideas in this field.

Tab 1 Approaches to improve antibiotics production in Actinomycetes.

1 strain breeding classical random mutagenic breeding chemical induced method physical induced method compound mutagenesis directive screening of mutant strains genetic engineering breeding transfer one or more key enzyme(s) of the biosynthesis pathway transfer positive regulatory gene(s) of the biosynthesis pathway inactivation of negative regulatory gene(s) of the biosynthesis pathway inactivation of accessory pathways of target antibiotic transportation of target toxic antibiotic out of cell expression in heterologous host protoplast fusion
2 Fermentation process optimization optimization of fermentation condition 3 Other approaches mixed culture
quorum sensing

1 strain breeding

Wild Actinomycetes strains typically produce limited quantities (1~100 μg/ml) of secondary metabolites and thus during the subsequently drug development process, there is a need of strain improvement to supply such promising products in significant quantities. Improvement of microbial strains for overproduction of bioactive products has been the hallmark of all pharmaceutical fermentation processes. At present, strain improvement could be achieved chiefly through classical random mutagenic breeding, protoplast fusion breeding and modern genetic engineering technology.

Classical random mutagenic breeding

Classical random mutagenesis continues to be an effective way to find out productive strains and has proven itself many times during the development of antibiotic industry making it the best option for strain improvement hitherto and in the foreseeable future. This approach acquired strain mutation through artificial means which could enhance low spontaneous mutation rate (10-5 to 10-8) more than hundred times with little understanding of related molecular genetic basis and screened forward mutant strain. Artificial means mentioned above mainly include physical treatments, chemical mutagens and compound mutagenesis. Physical mutagens include ultraviolet, X ray, gamma-ray radiation, fast neutrons, laser, micrewave, ion beam *et al.* and ultraviolet was the most frequently used mutagens among these agents. For example, UV irradiation was used to improve saadamycin production in *Streptomyces* sp. and to investigate the multiple pathways for acetate assimilation in *Streptomyces cinnamonensis.* [1, 2] P Rugthaworn obtained 35 improved Actinomycete isolates with anti-phytophatogenic fungi effect by gamma radiation. [3] Frequently adopted chemical mutagens are methylmethane sulfonate (MMS), hydroxylamine (HA), alkylating agents such as diethyl sulfate (DES), ethylmethane sulfonate (EMS) and nitrosoguanidine (NTG).

However, mutant stocks usually become inactivated to sole mutagen (flat-topped effect) in conventional mutagenic breeding. On the contrary, through accumulation and superposition productive strains tend to be acquired because the susceptive loci are not quite similar with different mutagens andtherefor multiple loci could be under susceptibility by a combination of different mutagens. It is widely accepted that compound mutagenesis possess synergistic effect and reasonable combination of mutagen(s) usually bring satisfactory result. The patterns of compound mutagenesis are two or more mutagens successive usage or simultaneous usage and repetition of the same mutagen. For example, UV irradiation and NTG were used to improve the tylosin productivty of *Streptomyces fradiae* strain. [3] And milbemycin-producing *treptomyces bingchenggensis.* [4]

Whichever physical, chemical or compound mutagenesis, techniques that efficiently select mutants with potential high-producing traits (prescreening) are fundamental to reducing efforts that would otherwise be spent in the subsequent screening process. Traditionally, titer testing of a large number of isolates was the screening criterion after mutagenesis. However, the considerable amount of time and efforts required for such a screening approach is a significant shortcoming. New strategies that can complement the traditional method to

increase the overall efficiency will lower both the costs and duration of the commercialization process. Directing screening was introduced to reduce the laborious work. Selection of biochemical mutants resistant to a desired metabolite, especially precusor, is one of the most common directing screening approaches. [5] Generally speaking, Actinomycetes' ability to synthesize antibiotics is closely related to their utilization ability of precusor and therefore, mutant strains selected through added precusor in medium are tend to possess higher antibiotic yield. For example, a strain *Streptomyces pristinaespiralis* with different precusors was obtained, and its production of pristinamycin increased 100 times compared to primitive strain. [6] The production of lincomycin was increased by addition of three amino acids (L-proline, L-tyrosine, L-alanine) which are the precursors of propylproline to the fermentation medium. [7]

Genetic engineering breeding

Although classical methods are still effective even without using genomic information or genetic tools to obtain highly productive strains, these methods are always time-and resource-intensive. [8] Moreover, in the process of classical random mutagenic breeding, manipulations are usually random and blind without specific and clear-out target and sites. As the rapid development of modern molecular biology, the biosynthesis genes of a great deal of important antibiotics have been successfully cloned and identified. Therefore, it is theoretically plausible to control antibiotic yields according to our wishesthrough gene manipulation. Manipulation of the specific biosynthesis/regulatory genes that govern secondary metabolite production is an important aspect of metabolic engineering that can efficiently improve fermentation titers. There are two prerequisites on which the success of any genetic engineering proposal heavily depends: (1) sufficient knowledge of the biosynthesis pathway of target antibiotic, and (2) anefficient set of genetic transformation system to ensure the proposed manipulation. With the great market demand and rapid development of Actinomycetes antibiotics, certain progresses of above mentioned information have been acquired through different researchers. Based on these progresses, antibiotics yield improvement hasbeen achieved by genetic engineering methods. For example: as for the approach of transfer one or more key enzyme(s) of the biosynthesis pathway to increase antibiotics yield, Maharjan S et al increased flaviolin production by expression of acetyl-CoA carboxylase (ACCase) to enhance the carbon flux through acetyl-CoA to malonyl-CoA because malonyl-CoA is the precursor of flaviolin in Actinomycetes. [9] As for the approach of transfer positive regulatory gene(s) of the biosynthesis pathway to

increase antibiotics yield, considering the gene *novE* and *novG* are the putative regulatory genes in the biosynthetic gene cluster of the amino-coumarin antibiotic novobiocin, overexpression of *novE* resulted in an increase of novobiocin formation. [10] The production of moenomycin was improved by overexpressing *relA*, the pleiotropic regulatory genes in streptomycetes. Partial duplication of the *moe* cluster also increased average moenomycin production. [11] In *Actinosynnema pretiosum*, transformants overexpressing *asm2* and *asm39* showed an increase in ansamitocin production. [12]

As for the approach of inactivation of negative regulatory gene(s) of the biosynthesis pathway to increase antibiotics yield, gene disruption of *wblA/spe* from the *S. peucetius* overproducing industrial mutant resulted in an approximately 70% increase in doxorubicin and daunorubicin productivity in that *wblA* gene was a pleiotropic down-regulator of antibiotic biosynthesis in Streptomyces species. [13] Lee K. M. et al discovered that the *sngR*-disruptants showed a 4.6-fold higher production of natamycin than the wild-type strain. The gene *sngR* encodes a c-butyrolactone autoregulator receptor, which has a common activity as DNA-binding transcriptional repressors controlling secondary metabolism and/or morphological differentiation in *Streptomyces.* [14]

As for the approach of inactivation of accessory pathways of target antibiotic, Yang Y.H. et al found that deletion of phosphomannomutase in *S. coelicolor* caused a dramatic increase in actinorhodin production. [15] To improve antibiotic productivity, several studies have been conducted on glycolysis, the main pathway to secondary metabolic processes, by controlling pathway enzymes and carbon flux. [16] For example, deletion of phosphomannomutase, which is important in controlling the flux of carbon into glycolysis, in *S. coelicolor* caused a dramatic increase in actinorhodin production. [17]

Since 1977, protoplast fusion has been developed for genetic recombination and up to today, fusion of Actinomycete protoplasts also induced a high frequency of genetic recombination. Intraspecific fusion of protoplasts of different *S. fradiae* strains allowed stable recombinant strains to be obtained, resulting as well in induction of synthesis of new antibiotics. Beltrametti F et al increased thiazolylpeptide antibiotic GE2270 through protoplast fusion in *Planobispora rosea.* [18] Although certain antibiotics yield increase has been achieved through genetics engineering methods, substantial increase of commercial strains' antibiotics yields is still difficult to be realized. We owe the appearance of so many related reports to novel approach compared to traditional methods. Single or several gene(s) manipulation could not

actually increase Actinomycetes antibiotics in that more studies have showed that the regulation of Actinomycetes antibiotics production is a network mode.

2 fermentation process optimization

For the purpose of antibiotic yield improvement in Actinomycetes, except for strain improvement, fermentation process condition also plays a critical role because it effects the formation, concentration and yield of a particular product. Therefore it is important to consider the optimization of fermentation process in order to maximize the profits from fermentation process. During its development, two lines of progress facilitate the process, one is the development of related user-friendly analytical equipment techniques such as high pressure liquid chromatography (HPLC) and mass spectroscopy which increased detect accuracy, and the second is the knowledge of metabolic analysis which provides a guiding tool in fermentation process optimization.

During the development of fermentation process optimization, conventional technique of one-factor-at-a-time manipulation has been applied as classical method. The advantage of this method lies on its easy and convenience which made it the most popular method for improving fermentation medium and process condition although it was not only time-consuming and expensive, but also failed to consider the interactions between different factors. And concerns to antibiotics producing, optimization of fermentation condition is usually perpetual because new mutant strains are continuously being introduced. Among the conditions, medium components, especially carbon source and nitrogen source usually play important role. Antibiotic biosynthesis in Actinomycetes appears to be regulated in response to nutritional status. [19] One of the most prominent characteristics of the regulation of antibiotic biosynthesis in streptomycetes is its diversity and complexity. Glucose is an excellent carbon source for growth, but a high concentration of glucose usually inhibits the biosynthesis of antibiotics. The basic mechanism(s) of the phenomenon is not understood completely. Moreover, the biosynthesis of antibiotics is also inhibited by rapidly utilized nitrogen sources such as ammonium and regulated by inorganic phosphate. [20] Because the one-factor-at-a-time approach frequently failed to identify the optimal conditions for the bioprocess, other approaches intending to consider multiple factors have been introduced. Factorial design approach is the improvement of one-factor-at-a-time approach and is the most frequently adopted method in optimization of fermentation. In this method, several factors with two or more levels are independently varied and the results of limited

experiments could provide a comparatively optimized fermentation condition. [21] Except for factorial design, central composite design, response surface design and artificial neural network are also the commonly used methods. Optimized conditions including nutritional conditions and physical parameters could be chosen through above mentioned methods.

3 other approaches

Except for strain breeding and optimization of fermentation, other strategies such as mixed culture and quorum sensing have attracted researchers' attention to attain the goal of antibiotics overproduction.

Microbes existed in their natural environments e.g., soil, terrestrial plant surfaces, and marine plant and animal surfaces are presumably under a high microbial burden. Surviving in such competitive environments likely requires strategies. Many microbes synthesize potentially antibiotics as microbial secondary metabolites, against which they protect themselves. Environmental conditions are therefore critical for microbes to the synthesis of microbial secondary metabolites. In this sense, mimicing the natural microbial environments, that is to say, grow them in the presence of other microbes might be an alternative strategy to improve antibiotics yields. Competition for limiting natural resources is believed to be the selective force that promotes biosynthesis of biologically active compounds. Several reports of mixed fermentation have showed the ability of added microbes to increase antibiotics yields or kinds. [22]

Quorum sensing (QS) is a regulatory system that allows geneexpression to be controlled in response tosmall diffusible signalingmolecules produced and released by bacteria. Quorum sensing was also observed among many Actinomycetes, as an important regulatory mechanism of secondary metabolite biosynthesis and/or cell differentiation. Up to now, several specific products of Actinomycetes have been identified as cell-signaling molecules which modulate the secondary metabolic activitiesin streptomycetes, probably by the mode of quorum sensing. [23, 24]

Conclusions and prospects for the future

Antibiotics produced by Actinomycetes have already played a pivotal role in human medicine and various strategies have been exploited to augment its yields in order to meet the market demand. However, each strategy, with its specific shortcoming, could only increase the yield of antibiotics to certain degree. For instance, although classical random mutagenic breeding

methods have succeeded in generating many industrial strains, they are time-consuming and high-cost processes. The development of molecular microbiology and recombinant DNA technology has led to a number of strategies for rational strain improvement known as genetic engineering breeding. However, the goal of genetic engineering breeding method was never fully achieved. As for fermentation process optimization method, systematic approaches have been applied to design suitable media and physical parameters. Multiple physical parameters including pH, temperature, dissolved oxygen, rotation speed, metal ion, nutrition composition and concentration, were all under consideration. These parameters might differ with different strain fermentation and even vary in different phase of the same strain. Therefore, it was theoretically plausible but actually difficult to achieve. Application of mixed fermentation or quorum sensing to antibiotics production augment seems obvious alternatives, but the fields are in their infancy, probably because of early fears of lack of reproducibility and sufficient knowledge. Nevertheless, continuous endeavours have been made to increase Actinomycetes antibiotics in various aspects and notable achievement has been gained. In the future, with countless possible knowledge accumulation in Actinomycetes growth dynamics, metabolic pathway, metabolic regulation, antibiotic gene cluster, the relationship with their niche and unexpected progress in other branches, the potential of Actinomycetes antibiotics yields meet production requirement seems quite promising.

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