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Improvement of Natamycin Production by Cholesterol Oxidase Overexpression in *Streptomyces gilvosporeus* [S]

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Natamycin is a widely used antifungal antibiotic. For natamycin biosynthesis, the gene *pimE* encodes cholesterol oxidase, which acts as a signalling protein. To confirm the positive effect of the gene *pimE* on natamycin biosynthesis, an additional copy of the gene *pimE* was inserted into the genome of *Streptomyces gilvosporeus* 712 under the control of the *ermE** promoter (p_{ermE}*) using intergeneric conjugation. Overexpression of the target protein engendered 72% and 81% increases in the natamycin production and cell productivity, respectively, compared with the control strain. Further improvement in the antibiotic production was achieved in a 1 L fermenter to 7.0 g/l, which was a 153% improvement after 120 h cultivation. Exconjugants highly expressing *pimE* and *pimM* were constructed to investigate the effects of both genes on the increase of natamycin production. However, the co-effect of *pimE* and *pimM* did not enhance the antibiotic production obviously, compared with the exconjugants highly expressing *pimE* only. These results suggest not only a new application of cholesterol oxidase but also a useful strategy to genetically engineer natamycin production.

Keywords: Natamycin, cholesterol oxidase, *Streptomyces gilvosporeus*, intergeneric conjugations

Introduction

Natamycin, also known as pimaricin, is a widely used polyene antifungal antibiotic [19]. Its antifungal activity is achieved by its ability to bind to steroidal components in the fungal cell membrane, leading to the leakage of cellular materials [23]. Natamycin is a very potent inhibitor of yeast and mold growth as well as of aflatoxin synthesis. Therefore, natamycin plays an important role in the treatments of many fungal diseases, such as broncho-pulmonary aspergillosis [6] and mycotic keratitis.

Natamycin is considered to be effective on a broad spectrum of fungi. It is highly effective at low doses against yeast and molds, but displays low toxicity to mammalian cells. Globally, natamycin is also a widely permitted food additive that is being legally used in over 50 countries, including the USA and Europe, for the safe preservation of a wide variety of food products, such as cheese, fermented meat, yogurt, beverages, wine, and baked foods, against yeasts and molds [4, 5, 17]. It is one of the very few antibiotics that have been recommended as food additives and belong to the Generally Regarded As Safe list of compounds by the US Food and Drugs Administration. Hence, this antibiotic has considerable commercial value.

Natamycin is produced in submerged culture under aerobic conditions by *Streptomyces natalensis* [14], *Streptomyces chattanoogensis* [7], and *Streptomyces gilvosporeus* [13]. Current industrial concerns include a significant improvement of its production to meet increasing commercial demand. Recently, the polyene macrolide antibiotic biosynthetic gene cluster was characterized [3]. Consequently, increasing attention has been given to genetic manipulation as a good

Table 1. Microbial strains and plasmids used in this study.

Microbial strains and plasmids	Relevant description	Source or references
E. coli DH5α	General cloning host	This study
E. coli ET12567 (pUZ8002)	Non-methylating donor for all intergeneric conjugations	[8]
pUZ8002	Nontransmissible *oriT* mobilizing plasmid with a *tra* gene	[8]
pSET152	Integrative plasmid, phage ΦC31 integrase (*int*) gene, attachment (*attP*) functions, apramycin resistace gene	This study
pUC57	The sourse of an erythromycin resistance gene promoter (p_{ermE*})	Shanghai Sangon Biotechnology Co. Ltd

approach to significantly improve the production of polyene antibiotics. As an activator, exconjugant overexpressing *pimM* showed a 240% increase in natamycin production of *S. natalensis* [12]. A significant enhancement in natamycin production was recently obtained by a stable integration of a *Vitreoscilla* hemoglobin gene (*vgb*) into the chromosome of an industrial natamycin-producing strain of *S. gilvosporeus* [24].

Cholesterol oxidase (PimE or ChoD), an extracellular enzyme produced by *Streptomyces*, is encoded by the gene *pimE*, which is one of the genes in the natamycin biosynthetic gene cluster. This enzyme from either endogenous or exogenous origin can restore the ability of natamycin production in a *pimE* mutant as a signalling protein [15]. PimE has been widely used in many areas of medical and agricultural research, with the analysis of serum cholesterol as the first addressed application [9]. This enzyme has been used as an insecticidal protein active against boll weevil larvae and other insect larvae [18]; however, limited information is available on PimE in the biosynthesis of any antibiotic. PimM (encoded by the gene *pimM*) is a positive regulator, which may regulate the expression of natamycin biosynthesis genes [2]. In this study, we constructed, for the first time, a recombinant strain overexpressing PimE by intergeneric conjugation to enhance natamycin production. Furthermore, PimE and PimM were highly expressed simultaneously to investigate their co-effects on antibiotic production.

Materials and Methods

Microbial Strains and Plasmids

Table 1 lists the microbial strains and plasmids used in this paper. The natamycin-producing strain, *S. gilvosporeus* 712, was mutated by ultraviolet irradiation and collected in our laboratory.

Effects of Supplementation with Escalating Concentrations of PimE

Commercial PimE (10.5 U/mg; *Brevibacterium*, USA) was added at the beginning of culture, at concentrations ranging from 1 to 50 mg/l, and its effect on natamycin production was studied [15].

Construction of Recombinant Plasmid and Strain

Standard genetic techniques with *Escherichia coli* and in vivo/in vitro DNA manipulations were performed [20]. Primer synthesis and DNA sequencing were carried out at Shanghai Sangon Biotechnology Co. Ltd. A 0.25 kb fragment that contained the *ermE** promoter and a 1.65 kb fragment that contained the entire *pimE* sequence without its native promoter were amplified by PCR (primer sequences are shown in Table 2). The gene *pimE* was fused after p_{ermE*}* via SOE PCR [11]. The p_{ermE*}*–*pimE* fragment after digestion was inserted into the XbaI sites of plasmid pSET152 to generate the recombinant vector pSET152-*pimE*. Plasmid pSET152-*pimE* was introduced into *S. gilvosporeus* 712 by the intergeneric conjugation from *E. coli* ET12567, following a regular protocol [10], to yield *S. gilvosporeus*–*pimE* (named *S. gilvosporeus* swjs-801). Meanwhile, plasmid pSET152 was integrated into the *S. gilvosporeus* 712 genome as the control, which was named *S. gilvosporeus*-pSET152.

For construction of a high-expression PimE and PimM vector, a

Table 2. Primer sequences of p_{ermE*}* and *pimE.*

Target gene	Primer sequences (5′-3′)	Amplicon size (bp)
p_{ermE*}	F: caggtcgact<u>ctagag</u>acgtccatgcgagtgtcc	221
	R: tgctggttctcgaacattggatcctaccaacc	
pimE	F: ggttggtaggatccaatgttcgagaaccagca	1,694
	R: gct<u>ctagac</u>tagt<u>ggtggtggtggtggtggtg</u>ataggcggccttgacgt	

1.0 kb *pimM* fragment including the whole ORF and its native promoter was inserted into the EcoRI and XbaI double-digested pSET152, generating the plasmid pSET152-*pimM*. Then a 1.9 kb p_{ermE}*–*pimE* fragment was brought into the XbaI-digested pSET152, generating the plasmid pSET152-*pimM*-*pimE*. Then, pSET152-*pimM*-*pimE* was introduced by conjugation into the original strain, *S. gilvosporeus* 712 (yielding *S. gilvosporeus-pimE-pimM*), as described above.

Production of Natamycin in Fermentation Culture

Spores of *Streptomyces* were harvested on MS (20 g/l mannitol, 20 g/l soya flour) agar medium. For fermentation, ~1 cm^2 agar pieces of *S. gilvosporeus* 712, *S. gilvosporeus*-pSET152, and *S. gilvosporeus* swjs-801 were activated in the seed medium (10 g/l glucose, 5 g/l peptone, 3 g/l yeast extract, and 3 g/l malt extract) for 20 h at 29°C. The obtained cells were inoculated into 30 ml of fermentation medium (20 g/l peptone, 4.5 g/l yeast extract, 2 g/l NaCl, 1 g/l MgSO$_4$, and 60 g/l glucose) in a 250 ml Erlenmeyer flask at a rate of 5% (v/v), and cultured for 120 h at 29 °C and 220 ×*g*. Aliquots were transferred to 700 ml of fermentation medium in a 1 L fermenter (INFORS, Switzerland), and grown at 29°C for 120 h with the aeration rate of 1 vvm and at least 30% dissolved oxygen, relevant to the agitation rate adjustment. Antifoaming agent was added once needed at a rate of 0.5% (v/v), and the glucose level was monitored at 20−30 g/l throughout the fermentation process by glucose feeding.

Comparison of natamycin production between *S. gilvosporeus* swjs-801 and *S. gilvosporeus-pimE-pimM* was conducted in the shake flask cultures as described above.

Detection of Protein Expression with His$_6$-tag

A His$_6$-tag sequence was added in the gene *pimE* for the detection of the protein. *S. gilvosporeus* 712 and *S. gilvosporeus* swjs-801 were cultured in shake flasks for 3 days. Subsequently, 10 ml of the culture broth was centrifuged for 20 min at 10,000 ×*g*. The supernatant was concentrated 100 times as an SDS-PAGE sample, which was then fractioned on a 10% polyacrylamide gel. Protein was detected using a western blot assay, with a mouse anti His$_6$-tag antibody (CW0286; CWBIO, China) and goat anti-mouse IgG conjugated with horseradish peroxdase (CW0102; CWBIO).

Analytical Methods

Preliminary screening of exconjugants was bioassayed using *Candida albicans* as the test fungi. Clarified supernatants (1 ml) from shake flask or bioreactor cultures (obtained by centrifugation at 10,000 ×*g* for 20 min) were used for the determination of the PimE activity. The PimE activity was determined using the hydrogen peroxide colorimetric method of Allain *et al.* [1]. Dry cell weight was assayed by washing the remaining cell pellets twice with distilled water, followed by drying to constant weight at 80°C. For natamycin determination, the culture broth (1 ml) was ultrasonically extracted for 20 min using 9 ml of absolute
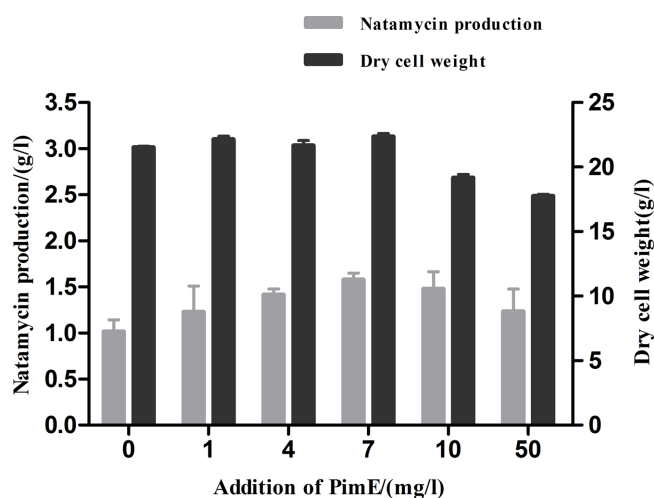
Fig. 1. Effect of commercial cholesterol oxidase addition on natamycin production by *S. gilvosporeus* 712 (values are the mean ± standard deviation, *n* = 3).

methanol and then centrifuged at 10,000 ×*g* for 10 min. HPLC analysis of natamycin was carried out using the method of Wang *et al.* [24].

Results and Discussion

Effects of Supplementation with Escalating Concentrations of PimE on Natamycin Production by *S. gilvosporeus* 712

Three parallel cultures were conducted to investigate the effects of supplementation with varying (1, 4, 7, 10, and 50 mg/l) PimE levels on natamycin production by *S. gilvosporeus* 712. As shown in Fig. 1, main culture supplementation with PimE stimulated natamycin production compared with the control. Initially, antibiotic production rose with increasing levels of PimE supplementation, thereby achieving peak natamycin production of 1.58 g/l at 7 mg/l PimE, which indicated 56.4% more natamycin production compared with the control. Further supplementation with PimE resulted in a reduced antibiotic production, although the accumulation of natamycin as well as cell productivity was still higher than that in the control. Cell growth was not affected by PimE supplementation at 1−7 mg/l, but higher levels resulted in progressively lower cell growth. These results collectively confirmed that the expression of PimE was insufficient for natamycin biosynthesis in *S. gilvosporeus* 712, and prompted us to investigate whether the homologous PimE can produce better effects.

Construction of *S. gilvosporeus–pimE* and *S. gilvosporeus-pimM-pimE*

Exconjugants were selected after at least three cycles of

single colony purification, and PCR verified the successful insertion in the plasmids pSET152-*pimE* and pSET152-*pimM-pimE*. Furthermore, the exconjugants have remained stable throughout several generations. The strain with the largest bacteriostatic ring during bioassay was then used in the experiments.

Detection of PimE-His$_6$

To distinguish the endogenous PimE from the recombinant PimE in *S. gilvosporeus*, the inserted gene (*pimE*) was tagged with a His$_6$-Tag. The *pimE* gene encompasses a 1,650 bp DNA and encodes 549 amino acids. As shown in Fig. 2, the PimE-specific band at the predicted molecular mass (60 kDa) was detectable in lane 2, but not in the control line, thereby confirming the expression of PimE in *S. gilvosporeus* swjs-801.

Effects of *pimE* Overexpression on Natamycin Production in Shake-Flask Cultures

To investigate the effects of *pimE* overexpression on natamycin production and kinetics of cell growth, *S. gilvosporeus* 712, *S. gilvosporeus*-pSET152, and *S. gilvosporeus* swjs-801 were cultured in 250 ml shake flasks of a 30 ml working volume for 120 h. *S. gilvosporeus*-pSET152, loaded with null plasmid, did not display any significant difference in cell growth and natamycin production compared with the control strain, which presented similarly as *Streptomyces* sp. US 24 and *S. gilvosporeus-vgb* [22, 24] (data not shown). Cell growth (Fig. 3B) and sugar consumption (Fig. 3C) curves were also similar to the counterparts in PimE-overexpressing and control strains, suggesting that the integration of the gene *pimE* did not affect growth or antibiotic biosynthesis. In the late stage of fermentation, natamycin production soared beyond expectation probably because of the tardy growth of the cells. However, the recombinant strain displayed a noteworthy increase in natamycin production (72% (p < 0.05); Fig. 3A) and bioproductivity (81% (p < 0.05); Fig. 3D).

Effects of *pimE* Overexpression on Natamycin Production in 1 L Fermenter

To further investigate the effects of *pimE* overexpression on the natamycin production and the kinetics of cell growth, *S. gilvosporeus* 712 and *S. gilvosporeus* swjs-801 were cultured in 1 L fermenters of a 700 ml working volume, with dissolved oxygen and glucose levels controlled. As shown in Fig. 4, in parallel with cell growth, natamycin accumulated in the culture. A significant increase in the
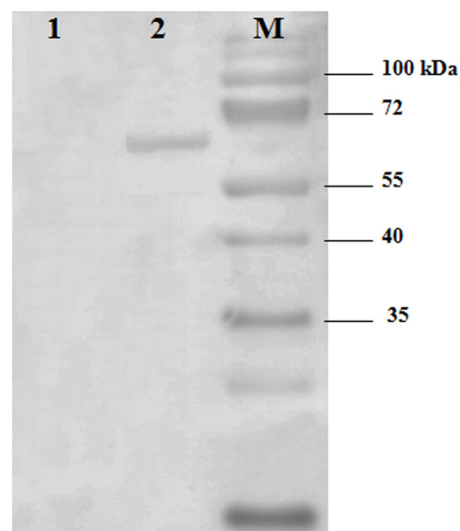

Fig. 2. Western blot analysis of PimE protein in the supernatant of *S. gilvosporeus* 712 (lane 1) and *S. gilvosporeus* swjs-801 (lane 2), with the Thermo Scientific PageRuler prestained protein ladder as the marker (lane M).

natamycin yield was observed (Fig. 4A), with the concentration reaching 7.0 g/l, which was 153% (p < 0.05) higher than that in the control. Generally, cell productivity was higher (Fig. 4D) in the fermenters, probably because of the continuous oxygen supply and better pH control. Remarkably, PimE activity was higher in the recombinant strain than that in the control (Fig. 4C), specifically in the late fermentation process. As reported by Mendes *et al.* [15], PimE is a signalling protein in natamycin production that relies on its enzymatic activity. Compared with heterogenous PimE, the homologous one strongly triggered natamycin production with a slightly higher enzymatic activity. Thus, PimE plays a key role in natamycin biosynthesis.

The PimE-encoding gene appears to work as a marker for the polyene antibiotic gene cluster that has 26–28 members [16, 21]. We infer that this gene could play a similar role as in the biosynthesis of polyene antibiotics. Our results provide a novel application of PimE in the biosynthesis of polyenes. However, medium composition, pH value, and dissolved oxygen also need to be optimized. Researchers are currently considering the insertion of *pimE*, together with *vgb* (*Vitreoscilla* hemoglobin gene), into the genome of *S. gilvosporeus* 712 to further improve natamycin productivity. However, the exact mechanisms underlying the role of PimE as a signalling protein in polyene antibiotic biosynthesis remains to be understood. Further experiments will provide the answer to this question.
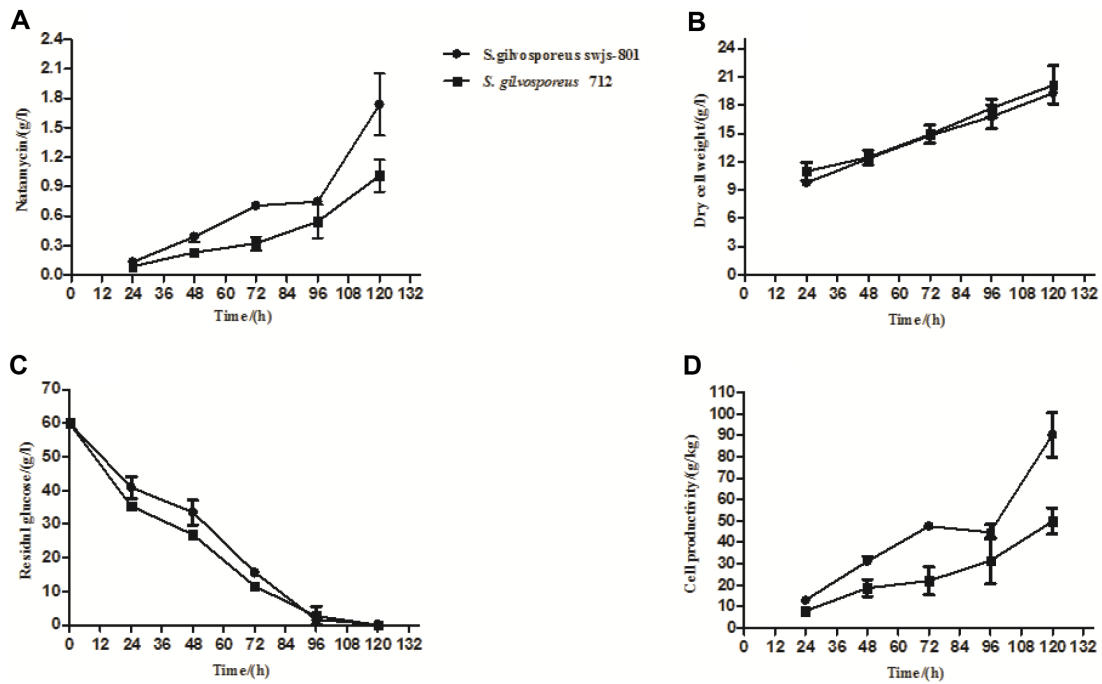
Fig. 3. Kinetics of cell growth and natamycin production by *S. gilvosporeus* 712 and *S. gilvosporeus* swjs-801 in shake-flask cultures. Natamycin production (**A**), dry cell weight (**B**), residual glucose (**C**), and cell productivity (**D**) were measured (values are the mean ± standard deviation, *n* = 3).
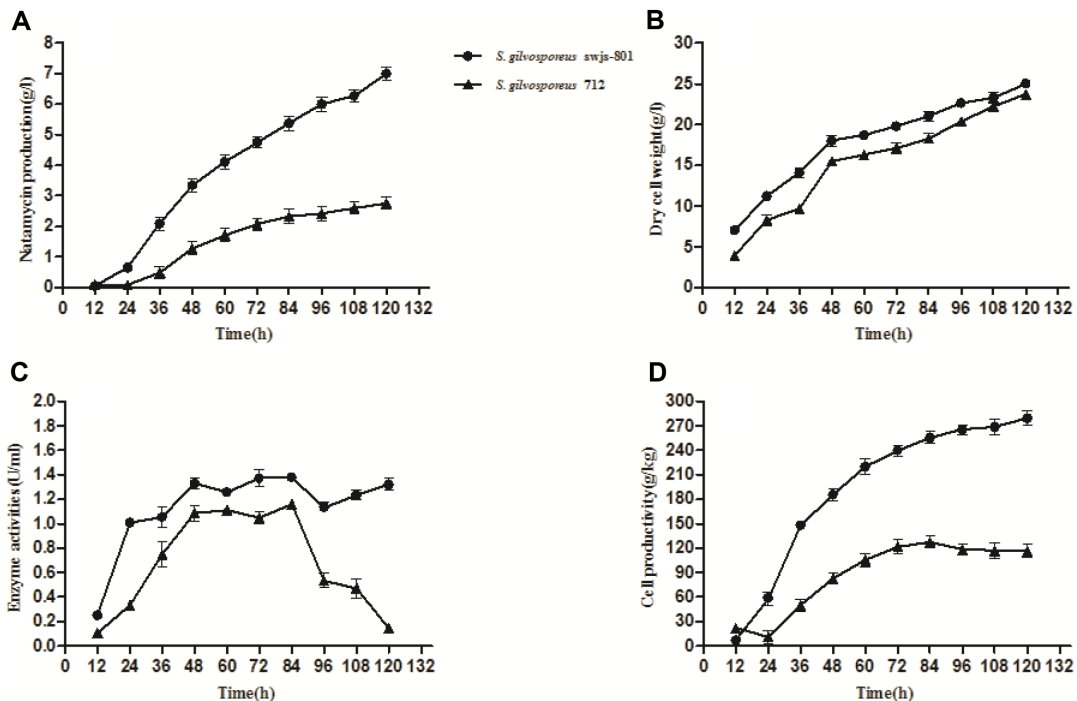
Fig. 4. Kinetics of cell growth and natamycin production by *S. gilvosporeus* 712 and *S. gilvosporeus* swjs-801 in 1 L fermenters. Natamycin production (**A**), dry cell weight (**B**), enzyme activities (**C**), and cell productivity (**D**) were determined (values are the mean ± standard deviation, *n* = 3).

Comparison of Natamycin Production Between *S. gilvosporeus* swjs-801 and *S. gilvosporeus-pimE-pimM*

To investigate the effects of PimE and PimM on natamycin production, *S. gilvosporeus* swjs-801 and *S. gilvosporeus-pimE-pimM* were cultured in 250 ml shake flasks of a 30 ml working volume for 120 h. The growth characteristics were similar in both exconjugants (data not shown), and otherwise exconjugants highly expressing PimE and PimM did not enhance natamycin production obviously (only 6.2% higher, shown in Fig. S1). PimM controls multiple natamycin biosynthetic promoters directly; however, it does not control that of *pimH* or *pimE* [2]. PimE acts as a signalling protein, which is being investigated [15]. Thus, we infer that PimM and PimE may act on the same genes of the natamycin biosynthetic gene cluster via different regulator circuits, consequently making the regulatory effects less obvious.

In summary, in response to increasing demands for natamycin, an additional copy of the gene *pimE* was integrated into the chromosome of *S. gilvosporeus*, a well-known natamycin producer. As a result of the gene insertion, PimE was overexpressed in *S.gilvosporeus* as an active form and significantly enhanced the natamycin yield. This effect was even more significant on the fermenter level. We believe that this study provides a better natamycin-producing industrial strain, as well as an excellent strategy to raise the yields of other secondary metabolites in industrial strains.

Acknowledgments

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