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Identification and Expression of Genes Involved in the Biosynthesis of Penicillin and Its Detection by HPLC in *Penicillium chrysogenum*

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Abstract

In this study, after identification of genes involved in biosynthesis of penicillin, we evaluated the expression of pcbAB and pcbC genes in *P. Chrysogenum*. A quantitative PCR (qPCR) approach was used to determine how these genes were expressed in different time courses. In addition, the produced penicillin content was measured using HPLC. qPCR analysis of mRNAs extracted from *P. chrysogenum* indicated that the expression levels of pcbAB, pcbC increased in seven days after inoculation compared to the expressed levels with others time courses, but this difference was not significant statistically. The analysis of chromatograms from injected showed that the highest content of penicillin in media observed in 7 days after cultivation, except in *P. chrysogenum* PTCC 5033. In addition, the results of comparative analysis among the tested strains showed that the penicillin content in 5, 7 and 11 days after cultivation was highest in *P. chrysogenum* PTCC 5037, *P. chrysogenum* PTCC 5031 and *P. chrysogenum* PTCC 5033, respectively. However, the results showed an evident relationship between the expression levels of penicillin biosynthesis genes and yielded penicillin.

Keywords: Secondary Metabolites; Penicillin; Gene Expression; HPLC.

Introduction

The production of the forty-five thousand tons of b-Lactam antibiotics, e.g., penicillin in the year 2000 [1], has revealed one of the largest-selling classes of drugs worldwide is the production of these antibiotics nowadays. The penicillin production can be active by environmental factors and endogenous metabolic signals [2-4], but the genetic basis for control of penicillin production is still unclear.

The sequencing results of the *Aspergillus* genome [5-7], and *Penicillium chrysogenum* [8], has revealed that their genomes along with numerous gene clusters can be encoded nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS), hybrid polyketide-nonribosomal peptide synthetases (HPS), isoprenoid synthetases, and other unconventional antibiotic-synthesizing enzymes.

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It was shown that *P. chrysogenum* and *Aspergillus nidulans* strains with increased penicillin production had increased the penicillin biosynthetic enzymes levels, number and volume fraction of microbodies in the cell [9, 10] that defects in microbodies associated with some of inherited human metabolic disorders [11].

According to the well-known *P. chrysogenum* biochemistry and molecular genetics, penicillin biosynthesis in *P. chrysogenum* is an excellent model for better understanding of complex molecules synthesis and the control of their production in cell [12].

Filamentous fungi are well-known producers of hundreds of bioactive secondary metabolites [13]. Although, secondary metabolites are low molecular weight, families of related compounds at restricted parts of the life cycle and, also, generally nonessential for survival [14], may positively have an effect on growth, physiology, or reproduction of the producing organism and often correlated with a specific stage of morphological differentiation.

In addition, several fungal secondary metabolites are of applied interest because of their role as toxins or their application as antibiotics [15-17]. The genes involved in fungal secondary metabolic pathways share a tendency towards physical clustering, with a preference for subtelomeric regions [5, 8]. Evidence is growing that this spatial organization contributes to control of these pathways.

The penicillin biosynthesis pathway includes three enzymes, δ (L-al-aminodipyl) - L-Cysteinyl-D-Valine (ACV) synthetase (ACVS), isopenicllin N synthetase (IPNS), and aminopenicillanic acid acyltransferase. The mentioned enzymes catalyze the formation of penicillin from its amino acid precursors [4].

The first step of the penicillin biosynthesis pathway is the non-ribosomal condensation of the three precursor amino acids, L-α-aminoadipic acid, L-cystein, and Lvaline, by the enzyme synthetase that correlated with membranes or small organelles.

The product of this reaction is a tripeptide δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), which is cyclized to isopenicillin N (IPN) by the activity of cytosolic enzyme, the isopenicillin synthase [9, 18]. The isopenicillin N is later modified by the enzyme isopenicillin acyltransferase that was located in organelles to form penicillin G (Figure 1).

The three enzymes are encoded by the pcbAB, pcbC, and penDE genes, which are organized as a cluster that including a 15-kb DNA region and the amplification has occurred within chromosome I, the largest P. chrysogenum chromosome [19-21]. The pcbC and penAB genes, which encode IPNS and ACVS, respectively, are expressed from a 1.16-kb intergenic region serving as a regulated promoter in opposite directions [19]. Also, the penDE gene has an essential role in the activity of isopenicillin N acyltransferase. In some cases, many of the secondary metabolite genes remain silent, although it is likely that they may be expressed under still-unknown conditions [22].

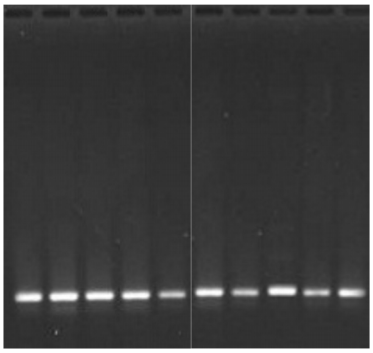

Figure 1. The amplification results of pcbAB (left) and pcbC genes (right) in Penicillium strains.

The expression of the three genes, pcbAB, pcbC and penDE, affect by a complex regulatory network, including nutritional and developmental factors [23, 24]. The previous studies have shown glucose and sucrose negatively regulate the penicillin biosynthesis in *P. chrysogenum*, whereas maltose, galactose and fructose had a less negative effect [24]. Also, in another study, the expression of pcbAB was higher in batch cultures supplemented with phenol as the carbon source; meanwhile, the expression of pcbAB was not significantly affected by phenolic compound [25].

The results of present work, give an insight into the expression levels of penicillin G in different time courses. Therefore, one of the purposes of this study was to obtain more information about the production of penicillin in *P. chrysogenum* strains during cultivation.

Materials and Methods

Strains and culture conditions

Fungi strains in this study belong to the Penicillium genus. The PTCC5031, PTCC 5033, PTCC5037 and PTCC5071 from P. Chrysogenum and the PTCC5074 from P. notatum was purchased from Iranian fungi and bacterial research center.

Identification of genes in penicillin synthesis

For colony isolation, samples were inoculated to on Potato dextrose broth (PDB) and incubated at 25°C for 7 days. Total DNA was extracted using CTAB containing Buffer. PCRs were performed with a 25Ml volume containing the following: 2.5 µl PCR Buffer;

3.5 mM MgCl2; a 200 µM concentration (each) of dATP, dCTP, dGTP, and dTTP (Fermentas); 1µM primer; 0.25 U of Taq DNA polymerase (Bioline); and 50 ng of genomic DNA. The following primers were used (pcbAB forward: 5′-CACTTGACGTTGCGC ACCGGTC-3′; pcbAB reverse: 5′-CTGGTGGGTGAG AACCTGACAG-3′) and (pcbC forward: 5′-AGGG TTACCTCGATATCGAGGCG-3′; pcbC reverse: 5′-G TCGCCGTACGAGATTGGCCG-3′). PCR was carried out using an initial denaturation step of 94°C for 5 min; followed by 35 cycles of 1 min at 94°C, 1 min at 34°C, and 2 min at 72°C; and a final extension of 72°C for 5 min. Amplified fragments were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

Gene expression analysis

Total RNA extraction and cDNA synthesis

Total RNA was extracted using Fermentas Kit from the mentioned fungi according to the manufacturer's instructions. The quantity and quality of extracted RNA were determined for each sample using agarose gel electrophoresis. Reverse transcription was performed using QuantiFast Revers -Transcriptase cDNA synthesis kit (QIAGEN, Cat. N0. 205311). Real time PCR was carried out with two specific primer pairs include (pcbAB forward: 5′-CACTTGACGTTGCGC ACCGGTC-3′; pcbAB reverse: 5′-CTGGTGGGTG AGAACCTGACAG-3′) and (pcbC forward: 5′-AGGG TTACCTCGATATCGAGGCG-3′; pcbC reverse: 5′-G TCGCCGTACGAGATTGGCCG-3′) using QuantiFast SYBER Green PCR kit (QIAGEN, Cat. No. 204052). Housekeeping Beta actin gene (forward: 5′- CTGGCC GTGATCTGACCGACTAC-3′, reverse: 5′- GGGGGA GCGATGATCTTGACCT-3′) was chosen as a reference gene. Amplification of the pcbAB and pcbC genes was performed for 40 cycles, which consisted of initial denaturation (95 °C, 5 min), denaturation in cycle (95°C, 10 s), combined annealing and extension (60°C, 30 s). The beta actin reference gene was amplified by 40 cycles under the same conditions in a different tube. In each PCR run, preparation of standard curve was carried out by serial dilution of pooled cDNA from samples. The relative expression ratio of the mentioned genes as a target gene was normalized to beta actin gene using 2-ΔΔct methods [26]. Quantification for each treatment group was performed in three replicates.

HPLC analysis

PEN G (sodium salt) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Formic acid of p.a. grade, Acetonitrile of HPLC grade from Merck (Darmstadt, Germany) and ammonium acetate of HPLC grade were purchased from Fluka (Steinheim, Germany), and Purified water (18.2 MOcm) was from Ariashimi (Tehran, Iran). The PTCC5031, PTCC 5033, PTCC5037 and PTCC5071 from *P. chrysogenum* and the PTCC5074 from *P. notatum* were fermented on a liquid culture in specific media of penicillin synthesis (3 gr yeast extract and 21 gr sucrose per one liter of pure distilled water (dd water)). Samples from the supernatant were taken after three time courses (5, 7 and 11 days after fermentation) at room temperature, with 120 rpm on a rotory shaker. Fifty micro liters of both the unprocessed supernatant and an ethyl acetate-extracted fractions were analyzed with HPLC (Waters Inc. USA) with a C18 column at 25C, and the flow rate was 0.2 mL/min. Penicillin G at a concentration of 0.1 mg/ml was used as the control sample. The mobile phase consisted of 0.1% formic acid in water with 1 mM ammonium acetate (solvent A, pH 2.7) and acetonitrile (solvent B).

Statistical analysis

Data were analyzed using GLM procedures of SAS software version 9.1. Differences between means were tested using Tukey test. Differences were considered significant at $P< 0.05$)

Results

Identification of genes in penicillin synthesis

In this study, the pcbAB and pcbC genes which encoded some enzymes that have a key role in the first step of penicillin synthesis were analyzed. Our results showed that these genes amplified well in all of the mentioned strains (Figure 1).

pcbAB gene expression

The expression of pcbAB and pcbC were quantified by RT-PCR assay and expressed relative to expression of beta actin. Expression of pcbAB and pcbC mRNA was done in three times courses (5, 7 and 11 days after cultivation). In the expression of pcbAB mRNA numerically was higher in PTCC 5037 strain than others whereas PTCC 5031 in 7 and 11days after cultivation, the expression pcbAB was in least , but this differences was not statistically significant ($P>0.05$).

In the effect of time in expression of pcbAB mRNA, our results indicated that the level of the expression of pcbAB gene in 7 days group increased as compared to 5 and 11 group in all of the mentioned fungi. Although, the level of gene expression in 7 days after cultivation group increased as other groups ($P<0.05$), this difference wasn't significant statistically ($P>0.05$).

The comparative analysis among the expression

levels between the mentioned fungi showed that *P. chrysogenum* PTCC 5033 and *P. chrysogenum* PTCC 5074 highest and P. chrysogenum PTCC 5031 had shown the least of expression levels in 5 days after cultivation. In addition, in 7 days after cultivation, *P. chrysogenum* PTCC 5031 was higher than others, and finally, our results showed decreased expression levels of all the mentioned fungi except *P. chrysogenum* PTCC 5037 in 11 days after cultivation. Also, *P. chrysogenum* PTCC 50331 and P. chrysogenum PTCC 5071 had the highest expression gene levels in 11 days after cultivation.

pcbC gene expression

The results of the evaluation of time course's effect in expression of pcbC mRNA was similar to the obtained results from pcbAB gene that the level of the expression of pcbC gene in 7 days group was higher than to 5 and 11 group in all of the mentioned fungi and this difference wasn't statistically significant (P>0.05). On the other hand, the results of comparative analysis among the mentioned strains showed the maximum expression levels in 5, 7 and 11 days after cultivation was belong to PTCC 5071, PTCC 5074 and PTCC 5031, respectively.

HPLC results

For quantifying the produced penicillin G by tested strains in different extractions the HPLC was used. So, the mentioned fungi were cultivated in specific media, and in 5, 7 and 11 days after cultivation extracted the antibiotic. Then, each extract with certain time of fungal growth injected to HPLC. The analysis of obtained chromatograms from injected samples showed that the

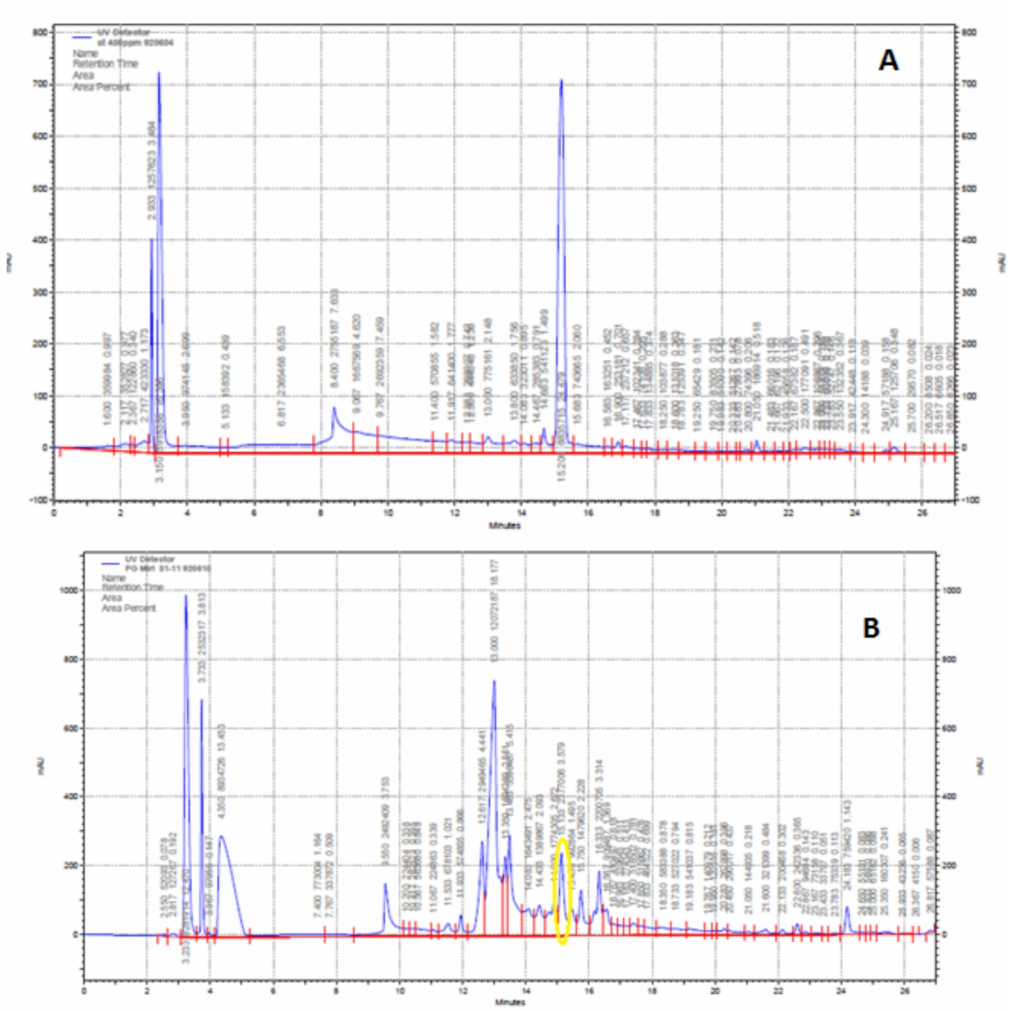

Figure 2. HPLC analysis of the supernatant of a fermentation of *P.chrysogenum* PTCC 5031 A) Control sample of penicillin G at a concentration of 0.1 mg/ml. The retention time for the penicillin G peak was 15.80 min. (B) Chromatogram of the unprocessed supernatant. The extraction with ethyl acetate partially purifies and enriches the penicillin G in the sample.

highest content of Penicillin in media was in 7 days after inoculation, except in *P. chrysogenum* PTCC 5033 strain. In addition, the results of comparative analysis among the mentioned strains showed that the penicillin content in 5, 7 and 11 days after cultivation was highest in *P. chrysogenum* PTCC 5037, *P. chrysogenum* PTCC 5031 and *P. chrysogenum* PTCC 5033, respectively.

Discussion

In general, the results of gene expression for both surveyed genes in most strains showed the expression levels of these genes in 7th day after inoculation was higher than other treatments The demand for semisynthetic antibiotics is still increasing that can be noticed to a world market of more than 60000 tons each year. Therefore, it would be valuable to optimize the industrial production and increasing yields for the β–lactam producing companies. One of the best choices to increase of related genes expression is the carbon precursor's supply during biosynthesis of penicillin. The previous studies had shown that the production of penicillin is not sensitive to carbon supply precursors by primal metabolism, but they revealed providing and availability of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) co-factors is more important than carbon precursors in the production of penicillin [27, 28]. In some samples that the level of gene expression and penicillin content was low, it may be due to the increased production of by products that causes the most available energy of cell consume for the production of these products [29]. To evaluate the molecular genetic basis of the high production of penicillin, some attempts that obtained from strain development programs were done. It seems that in *P. chrysogenum*, the copy number of the whole penicillin gene cluster is the best reliable reason for increasing the penicillin production. There are some problems in transformation of structural gene with large copy numbers [30-32]. At some regions, the increase of copy numbers is not the cause of increased penicillin production. Although, genetic studies have considered the increase of IPNS and ACVS activities for increment of penicillin production, there is some limitation in further activities of these enzymes, because in a certain step, providing carbonic precursors and co-factors will be the limitation of speed. In previous studies, Van Golik et al (2000) in a study based on the production of penicillin in some strains using industrial species with high production of *P. chrysogenum*, reported that it seems the production or removal of NADPH is a restricted factor in penicillin biosynthesis [27]. The demand for NADPH is increasing stepwise by cell cultivate in glucose or xylose as the carbon source blended with ammonia or nitrate as nitrogen source, that caused the decreased penicillin biosynthesis gradually, that may be due to the low content of NADPH. After that it can effect on ATP supply from primary metabolism as limiting factor for penicillin biosynthesis. Almost, most of studies on primal metabolism and penicillin synthesis have been done in fluxome levels and there is no study at the metabolome levels. Several transcriptome studies on *P.chrysogenum* were done in recent years that have shown the candidate genes for protein transfer are genes which up regulated in penicillin production condition. In addition, DNA sequencing could be used to evaluate the translated protein content in microorganisms (proteome) using MS technique. Although, finding a dialectician technique for proteome studies is very hard, the recent progress in MS technique provides the possibility of the first proteome study in *P.chrysogenum* [33, 34]. In a study, the translation and transcription of three produced antibiotic genes in *P.chrysogenum* carried out [35]. The effect of peroxisome on penicillin synthesis in *P. chrysogenum* was studied. The results of the mentioned study showed that 6-amino penicillanic acid acyltransferase (AT) and acyl coenzyme A (coA) enzymes are effective in biosynthesis of penicillin. Also, the researchers had shown increase of the number of peroxisome is caused to increase of penicillin synthesis.

In 2012, a study was performed based on effect of phenolic compounds and osmotic pressure on the expression of penicillin genes using qPCR assay in *P. chrysogenum var. halophenolicum*. In addition, the effect of salt on ability of these fungi was analyzed by HPLC. Moreover, there is no significant relation between the expression gene and penicillin production. It seems that the existence of phenol and phenolic compounds has a positive effect on antibiotic synthesis. These results showed that phenol, phenolic compounds and high density of salt could act as a stress factor for *P. chrysogenum var. halophenolicum* during high antibiotic production [25]. Using NMR, Spectrophotometry, two hundred of Penicillium isolates which obtained from fifteen soil samples were analyzed. In these fungi antimicrobial activities were observed and also butanol and acetate ethyl solutions were used for antibiotics extraction. In the mentioned study, the antimicrobial of both extraction methods was performed by DISC and only in one of them which acetate ethyl solution was checked, the antimicrobial activities was observed [36]. In a study, the survival of *P. chrysogenum* spores against UV ray and the production of antibiotic products were investigated. The produced penicillin content was analyzed by HPLC. The results of

this study revealed that mutation in *P. chrysogenum* could increase the production of penicillin which used for industrial products [37]. Another study showed that macromolecules would cause emulsion if the macromolecules such as polysaccharides and proteins, have not filtered in penicillin extraction process by solutions. Also, the researchers reported that it can be prevented from emulsion by ultra-filtration and thus improved quality and quantity of antibiotic production [38]. Babae et al (2008) reported that only Streptomyces which were separated from dessert areas have shown antibacterial activities. Also, the effects of ecological parameters such as temperature, acidity and the sources of carbon and nitrogen on antibacterial activities were studied [39]. The evaluation of the mentioned environmental parameters revealed the regulation of acidity in range 6.3- 7.2, temperature to 33 – 36 C, and addition of 10 gr glucose, 10 gr lactose and 1 gr methionine and tryptophan per liter as a carbon and nitrogen sources could be increased antibacterial activity.

Many factores have positive effect of penicillin production. Related researches showed different internal, external and genetic sources have influence on penicillin increase in laboratory and pilot conditions. Some of effective factores have minor or major role in penicillin production.

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