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TRANSFORMATION OF GLYCOSYLTRANSFERASE *DNR*S AND AUXILIARY *DNR*Q GENES INTO THE *STREPTOMYCES PEUCETIUS* MH9.2 FOR THE ENHANCE OF DOXORUBICIN PRODUCTION

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SUMMARY

Doxorubicin antibiotic produced by *Streptomyces peucetius* is a very crucial antibiotic with a large spectrum used as an anticancer drug and in clinical treatment. Production of doxorubicin from wild type strain by microbiological fermentation process is nomally very low yield and limited. The enhancement of doxorubicin production (DXR) by the genetically engineering of the auxiliary genes involved in the pathway of doxorubicin biosynthesis from *Streptomyces peucetius* has being focused by many research groups. The gene cluster responsible for biosynthesis of doxorubicin had been isolated, sequenced and characterized. Among them, the *dnr*Q and *dnr*S genes belong to the gene cluster encoded for the glycosyltransferase and helper enzymes, which are responsible for attachment of sugar moiety to **ε**-rhodomycinon intermediate and regulation of doxorubicin production, respectively. In this research, the *dnr*Q and *dnr*S genes were isolated and cloned in the p25.1 vector containing the strong promoter (*ermE**) to generate the p25.1QS as a recombinant vector. The obtained plasmid was transferred into the host strain *Streptomyces peucetius* MH9.2 by the protoplast transformation method to generate the *S. peucetius* MH9.2SQ strain. Doxorubicin production from the recombinant *S. peucetius* MH9.2SQ and the parent strain were extracted and purified by using chlorofrom and methanol solvents. The products were confirmed by HPLC, LC-mass analysis and antibacterial bioassay. The results showed that the over production of doxorubicin was accumulated by the recombinant strain from the fermentation broth. In the *S. peucetius* MH9.2SQ, the doxorubicin productivity was 3.1 to 3.6 folds higher than in the parent strain. This recombinant strain could be a potential candidate for further research in enhancement and larger biomass scale of DXR production.

Keywords: *Doxorubicin production,gene cloning, MH9.2SQ strain, Streptomyces peucetius.*

INTRODUCTION

Doxorubicin (DXR) (adriamycin or 14-hydroxydaunorubicin) is an anthracycline antibiotic produced from *Streptomyces peucetius* ATCC 27952 and used in chemotherapy for treatment of various cancers. DXR was first isolated and studied by the Arcamone group (Arcamone *et al.,* 1997). The wild type strain is nomaly to produce very low yield (2 $mg.l^{-1}$) due to very toxic compound and it causes the death for host cell (Mallar *et al*., 2010). To improve the production of doxorubicin, many researchers have had efforts to study the biosynthesis pathway and the gene regulation for higher yield of DXR by genetical modification and recombinant methods of wild type strain. Many of the published studies on the enhancement of secondary metabolites have focused either on the over expression of one positive regulator or by the deletion of one negative regulator at a time. Two classes of regulatory genes were reported as pathway specific and global regulatory involved in the production of secondary metabolites (Chater *et al*., 1996). Some other regulatory genes generally located outside the biosynthetic gene cluster may play a regulatory role in the cluster showing effects on the production of multiple secondary metabolites. Over expression of regulators increase the production of different secondary metabolites such as actinorhodin in *S. coelicolor* by *actII-orf4* and *redD* (Narva *et al.,* 1990; Fernández-Moreno *et al*., 1991), nikkomycin in *S. ansochromogenes* by *sanG* (Liu *et al*., 2005),

clavulanic acid in *S. clavuligerus* by *ccaR* (Hung *et al.,* 2007).

Doxorubicin biosynthesis gene cluster had been isolated and sequenced, among them the *dnr*S and *dnr*Q genes play a very crucial role for the post biosynthesis doxorubicin as glycosylation step. The gene *dnr*S encodes for glycosytransferase and is responsible for the attachment of deoxysugar 2,3,6-trideoxy-3-aminohexose, daunosamine to the rhodomycinone core (Dreier *et al*., 2007; Jiang *et al.,* 2006). The gene *dnr*Q encodes the auxiliary protein to help the activity of DnrS and for the complement of DnrQ/S activity (Singh *et al*., 2010) (Fig. 1). In addition, glycosyltransferase and auxiliary gene have thought to act as regulator for the enhancement of production. The expression of *dnr*S or *dnr*Q separately had not given any positive regulation for the DRX production. However, the over expression together of *dnr*S/Q led to remarkably increase the DRX amount in the *S. peucetius* host cells via in *vivo* experiment (Sharee *et al*., 1995; Sailesh *et al.,* 2009).

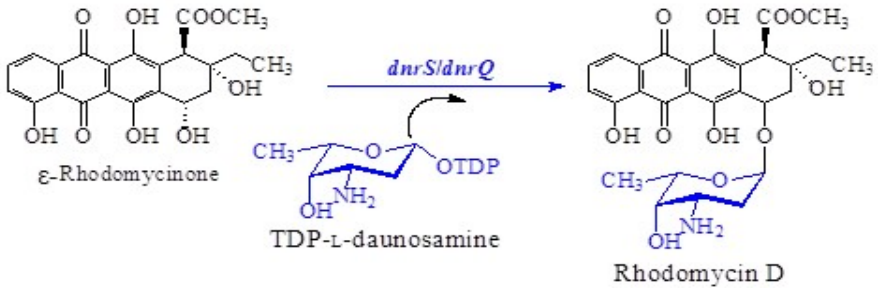

Figure 1 The mechanism of dnrQ/S activity in the biosynthesis of DXR.

The aim of this study was to develop recombinant strains of *S. peucetius* which are improved DXR production by the genetic engineering *dnr*S/*dnr*Q. The genes were cloned and expressed in the *S. peucetius* host cells. The screening process is to better understand that the *dnr*Q and *dnr*S works as regulation role for higher amount of DXR and the selection the engineered *S. peucetius as* potentical candidate used in the production on scale.

MATERIALS AND METHODS

Materials

DXR standard was provided by the Genechem Co. (Daejeon, Korea). HPLC grade methanol was acquired from Mallinckrodt Baker (Phillipsburg, NJ, USA). The PCR premix (Genotech, Korea) was used for the preliminary screening or optimizing the PCR conditions. Glucose, 22.5 g; Difco yeast extract, 5.04 g; $NaNO_3$, 4.28 g; K_2HPO_4, 0.23 g; HEPES, 4.77 g; $MgSO_4.7H_2O$, 0.12 g; NaOH, 0.4 g; trace element solution; PEG, thiostrepton antibiotics, and other chemicals for experiment were purchased from Sigma Co. Ltd. *S. peucetius* MH9.2, *S.peucetius* wild strains supported from the Central of Research and Applied Biotechnology, Ha Noi Open University was used as a parental strain.

Methods

DNA manipulation, gene cloning and polymerase chain reaction (PCR)

Standard methods were used for DNA cloning, plasmid isolation, and restriction enzyme digestion followed by Kieser's protocol (Kieser *et al.,* 2000). The nucleotide sequences were analysed by using DNA strider. Potential open reading frames (ORFs) were identified using Frame Plot (Sambrook *et al.,* 2001), and were searched for homologies using NCBI BLAST server (www.ncbi.nlm.nih.gov/BLAST/).

Oligonucleotide primers for the amplification of desired ORFs using a thermo cycler (Takara) used in this study are summarized in Table 1. The reaction mixture contained 20 pM primers, 0.125 mM deoxynucleotide triphosphates (dNTPs), 10% dimethyl sulfoxide (DMSO), appropriate amount of template DNA, 2 unit of La Taq polymerase, 2.5 mM $MgCl_2$, in the PCR buffer supplied with enzyme. The PCR conditions: denaturation temperature at 94ºC for 7 min then followed by 30 cycles of 94ºC for 1 min, annealing temperature

range from 55ºC to 68ºC for 1 min depending upon the PCR primers and the template and 72ºC for 1 min; and 72ºC for 10 min for the last extension cycle.

Table 1. Primers for PCR.

Name	Sequence	Restriction site
*dnr*Q-F	5'- GAATTCGTGTGAGGAGCACGACG -3'	*EcoR*I
*dnr*Q-R	5'-GGATCCGGTCACCCTCCCTGGG -3'	*BamH*I
*dnr*S-F	5'- CTGCAGCAGGGAGGGTGACCCC -3'	*Pst*I
*dnr*S-R	5'-AAGCTTATCCAAACGAAAGCCC- 3'	*Hind*III

Construction of expression plasmid p25.1SQ

Isolation and purification of dnrS and dnrQ

To amplify the *dnrS* (1296 bp) and *dnrQ* (1317 bp) from *S. peucetius* genomic DNA, the PCR process was carried out as protocol described above. The primers *dnr*Q-F/*dnr*Q-R were used for cloning *dnr*Q; *dnr*S-F/*dnr*S-R used for cloning *dnr*S gene. The PCR products of *dnr*Q and *dnr*S were digested by *EcoR*I/*BamH*I and *Pst*I/*Hind*III respectively, which were then cloned into the cloning vector with high copy number, namely, pGEM-3Z(+) producing the pGEM-QS vector containing *dnr*QS fragment for the further other analysis and experiments. All of the PCR products were cloned into pGEM-T vector (Promega, USA) and sequenced prior to cloning into the expression vector to verify that no mutations had been introduced during the PCR amplification.

Cloning dnrS and dnrQ into p25.1 vector

Originally, the transformation into *Streptomyces* cell has been challenged many research groups, so iBR25 vector was constructed as a special vector used to transform into *Steptomyces* cells (the size 7.5kb). Vector modified p25.1 (9.7kb) has been completely created which is bared *des*III and *des*IV genes form *S. venezuela* genome and strong promotor isolated from Erythromycin gene cluster. The *des*III and *des*IV genes are functioned as participating in the deoxysugar biosynthesis of antibiotics and increasing the antibiotic biosynthesis production (Thuy *et al.,* 2014).

Protoplast transformation and screening

For protoplast transformation, regeneration, and DNA transformation were slightly modified from the methods of Kieser et al. (2000) and followed by Sailesh's protocol (Sailesh *et al*., 2010). *S. peucetius* was grown in a 500 ml baffled flask containing 50 ml R2YE medium at 28°C for 48 – 60 hrs. Mycelia were harvested by centrifugation and washed with 10.3% sucrose then with P-buffer. The protoplast and mycelia mixture were filtered through cotton plug to remove cell debris. The protoplasts were gathered by centrifugation and washed twice with P-buffer. A 125 µl of protoplasts were treated with 0.1 mM aurintricarboxylic acid (ATA) for 10 min and 12 µl to 15 µl of plasmid DNA was added. Immediately, 200 µl of 40% (wt/vol) polyethylene glycol 1000 (PEG) solution was added and then, centrifuged briefly to remove PEG. These transformed protoplasts were plated in R2YE regeneration plate, and was incubated at 28°C for 24 hrs. The lawn colonies were overlaid by 0.3% agar solution containing appropriate antibiotics (20 - 100 µg ml^{-1} thiostrepton). After overlaying, selected colonies were screened on growing media with antibiotics as per the required condition.

Production of DRX by engineered strain

The *S. peucetius* engineered strains were inoculated into 50 ml NDYE-glucose medium [(per liter) glucose 22.5 g; Difco yeast extract 5.04 g; $NaNO_3$ 4.28 g; K_2HPO_4 0.23 g; HEPES 4.77 g; $MgSO_4.7H_2O$, 0.12 g; NaOH 0.4 g; and trace element solution, 2 ml] at 28°C and 230 rpm. After 48 h of incubation, 1 ml of seed culture was transferred to 50 ml of NDYE-maltose medium, which comprises of (per liter) Difco maltose, 22.5 g instead of glucose in NDYE-glucose medium. The incubation was carried out at 28°C and 230 rpm for 5 days.

Isolation of doxorubicin from fermentation broth

Aliquots of culture broth containing mycelia were extracted with twice volume of chloroform-methanol (9:1) (Parajuli *et al.,* 2005). The extracted solvents were evaporated until dryness by the method of rotary evaporator. Thus obtained residue extract was reconstituted in 1 ml of methanol and

subsequently subjected to HPLC and LC-MS analysis. For the comparison of DXR production, 1 ml culture media of broth strains were extracted with double volume of the mixture of chloroform: methanol (9:1) after 84, and 96 h of incubation. The extraction-solvent was evaporated *in vacuumm* and dissolved in 50 µl of methanol. A 15- 20µl sample of the extract was injected to HPLC.

Analysis of DXR production

The HPLC analyses were performed by injecting 15 µl of extracts sample fitted with a Mightysil RP-18 GP column (4.6 x 250nm x 5 µm, Kanto Chemical Tokyo). The elution was carried out with water (at pH 2.3 by trifluoro acetic acid) at the flow rate of 1 ml min^{-1} by the following method: 0-50 min (0-100% B), 50-60 min (100% B) and 60-70 min (100% A). Detection was carried out by UV light absorption at 254 nm wavelengths. Authentic DXR and DNR were taken as reference.

The HPLC standard cover of doxorubicin was used for the quantity analysis which was set up with the range of doxorubicin concentration from the 100, 200, 300, 400, and 500 $\mu g.ml^{-1}$ (Table 2).

Antibacterial assay

Bacillus subtilis was used for test of antibacterial activity. Specifically, 5 µl aliquots of the chloroform-methanol whole-cell extracts were added to paper discs and placed on a lawn of test strain (*B. subtilis*) overlaid onto nutrient agar plates. The plates were incubated at 37°C overnight. The antibacterial activity of the secondary metabolites was observed by the appearance of the diameter of clear inhibition zones surrounding the paper disc. Methanol was used as negative control.

Table 2. Concentration of doxorubicin for the standard cover for HPLC analysis.

Concentration of doxorubicin ($\mu g.ml^{-1}$)	Pick area
100.0	4615969
200.0	9310344
300.0	14198707
400.0	18333907
500.0	23221173

RESULTS AND DISCUSSION

Isolation and sequence analysis of *dnr*S and *dnr*Q

The *dnr*S and *dnr*Q genes were amplified by the PCR method as described in the protocol above (Materials and Method). All of the PCR products were cloned into pGEM-T vector (Promega, USA) and sequenced prior to cloning into the expression vector to verify that no mutation had been introduced during the PCR amplification. The open reading frame *dnrQ* (1,317 bp) flanked by *dnrS* (1,296 bp) are located within the doxorubicin biosynthesis gene cluster downstream of the polyketide synthase (PKS) genes. The *dnr*Q and *dnr*S genes were flanked upstream by *dnr*P and downstream by *dnr*C. Using the ClustalW program, the determined sequence analysis of these genes revealed a deduced amino acid sequence of *dnr*S (432 aa) that encodes glycosyltransferase and showed a very high degree of similarity to a number of the known GTs in the GenBank, including GT from *Streptomyces prasinopilosus* (94% identity) (GenBank accession no. WP_055571862.1); glycosyltranferase from *Streptomyces sp.* ScaeMP-e83 (87% identiy) (GenBank accession no WP_093692494.1); and GT from *Streptomyces kasugaensis* (61% identity) (GenBank accession WP_094791596.1). In addition, analysis of the amino acid sequences of dnrQ encoded for the auxilary protein to help the activity of dnrS (Singh *et al.,* 2010) that show high similarity to protein helper of *Streptomyces prasinopilosus* (GenBank Accession no. WP-055693204.1); activator protein from *Streptomyces sp.* ScaeMP-e83 (GenBank accession no. WP_093692495.1).

Construction of expression plasmid p25.1SQ

To construct recombinant expression plasmid, the 2.5 kb DNA fragment containing the *dnr*SQ was digested by *Eco*RI/*Hind*III from the pGEM-SQ vector and the cloned into modified p25.1 vector digested with the same restriction enzymes as well as by the ligation reaction.

The screening process was carried out by the transformation of ligation mixture into *E.coli* XL-blue and growing in the Apramycin antibiotic medium for the selection of correct colonies which are bearing the expression plasmid containing *dnr*QS fragment named p25.1SQ. Finally, the obtained recombinant expression plasmid p25.1SQ was examined for the correct clone by restriction enzyme digestion (*Eco*RI/*Hind*III). The bands of DNA

running in the agarose electrophoresis have shown the correct clone of the *dnr*QS (2.6 kb) into the expression plasmid (9.7 kb).

The appropriated conditions for transformation of p25.1.SQ into *S. peucetius* MH9.2

The plasmid was firstly propagated into *E. coli* ET-12567 host to get demethylated plasmid DNA and to get of risk the degradation of foreigner DNA. Then isolated plasmid from *E. coli* ET was transformed into *S. peucetius* MH9.2 host by using PEG-mediated protoplast transformation. For screening process, the transformed protoplast mixture was plated onto the R2YE medium. The plates were overlaying with 20 - 1000 $\mu g.ml^{-1}$ thiostrepton (tsr) after exactly 20 hrs of inoculation. The correct recombinant strains which is harbored the expression plasmids named as *S. peucetius* MH9.2QS) were selected by showing growing and resistant thiostreptone resistance.

The factors, have effects on the transformation yield, include concentration of PEG, the time period for lysozyme inoculation and thiostreptone antibiotic overlaying after transformation. The results have shown that there was very high growth rate but no transformation occurred if the antibiotic, PEG and lysozyme were applied at very low concentration. In contrast, all cell deaths were observed when those condition and concentration increased. The best condition for transformation was discovered for recombinant strain including 80 $mg.mg^{-1}$ tsr, 40% v.v PEG and lysozyme inoculation for 50 min, respectively (Table 3).

Table 3. Selection of condition for transformation of the p25.1SQ plasmid into *S. peucetius* MH9.2.

Thiostrepton		**Inoculation cell in PEG**		**Inoculation of lyzozyme**	
Tsr Conc. $\mu g.ml^{-1}$	Growth of transformed colonies	PEG Conc (%)	Growth of transformed colonies	Time (min)	Growth of transformed colonies
50	++	10	-	30	High growth rate No transformation occurred
60	+	20	-	40	High growth rate No transformation occurred
70	**-**	30	**+**	**50**	**Normal growth rate** **Transformation successes**
80	-	**40**	**++**	60	Very low growth rate Transformation successes
90	-	50	-	70	Death cell
100	**-**	60	-		Death cell

Antibacterial bioassay and thin layer chromatography (TLC) of doxorubicin

For the preliminary analysis of production from recombinant strain, the fermentation of the recombinant was carried out for 84 hrs and the host strain as control sample was culture for 60 hrs, 72 hrs, 84 hrs and 96 hrs, respectively. The solvent system of $CHCl_3$:MeOH was applied to the whole cell extracts, then extraction was centrifuged to remove the medium and collect the solvent containing crude doxorubicin extraction. The TLC and bioassays analysis were performed. TLC analysis indicated that *S. peucetius p*25.1 QS produced doxorubicin compound in higher amount in comparison with the *S. peucetius* MH9.2 (Fig. 2B). Similarly the bioactivity test of the compounds indicated the greater inhibition zone in case of *S. peucetius* 25QS than *S. peucetius* MH9.2 (Fig. 2A).

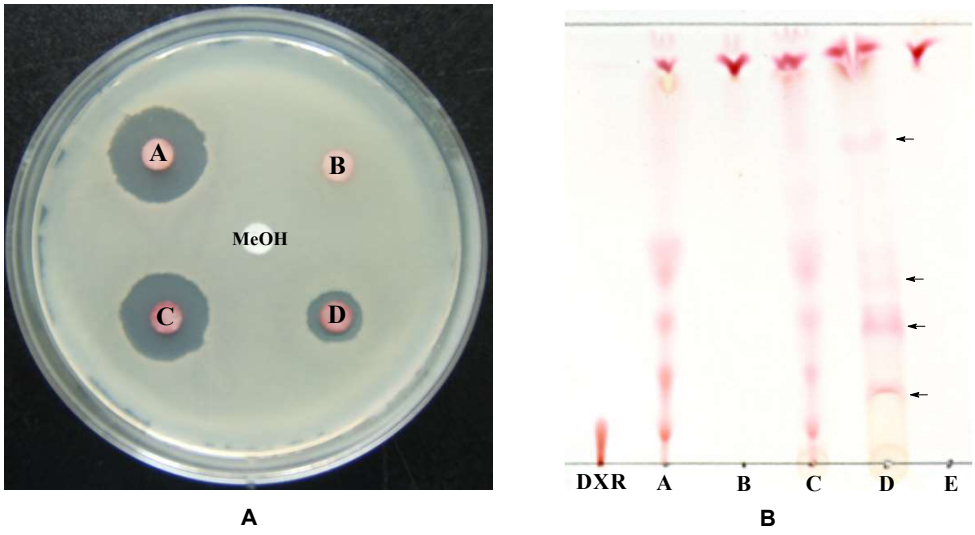

Figure 2. A: Antibacterial assay of extracted compound from *S. peucetius* against *B. subtilis*; B: TLC of total anthracycline chloroform:methanol:acetic acid (9:1:1); DXR: doxorubicin standard; A crude from the recombinant strain *S*.p25.1QS); C,D,B,E crude extract from S.MH9.2 isolated after 60, 72, 84 and 96 hrs fermentation.

Analysis of DXR production from engineered strain

To test the level accumulation of doxorubicin, four colonies of *S. peucetius* MH9.2QS recombinant strains and MH9.2 as control strain were selected for analysis and measuring DXR production. The fermentation of strains in the production medium (NDYE) were carried out for biomasses of both recombinant and parental strains.

The HPLC and LC-mass analysis of the isolated doxorubicin product from the recombinant strain have shown the significant amount increased more than three times in comparison with parental strains. HPLC profile showed the compound having molecular ion with peak at a retention time of 32.5 min in HPLC (Figs. 3A, 3B, 3C). The amount of doxorubicin produced by recombinant strains, which were calculated in the biomass fermentation, was from 6.8 to 7.25 mg.l^{-1}. Meanwhile the amount produced by the parental strain was only 2.3 mg.l^{-1} (Table 4).

Profile with *m/z* = 544.6 [M+H$^+$] in LC-ESI/MS analysis which corresponded to DXR (calculated mass 543.53). Identification of DXR was also verified by comparison with standard (Figs. 4A, 4B). The results indicated that the DXR productivity was enhanced more by over expression of the cognate GT pairing of *dnrS*/*dnrQ* in the host strain *S. peucetius*. The recombinant strain could be potential for the other phase research.

Table 4. Production analysis by HPLC of the recombinant strains.

Name	Y	X	Yields (µg.ml^{-1})
S. peucetius MH9.2	335994	5,84342692	2,337371
*S. p*MH9.2QS02	887320	17,76811437	7,107246
*S. p*MH9.2QS05	901007	18,06415192	7,225661
S.p MH9.2QS03	852834	17,02221309	6,808885
*S. p*MH9.2QS06	915898	18,38623091	7,354492

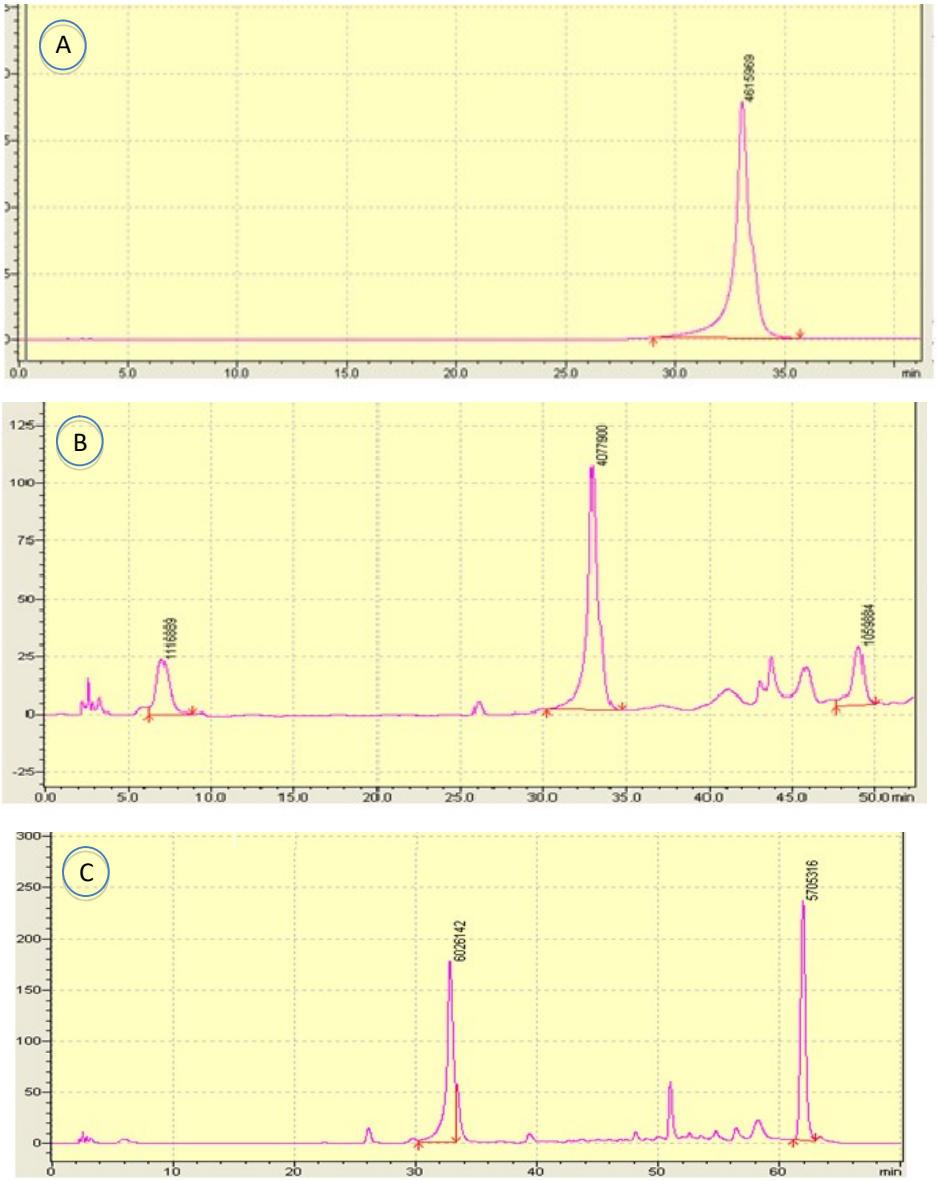

Figure (3). HPLC chromatograms of extracted doxorubicin from *S. peucetius*, perantal and recombinant strains. (A) Standard DXR, (B) *S. peucetius* MH9.2, C) *S. peucetius* 25.1MH9.2SQ.

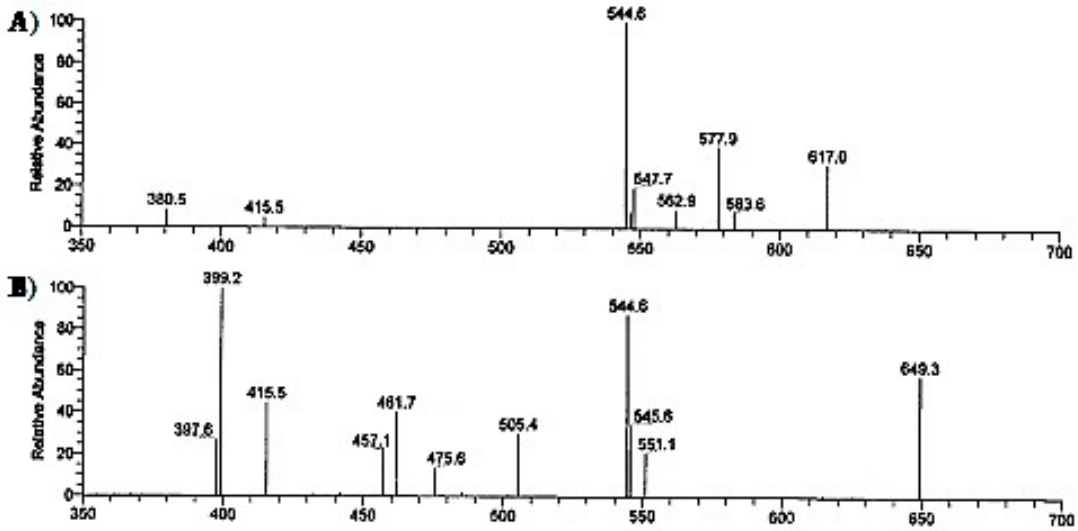

Figure 4. ESI-mass analysis of the doxorubicin standard in positive mode with the molecular weight (mz +) 554.6; (A) DXR standard (B) S. peucetius 25.1MH9.2QS.

CONCLUSION

Over production of doxorubicin from recombinant strain by genetically engineering, in which the *dnr*Q and *dnr*S genes were cloned into p25.1 and then transformed into the host *S. peucetius* MH9.2, has given the significant effect on DXR biosynthesis. The production of DXR was enhanced up to 7.3 mg l^{-1} (3.5 folds if compared to parental strain) whereas, the parental strain produced 2.2 mg l^{-1}. The strain S.p.MH9.2QS has the higher titers of DXR because of higher TDP-L-daunosamine pool and efficient glycosylation and to reduce the toxic nature of DXR against cell itself.

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BIẾN NẠP GENE GLYCOSYLTRANSFERASE *DNR*S VÀ GEN HỖ TRỢ *DNR*Q VÀO TẾ BÀO *STREPTOMYCES PEUCETIUS* MH9.2 LÀM TĂNG KHẢ NĂNG TỔNG HỢP DOXORUBICIN

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TÓM TẮT

Doxorubicin (DXR) là kháng sinh quan trọng được sử dụng điều trị ung thư, được sinh tổng hợp bởi *Streptomyces peucetius*. Quá trình sản xuất doxorubicin bằng phương pháp lên men từ chủng vi sinh vật gốc thường thu được hiệu suất thấp. Có nhiều nhóm nghiên cứu đã và đang tập trung nghiên cứu để làm tăng khả năng sinh tổng hợp doxorubicin từ chủng gốc bằng kỹ thuật tách dòng, biểu hiện nhóm gen hỗ trợ và điều hòa của chủng gốc. Nhóm gen *dnr*Q và *dnr*S tham gia con đường sinh tổng hợp doxorubicin có chức năng là vận chuyển gốc đường khử *dnr*S, và gen hỗ trợ đi kèm là *dnr*Q hay còn được gọi là gen điều hòa. Nhóm gen *dnrS/Q* được tách dòng và sau đó được chuyển vào vector p25.1 có mang promoter mạnh (*ermE**). Vector này được biến nạp chuyển vào chủng gốc *S. peucetius* MH9.2 bằng phương pháp tế bào trần đã tạo được chủng tái tổ hợp *S. peucetius* MH9.2SQ. Khả năng sinh tổng hợp doxorubicin từ chủng tái tổ hợp đã tăng vượt trội từ 3,1 đến 3,6 lần so với chủng gốc ban đầu. Các kết quả được khẳng định bằng phương pháp phân tích sắc ký lỏng cao áp (HPLC), sắc ký lỏng khối phổ (LC-mass) và đánh giá sinh học. Kết quả này cho thấy chủng tái tổ hợp là tiềm năng ứng dụng cho các nghiên cứu tiếp theo để tạo sản phẩm doxorubicin với qui mô lớn hơn.

***Từ khóa*:** *chủng tái tổ hợp di truyền, sinh tổng hợp Doxorubicin, Streptomyces peucetius, tách dòng gene.*