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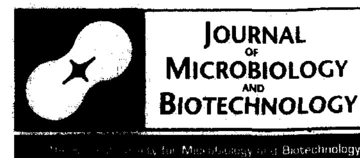

Doxorubicin Productivity Improvement by the Recombinant *Streptomyces peucetius* with High-Copy Regulatory Genes Cultured in the Optimized Media Composition

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Abstract Doxorubicin is a clinically important anticancer polyketide compound that is typically produced by *Streptomyces peucetius* var. *caesius*. To improve doxorubicin productivity by *S. peucetius*, a doxorubicin pathway-specific regulatory gene, *dnrI*, was cloned into a high-copy-number plasmid containing a catechol promoter system. The *S. peucetius* containing the recombinant plasmid exhibited approximately 9.5-fold higher doxorubicin productivity compared with the wild-type *S. peucetius*. The doxorubicin productivity by this recombinant *S. peucetius* strain was further improved through the optimization of culture media composition. Based on the Fractional Factorial Design (FFD), cornstarch, K_2HPO_4, and $MgSO_4$ were identified to be the key factors influencing doxorubicin productivity. The Response Surface Method (RSM) results based on 20 independent culture conditions with varying amounts of key factors predicted the highest theoretical doxorubicin productivity of 11.1 mg/l with corn starch of 46.33 g/l, K_2HPO_4 of 4.63 g/l, and $MgSO_4$ of 9.26 g/l. The doxorubicin productivity of the recombinant *S. peucetius* strain with the RSM-based optimized culture condition was experimentally verified to be 11.46 mg/l, which was approximately 30.8-fold higher productivity compared with the wild-type *S. peucetius* without culture media optimization.

Key words: Doxorubicin, *Streptomyces peucetius*, regulatory gene, media composition optimization

Doxorubicin is one of the most clinically important anticancer drugs, which belongs to a structural family of type II polyketide compound produced by *Streptomyces peucetius* var. *caesius* [18, 25]. Like other secondary metabolites biosyntheses in *Streptomyces* species, the doxorubicin biosynthesis in *S. peucetius* is also tightly regulated at the very complicated genetic levels [1, 9, 14, 22]. Because of the complex and tight regulation mechanism of doxorubicin biosynthesis, the wild-type *S. peucetius* hardly produce any detectable amount of doxorubicin in a typical *Streptomyces* culture [22]. For many years, people have tried to improve the doxorubicin productivity through recursive random mutagenesis as well as culture condition optimization. However, this kind of traditional *Streptomyces* strain improvement strategy was pursued based on trial-and-error approaches, without any consideration of genetic background of the strain related to doxorubicin biosynthetic regulation [21, 25].

In this short communication, a doxorubicin pathway-specific regulatory gene, *dnrI*, was cloned into a high-copy-number plasmid containing a catechol promoter system [20] to improve doxorubicin productivity by the recombinant *S. peucetius*. The doxorubicin productivity by this recombinant *S. peucetius* strain was further improved through the optimization of culture media composition using Fractional Factorial Design (FFD) and Response Surface Method (RSM) [6, 8, 11, 23]. The doxorubicin productivity of the recombinant *S. peucetius* with the RSM-based optimized culture condition was experimentally proven to be consistent with its theoretical value, implying an efficient strategy to optimize doxorubicin productivity by combining the genetic regulatory system and culture media composition.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Streptomyces peucetius var. *caesius* (ATCC27952) was purchased and maintained as a 20% glycerol stock solution stored at - 20°C. The culture media composition for doxorubicin

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production by *S. peucetius* was corn starch 10 g; NaCl 1 g; $(NH_4)_2SO_4$ 2 g; K_2HPO_4 1 g; $CaCO_3$ 2 g; $MgSO_4$ 2 g; tryptone 2 g; Inorganic solution ($FeSO_4 \cdot 7H_2O$ 1 mg; $MgCl_2 \cdot 6H_2O$ 1 mg; $ZnSO_4 \cdot 7H_2O$ 1 mg; H_2O 1,000 ml) 1 ml; H_2O 1,000 ml [21]. To produce doxorubicin, 10% (v/v) of *S. peucetius* stock solution was inoculated into a 25 ml culture medium in a 250-ml baffle flask containing small glass beads, followed by the constant shaking with 200 rpm at 30°C. Thiostrepton (5 µg/ml) was added into the culture medium for the recombinant *S. peucetius*. The bacterial cells including *S. lividans* TK21 and *E. coli* DH5α, and the plasmids including pGEM T-easy (Promega, WI, U.S.A.) and a streptomycetes-*E. coli* shuttle vector pWHM3 [10], were used for cloning and expression experiments, following the standard molecular biology procedures [10].

Gene Cloning and Genetic Manipulation

For total DNA isolation, the *S. peucetius* stock suspension was inoculated into 25 ml of R2YE liquid media [13, 15, 16] and cultured for 4 days at 30°C. The *Streptomyces* total DNA isolation method was as previously described [13, 15, 16]. Two genes, *dnrI* and *catR* including its promoter, were individually amplified by PCR (Idaho Technology, ID, U.S.A.) using each pair of primers. The forward and reverse primers for *dnrI* were 5'-GAGCACACCGCATAT-GAAGATCAATA-3' and 5'-ACGTAGGTGGAAGCTTG-ACTCCTTGT-3', respectively. The forward and reverse primers for *catR* were 5'-AGATCTCCTCGAATTCGAA-GATCCGG-3' and 5'-GCGTGATCTTCATATGCTGAC-CTCGT-3', respectively. The PCR condition for high G+C DNA was described elsewhere [20]. Each of the PCR-amplified products was cloned into a pGEM T-easy plasmid (Promega, WI, U.S.A.) and confirmed by sequencing. The *dnrI* gene was then subcloned into an *Eco*RI-digested pWHM3, resulting in pESK401 (Fig. 1). The *catR* gene including its promoter region was translationally coupled with *dnrI*, followed by subcloning into an *Eco*RI-*Hin*dIII double-digested pWHM3, resulting in pESK402 (Fig. 1).

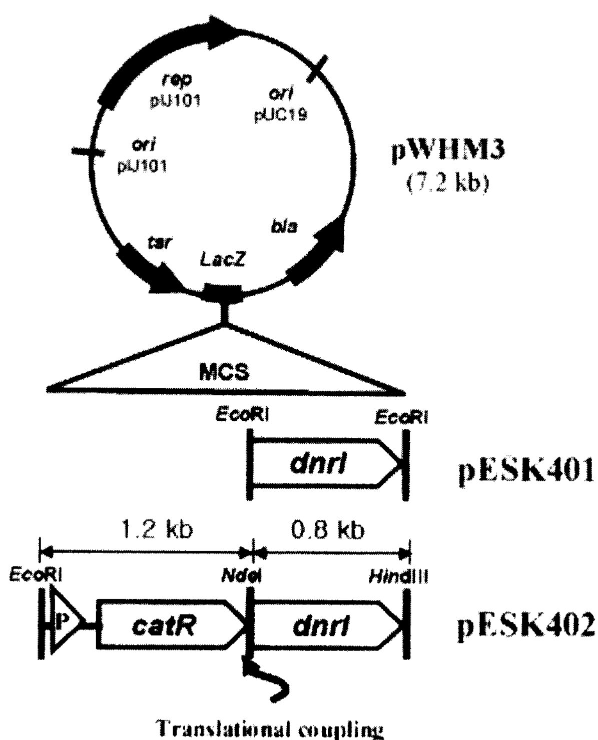

Fig. 1. Schematic maps of pWHM3, pESK401, and pESK402.

HPLC Assay for Doxorubicin Quantification

The *S. peucetius* culture was extracted with an equal volume of a mixture (iso-propyl alcohol:30% HCl=50:1), followed by a 10 min centrifugation at 15,000 rpm. The resulting supernatant was injected into an HPLC system (Young-In SP903D, Seoul, Korea) for doxorubicin quantification using a reverse-phase C_{18} column (4.6×250 mm, Symmetry, Milford, U.S.A.) with an isocratic mobile phase buffer of acetonitrile:water (1:1 v/v) containing sodium dodecyl sulfate (1.327 g/l) and phosphoric acid (0.68 ml/l). The column temperature was maintained at room temperature, and doxorubicin was monitored at 254 nm. The flow rate was kept constant at 1 ml/min during the entire HPLC assay. The standard doxorubicin was purchased from Sigma Co. (St. Louis, U.S.A.).

Optimized Media Composition Analysis

In order to optimize the doxorubicin media composition based on Response Surface Methodology (RSM), a Fractional Factorial Design (FFD) method was employed to find out the key factors influencing doxorubicin productivity in the recombinant *S. peucetius* [2, 4, 6, 8, 11, 23]. The statistical analysis was performed using Minitab software (version 13.20, Minitab Co., PA, U.S.A.). The contour plot was calculated based on the method proposed by Myers and Montgomery [17].

RESULTS AND DISCUSSION

Productivity Improvement by Doxorubicin-Specific Regulatory Gene

It has been documented that some regulatory genes involved in *Streptomyces* antibiotic biosynthesis could enhance the antibiotic productivity if the target regulatory gene was overexpressed as a multicopy plasmid form [12] or a chromosomally integrated form [Kim *et al.*, unpublished data]. In order to enhance the doxorubicin productivity *via* an improved regulatory genetic system, the *dnrI*, a doxorubicin-specific positive regulatory gene, was PCR-amplified and subsequently cloned into a streptomycetes-

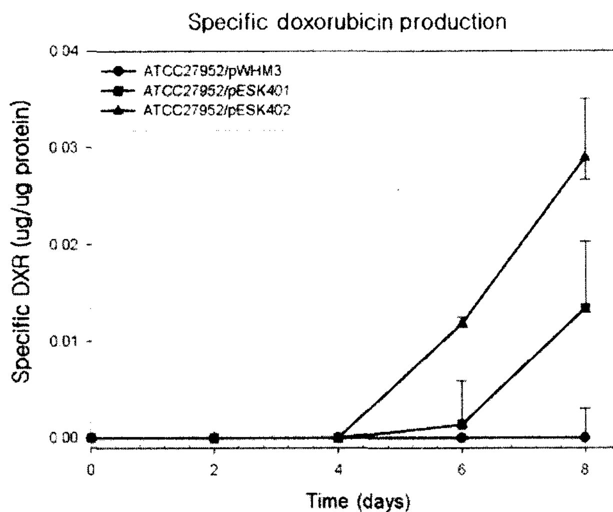

Fig. 2. Specific doxorubicin concentration by pESK402-containing *S. peucetius* var. *caesius* ATCC27952 (■), pESK401-containing *S. peucetius* var. *caesius* ATCC27952 (▲), and pWHM3-containing *S. peucetius* var. *caesius* ATCC27952 (●).

E. coli shuttle plasmid pWHM3, resulting in pESK401 (Fig. 1). The *dnrI* is a pathway-specific regulatory gene stimulating a doxorubicin biosynthetic pathway, and has been successfully applied to the *S. peucetius* ATCC29050 to improve the productivity of a doxorubicin precursor, daunorubicin [19]. As expected, the recombinant *S. peucetius* containing pESK401 showed approximately 5.2-fold higher doxorubicin specific-productivity than the pWHM3-containing wild-type strain (Fig. 2). Because the cloned *dnrI* gene in pESK401 did not contain its own promoter, it is believed that the *dnrI* expression is derived from the tiostrepton promoter located in the pWHM3 vector [10]. To ensure the stable expression of *dnrI* in the plasmid, the previously characterized catechol promoter system [21] was translationally coupled with the *dnrI*, resulting in pESK402 (Fig. 1). The recombinant *S. peucetius* containing pESK402 exhibited approximately 9.5-fold higher doxorubicin specific-productivity than the pWHM3-containing wild-type strain (Fig. 2). It is highly probable that the cloned *dnrI* gene in pESK402 was more stably expressed under the *catR* promoter system comparing with the promoter-less *dnrI* in pESK401. The *catR* promoter system was previously characterized to be induced at the stationary phase in the absence of any inducer, even though the induction level was enhanced in the presence of some inducers including catechol or 4-chlorophenol [21]. The addition of aromatic inducers in the doxorubicin culture, however, significantly inhibited the growth of the recombinant *S. peucetius* stain, implying that the further modification of *S. peucetius* strain to be resistant to aromatic compounds is necessary to fully maximize the potential inducibility of the catechol promoter system.

Table 1. Experimental range and levels of culture media components.

Nutrient (g/l)	- point	0 point	+ point
Cornstarch	10	20	30
NaCl	1	2	6
$(NH_4)_2SO_4$	2	4	3
K_2HPO_4	1	2	6
$CaCO_3$	2	4	6
$MgSO_4$	2	4	6
Tryptone	2	4	6

Optimization for Culture Media Composition

It was previously confirmed that the culture condition and the media composition significantly influence the doxorubicin productivity in *S. peucetius* [21]. It is believed that some of the culture media components may stimulate the complicated doxorubicin regulatory systems either directly or indirectly, of which the detailed mechanisms are not yet completely understood. Based on these speculations, it is highly expected that the recombinant *S. peucetius* with an overexpressed regulatory gene could be further improved to produce more doxorubicin once the culture media for the recombinant *S. peucetius* strain is optimized. In order to maximize the doxorubicin productivity by the pESK402-containing *S. peucetius*, a Response Surface Methodology (RSM) and a Fractional Factorial Design (FFD) were employed to find out the key factors influencing doxorubicin productivity in the recombinant *S. peucetius*. The FFD based on Table 1 and the experimental results shown in

Table 2. Complex analysis of variance model.

Run	Cornstarch	NaCl	$(NH_4)_2SO_4$	K_2HPO_4	$CaCO_3$	$MgSO_4$	Tryptone	DXR (mg/l)
1	+	+	-	+	-	-	-	2.57536
2	+	+	+	+	+	+	+	8.58678
3	-	+	-	-	+	-	+	0
4	-	-	+	+	-	-	+	0
5	-	-	-	+	+	+	-	6.78486
6	+	-	-	-	-	+	+	2.35998
7	-	+	+	-	-	+	-	0.86406
8	+	-	+	-	+	-	-	2.38

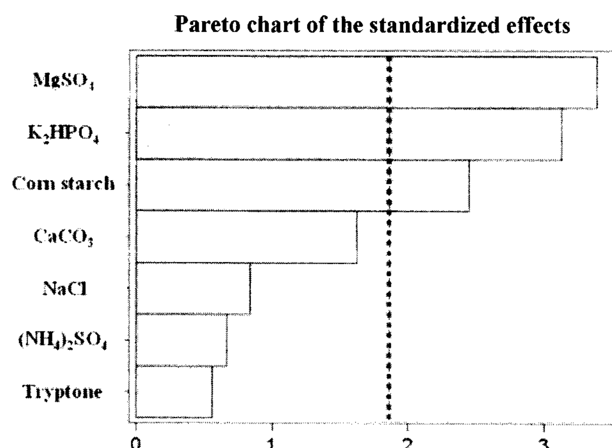

Fig. 3. Pareto chart of the standardized effect on the doxorubicin productivity.

Table 2 suggested that there are three key components in the medium; $MgSO_4$, K_2HPO_4, and cornstarch (Fig. 3). To find out the optimum concentrations of the key components, the range and the levels of the variables were selected (Table 3). The central values (zero level) chosen for experimental design were starch=30 g/l, K_2HPO_4=3 g/l, $MgSO_4$ =6 g/l. The doxorubicin productivities were obtained from the experimental results of 20 independent culture conditions (Table 4). Based on 2D contour plots, the interactions between K_2HPO_4 and cornstarch, and the relationship between $MgSO_4$ and cornstarch were not very significant for doxorubicin productivity (data not shown). However, the relationship between K_2HPO_4 and $MgSO_4$ was found to be critical for doxorubicin productivity, revealing optimum concentrations for K_2HPO_4 and $MgSO_4$ at 1- 1.5 g/l and 6-8 g/l, respectively (Fig. 4). The application of RSM yielded the following regression equation that is an empirical relationship between doxorubicin concentration (Y) and the test variables in coded unit.

$$Y=7.4473+1.2517X_1-0.3471X_2+0.4080X_3+0.3036X_1^2-0.0534X_2^2-0.2508X_3^2+0.4932X_1X_2-0.0401X_1X_3+0.0796X_2X_3 \quad (1)$$

where Y is the doxorubicin concentration, and X_1, X_2, and X_3 are the coded values of the test variables of cornstarch, K_2HPO_4, and $MgSO_4$, respectively. Using the method proposed by Myers and Montgomery [17], the maximum doxorubicin concentration (Y) was calculated, where the coded values were X_1=1.633, X_2=1.633, and X_3=0.955, respectively. The natural values obtained by putting the respective values in Eq. (1) are cornstarch (X_1)= 46.33 g/l, K_2HPO_4 (X_2)=4.63 g/l, and $MgSO_4$(X_3)=7.64 g/l. The model predicts that the maximum concentration of doxorubicin that can be obtained using the above optimum concentrations of the variables is 11.1 mg/l. The final verification of the results using the optimized medium was accomplished by carrying out the doxorubicin culture by the recombinant *S.*

Table 3. Experimental range and levels of the independent variables.

Variables (g/l)	Symbol		Range and levels				
	Real	Coded	- 1.633	- 1	0	+1	+1.633
Cornstarch	U_1	X_1	13.67	20	30	40	46.33
K_2HPO_4	U_2	X_2	1.37	2	3	4	4.63
$MgSO_4$	U_3	X_3	2.73	4	6	8	9.26

Table 4. CCD plan in coded value and observed response (doxorubicin).

Run	X_1	X_2	X_3	DXR (mg/l)
1	- 1	- 1	- 1	8.916
2	1	- 1	1	9.666
3	- 1	- 1	1	6.861
4	- 1	1	- 1	3.793
5	- 1	1	1	6.001
6	1	1	1	7.568
7	1	1	- 1	8.731
8	1	- 1	- 1	7.936
9	1.633	0	0	10.727
10	0	1.633	0	8.002
11	0	0	1.633	8.134
12	- 1.633	0	0	6.057
13	0	- 1.633	0	6.824
14	0	0	- 1.633	5.693
15	0	0	0	8.916
16	0	0	0	7.864
17	0	0	0	6.046
18	0	0	0	7.554
19	0	0	0	6.001
20	0	0	0	8.194

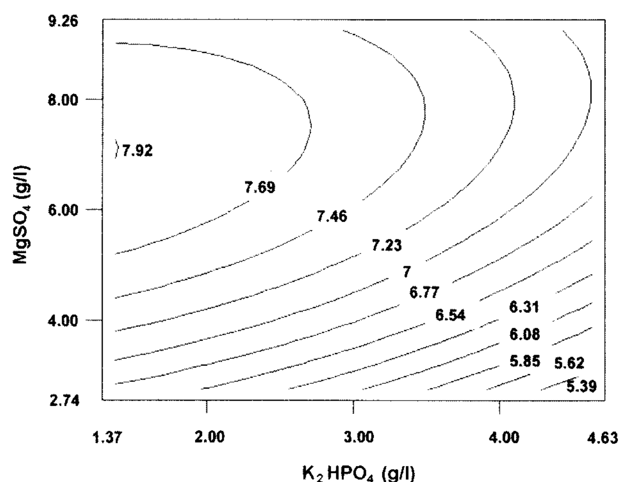

Fig. 4. Contour plot of doxorubicin concentration (mg/l): the effect of K_2HPO_4 and $MgSO_4$ on doxorubicin production. Other variables are held at zero level.

Table 5. Experimental verification of the combined effect of an optimized medium on the response of doxorubicin.

Variables	Levels before optimization (g/l)	Level after optimization		Doxorubicin (mg/l)		
		Code	Uncode (g/l)	Before optimization	After optimization	
					Predicted	Experimental
Cornstarch	10	1.633	46.33	4.0384	11.1	11.46
K_2HPO_4	1	1.633	4.63			
$MgSO_4$	2	0.955	7.64			

peucetius containing pESK402. As shown in Table 5, the doxorubicin productivity was measured as 11.46 mg/l, which was very close to the theoretical value. In conclusion, the recombinant strain from the optimized culture exhibited approximately 2.8-fold higher doxorubicin productivity than the same recombinant strain from an un-optimized culture, and even 30.8-fold higher than the wild-type strain from an un-optimized culture.

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