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Genetic Engineering of *Streptomyces natalensis* VTCC-A-3245 to Improve Its Natamycin Production

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Abstract: Natamycin, a polyene compound with broad-spectrum activity against yeasts and fungi, was firstly found in *Streptomyces natalensis*. Because of its low toxicity to mammalian cells, natamycin is widely used in food industry and medicine to prevent fungal growth. Although natamycin has been used worldwide, this antifungal compound has not been produced in Vietnam. One of the reasons is that we do not own any industrial-scale production strain. In order to develop such production strain, strain improvement must be involved. Therefore, we carried out the study "Genetic engineering of *Streptomyces natalensis* VTCC-A-3245 to improve its production of natamycin". In this study, we introduced a copy of the gene *pimM*, a positive regulator gene in the natamycin biosynthetic pathway, into *S. natalensis* chromosome, hence boosting the expression of the structural genes, resulting in the increase of natamycin production. As a result, a recombinant pSET152 plasmid containing *pimM* was constructed and transformed successfully into *E. coli* ET12567. After conjugation, a *S. natalensis* mutant carrying an additional copy of *pimM* was obtained. The result showed that the level of natamycin produced by the *S. natalensis* mutant strain increased 3 fold compared to the *S. natalensis* wildtype strain.

Keywords: Streptomyces natalensis, natamycin, strain improvement, pimM.

1. Introduction

The actinomycetes are a large group of gram-positive bacteria which are characterized by the high G + C content in their DNA [1]. Actinomycetes are known as an important group of microorganisms because they provide large amounts of secondary metabolites including antibiotics, anti-fungal, and anti-cancer agents which have significant applications in agriculture, clinic and industry[2]. Among them, natamycin, an antifungal compound, has been used world-wide in food industry and medicine [3]. Natamycin was first isolated from *Streptomyces natalensis* in 1955 [4]. Natamycin is a polyene macrolide with the molecular formula of $C_{33}H_{47}NO_{13}$ and a molecular weight of 665.75 [5]. Natamycin shows broad-spectrum activity against yeasts, fungi and is able to inhibit aflatoxin production [3]. Natamycin is believed to bind with ergosterol, the primary sterol in fungal cell membranes and inhibit amino acid and glucose transport across the plasma membrane [6].

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In general, wild type strains isolated from nature usually produce only a low level of bioactive compounds (1~100 µg/ml) [7]. Therefore, strain improvement is very important to produce the industrial- scale production strains. Classical methods involving random mutation by physical or chemical mutagens are considered labor-intensive [8]. Meanwhile, genetic engineering for strain improvement has created new opportunities to engineer microorganisms for the production of natural products with high yields. The secondary metabolites can be increased by several approaches such as: engineering regulatory network, genome shuffling and expression of secondary metabolite genes in heterologous hosts. As a result, the secondary metabolites may be enhanced from 2 to several 10 folds, even to 100 folds [9]. One of a powerful tool to enhance the production of bioactive substances in *Streptomyces* is to introduce positive regulator genes into its genome by intergeneric conjugation from *E. coli* [10]. In this method, based on the capable of conjugal transfer from *E. coli* to *Streptomyces*, plasmids containing DNA fragment can be integrated into *Streptomyces* chromosome either site-specifically at the ϕC31 or pSAM2 attachment sites or via insert-directed homologous recombination [11]. This method is considered simple and does not require protoplast preparation. Besides, there are a variety of vectors that have been developed that permit site-specific or insert-directed chromosomal integration. Moreover, these vectors replicate in *E. coli*, hence, the production of required constructs is considerably facilitated [12].

The sequence of the natamycin biosynthetic gene cluster has been published with 18 open reading frames spanning 84 985 bp of the *S. natalensis* genome. This cluster includes 13 polyketide synthase (PKS) modules and 13 additional proteins that presumably govern post-PKS modification of the polyketide skeleton, export and regulation of gene expression [13]. In this study, we chose to introduce another copy of a positive regulator gene of the natamycin biosynthetic gene cluster, *pimM*, into *S. natalensis* chromosome, hence boosting the expression of the structural genes, resulting in the increase of natamycin production.

2. Materials and methods Microorganisms

Strain *Streptomyces natalensis* VTCC-A-3245 (= JCM 4693) was obtained from the Vietnam Type Culture Collection (VTCC), Institute of Microbiology and Biotechnology (IMBT), Vietnam National University, Hanoi. Indicator strain, *Saccharomyces cerevisiae* VTCC-Y-62, was also obtained from VTCC. *E. coli* DH5α and *E.coli* ET12567 [pUZ8002] were a gift from Prof. Takuya Nihira (Osaka University, Japan).

Extraction of genomic DNA from S. natalensis

S. natalensis cells from 3 ml of culture broth was lysed with 0.2 ml lysis buffer (100 mM Tris HCl, 100mM Na_2EDTA, 1.5 M NaCl, 1% cetyltrimethyl ammonium bromide , pH 8.0), 50 µl lysozyme (30 mg/ml) and 50 µl SDS 20% at 65^oC for 2 hours. The mixture was centrifuged at 8,000 g for 10 min, the supernatant was then collected and added an equal volume of chloroform: isoamyl alcohol (24 : 1). After centrifugation at 16,000 g for 5 min, the upper phase was transfer into a new tube. This step was repeated three times. One volume of isopropanol was added, DNA was precipitated by centrifugation and resuspended in 50 µl water. RNA was removed by RNase.

Amplification of pimM

The *pimM* gene was amplified from the genomic DNA of strain *S. natalensis* by PCR with primers PMD (5'-TCCTGGATCCGCCCTGTGCCCGCTCACT TCACGAAG-TCG-3') and PMR (5'-GGTTGGATCCTTGCGGTCGGTGGTGC-GGGCATTACGG- 3'). *Bam*HI restriction sites were underlined. The PCR condition was 95°C,

5 min; 30 cycles of 95°C, 30 s, 62°C, 15 s and 72°C, 1 min 30 sec and a final extension cycle at 72°C, 7 min, then stored at 4°C until electrophoresed and tested on gel agarose 1%.

Construction of recombinant plasmid pSETpimM

Plasmid pSET152 and *pimM* PCR product were digested with *Bam*HI restriction enzyme. The reaction contained 28 µl template, 10 µl 10X buffer, 2 µl *Bam*HI and 60 µl H_2O. Incubated at 37°C, overnight. *Bam*HI digested-*pimM* and pSET152 were ligated by T4 ligase. The ligated plasmid was transformed into *E. coli* DH5α by heat-shock at 42°C for 60 second. The *E. coli* DH5α colonies containing recombinant plasmid pSETpimM were screened and selected by apramycin (Apr) as well as blue/white colonies using IPTG and X-gal. The recombinant plasmid pSETpimM was extracted and submitted to sequencing.

Intergeneric conjugation between E. coli ET 12567 [pUZ8002] containing pSETpimM and S. natalensis

The recombinant plasmid pSETpimM was transformed into *E. coli* ET 12567 by heat-shock at 42°C for 60 second. The *E. coli* ET12567 [pUZ8002] colonies containing recombinant plasmid pSETpimM were screened and selected by Apr, kanamycin and chloramphenicol as well as blue/white colonies using IPTG and X-gal**.** The selected colonies were was checked by PCR to confirm the presence of *pimM*. Then the *E. coli* ET 12567 strain containing pSETpimM was used for the conjugation experiments. The donor *E. coli* ET 12567 [pUZ8002] containing pSETpimM grown in 20 ml LB with glucose to an OD_{600} of 0.61 at 37°C. The cell was collected by centrifugation, washed twice, and resuspended in 500 µl of LB, kept on ice. For each conjugation reaction, 10^7 *S. natalensis* spores were added to 500 µl 2×YT broth (tryptone 16 g, yeast extract 10 g, NaCl 5 g, water 1L), incubated at 45°C for 10 min, then kept on ice. After that, the *E. coli* and the *S. natalensis* spores were mixed together, left at room temperature for 10 min. The cell pellet was then collected by centrifugation, resuspended in 50 µl residual liquid and spread on dried MS agar plates (mannitol 20 g, soya flour 20 g, tap water 1 L, agar 20 g) supplemented with 10mM $MgCl_2$. Plates were incubated at 30°C for 16-20 h, and then overlaid with 0.5 mL of sterile water containing 500 µg nalidixic acid and 10 µl of Apr (50 mg/ml. After that, plates were incubated further for 7–10 days until actinomycete colonies appeared. The exconjugants were streaked on YS plates containing 20 µl Apr (50 mg/ml) and 20 µl nalidixic acid (25 mg/ml) for selection. The intergration of the plasmid into the *Streptomyces natalensis* genome was confirmed by the amplification of *Apr* gene by PCR.

Comparison of the level of natamycin production in mutant strains and wild type strain

A mutant strain and the wildtype strain were cultured in natamycin production medium (glucose 60, soybean meal 10, peptone 5, yeast extract 5, beef extract 5, NaCl 2, $CaCO_3$ 5, $MgSO_4$ 1 g/l) shaked at 160 rpm, 30°C for 4 days. Natamycin was extracted within the same volume of n-butanol. The amounts of natamycin in the two samples were compared using agar diffusion assay with *Saccharomyces cerevisiae* VTCC-Y-62 as the testing organism. The assay was performed at 30°C and the diameters of the inhibition zones were recorded after 24 h. In addition, natamycin production was quantified by HPLC using Cadenza C18 column (3 µm, 75 × 4.6 mm) (Imtakt, USA) and an increased gradient of acetonitrile. The detection wavelength was set at 304 nm.

3. Results and discussion

Amplification of pimM from S. natalensis genomic DNA

pimM and its promoter (~1 kb) were amplified from the genomic DNA of *S. natalensis* by PCR. The PCR reaction was performed as described in the method section with primers PMD and PMR. The PCR product was analyzed by electrophoresis on 1% agarose (Figure 1). The result showed that an 1 kb PCR product was obtained as expected.

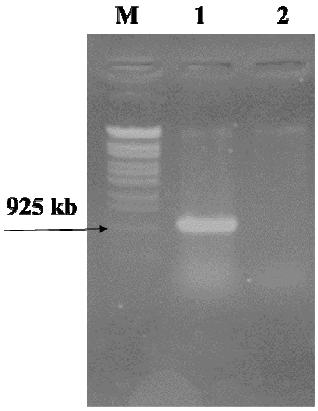

Figure 1. Agarose gel electrophoresis of *pimM* PCR product.
M: λ Marker; **1**: *pimM*; **2**: Negative control

Construction of the recombinant vector pSETpimM

The *pimM* PCR product and the pSET152 plasmid were treated with *Bam*HI restriction enzyme. The recombinant vector pSETpimM was constructed by ligation of *Bam*HI-digested *pimM* and *Bam*HI-digested pSET152. The ligated vector pSETpimM was then transformed successfully into *E. coli* DH5α strain by heat-shock method. One white colony was selected and grown in LB medium containing apramycin. The transformed plasmid was extracted and checked on agarose gel electrophoresis in order to check the presence of the recombinant plasmid pSETpimM (Figure 2). As expected, the recombinant plasmid pSETpimM (lane 2) was bigger than the original plasmid pSET152 (lane 1), proving the presence of the insert in the vector.

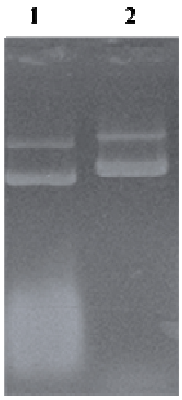

Figure 2. Agarose gel electrophoresis of pSETpimM plasmid extracted from transformed *E. coli* DH5α colony. **1**: Control - pSET152; **2**: pSETpimM

In addition, in order to confirm the correct sequence of the inserted *pimM*, the plasmid pSETpimM was sent for sequencing. The sequence result showed 100% identity to *S. natalensis pimM* gene (AM493721.1) using BLAST search. This result indicated that there was no mutation in the inserted *pimM* gene.

Conjugation of E. coli ET 12567 [pUZ8002] containing pSETpimM and S. natalensis

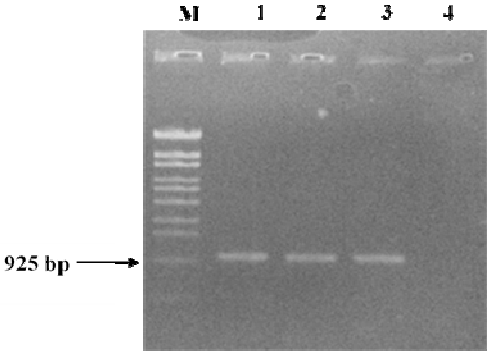

Figure 3. Agarose gel electrophoresis of *pimM* PCR product from transformed *E. coli* ET12567 [pUZ8002] colonies.
M: λ Marker
Lane 1: Positive control (pSETpimM as template)
Lane 2: *pimM* PCR product from colony 1
Lane 3: *pimM* PCR product from colony 2
Lane 4: Negative control (pSET152 as template)

The recombinant vector pSETpimM was then transformed into *E. coli* ET12567 [pUZ8002] by heat-shock method. The presence of the recombinant vector pSETpimM in the selected colonies was test by amplification of *pimM* with primers PMD and PMR. The PCR product was checked on agarose gel electrophoresis (Figure 3). Both selected colonies (lane 2 and 3) showed a clear DNA band similar to the positive control. Therefore, pSETpimM was successfully transformed into *E. coli* ET12567 [pUZ8002].

In order to introduced an additional copy of *pimM* gene into the *S. natalensis* chromosome, pSETpimM-containing *E. coli* ET12567 [pUZ8002] was conjugated with *S. natalensis* spores. By using 10^7 *S. natalensis* spores per each conjugation experiment, there were approximately 80 colonies grown on the MS agar plate after 5 days (Figure 4).

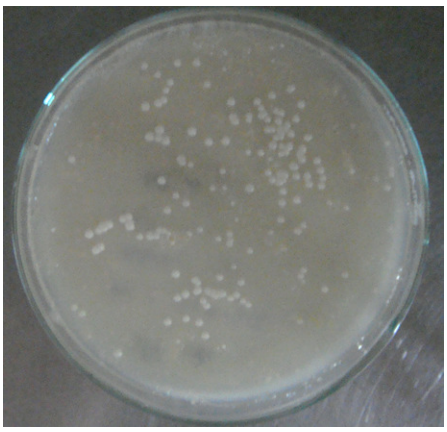

Figure 4. The exconjugants appeared on MS agar.

Screening of the mutant strains within an additional a copy of pimM gene

An exconjugant colony was selected and grown in YS medium. The insertion of an additional copy of *pimM* gene into the *S. natalensis* chromosome was checked by amplification of the *Apr* gene (~ 1 kb) by PCR. The PCR product was tested on agarose gel electrophoresis (Figure 5). The result showed that the *S. natalensis* mutant strain contained *Apr* gene, indicating the successful insertion of the pSETpimM plasmid into *S. natalensis* genomic DNA.

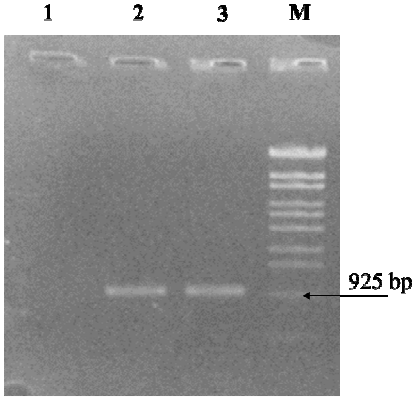

Figure 5. Agarose gel electrophoresis of *Apr* gene PCR product from transformed colonies.
Lane **1**: Negative control (*S. natalensis* genomic DNA as template)
Lane **2**: Mutant strain (*S. natalensis* mutant genomic DNA as template)
Lane **3**: Positive control (pSET152 as template)
M: λ Marker.

Evaluation of natamycin production in mutant and wild type strains

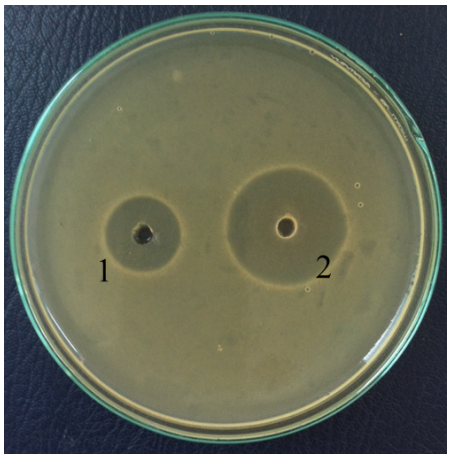

Figure 6: Agar diffusion assay using *Saccharomyces cerevisiae* as the testing strain
1: *S. natalensis* wild type strain; 2: *S. natalensis* mutant strain

In order to check the natamycin production of *S. natalensis* wild type and *S. natalensis* mutant strains, agar diffusion assay using *Saccharomyces cerevisiae* as the testing strain was performed. The result showed that the diameter of the inhibition zone by *S. natalensis* wild type strain was 17 mm while the diameter of the inhibition zone by *S. natalensis* mutant strain was 28 mm. This result proved that *S. natalensis* mutant strain produced a higher level of natamycin compared to *S. natalensis* wild type strain (Figure 6).

However, this agar diffusion assay could not provide quantitative data. Therefore, natamycin was extracted and quantitated by HPLC method. The HPLC result was shown in Figure 7. The retention time of natamycin was 19.5 minute and the natamycin peak had the typical UV-visible wavelength absorption profile of a polyene compound with three maximum absorption wavelength of 240, 304, 360 nm (Figure 8). The area under the curve of natamycin peak in two samples was calculated, showing that the mutant strain produced higher amount of natamycin than wild type strain by 3 folds.

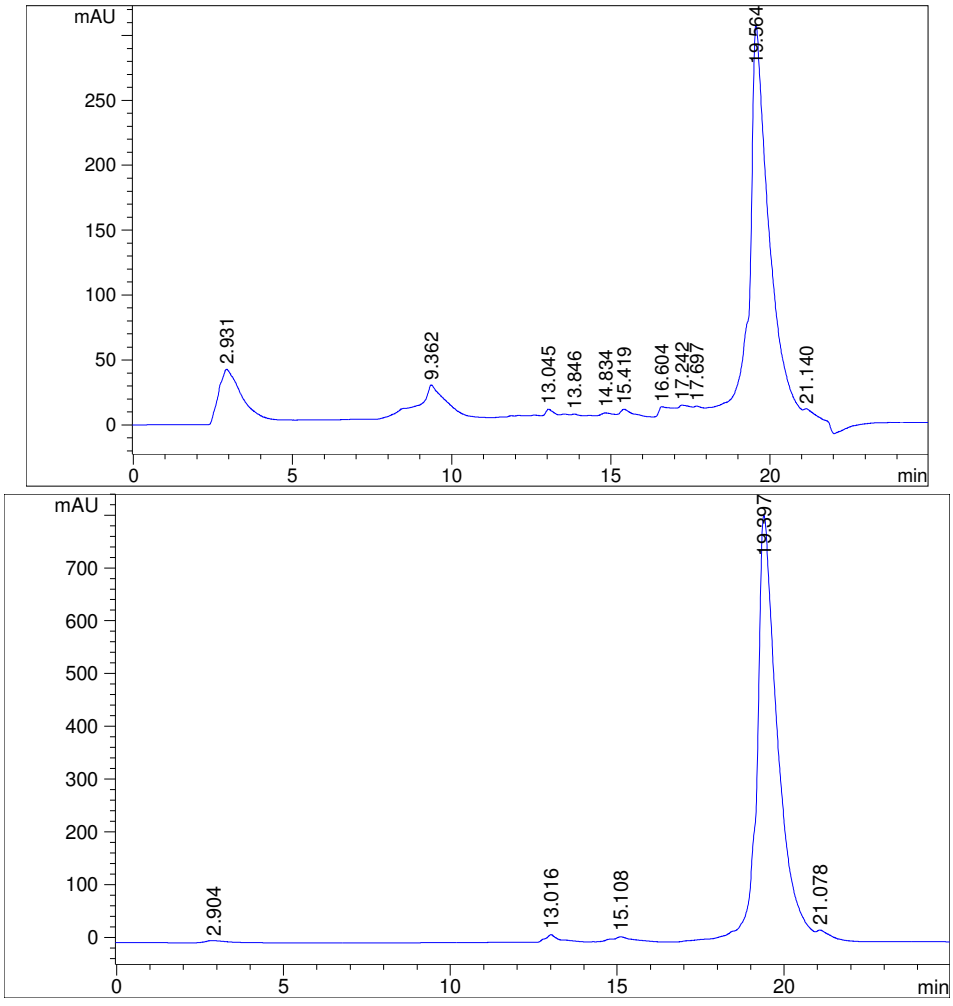

Figure 7. HPLC profiles of butanol-extracted broths from wild type *S. natalensis* (top) and *S. natalensis* transformed with pSETpimM (bottom).

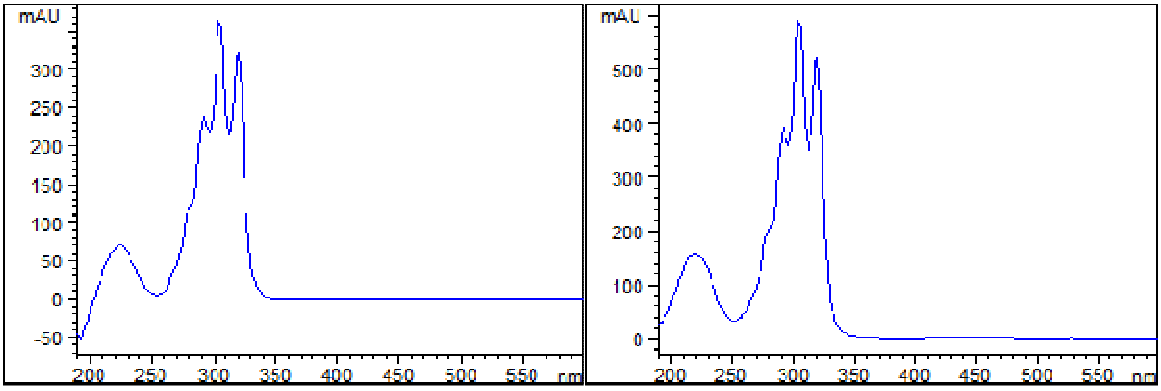

Figure 8. Absorption profile of natamycin peak from wild type *S. natalensis* (left) and *S. natalensis* transformed with pSETpimM (right).

In comparison with the reference from Antón et al. (2007), the increase in natamycin production by introducing a copy of pimM to the genome of S. natalensis ranged from 2.4 folds after 48 h of growth to 1.5 folds after 96 h of growth [13]. Therefore, a higher increase (3 folds) in natamycin yield was obtained in the S. natalensis mutant strain in this study. This result once again confirmed that pimM is a positive regulator of the natamycin biosynthesis pathway. However, in order to produce a producing strain, other genetic modifications should be applied to increase the yield of natamycin produced by S. natalensis.

4. Conclusions

In this study, gene *pimM*, a positive regulator gene in the natamycin biosynthetic pathway, was amplified from *S. natalensis* chromosome. A recombinant pSET152 plasmid containing *pimM* (pSETpimM) was constructed and transformed successfully into *E. coli* ET12567. By conjugation of pSETpimM-containing *E. coli* ET12567 and *S. natalensis* spores, a copy of the gene *pimM* was introduced into *S. natalensis* chromosome. As a result, a *S. natalensis* mutant carrying an additional copy of pimM was obtained and the level of natamycin produced by the *S. natalensis* mutant strain increased 3 folds compared to the *S. natalensis* wild type strain.

Acknowledgements

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improvement of an antibiotic-overproducing industrial strain of *Streptomyces albus*". *Applied and environmental microbiology*. 69(11): p. 6412-6417.

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Cải biến di truyền chủng *Streptomyces natalensis* VTCC-A-3245 nhằm tăng khả năng sinh hoạt chất Natamycin

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Tóm tắt: Natamycin, một hợp chất dạng polyene có khả năng kháng nấm sợi và nấm men, được tìm thấy lần đầu tiên từ loài *Streptomyces natalensis*. Bởi vì natamycin ít gây hại cho tế bào động vật nên natamycin được sử dụng rộng rãi trong bảo quản thực phẩm và y học. Mặc dù natamycin đang được sử dụng phổ biến trên thế giới, hợp chất này chưa được sản xuất ở Việt Nam. Một trong các lý do là bởi vì Việt Nam chưa sở hữu chủng sản xuất ở quy mô công nghiệp. Để tạo được một chủng sản xuất như vậy, các bước cải biến di truyền cần được thực hiện. Vì vậy, chúng tôi thực hiện nghiên cứu "Cải biến di truyền chủng *Streptomyces natalensis* VTCC-A-3245 nhằm tăng khả năng sinh hoạt chất natamycin". Trong nghiên cứu này, một bản của gen điều hòa dương *pimM* của con đường sinh tổng hợp natamycin được chèn thêm vào hệ gen của chủng *S. natalensis* nhằm tăng quá trình phiên mã của các gen cấu trúc, dẫn đến tăng lượng hoạt chất natamycin sản sinh. Kết quả cho thấy một vector tái tổ hợp pSETpimM đã được xây dựng và biến nạp thành công vào chủng *E. coli* ET12567. Bằng cách tiếp hợp chủng *E. coli* ET12567 chứa vector tái tổ hợp pSETpimM với bào tử chủng xạ khuẩn *S. natalensis*, một chủng *S. natalensis* cải biến VTCC-A-3245 có chứa thêm một bản của gen *pimM* vào hệ gen được chọn lọc. So sánh khả năng sinh natamycin giữa chủng tự nhiên và chủng cải biến cho thấy chủng cải biến có lượng natamycin cao hơn chủng hoang dại 3 lần.

Từ khóa: Streptomyces natalensis, natamycin, cải biến di truyền, *pimM*.