Full Length Research Paper

Enhancement production of qinlingmycin by a soil-derived *Streptomyces* No. 24 using protoplast fusion technology and assessment of antibacterial activity

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This study was aimed at improving qinlingmycin production by protoplast fusion technology for *Streptomyces* No. 24 and mutant strain Ms-24. In the protoplast preparation, the optimal glycine concentration, lysozyme concentration and lytic time were 0.25%, 1 mg/ml and 1 h, respectively. In the process of protoplast fusion, the weight of polyethylene glycol (PEG) molecule had little effect, while the top fusion efficiency was observed at 50% PEG. By protoplast fusion, the three high qinlingmycin producing fusants Pf77, pf126 and pf138 were fortunately obtained from 163 fusant strains. *In vitro* antibacterial activities of the three fusants than *Streptomyces* No. 24 were increased by 43.48, 60.87 and 65.22%, respectively. The results by HPLC detection analysis showed qinlingmycin productions of the fusants fermented broths were increased by 36.59, 74.39 and 91.46%, respectively, than that of *Streptomyces* No. 24. The fusants showed good heredity stability in continuing transferred ten generations test.

Key words: Protoplast fusion, *Streptomyces* No. 24, mutant Ms-24, qinlingmycin, antibacterial activity.

INTRODUCTION

β-Lactam antibiotics, containing *β*-lactam ring in the molecules structure, have been divided as six types including penicillin, cephems, cephams, clavams, carbapenems and monobactams. These antibiotics play a very important role for human health in a long period; however, the wide-spread occurrence of antibiotic resistance is threatening human health (Sabiha, 2001; Fulgueira et al., 2004)). Therefore, the discovery and development of new antibiotics is essential to combat drug-resistant pathogens. In the course of a screening program for new antibiotics from microbial products, an actinomycete *Steptomyces* No. 24, isolated from soil samples of Qinling Mountains of Shaanxi province, was found to produce a novel *β*-lactam antibiotic, namely qinlingmycin, which showed strong antibacterial activity *in vitro* (Long, 2006).

Protoplasts are the cells of which cell walls are removed and cytoplasmic membrane is the outermost layer in such cells (Bhojwani et al., 1977; Dai et al., 2005). Protoplast fusion is a physical phenomenon, during fusion two or more protoplasts come in contact and adhere with one another either spontaneously or in the presence of fusion inducing agents (Gallmetzer, 1999). In protoplast fusion technology, two genetically different protoplasts isolated from the somatic cells are experimentally fused to obtain parasexual hybrid protoplasts which contained heteroplasoic cytoplasm and two fused parent nuclei. By protoplast fusion, it is possible to transfer some useful genes such as disease resistance, rapid growth rate, more product formation rate, protein quality, drought resistance, herbicide resistance from one species to another (Wang et al., 2003; Yu et al., 2001; Mahadevan et al., 1997). At

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present, protoplast fusion is relatively a new versatile technique to induce or promote genetic recombination in a variety of prokaryotic and eukaryotic cells (Bhojwani et al., 1977; Javadekar, et al., 1995; Collonnier et al., 2003), especially, industrially useful microorganisms (Murlidhar et al., 2000; Zhou et al., 1990; Vazquez et al., 1997; Siemens et al., 1995, Shafiee et al., 2001; Izgu, et al., 2004), such as *Streptomycete ambofaciens*, *Micromonsporae chinospora* (Hopwood et al., 1979). Because it breakdowns the barriers to genetic exchange imposed by conventional mating systems.

The aim of this study was to investigate qinlingmycin production in *Streptomyces* No. 24, mutant strain Ms-24 and fusants as well as evaluating antibiotic production and antibacterial activity of the fusants.

MATERIALS AND METHODS

Microorganism

Streptomyces No. 24 was isolated from soil samples of Qinling Mountains in Shaanxi province of China and mutant strain Ms-24 was obtained by ultrasonic wave mutagenesis. The two strains were preserved in Gause's synthetic agar (GS) slants at 4°C.

Preparation of spore suspension

Streptomyces No. 24 and mutant strain Ms-24 were inoculated into the GS medium plates under dark in an incubator at 28°C, respectively. After 4 days old, mature spores were clearly observed by an optical microscope, 5 ml sterile water was added to GS plates, washing water was poured into Eppendoff tubes and spore suspension was obtained after sharp oscillation by an oscillator and filtering by sterile absorbent cotton.

Mycelium culture and protoplast preparation

0.1 ml spore suspension (1×10^6 spores/ml) was inoculated into 50 ml mycelium medium (soluble starch 4 g, maltose 10 g, yeast extract 5 g, distilled water 1000 ml, pH 5.5) in 250 ml Erlenmeyer flasks to incubate for 2 days in rotary shaker (210 rpm) at 28°C, ten percent of these inoculums were transferred into 50 ml mycelium medium containing 0.5% glycine (Gly) and were sustainingly incubated for 2 days. 30-ml culture suspension was taken out and centrifuged for 15 min at 3000 rpm, supernatant liquid was removed and then mycelium was resuspended into 10.3% sucrose solution. The process of treatment was carried out once again and then mycelium deposits were obtained. The harvested mycelium was dissolved into lysozyme solution (2 ml, Sigma, China) after degerminged by using 0.22 μl filters; the solution was kept for 60 min at 30°C in an electro-thermostatic water bath. Filtered by absorbent cotton, the supernatant was centrifuged for 15 min at 3000 rpm and the deposits were resuspended into buffer solution, namely the protoplast solution.

Double staining fluorescence label of two parent protoplast

The protoplast of *Streptomyces* No. 24 was stained by using phensafranine reagent (Sigma, China) described as Lin et al. (2005). Briefly, the one milliliter mixture of the 10% (volume)

obtained protoplast solution of *Streptomyces* No. 24 and 90% (volume) P buffer solution (sucrose 103 g, K_2SO_4 0.25 g, $MgCl_2 \cdot 6H_2O$ 2.02 g, trace elements solution 2 ml, distilled water 800 ml, autoclaving for 30 min at 115°C; trace elements solution: $ZnCl_2$ 40 mg, $FeCl_3 \cdot 6H_2O$ 10 mg, $CaCl_2 \cdot 2H_2O$ 10 mg, $MnCl_2 \cdot 4H_2O$ 10 mg, $Na_2B_4O_7 \cdot 10H_2O$ 10mg, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 10mg, distilled water 1000ml) was stained by 0.01% phensafranine solution. The living protoplast was dyed as red, while death was not dyed.

The protoplast of *Streptomyces* No.24 was stained by using Evans blue reagent (6,6'-[3,3'-dimethyl(1,1'-biphenyl)-4,4'-diyl]bis(azo)-bis-[4-amino-5-hydroxy-1,3-naphthale-nedisulfo], Sigma, China) described as Ulrike et al. (1999). Briefly, the one milliliter mixture of the 10% (volume) obtained protoplast solution of mutant strain Ms-24 and 90% (volume) P buffer solution was stained by 0.01% Evans blue solution. The living protoplast was dyed as blue while death was not dyed.

Fusion, regeneration and screening of protoplast and evaluation of the fusants stability

The 50 μl dyed protoplast solution of *Streptomyces* No. 24 was sufficiently mixed in the tube with 15ml dyed protoplast solution of mutant strain Ms-24. The mixture was centrifuged for 15 min at 3000 rpm and the supernatant was eliminated. Light touching the tube, the deposited protoplast solution was suspended in the remaining buffer solution. Washed by adding 15 ml buffer solution, the deposited protoplast solution were resuspended and added by two milliliter fifty percent polyethylene glycol (PEG) buffer solutions. After staying for 5 min at 35°C, the mixture was diluted by adding 15 ml buffer solution and centrifuged to remove the supernatant. Finally, the sediment was equably dissolved in buffer solution.

The stated protoplast mixture solution was uniformly spread in the optimal regeneration medium (sucrose 103 g, glucose 2 g, peptone 2 g, yeast extract 2 g, casein hydrolysate 4 g, KH_2PO_4 0.05 g, $MgCl_2 \cdot 6H_2O$ 10.12 g, $CaCl_2 \cdot 2H_2O$ 2.9 g, L-His 0.776g, L-Glu 2.5 g, trace element 2 ml, agar 18 g, distilled water 1000 ml, pH 7.2) and was then, incubated in a incubator for 4 days under dark at 28°C. The single clone by sterile toothpicks was inoculated to incubate according to the earlier mentioned condition. The fusant contained red-blue clone was determined and by an optics-microscope (Wu, 2004).

The obtained fusants were continuously inoculated ten generations in GS medium plates. The 10th generation fusant clones were inoculated into fermented medium (millet 10 g, glucose 10 g, $CaCO_3$ 2 g, NaCl 2.5 g, peptone 3 g, distilled water 1000 ml, pH 7.2 to 7.4) and the fermented broths were harvested and evaluated *in vitro* against *Bacillus subtilis*.

Extraction, purification and HPLC analysis of qinlingmycin

The 100 ml fermented broths of the *Streptomyces* No. 24 and the fusants were statically absorbed onto 20 g (dry weight) macroporous resin HPD100 (Cangzhou Baoen, Co. Ltd., China), firstly diluted by distilled water (500 ml) and followed by the elution with methanol. The methanol fractions were evaporated at 40°C in vacuum to yield dark red extracts. The trace extracts were sufficiently dissolved in the methanol (GR) for analysis by HPLC.
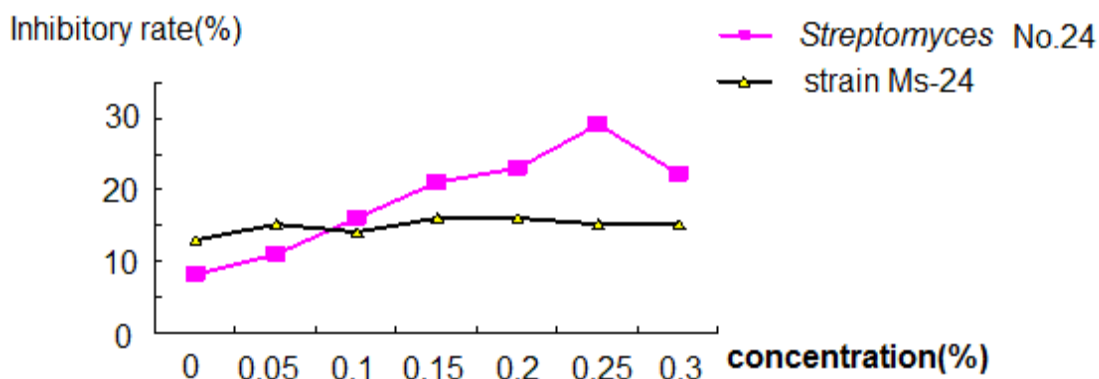
The analysis of the qinlinmycin production was performed on a Nucleosil C_{18} column (250×4.6 mm, 5.0 μm, Macherey-Nagel, German) maintained at 25°C with UV detector at 340 nm, using methanol/H_2O(75/25,v/v) as the solvent, flow rate 0.8 ml/min.

Antibacterial activity of qinlingmycin and the fusants fermented broth

Firstly, one milliliter spore suspension (1×10^6 spores/ml) was

Table 1. MICs of qinlingmycin, penicillin and cephradine against six bacteria.

Sample	MIC (mg/l)					
	S. Luteaschroeter	B. cereus	B. cirulans	S. aureus	P. aereuginosa	E. coli
Penicillin	1.56	2.50	4.00	1.50	1.25	1.12
Cepradine	1.10	2.00	3.50	0.55	1.00	0.70
Qinlingmycin	2.00	1.20	1.50	0.65	0.75	1.85

Figure 1. Effect of different concentration glycine on protoplast formation.

inoculated into 50 ml seed medium in 250 ml Erlenmeyer flasks and incubated for 24 h in rotary shaker (180 rpm) at 28°C and then ten percent of seed medium were transferred into inoculated into 50 ml production medium in a 250 ml Erlenmeyer flask. The strain/fusant was incubated at 28°C with 180 rpm on a rotary shaker for 108 h. After fermentation, the production medium was collected and centrifuged at 5000 rpm for 10 min at room temperatures to separate the supernatant and mycelium. The supernatant was filtered by 0.22 µl filters for eliminated contamination before evaluation of antibacterial activity.

Minimal inhibitory concentration (MIC) of qinlingmycin was measured by micro-broth dilution method in 96-well culture plates employing Mueller-Hinton broth (Hangzhou Microbial Reagent Co. Ltd), according to the Standard of National Committee for Clinical Laboratory (NCCLS, 2003). Briefly, the tested bacteria were incubated in MH broth for 12 h at 30°C in 190 rpm, and the cell/spore concentration were diluted to approximately 1×10^5-1×10^6 CFU with MH broth. After incubation for 24 h at 30°C, the MICs were examined.

The antibacterial activity of the filtered supernatant of the fusants was tested by agar diffusion assay (Servin AL, 2004). The plates were incubated at 37°C for 24 h during which activity was evidenced by the presence of a zone of inhibition surrounding the well. Each test was repeated three times and the antibacterial activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the secondary metabolite when compared with the controls. The control antibiotics penicillin and cefradine were purchased from Sigma of China.

RESULTS

Evaluation of antibacterial activity of qinlingmycin

The antibacterial activity of qinlingmycin, produced by a soil-derived actinomyces *Streptomyces* No. 24, was carried out in Table 1. The results showed that the inhibitory effect of qinlingmycin were stronger than penicillin and cefradine against *Bacillus cereus*, *Bacillus cirulans*, *Pseudornonas aereuginosa*, while sweaker again-t *Sarcina Luteaschroeter*. Especially, the MIC values of qinlingmyces against *B. cirulans*, was 1.50 mg/l, which was less than half of MICs of penicillin and cefradine.

Effect of glycine concentration on protoplast preparation

Influence investigation of glycine concentrations on protoplast preparation was illustrated in Figure 1. Under the concentration of 0.25% glycine, with increasing glycine concentration, the protoplast preparation ratio of *Streptomyces* No. 24 was increasing and the maximum increased as thirty percent. However, with increasing more than 0.25% glycine concentration, the preparation ratio of protoplast decreased dramatically, this study thus, revealed the optimal glycine concentration for protoplast preparation of *Streptomyces* No. 24 was 0.25%. While no effect was obviously showed between the glycine concentration and the protoplast preparation ratio of strain Ms-24.

Influence of lysozyme concentration on protoplast formation and regeneration rate

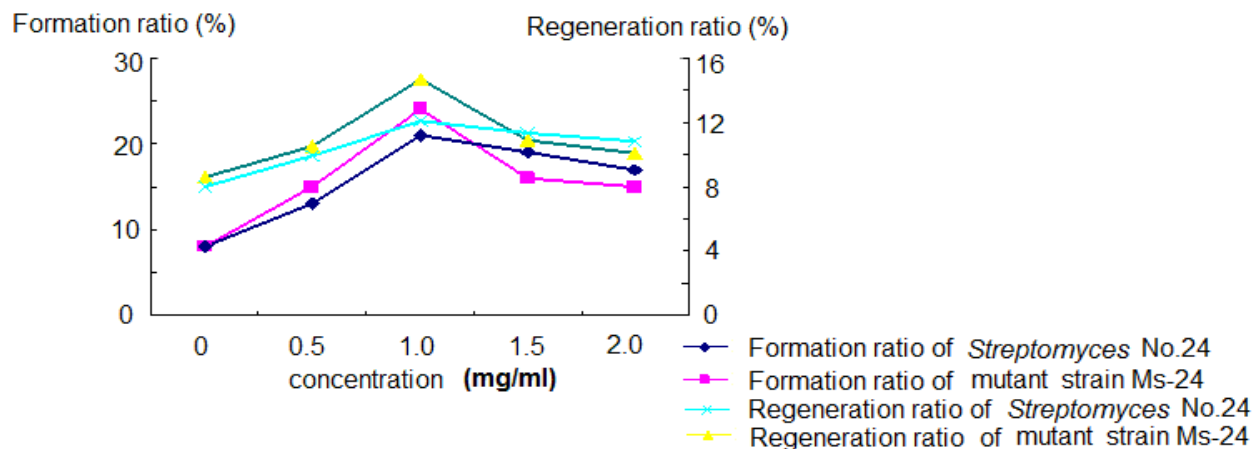Investigation of lysozyme concentration on protoplast for-

Figure 2. Effect of different lysozyme concentration on protoplast formation and regeneration rate.
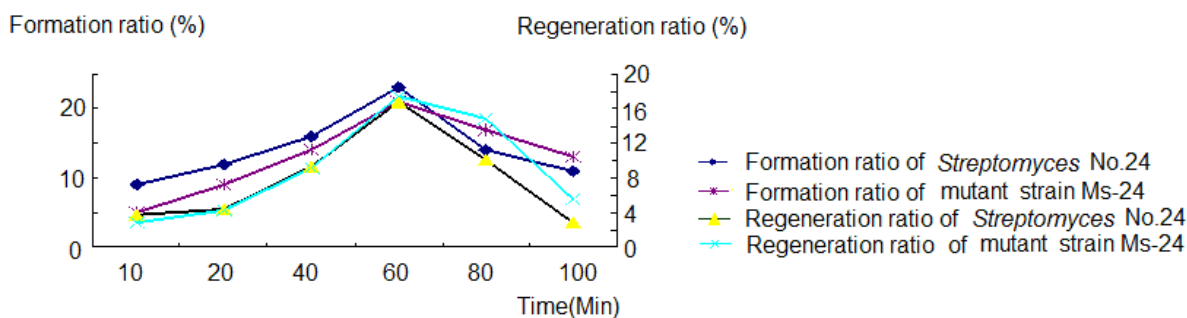

Figure 3. Effect of lytic time on protoplast formation and regeneration rate.

Table 2. Effect of different molecule weight PEG on protoplast fusion.

Concentration	Fusion rate (%, mean ± SE)					
(%)	PEG1000	PEG2000	PEG3000	PEG4000	PEG5000	PEG6000
10	0.8±0.1	1.1±0.2	1.0±0.2	0.9±0.2	0.8±0.1	1.4±0.2
20	1.5±0.2	1.9±0.1	2.1±0.1	1.8±0.1	1.6±0.2	2.2±0.1
30	2.8±0.4	2.3±0.2	2.5±0.3	2.0±0.2	1.9±0.3	2.7±0.5
40	3.2±0.4	3.5±0.1	2.9±0.4	3.4±0.3	3.0±0.5	3.6±0.4
50	6.1±0.2	5.9±0.4	5.5±0.5	6.2±0.5	5.8±0.5	6.8±0.3
60	5.0±0.3	4.7±0.3	5.3±0.3	4.9±0.2	5.1±0.4	5.8±0.5

mation and regeneration rate is clearly display in Figure 2. The top values of both the protoplast formation ratio and regeneration ratio were observed for *Streptomyces* No. 24 and mutant strain Ms-24 at lysozyme concentration of one mg/ml.

Influence of lytic time on protoplast formation and regeneration rate

The maximum values of both the protoplast formation ratio and regeneration ratio were obviously observed

while lytic time of *Streptomyces* No. 24 and mutant strain Ms-24 was sustained for 60 min. Influence of lytic time on protoplast formation and regeneration rate is clearly display in Figure 3.

Effect of polyethylene glycol (PEG) on protoplast fusion

Effect of the weights and concentrations polyethylene glycol (PEG) on protoplast formation and regeneration
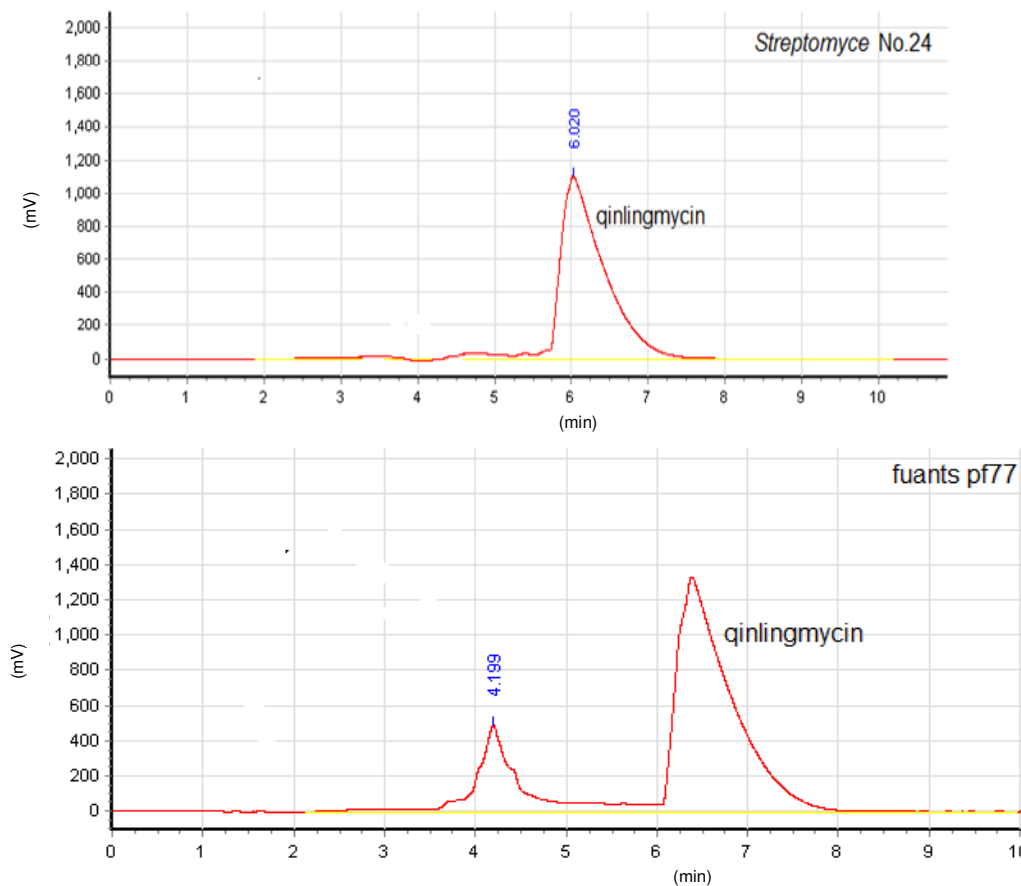
Figure 4. HPLC analysis pictures of qinlingmycin of *Streptomyces* No. 24 and the fuants pf77, pf126 and pf138.

rate was in detail carried out in Table 2. The results showed that molecule weight of PEG had little effect on protoplast fusion under the same concentration. Nevertheless, sufficient effect was exhibited at different the concentration under the same molecule weight of PEG. Especially, the top values of fusion rates were obtained in all tested PEG at the fifty-five percent concentration of PEG.

HPLC detection of qinlingmycin production of two parent strain and the fusants

HPLC detection pictures of qinlingmycin production of two parent strains and the fusants were carried out in Figure 4 and the qinlingmycin production of the three fusants strains were significantly enhanced by naked eyes observation. The earlier mentioned conclusion also was confirmed by analysis data according to superficial unitary method in Table 3. The qinlingmycin production values of three fusants strain pf77, pf126 and pf138 were 1.05, 1.43 and 1.57 mg/ml, respectively and increased by 36.59, 74.39 and 91.46% than that of the parent strain *Streptomyces* No. 24.

Generations' stability of the fusants pf77, pf126 and pf138

The obtained fusants pf77, pf126 and pf138 were continuously inoculated ten generations in GS medium plates. The 10 generations strains of the fusants pf77, pf126 and pf138 were evaluated against *B. subtilis* in Table 4. All ten generations strains of the three fusants pf77, pf126 and pf138 *in vitro* exhibited stronger activities than the parent strain *Streptomyces* No. 24 against *B. subtilis*. The results showed that the three fusants pf77, pf126 and pf138 had the stabile generations' transitivity.

DISCUSSION

Protoplast fusion technology has widely applied in the various areas of modern biology, however, the mechanism of protoplast fusion is not fully known. At present, one is widely explained, briefly, when the protoplasts are closely adhered, the external fusogens cause disturbance in the intramembranous proteins and glycoproteines. (Teeradakorn et al., 1998; Urano et al., 1998), This increases membrane fluidity and creates a
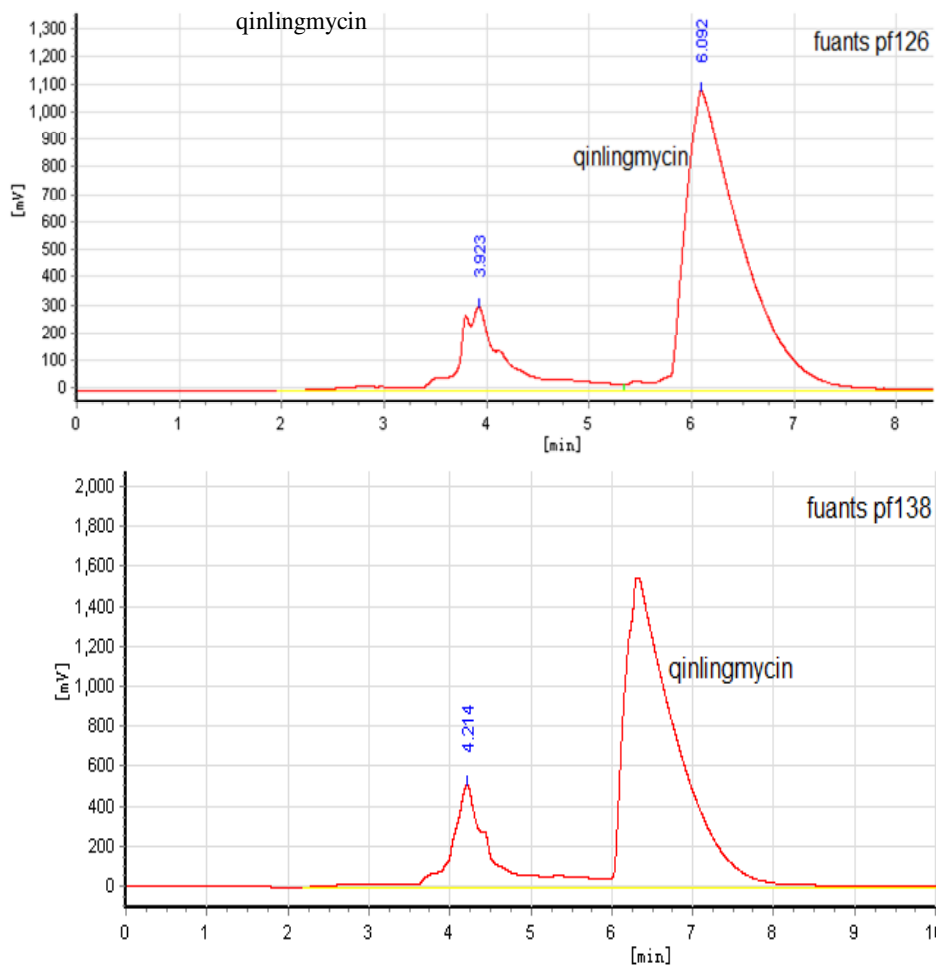
Figure 4 Contd. HPLC analysis pictures of qinlingmycin of *Streptomyces* No. 24 and the fuants pf77, pf126 and pf138.

Table 3. Content of qinlingmycin of the fusants fermented products.

Strain number	No. 24	pf77	pf126	pf138
Apex area	369900±9022	474531±13558	646831±4523	710099±13569
Qinlingmycin (mg/ml)	0.82±0.02	1.05±0.03	1.43±0.01	1.57±0.03

Table 4. Antibacterial activity of ten generations of three fusants against *B. subtilis*.

Strain	Inhibition zone(mm/diameter)									
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
pf77	33±1	33±2	33±2	33±3	33±1	33±1	33±3	33±2	31±1	31±2
pf126	37±2	37±1	37±2	37±3	37±2	37±3	37±3	37±1	37±3	37±1
pf138	38±1	38±3	38±1	38±2	38±3	38±1	38±2	37±3	37±2	37±1
No.24	23±1	23±1	23±2	23±1	23±1	23±2	23±3	23±2	23±1	23±1

region where lipid molecule intermix, allowing coalescence of adjacent membranes. The negative charge carried by protoplast is mainly due to intramembranous phosphate groups. The high molecular weight polymer (1000 to 6000) of PEG acts as a molecular bridges connecting the protoplasts (Peberdy, 1980). Calcium ions linked the negatively charged PEG and membrane surface. On elution of the PEG, the

surface potential are disturbed, leading to intramembrane contact and subsequent fusion, Besides this, the strong affinity of PEG for water may cause local dehydration of the membrane and increase fluidity, thus inducing fusion. Protoplast fusion takes place when the molecular distance between the protoplasts is 10A or less. This indicates that protoplast fusion is highly a traumatic event (Jogdand, 2001; Narayanswamy, 1994).

In this study, the three high qinlingmycin-producing fusants pf77, pf126 and pf138 were fortunately obtained by using protoplast fusion technology. In the process of the protoplast preparation, it is investigated for the appropriate concentration of glycine, lytic time and fusion condition. Glycine concentration is a sensitive factor for the protoplast preparation of *Streptomyces* No. 24, which is extremely similar with the reported conclusion (Lin et al., 2005). However, changes of glycine concentration are not obviously effect on protoplast preparation of mutant strain Ms-24, the reasons of which need to be explored in further study. For protoplast fusion, it is important that the cell wall of microorganisms is degraded and hence, various enzymes used for this process. *Streptomyces* cell walls are degraded by the action of lysozyme (Jogdand, 2001; Kim, 2000). However, commercial lysozyme unusually contains other trace harmful enzymes to protoplast, such as peroxidase and ribonuclease. With the volume of harmful enzymes, the activity of the protoplast could be reduced (Homann et al., 1999). Lyric time is another important factor. Long lyric time results in plasma membrane damage to cause break-down of the protoplast, accompanying by reducing activity of the protoplast. In the protoplast preparations of the *Streptomyces* No. 24 and mutant strain Ms-24, the optimal lysozyme concentration and lyric time are one mg/ml and 60 min, respectively. PEG molecules contain ether bond, which produces hydrogen bond along with H_2O, proteins and saccharides and leading to building effective cohesion between protoplast membranes (Jogdand, 2001).

By using protoplast fusion technology, three fusants pf77, pf126 and pf138 were discovered and showed higher antimicrobial activity and higher production of qinlingmycin than the parents' strains. It is confirmed that protoplast fusion is an important and useful tool in the improvement of microorganism strains and protoplast fusion in future will be one of the most frequently used research tools in the microorganisms breeding.

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