Transcriptome analysis of the regulation of natamycin biosynthesis in Streptomyces natalensis HW-2 by fungal elicitor

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Abstract

Background Natamycin is a polyene macrolide polyketide antibiotics and used in 150 countries as a natural food preservative. Streptomyces natalensis is an important producer. Elicitation had been approved to be an effective method to improve the biosynthesis of secondary metabolites. Fungal elicitor from Penicillium chrysogenum AS 3.5163 showed inductive effect on the biosynthesis of natamycin in S. natalensis HW-2 fermentation. However, regarding the global gene expression of natamycin in response to fungal elicitor is not still reported. Results RNA-Seq analysis showed that there were 1265 differential expression genes (DEGs) at 40 h and 2196 DEGs at 80 h. The fungal elicitor had stronger effects on the transcription level of S. natalensis HW-2 at 80 h than that at 40 h. Gene Ontology (GO) enrichment analysis of DEGs showed significant enrichment in biological processes. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that the fungal elicitor mainly affected the expression levels of some genes about cellular process, metabolism and genetic information, especially in pentose phosphate pathway (PPP), glycolytic pathway (EMP) and tricarboxylic acid cycle (TCA). KEGG pathway showed that fungal elicitor had a greater influence on the metabolism of branched-chain amino acids (BCAAs). Among them, 23 DEGs associated with BCAAs metabolism were up-regulated or down-regulated. The supplementation experiment with BCAAs confirmed that 0.2 g/L of L-Ile and 0.5 g/L of L-Val increased natamycin yield by 17.6% and 37.8%, respectively. Fungal elicitor also up-regulated the transcriptional levels of most of the enzymes associated with the biosynthesis of natamycin and two important transcription regulators (pimR and pimM). To confirm the accuracy of RNA-Seq, the results of qPCR showed that these gene expression levels were in agreement with the transcription changes by RNA-Seq. Conclusion In this study, the change of transcriptional levels in S. natalensis HW-2 under treated with the fungal elicitor was firstly reported. The major finding of our comparative transcriptome analysis is that the fungal elicitor improves the supply of precursor, and alters the expression of natamycin related genes and regulator of secondary metabolism. From our results, we conclude that regulatory alterations are important factors for the enhancement of natamycin.

Background

Actinomycetes are a diverse family of filamentous bacteria that produce a plethora of natural products relevant for agriculture, biotechnology and medicine, including the majority of the antibiotics [1]. Natamycin, also known as pimaricin, was synthesized by the type I modular polyketide synthase (PKS I) and produced by Streptomyces natalensis, S. gilvosporeus and S. chattanoogensis. It was a polyene macrolide polyketide antibiotics, and showed high efficient, broad-spectrum antifungal activity [2]. It was biosynthesized through acetate malonate pathway which was common to all macrocyclic polyketides. As similar as the biosynthesis of the fatty acids, the units derived from two-carbon or three-carbon substances were assembled to form the polyketide chain by a polyketide synthase [3]. Because natamycin could lead to the change of cell membrane structure and the leakage of cellular materials in fungi, it could effectively inhibit the growth of fungi with low dose and high efficiency. In 1978, it was the first antifungal agent as food preservative which was approved by FDA of the United States [4]. So, it was

widely used to inhibit fungal contamination of non-sterile foods [5]. Among the major applications of natamycin, it is used in 150 countries as a natural food preservative. In addition, it can be used as agent to treat the fungal conjunctivitis, scleritis and endophthalmitis [6] and as the first drug for the treatment of fungal keratitis [7]. Moreover, it is also used to prevent mould contamination in agricultural cultivation [8]. So, the increasing need of healthy processed foods with natural preservative assures that the demand of natamycin will continue to grow in the future.

The structure of natamycin was a 26-membered macrolactone ring with four double bonds. The macrolide skeleton ring was obtained by catalyzing the condensation of 12 acetate and one propionate units by PKS I [9]. Natamycin was mainly produced by the submerged fermentation. In order to improve natamycin production, many methods had been used, which included improvement of cultivation conditions [10], optimization of medium composition[11], addition of precursors [12], gene engineering of natamycin biosynthetic genes [13] and genome shuffling [14]. Elicitation had been approved to be an effective method to improve the biosynthesis of secondary metabolites in some actinomycetes [15]. It was reported that the fungal elicitor from the broth extract of P. chrysogenum could enhance the yield of natamycin with S. natalensis HW-2 as production strain [16]. The further study showed that the fungal elicitor not only influenced the fermentation process of S. natalensis HW-2, but the physicochemical characteristics of the strains were significantly changed, such as morphology of the colony, the activities of critical enzymes and the levels of intracellular reactive oxygen species (ROS) and Ca^{2+} ions [17]. The elicitor showed a positive correlation with natamycin biosynthesis.

Now, the eliciting mechanism of elicitors was clearly in plants and fungal cells, but it was unknown in bacterial cells [18]. It was guessed that the elicitor probably influenced the transcription level of the secondary metabolite gene cluster and induce some transcriptional activators of the important gene cluster [19]. Like most secondary metabolites synthesized by actinomycetes, natamycin production was regulated by complex regulatory networks that respond to some environmental and physiological factors [20, 21]. One of these networks was transcriptional regulation. There were several researches to report the transcriptional regulation by the elicitors in fungi [22, 23], but little is known about the response to the elicitors in actinomycetes. Recently developed transcriptomic analysis based on RNA-Seq can provide more information for expression levels for different genes, and will be a better way to study the transcriptional regulation [24]

Our laboratory is interested in elicitation of secondary metabolism in Streptomyces. In the present work, the effect of fungal elicitor on the transcriptome profiling of S. natalensis HW-2 was investigated using high-throughput RNA-Seq method. We searched for genes that are differentially regulated with elicitor, and analyzed their functions and metabolic pathways by GO and KEGG. We believe that the results will establish an important link between the fungal elicitor and the change of natamycin biosynthesis pathway. The study will provide a good foundation for the application of fungal elicitor and the discovery of induction mechanism.

Results

Growth and natamycin production kinetics

To investigate the growth and natamycin production kinetics of *S. natalensis* HW-2 treated with and without the fungal elicitor, the fungal elicitor was added into the broth after fermentation for 24 h, and the production of natamycin and dry cell weight (DCW) were measured. Figure 1a showed that the logarithmic period and the stability period of *S. natalensis* HW-2 were 36-72 h and 72-108 h, respectively. Figure 1b showed that the biosynthesis of natamycin started at 24 h, and the maximum natamycin yield was 1.25 g/L at 120 h in the control and 1.88 g/L at 120 h in the experiment group. The yield was increased by 50.4%. According to the growth and antibiotic production kinetics, the checked points to the transcriptome of *S.natalensis* HW-2 were chosen in this study.

Transcriptome analysis of *S. natalensis* HW-2

For a global analysis of fungal elicitor-induced genes, *S. natalensis* HW-2 was cultured for 24 h and the fungal elicitor was added into the fermentation broth. Then, the strain was grown up to the mid-exponential growth phase (40 h) and early period of stationary phase (80 h) (see figure 1). The mycelium was collected. Total RNA in control groups and experimental groups were isolated and the transcriptome was studied. *S. natalensis* ATCC 27448 genes were publically available and used as reference genes. The change of all genes was represented in a volcano plot (Figure 2). Two groups were divided into 40 h and 80 h. These genes with a fold change ≥ 2 and $p<0.05$ were considered as differential expression genes (DEGs). Sequence reads were submitted to GenBank GEO database under accession number GSE112559, with a link at http://www.ncbi. nlm.nih.gov/geo/ query/acc.cgi?acc= GSE112559.

In response to fungal elicitor, the transcriptome of *S.natalensis* HW-2 showed that the transcripts of 7578 genes were detected and 1265 genes had significantly changed at 40 h (Figure 2a). Among them, 949 DEGs were up-regulated and 316 DEGs were down-regulated. As we can be seen from Figure 2b, 7500 genes were detected. Among them, 2196 genes had significantly changed at 80 h, in which 1940 DEGs were up-regulated and 256 DEGs were down-regulated (Table S2). The results illustrated that the fungal elicitor had significant effects on the transcriptional level of *S.natalensis* HW-2 at 40 h and 80 h. The transcriptome data showed greater change at the second time point than that at the first time point. Some of up-regulated and down-regulated DEGs were shown in Table 1. It can be seen that the transcriptional levels of family transcription factors, such as MarR, TetR, LyseE, GntR, MerR and LacI, were greatly up-regulated. The transcriptional levels of the some enzymes related to the degradation of short-chain fats, including acetyltransferase and CoA transferase, were significantly increased..

Gene ontology (GO) annotation and enrichment analysis

GO is an international standardized gene functional classification system. In this study, Go was used to comprehensively describe the properties of genes in the transcriptome library of *S. natalensis* HW-2. According to the results of sequence alignments (Figure 3a), there were 1265 DEGs at 40 h and they were classified into 46 functional groups which belonged to three main categories: biological processes (53.02%), cellular components (22.90%) and molecular functions (24.08%). In the biological processes

category, many DEGs were involved in biological regulation, metabolic processes, cellular process, single-organism process and responses to stimuli. In the cellular component category, most of DEGs were localized to cell part, cell and membrane. In the molecular function category, a large number of DEGs were involved in catalytic activity, transporter activity and binding.

Figure 3b showed that there were 2196 DEGs and they were classified into 33 functional groups at 80 h which belonged to three main categories: biological processes (47.91%), cellular components (32.55%) and molecular functions (19.54%). In the category of biological processes, many DEGs were involved in biological regulation, responses to stimuli, metabolic processes, single-organism process and localization. In the cellular component category, most of DEGs were localized to membrane part and membrane, followed by cell and cell part. In the molecular function category, a lot of DEGs were involved in binding, catalytic activity and nucleic acid binding transcription factor activity. In total, the results indicated that most of the identified DEGs were responsible to fundamental processes which were associated with biological regulation and metabolism.

GO enrichment analysis of DEGs showed significant enrichment in biological processes. The results showed that 12 biological processes were significantly enriched at 80 h. Among them, organic cyclic compound biosynthetic process, aromatic compound biosynthetic process and heterocyclic biosynthetic process showed enrichment (Table S3). These processes had important relationship with the biosynthesis of some secondary metabolites.

Kyoto encyclopedia of genes and genomes (KEGG) pathway mapping and enrichment analysis

To understand the interaction of genes and metabolic biological functions, different unigenes which had significant match in KEGG database using BLASTx were assigned to some different pathways. KEGG analysis showed that the fungal elicitor affected the expression level of some genes about cellular process, metabolism and genetic information, especially EMP, TCA and amino acid metabolism. The results showed that 988 unigenes could be assigned to 123 pathways at 40 h, and they were grouped into four groups which were cellular process, environmental information process, metabolism and genetic information process, respectively. In metabolism pathway, 887 unigenes were divided into 12 sub-categories, in which most of the representation unigenes were global and overview maps (447), carbohydrate metabolism (112) and amino acid metabolism (100), respectively. The DEGs of the biosynthesis pathway of siderophore group nonribosomal peptides were significantly enriched.

At 80 h, 891 unigenes could be assigned to 113 pathways which were divided into four groups. The metabolism group showed more significant by treated with the elicitor, followed by genetic information process and environmental information process. In metabolism pathway, 769 unigenes were divided into 12 sub-categories which included amino acid metabolism (155), global and overview (149) and carbohydrate metabolism (141).

The change of transcriptional levels of glycolytic pathway (EMP) and Pentose Phosphate Pathway (PPP) related genes

EMP and PPP are important to the biosynthesis of natamycin by providing the carbon flux. Transcriptome analysis showed that the transcriptional levels of some genes related to PPP and EMP were enhanced at the both points, especially in 80 h (Table 2). At 40 h, the transcription levels of SNA_RS15280, SNA_RS33595, SNA_RS17980 and SNA_RS18050 in EMP were significantly enhanced, but the expression levels of SNA_RS05450, SNA_RS32155, SNA_RS02615 and SNA_RS02620 decreased. SNA_RS33595 which codes fructose-bisphosphate aldolase was the important gene. The enzyme can influence the utilization of glucose in EMP. Its value of $\log_2$FC increased by 33% compared with the control. In PPP, the levels of SNA_RS08865 (fructose 5-dehydrogenase) was enhanced, but SNA_RS02355 (6-phosphogluconate dehydrogenase) and SNA_RS34475 (gluconate kinase) decreased. At 80 h, the levels of SNA_RS 09030, SNA_RS 16550, SNA_RS 05360, Pgk, SNA_RS 08155, SNA_RS 36040 and SNA_RS10315 were significantly enhanced. But, there were only two genes, SNA_RS 02685 (2-oxoisovalerate dehydrogenase) and SNA_RS 04695 (acetyl-CoA synthetase) decreased in EMP. In PPP, the levels of SNA_RS 33825, SNA_RS 27695, SNA_RS 10335, SNA_ RS16550, SNA_RS13075, SNA_RS 06470 were enhanced. There was no gene to decrease. The $\log_2$FC value of SNA_RS 27695 (2-dehydro-3-deoxy-phosphogluconate aldolase) and SNA_RS 10335 (6-phosphogluconolactonase), increased by 107% and 200% compared with the control. The results conformed that the fungal elicitor enhanced the carbon source utilization. The conclusion agreed with the result of our previous report.

Effects of fungal elicitor on transcription levels of tricarboxylic acid cycle (TCA) related genes

Fungal elicitor had less effect on TCA compared with that on EMP (Table S4). There were 7 DEGs at the both time. SNA_RS33855, SNA_RS06030, SNA_RS33475 and SNA_RS07675 were up-regulated at 40h. SNA_RS 34985 and SNA_RS 34190 were up-regulated, and SNA_RS 33480 was down-regulated at 80 h. The value of $\log_2$FC of SNA_RS33855 which encodes phosphoenolpyruvate carboxykinase (PEPC) was 1.79-fold increase compared with the control. The enzyme catalyzed the conversion of phosphoenolpyruvate (PEP) into oxaloacetic acid (OOA) which was one precursor to macrocyclic antibiotics. OAA was an important enzyme to produce malonyl CoA and methylmalonyl CoA which were the precursors of natamycin. SNA_RS07675, encoding pyruvate dehydrogenase, was enhanced a little and the enzyme catalyzed the conversion of pyruvate into acetyl-CoA which was an important precursor to macrocyclic antibiotics. The expression levels of citrate synthase and fumarate reductase were also up-regulated. The results showed that the fungal elicitor improved the flux of TCA.

At 80 h, the transcriptional level of SNA_RS 34190, encoding NAD(P)H dehydrogenase, increased by 136%, but that of SNA_RS 33480, encoding succinate dehydrogenase, decreased by 143%. The two genes were related to the respiratory chain which attended the energy metabolism. Interestingly, the fungal elicitor could lead to the former increase, but the latter decrease. The change of energy levels is not studied in this work, and needs to further study. These results suggested that fungal elicitor influenced the providing of precursors and energy metabolism in *S.natalasis* HW-2.

Effects of fungal inducer on transcriptional levels of branched-chain amino acids (BCAAs) metabolism related genes

BCAAs, including Isoleucine (Val), Leucine (Leu) and Valine (Ile) are often used in the antibiotics fermentation to stimulate the antibiotics production. The degradation of BCAAs can provide many important precursors for polyketide biosynthesis, such as acetyl-CoA, propionyl-CoA and butyryl-CoA [25]. In this study, KEGG pathway showed that fungal elicitor significantly influence the transcription level of some genes related to the synthesis and metabolism of BCAAs. As we can see from Table 3, 10 DEGs associated with BCAAs biosynthesis were up-regulated and 13 DEGs associated with BCAAs degradation were down-regulated at the two time points.

At 40 h, l the transcriptional levels of SNA_RS29605 and SNA_RS29610, encoding 3-isopropylmalate dehydratase large subunit and small subunit, increased by 806% and 494%, respectively. The level of SNA_RS29600 which encoded pyruvate carboxyltransferase also increased by 948%. Inversely, acetolactate synthase gene, encoding BCAAs synthesis protein, was down-regulated. Among the genes of BCAAs degradation, 4 DEGs were up-regulated. Comparing with the control, the transcriptional levels of SNA_RS24915 (acyl-CoA dehydrogenase), SNA_RS05930 (methylcrotonoyl-CoA carboxylase), SNA_RS05920 (hydroxymethylglutaryl-CoA lyase), SNA_RS35365 (acetyl-CoA carboxylase) and SNA_RS32205 (methylmalonyl-CoA mutase) increased by 150%, 223%, 158%, 39% and 139%, respectively. These enzymes catalyzed BCAAs to convert into acetyl-CoA and methylmalonyl-CoA.

At 80 h, 3 DEGs associated with the biosynthesis of BCAAs were up-regulated. The transcriptional level of SNA_RS 31700 increased from 742 to 2072 (log_2FC=1.41). The gene encoded acetolactate synthase large subunit and the enzyme is important in BCAAs to catalyze pyruvic acid to acetyl lactate. The transcriptional level of SNA_RS 31630, encoding branched chain amino acid aminotransferase (BCAT), increased from 141 to 348 (log_2FC=1.23). The BCAT enzyme could catalyze the conversion of BCAAs and α-ketoglutarate into glutamate and branched chain α-keto acid.

To further assess the effect of BCAAs in the biosynthesis of natamycin, L-Ile, L-Leu and L-Val with different concentrations (0.2, 0.5 and 1 g/L) were added into the fermentation medium of *S. natalensis* HW-2 and natamycin production was measured at 120 h. As shown in Fig.4, the yield of natamycin was enhanced after supplementation with L-Val and L-Ile. As compared with the control, 0.2 g/L of L-Ile and 0.5 g/L of L-Val increased the yield of natamycin by 17.6% (1.4 g/L) and 37.8% (1.64 g/L), respectively. However, there is no obvious effect with the addition of L-Leu. These results showed that the concentration of BCAAs was an important factor to natamycin production in *S. natalensis* HW-2.

Effect of the fungal elicitor on the transcriptional levels of natamycin biosynthesis genes

The biosynthesis of natamycin in *S. natalensis* requires a complex type I modular polyketide synthase (PKS I) and additional modification enzymes. The gene cluster encodes 13 PKS modules within five multifunctional enzymes (PimS0, PimS1, PimS2, PimS3, PimS4), and 12 additional proteins that catalyze post-PKS modifications of the polyketide skeleton. *PimR* and *pimM* were two important transcriptional regulators in natmaycin biosynthesis [2,26]. In the study, the transcriptional levels of natamycin biosynthesis genes were checked at 40 h and 80 h. As shown in Table 4, 16 DEGs which belongs to the

natamycin biosynthetic gene cluster showed differential transcription at the both times. The expression level of PKS I was enhanced and the values of $\log_2$FC were 0.86 and 1.56 at 40 h and 80 h, respectively. According to the results, 9 natamycin biosynthesis genes were up-regulated and 3 genes were down-regulated at 40 h. *pimD* and *pimJ* were significantly up-regulated. At 80 h, all of 12 genes changed. Among them, 9 genes were significantly up-regulated. The two transcriptional regulator, *pimR* and *pimM*, were up-regulated at the both time. The transcriptional levels of *pimR* were increased by about 1.5-fold at 40 h and 8-fold at 80 h. The transcriptional level of *pimM* increased by about 2.3-fold at 40 h and 4.7-fold at 80 h. The data demonstrated that the fungal elicitor had stronger effect on the transcriptional levels of natamycin biosynthesis genes at 80 h than that at 40 h. The results confirmed that the natamycin biosynthesis was at stationary phase and the expressions of the related genes were mainly at late exponential phase.

Validation of transcriptome data by quantitative RT-PCR

The dependence of transcription changes of selected genes on the fungal elicitor was validated by quantitative RT-PCR (qPCR; see Fig. 5). For qPCR, 14 functional genes were randomly selected, i.e. SNA_RS12245, SNA_RS18705, SNA_RS26515, SNA_RS26355, SNA_RS16550, SNA_RS11825, SNA_RS29280, SNA_RS09665, SNA_RS25540, SNA_RS05940, SNA_RS15480, SNA_RS05805, SNA_RS22675 and SNA_RS21375. These selected genes included the related genes of strain growth and biosynthesis of natamycin. The result was shown in Figure 4. There were some differences in the degree of gene change, but these gene expression trends agreed with the changes of transcript abundance by RNA-Seq. To sum up, the study indicated the accuracy and quality of DGEs sequencing, and it was a true reflection of the transcriptome level changes on *S. natalensis* HW-2 with the fungal elicitor.

Discussion

Natamycin is an interesting agent and food preservative which attracts more and more attentions. As similar as other macrocyclic polyketides, natamycin is synthesised by the action of PKI synthases which assemble carbon chains from small two-carbon and three-carbon precursors, in a fashion that mechanistically resembles fatty acid biosynthesis [3]. The production of natamycin is affected by a lot of environmental and physiological factors [20, 27]. So, many methods were used to enhance natamycin production in the fermentation of S. natalensis and S. chattanoogensis. Elicitation is an effective method to enhance the production of metabolites in actinomycetes. The research reported that the natamycin biosynthsis could be elicited by 2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol (PI factor) in S. natalensis [28]. Other compounds, such as glycerol and ethylene glycol, also had induction effect on natamycin biosynthesis [29]. Previously, we reported that fungal elicitors from P. chrysogenum induced the increase of natamycin yield in S.natalensis HW-2.

There are many researches to report the inducing mechanism of the fungal elicitor. The bacterial cells are probably sensed to the elicitors as chemical signals [30]. Elicitor might bring about some changes in the intracellular molecules such as Ca^{2+} and ROS which were as internal signals to control the regulation of

secondary metabolism through yet unknown mechanism [31, 32]. The elicitor could either directly influence the transcription of the secondary metabolite gene clusters or induce a transcriptional activator of the target gene cluster [33]. There were some researches about activating silent gene clusters in fungi and myxobacteria, but the report about the activation of gene clusters in actinomycetes by elicitation method was little known [22, 38]. Our previous studies found the addition of fungal elicitors from P.chrysogenum influenced the morphology of the colony and mycelium of S. natalensis HW-2, and the activities of some important enzymes in EMP and TCA. And, it led to the increase of the levels of intracellular ROS [17]. Some researchers confirmed a positive correlation between intracellular ROS levels, particularly H_2O_2, and natamycin production in S.natalensis ATCC 27448. And, these authors identified cellular NADPH/NADH ratio and the availability of biosynthetic precursors via BCAAs metabolism as the main natamycin biosynthetic bottlenecks under high level of ROS [35]. Ca^{2+} is as an important second messenger in cells. Under the high concentration of Ca^{2+}, the bacteria generated a rapid and efficient response to new situations by signaling pathway. In our previous research, addition of elicitor improved the level of intracellular Ca^{2+} by 100% in S. natalensis HW-2. In this work, the transcription level of SNA_RS07090, encoding calcium-binding protein, increased from 4 to 47 ($log_2FC = 3.48$) in 80 h. The results suggested that the fungal elicitor activates the signaling mechanisms in S. natalensis, which is a mechanism different from that of plants.

Transcriptional regulation is a complex process including many signals and network of regulators that cross talk with each other. In this study, high-throughput RNA-Seq technology, which is a fast, efficient, and cost-effective way to characterize the transcriptome, was used to research the change of transcription level of S. natalensis HW-2 with the treatment of fungal elicitor at two fermentation time points (40 h and 80 h). At the first time point, there are 1265 DEGs, including 949 up-regulated and 316 down-regulated DEGs. After adding the fungal elicitor, S.natalensis HW-2 was stimulated and the intracellular environment balance was broken, then the metabolism speed and biological regulation ability were also enhanced. At the second time point, there were 1940 up-regulated and 256 down-regulated DEGs. These differential genes were classified into 33 functional groups. At this time, the synthesis rate of natamycin was improved. Some BCAAs continued to be synthesized, which provided many precursors for the synthesis of natamycin.

Amino acids, especially BCAAs, are often used in the antibiotics production. It was reported that enhancement of precursor amino acid supplies improved bacitracin production by activation of branched chain amino acid transporter BrnQand deletion of its regulator gene lrp in Bacillus licheniformis [36]. BCAAs were used in improving secondary metabolites of Streptomyces spp, for example bitespiramycin [37]. A large number of CoA products, such as acetyl-CoA and propanyl-CoA can be produced in the process of amino acid metabolism. These substances act as precursors to secondary metabolites, especially compounds synthesized by the polyketonase pathway [38]. In this study, we demonstrated that the supplementation of L-Val influenced the biosynthesis of natamycin and fermentation process of S. natalensis HW-2. Natamycin yield increased to 1.45 g/L with 0.5 g/L L-Val, which was 50% higher than

that of the control. The result agreed with the report by Sharma et al [39]. So, we thought that the fungal elicitor mainly improved the supply of precursor by BCAAs metabolism.

Natamycin biosynthesis is through the acetic acid-malonic acid pathway (AA-MA pathway). Firstly, the precursors, including 12 acetate and one propionate units, were produced. Secondly, the macrolide skeleton construction of natamycin was formed by PimS0, PimS1, PimS2, PimS3 and PimS4. Thirdly, mycosamine was connected to the macrolide by the glycosyltransferase (pimK). Finally, natamycin was exported by two ATP-binding cassette (ABC) transporter proteins which were encoded by pimA and pimB. PimR and pimM are very important to natamycin biosynthesis. They are the LuxR family regulator [40]. When one or both of them were deleted, S. natalensis was lack of the ability to produce natamycin. PimM could govern some processes, including genetic information processing, energy and cofactor metabolism, carbohydrate and vitamin metabolism, lipid and amino acid metabolism, transcriptional regulation and secondary metabolite biosynthesis [2]. In this work, the transcriptional levels of natamycin related genes were mostly up-regulated after adding fungal elicitor. The two transcriptional regulators were significantly up-regulated at 80 h. But, the reason why the fungal elicitor could improve the transcriptional levels of most of the enzymes associated with natamycin biosynthesis was still unknown.

Elicitation involved chemical and biological elicitation. Biological elicitation mainly caused by microbial co-cultivation, microbial lysates and microbial cell components. Cell wall constituents, signaling molecules and carbohydrates which were derived from cells could be as elicitors [N-acetylglucosamine could elicit the production of actinorhodin and undecylprodigiosin in Steptomyces coelicolor [41]. PI factor (2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol) produced by S.natalensis, glycerol and 1,2-propanediol elicited the improvement of 15]natamycin production in S.natalensis [28, 29]. The elicitation response depends on the regulation of some metabolism pathways, up-regulation of antibiotic activator and the expression of some silent genes. Even though the exact mechanisms of elicitation remain unknown, biological and chemical elicitations are effective method to provoke the expression of bioactive metabolites [42].

In this study, the results suggested that natamycin production was increased because the fungal elicitor from P.chrysogenum AS 3.5163 not only activates the signaling mechanisms by improving the Ca^{2+} level, but improves the supply of precursors by BCAAs metabolism. However, the lack of information on the precise elicitation mechanism makes the research on the global regulatory networks to be a challenging task. So, further studies are needed to identify the structure of the elicitor through LC-MS, MS/MS and NMR. Also, our work supports the use of biotic elicitation in order to enhance the production of secondary metabolites for industrial-scale applications.

Conclusion

In this study, RNA-Seq was used to check the effect of fugal elicitor on the transcriptional levels of S. natalensis HW-2. The results showed that the fungal elicitor had stronger effects on the transcription level of S. natalensis HW-2 at 80 h than that at 40 h. GO enrichment analysis of DEGs showed significant

enrichment in biological processes. KEGG analysis showed that the fungal elicitor mainly affected the expression levels of some genes about cellular process, metabolism and genetic information. KEGG pathway showed that fungal elicitor had a greater influence on the metabolism of BCAAs. The supplementation experiment with BCAAs confirmed that L-Ile and L-Val increased natamycin yield. Fungal elicitor also up-regulated the transcriptional levels of most of the enzymes associated with the biosynthesis of natamycin and two transcription regulators. The results of qPCR showed that these gene expression levels were in agreement with the transcription changes by RNA-SEq. The major finding of our comparative transcriptome analysis is that the fungal elicitor improves the supply of precursor, and alters the expression of natamycin related genes and regulator of secondary metabolism. From the results, we conclude that regulatory alterations are important factors for the enhancement of natamycin.

Materials And Methods

Strains and culture media

Streptomyces natalensis HW-2 was the natamycin producer in the study [43]. Penicillium chrysogenum AS 3.5163 is from China General Microbiological Culture Collection Center. The seed medium and the natamycin fermentation medium of S. natalensis HW-2 was described by Wang et al [16]. The elicitor production medium of P. chrysogenum AS 3.5163 consisted of: glucose 30 g/L, beef extract 10 g/L, yeast extract 2 g/L, H_2O 1000 mL.

Cultivation conditions

Batch fermentation was made in 250 mL flasks containing 50 mL fermentation medium. The spores of S. natalensis HW-2 (1×10^8 spores/mL) were inoculated into the seed medium and was cultured at 28 °C, 180 rpm for 2 d. Then, S. natalensis HW-2 was cultured in fermentation medium at 28 °C, 200 rpm for 132 h. P. chrysogenum AS 3.5163 was cultured in fermentation medium at 28 °C, 180 rpm for 2 d.

Preparation and addition of elicitor extract

After the end of P. chrysogenum AS 3.5163 cultivation, the broth was filtrated with filter paper. The filtrate was obtained after centrifuging at 10000 rpm for 10 min. The supernatant was extracted with three-time ethyl acetate and the solvent was evaporated at 45 °C with a rotary evaporation. The extract was isolated by chromatography using a Sephadex LH-20 column (15 mm × 500 mm, particle diameter 5.0 μm) with 20–50% (v/v) methanol at flow rate 2 mL/min. The fraction eluted with 30% methanol was purified using a preparative RP-HPLC with a C_{18} column (Sephax, USA, 10 mm × 150 mm, particle diameter 5.0 μm), and mobile phase was 20% (v/v) methanol at flow rate 3 mL/min. Methanol was evaporated at 45 °C with a rotary evaporation. The residue was dried by vacuum freeze-drying and the elicitor was obtained. The elicitor was added to the broth of S. natalensis HW-2 according to the design of experiment.

Determination of natamycin

The quantitative determination of natamycin was carried out using HPLC method. After fermentation, the broth was extracted with nine times methanol and the mixture was shaken for 2 h with an oscillator. Then, it was centrifuged at 10000 rpm for 10 min. Natamycin was quantified using an Agilent 1260 system (Agilent technologies, USA) according to previous reports [16].

Transcriptome analysis RNA extraction

S. natalensis HW-2 was cultured in the natamycin production medium for 24 h, then the elicitor was added into the fermentation broth. The medium of P. chrysogenum AS 3.5163 was added into the fermentation broth in the control. The mycelia at late exponential phase (40 h) and at early stationary phase (80 h) were taken. Three biological replicates for each experimental condition were included in the analysis. For RNA analysis, three samples in every condition were mixed, then the mixture was quickly centrifuged at 4 °C and the mycelia was immediately frozen in liquid nitrogen until RNA extraction. Total RNA was extracted using a cetyltrimethylammonium bromide-based protocol and the purification was done with the RNeasy Mini Kit (Qiagen, Germany). Further steps in RNA extraction were carried out following the manufacturer's instructions. RNA purity was measured with the NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, USA). RNA quantity and integrity were determined using an Agilent RNA 6000 Nano Assay Protocol on an Agilent Bioanalyzer 2100 (Agilent technologies, USA). RNAClean XP Kit (Beckman Coulter, Inc, USA) and RNase-Free DNase Set (Qiagen, Germany) were used to further purify the total RNA.

cDNA library construction and sequencing

Sequencing libraries were performed following the instructions provided by the manufacturer. The total RNA was obtained and rRNA was removed using Bacteria Ribo-Zero rRNA Removal Reagents (Epicentre, USA). The mRNA was fragmented using fragmentation buffer. First strand cDNA was generated using reverse transcriptase and random primers. After second strand cDNA synthesis, the double-strand cDNA was purified using Agencourt® AMPure XP Bead(Beckman, USA). The suitable fragments were selected and amplified with PCR. The libraries were checked using an Agilent 2100 Bioanalyzer and sequenced by an Illumina HiSeq 4000 sequencing platform (Illumina, USA). The preparation of libraries and sequencing projects were performed at Shanghai Bohao Biotechnology Co., LTD (Shanghai, China).

Assessment of differential gene expression and gene annotation

High-quality reads were obtained by removing low-quality reads. Differential expression was tested with the software package edge R. Gene expression was measured by calculating Fragments Per Kilobase of transcripter Million mapped reads (FPKM) [44]. Log_2 values of Fold Change (log_2FC) were calculated and transcripts with an absolute $log_2FC \geq 1$ were considered to be significantly differential expression. For annotation, all of assembled unigenes were searched against public databases, including NCBI and SWISS-PROT databases. The genome of S. natalensis ATCC 27448 was used as the reference strain. Unigenes annotated were listed and the duplications were removed. GO annotation was performed using

the Blast2GO software. Pathway assignments were carried out based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Quantitative real-time PCR Analysis

For gene expression analysis by qPCR, 1 mg of total RNA from 80 h sample was treated with DNase I (Takara, China). The cDNA synthesis was performed using Prime Script II 1st Strand cDNA Synthesis Kit (Takara, China) following the manufacturer's instructions. The primer pairs in the qPCR amplifications were listed in Supplementary file 1: Table S1. qPCR was performed using SYBR Premix Ex Taq II (TaKaRa, China) on a Line Gene 9600 Real-Time PCR detection system (Bioer, China) with the following amplification conditions: 95 ^{o}C for 30 s (1 cycle), 95 ^{o}C for 5 s and 60 ^{o}C for 20 s (40 cycles). Reactions for each gene were carried out in triplicate. A melting curve was analyzed at the end of each qPCR to avoid the formation of non-specific products. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal reference gene [45]. The relative expression levels of genes were calculated using $2^{-\Delta\Delta C_t}$ method [46].

Statistical analysis

Experiments were done in three repeats. The data was analyzed with Student's t test. P values < 0.05 were considered significant.

Abbreviations

DEGs: differential expression genes; GO:Gene Ontology; KEGG:Kyoto Encyclopedia of Genes and Genomes; BCAAs:branched-chain amino acids; ROS:reactive oxygen species; DCW:dry cell weight; log_2FC:Log_2 values of Fold Change.

Declarations

Acknowledgements

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Authors' contributions

Dahong Wang and Jiangfeng Yuan designed the experiments and data process. LanLan Wei and Ying Zhang performed all experiments. Wenhao Shen drafted the manuscript. All authors read and approved the fnal manuscript.

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Availability of data and materials

RNA-Seq data were deposited to GenBank GEO database under accession number GSE112559, with a link at http://www.ncbi. nlm.nih.gov/geo/query/acc.cgi?acc= GSE112559.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Expression of some DEGs at 80h

Gene ID	Description	log_2FC
Up-regulated DEGs		
SNA_RS26515	MarR family transcriptional regulator	12.76
SNA_RS01540	short-chain dehydrogenase	12.37
SNA_RS18705	TetR family transcriptional regulator	11.88
SNA_RS12245	LysE family translocator protein	11.47
SNA_RS32435	phosphohydrolase	11.27
SNA_RS12165	nucleotidyltransferase	11.12
SNA_RS25180	Glutamine amido transferase	11.11
SNA_RS19005	acetyltransferase	11.10
SNA_RS09180	methyltransferase type 12	11.02
SNA_RS05520	ABC transporter permease	10.94
SNA_RS22595	serine/threonine protein phosphatase	10.39
SNA_RS27910	GntR family transcriptional regulator	10.39
SNA_RS26035	MerR family transcriptional regulator	10.38
SNA_RS19265	thiamine biosynthesis protein	10.18
SNA_RS04810	CoA transferase	9.83
Down-regulated DEGs		
SNA_RS15250	oxidoreductase	-3.96
SNA_RS15275	3-dehydroquinate synthase	-3.59
SNA_RS25540	AraC family transcriptional regulator	-3.59
SNA_RS15285	salicylate 1-monooxygenase	-3.14
SNA_RS10485	NAD-dependent dehydratase	-3.06
SNA_RS05650	ABC transporter permease	-2.98
SNA_RS27880	carbon monoxide dehydrogenase	-2.90
SNA_RS20585	MBL fold metallo-hydrolase	-2.86
SNA_RS27885	oxidoreductase	-2.85
SNA_RS06085	2-keto-myo-inositol dehydratase	-2.83
SNA_RS06050	GCN5 family acetyltransferase	-2.66
SNA_RS25675	carboxymuconolactone decarboxylase	-2.61
SNA_RS27220	crotonyl-CoA reductase	-2.51
SNA_RS32170	cholesterol esterase	-2.25
SNA_RS02770	butyryl-CoA dehydrogenase	-2.24

Table 2 The significant change of transcriptional levels of EMP and PPP related DEGs

Time	Gene ID	Description	log_2FC
EMP related DEGs			
40 h	SNA_RS15280	fructose-bisphosphate aldolase	2.34
	SNA_RS33855	phosphoenolpyruvate carboxykinase	1.79
	SNA_RS33595	fructose 1,6-bisphosphatase	1.33
	SNA_RS18050	NAD(P)-dependent alcohol dehydrogenase	1.30
	SNA_RS17980	aldehyde dehydrogenase	1.07
	SNA_RS10330	glucose-6-phosphate isomerase	-1.03
	SNA_RS05450	PTS sugar transporter	-1.17
	SNA_RS32155	pyruvate kinase	-1.60
	SNA_RS02615	pyruvate dehydrogenase alpha subunit	-5.65
	SNA_RS02620	alpha-ketoacid dehydrogenase beta subunit	-5.82
80 h	SNA_RS 09030	glucokinase	2.05
	SNA_RS 16550	6-phosphofrutokinase	1.70
	SNA_RS 05360	dihydrolipoamide dehydrogenase	1.61
	pgk	phosploglycerate kinase	1.57
	SNA_RS 08155	acid phosphatase	1.50
	SNA_RS 36040	phosphoglyceromutase	1.22
	SNA_RS10315	Triose-phosphate isomerase	1.05
	SNA_RS 02685	2-oxoisovalerate dehydrogenase	-1.32
	SNA_RS 04695	acetyl-CoA synthetase	-1.61
PPP related DEGs			
40 h	SNA_RS08865	fructose 5-dehydrogenase	2.45
	SNA_RS02355	6-phosphogluconate dehydrogenase	-1.14
	SNA_RS34475	gluconate kinase	-2.04
80 h	SNA_RS 33825	carbohydrate kinase	2.28
	SNA_RS 27695	2-dehydro-3-deoxy-phosphogluconate aldolase	2.07
	SNA_RS 10335	6-phosphogluconolactonase	3.0
	SNA_RS 16550	6-phosphofructokinase	1.70
	SNA_RS 13075	ribulose-phosphate 3-epimerase	1.24
	SNA_RS 06470	ribose-5-phosphate isomerase	1.35

Table 3 Effect of fungal elicitor on transcription levels of BCAAs metabolism related genes

Time	Gene ID	Description	log_2FC
BCAAs synthesis related genes			
40h	SNA_RS19260	thiamine pyrophosphate-binding protein	0.53
	SNA_RS29605	3-isopropylmalate dehydratase large subunit	**3.18***
	SNA_RS29610	3-isopropylmalate dehydratase small subunit	**2.57***
	SNA_RS29600	pyruvate carboxyltransferase	**3.39***
	SNA_RS31695	acetolactate synthase small subunit	-0.28
	SNA_RS31700	acetolactate synthase large subunit	-0.21
80h	SNA_RS 34040	L-serine/L-threonine ammonia-lyase	**2.57***
	SNA_RS 31700	acetolactate synthase I/II/III large subunit	**1.41***
	SNA_RS 31595	2-isopropylmalate synthase	**1.59***
BCAAs degradation related genes			
40h	SNA_RS24915	acyl-CoA dehydrogenase	**1.32***
	SNA_RS05930	methylcrotonoyl-CoA carboxylase	**1.69***
	SNA_RS05920	hydroxymethylglutaryl-CoA lyase	**1.37***
	SNA_RS35365	acetyl-CoA carboxylase subunit alpha	0.48*
	SNA_RS32205	methylmalonyl-CoA mutase	**1.26***
80h	SNA_RS 31630	branched-chain amino acid aminotransferase	**1.23***
	SNA_RS 05360	dihydrolipoamide dehydrogenase	**1.61***
	SNA_RS 35355	enoyl-CoA hydratase	**3.10***
	SNA_RS 07585	3-hydroxyisobutyrate dehydrogenase	**1.26***
	SNA_RS 25310	3-hydroxyacyl-CoA dehydrogenase	**-1.29***
	SNA_RS 07925	acetyl-CoA C-acetyltransferase	**-1.52***
	SNA_RS 06110	malonate-semialdehyde dehydrogenase (acetylating)	**-2.22***
	SNA_RS 32255	methylmalonyl-CoA/ethylmalonyl-CoA epimerase	**-1.28***

*values highlighted in bold have |FC| ≥ 2and p-value ≤ 0.05 in the RNA-Seq data.

Table 4 Effects of the fungal elicitor on the transcriptional level of natamycin biosynthesis related genes

Gene ID	Description	log_2FC	
		40 h	80 h
SNA_RS21375	type I polyketide synthase (PKSI)	0.86	**1.56***
SNA_RS02000	ABC transporter (*pimA*)	0.29	0.54
SNA_RS01285	ABC transporter (*pim B*)	0.56	0.79
SNA_RS22675	aminotransferase (*pimC*)	-0.5	**1.76***
SNA_RS05805	monooxygenase (*pimD*)	**1.11***	**1.08***
SNA_RS16480	cytochrome P450 (*pimE*)	0.16	**3.34***
SNA_RS14670	ferredoxin reductase (*pimF*)	0.5	**3.17***
SNA_RS07895	monooxygenase (*pimG*)	-0.8	**1.05***
SNA_RS35625	efflux pump (*pimH*)	-0.74	**1.96***
SNA_RS09665	thioesterase (*pimI*)	0.32	**1.83***
SNA_RS22185	AMP-ligase (*pimJ*)	**1.63***	**1.43***
SNA_RS15480	glycosyl transferase (*pimK*)	0.38	**3.40***
SNA_RS02075	tyrosine phosphatase (*pimL*)	0.64	0.62
SNA_RS19755	PAS-LuxR regulator (*pimM*)	**1.22***	**2.23***
SNA_RS07640	SARP-LAL regulator (*pimR*)	0.55	**3.01***

*values highlighted in bold have |FC| ≥ 2 and p-value ≤ 0.05 in the RNA-Seq data.

Figures

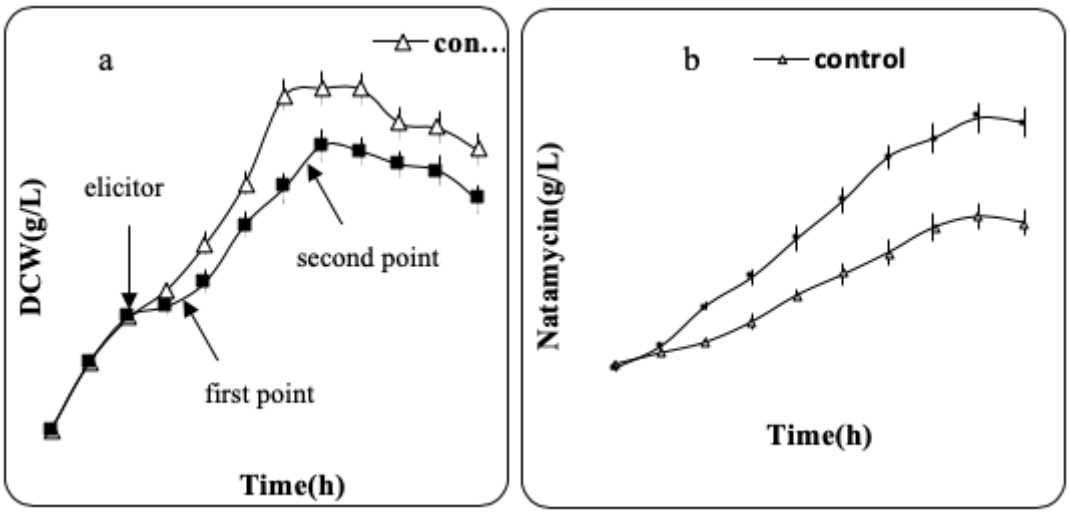

Figure 1

Effects of fungal elicitors on the dry cell weight (a) and natamycin production (b) in fermentation process of S. natalensis HW-2. Black triangle represents the experimental group and black cycle represents the control. The elicitor was added to the fermentation broth of S. natalensis HW-2 after fermentation for 24 h at 28 °C with the shaking of 200 rpm. The fermentation was continued to be done for another 108 h. The medium of P. chrysogenum AS 3.5163 was added into the fermentation in the control. The mycelia at late exponential phase (40 h) and at early stationary phase (80 h) were taken.

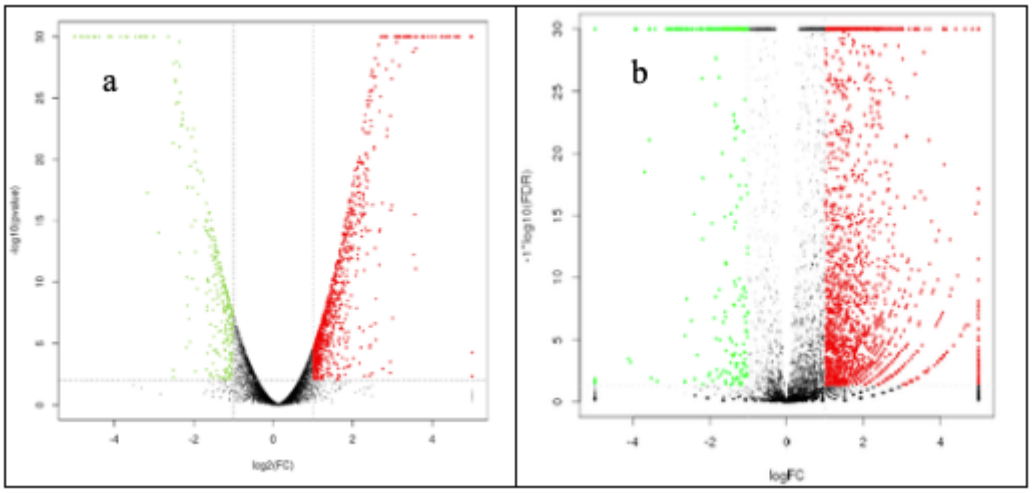

Figure 2

The volcanic plot with DEGs of S. natalensis HW-2 at 40 h and 80 h. Genes up-regulated with log2FC ≥1 were shown in red. Genes down-regulated with log2FC≤ -1 were shown in green. Genes with -1 log2FC

1 were shown in black represents. a: fermentation for 40 h. b: fermentation for 80 h.

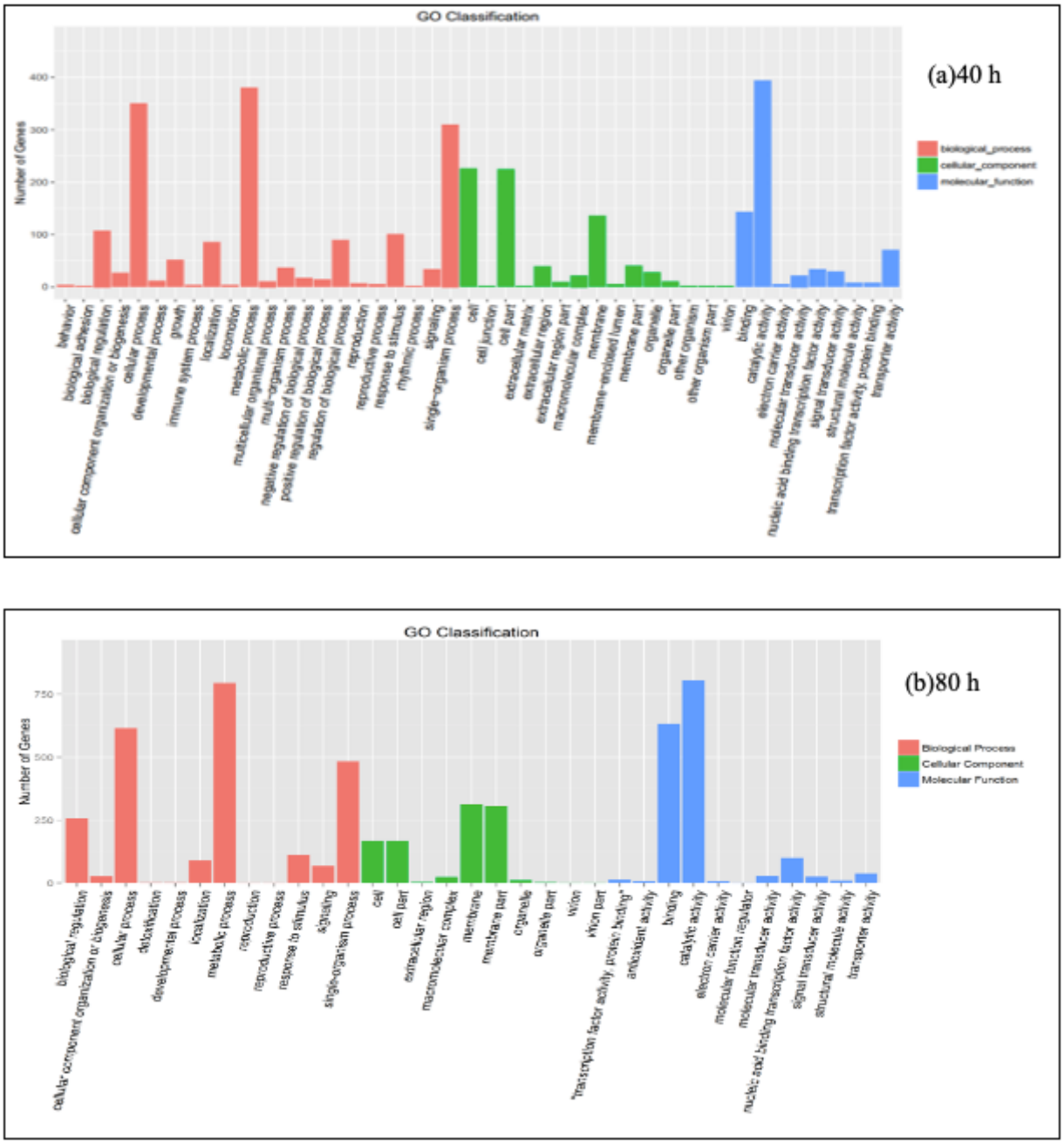

Figure 3

GO enrichment analysis of DEGs of S. natalensis HW-2 for different fermentation time. The results were summarized in three main categories: biological process, cellular component, and molecular function. a: fermentation for 40 h. b: fermentation for 80 h. The elicitor was added to the fermentation medium of S. natalensis HW-2 after fermentation for 24 h.

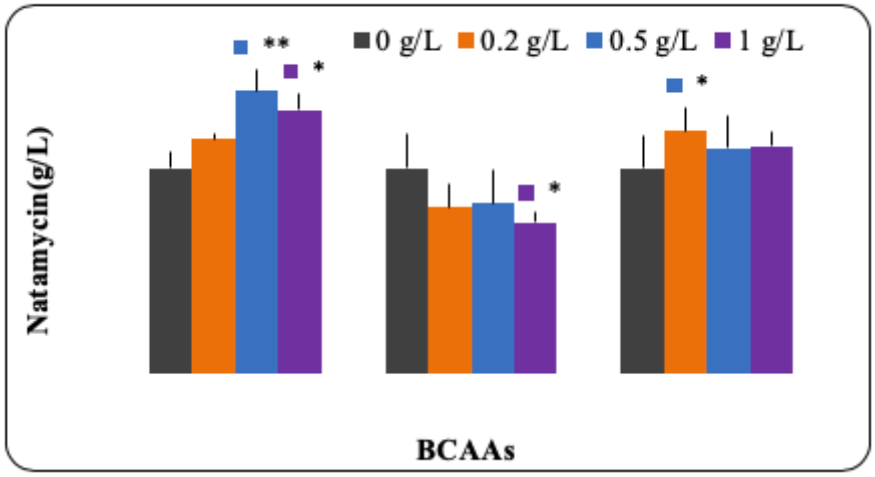

Figure 4

Effects of different types and concentrations of BCAAs on natamycin production on shake-flask cultivation of S. natalensis HW-2. L-Val, L-Leu and L-Ile were added into the fermentation broth of S. natalensisHW-2 after fermentation for 24 h at 28°C with the shaking of 200 rpm. The strain was continued to incubate for another 96 h. The first concentration 0 g/L is the control. *P < 0.05; **P < 0.01.

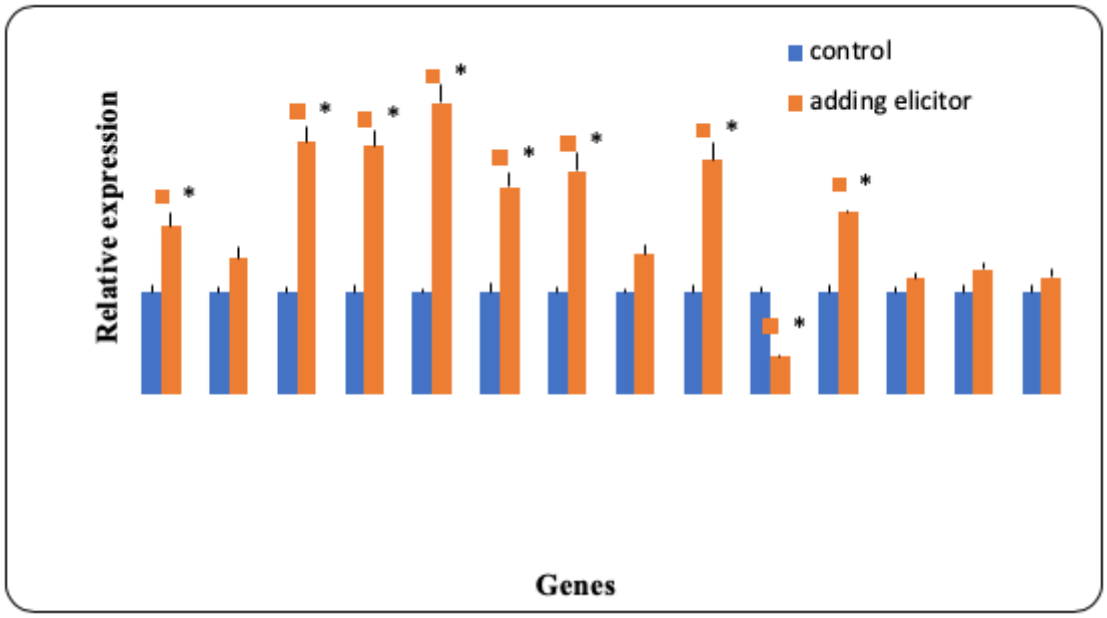

Figure 5

Verification of transcriptome results by quantitative RT-PCR. S.natalensis HW-2 was incubated for 80 h with or without fungal elicitor. The validation of expression levels of selected 14 target genes by RT-qPCR. Specific primers were used for amplification the target genes. Relative fold transcription of genes was calculated. Blue bars represent the expression in control and the values were set as 1. Red bars represent the relative expression with the elicitor comparing the control. The statistically significant difference (|FC| ≥ 1.5 and p-value ≤ 0.05) indicated with an asterisk (*).