IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
ISSN: 2278-3008. Volume 4, Issue 6 (Jan. – Feb. 2013), PP 23-27
www.iosrjournals.org

Nitrosoguanidine Mutagenesis and Genome Shuffling Enhanced the Oxytetracycline Production of *Streptomyces rimosus*

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***Abstract*:** *This research is aimed to improve the oxytetracycline production from Streptomyces rimosus. Five relatively high oxytetracycline yielding strains were obtained by nitrosoguanidine mutation of the protoplast of initial oxytetracycline producing strain Streptomyces rimosus JH and then subjected to genome shuffling as parental strains. The protoplasts of the five parental strains were prepared and inactivated before fusion, after recursive protoplast fusion of six rounds, the oxytetracycline production of all recombinants in shaking-flask fermentation was analyzed by ultraviolet spectrophotometer method comparing with the initial strain IH. The result indicated the yield of oxytetracycline was enhanced. The highest production of recombinant 26173 was about 186% higher than that of the highest-production parent strain and 339.8% higher of the initial strain. In result, genome shuffling is a good technique to rapidly improve oxytetracycline production in Streptomyces rimosus.*

Keywords: *Oxytetracycline, Genome shuffling, Streptomyces rimosus, nitrosoguanidine mutagenesis*

I. Introduction

The aromatic polyketide antibiotic, oxytetracycline (OTC) effective against a broad spectrum of microorganisms, is produced by *Streptomyces rimosus* as an important secondary metabolite[1].Oxytetracycline is a member of the tetracycline family. Tetracyclines are broad-spectrum antibiotics used in a variety of infections caused by Gram-positive and Gram-negative bacteria, rickettsias, trachoma, coccidia, amoebae, balantidia and mycoplasma. They are also used in non-therapeutics, for control of plant diseases, stimulation of aminoacid fermentation and inhibition of material biodeterioration[2]. Tetracyclines are one of the cheapest classes of antibiotics available today, making them attractive for use in developing countries with limited health care budgets. Today, oxytetracycline is widely used for semisynthesis of doxycycline and metacycline for medical use. But oxytetracycline is mainly used for animal disease prevention and control and is used as animal feed supplements[3].

Genome shuffling is a kind of reorganization genome technology without the detailed information about the whole genome[4]. Using the recursive multiparental protoplast fusion, genome shuffling is one of the most efficient methods for engineering complex phenotypes, as demonstrated in several other examples of microbial strains development[5], which offers the advantages of accumulated beneficial mutations genes associated with production and the good phenotypes can be obtained. This technique has been used successfully to improve the production of the polyketide antibiotic tylosin in *Streptomyces fradiae* [6]. In this research, classical genome shuffling method was used to increase the oxytetracycline production of *Streptomyces rimosus* strains. After six rounds of genome shuffling, the best performing strain was isolated by ultraviolet spectrophotometer [7].

II. Materials and Methods

2.1 Bacteria strain

The initial oxytetracycline producing strain *Streptomyces rimosus* JH was screened and preserved by our laboratory.

2.2 Mutagenesis and screening

The mutagenesis of *Streptomyces rimosus* JH was carried out by treating the protoplast of with nitrosoguanidine (NTG).

2.2.1 Protoplast preparation

The strain was cultured on the YM agar slant for 7 days at 28°C until spores formed. YM agar medium contains (per liter distilled water): 10g glucose, 3g yeast powder, 5g peptone, 3g maltose and 15g agar, the pH was monitored and adjusted to 7.0. Fresh mycelia for protoplast preparation was obtained by growing spores in liquid YM medium supplemented with 2.0% glycine for 12h at 28°C and harvested by centrifugation at 3,000 ×

g for 10 min at 4°C. The pellet was washed three times with 10.3% sucrose solution, re-suspended with P buffer which contains (per liter distilled water):103g sucrose, 0.25g K_2SO_4, 2.02g $MgCl_2 \cdot 6H_2O$, 0.05g KH_2PO_4, 3.68g $CaCl_2 \cdot 2H_2O$ and 5.37g N-[Tris(hydroxymethyl) metyl]-2-amino propanesulfonic acid (TES). Then 20mg lysozyme was mixed with 10 mL of the suspension and incubated for 2 h at 37°C. The appearance of spherical cells as judged by light microscopy was used as an indicator of protoplast formation. Protoplasts were centrifuged for 10 min at 2,000 × g, washed twice with P buffer to remove the lysozyme, and re-suspended with 10 mL P buffer[7-10].

2.2.2 Mutagenesis treatment and screening

NTG was used for mutagenesis of *Streptomyces rimosus* protoplast. 10 mL protoplast was treated with 0.6 mg/mL NTG for 30 min at 28°C. The NTG treated protoplast was mixed with R2 medium of 40°C rapidly and pour on the pre-poured R5 plate and cultivate for 4 days. The R2 medium contains (per liter distilled water): 103g sucrose, 0.25g K_2SO_4, 5.73g TES, 2.22g $CaCl_2 \bullet 2H_2O$, 5g agar, supplemented with 2 mL trace elements solution, pH 7.0. The R5 medium contains (per liter distilled water): 103g sucrose, 0.25 g K_2SO_4, 10.12 g $MgCl_2 \bullet 6H_2O$, 0.1g casein hydrolysate, 10g glucose, 5g yeast extract, 5.73 g TES, 3g L-proline, 0.05 g KH_2PO_4, 2.94 g $CaCl_2 \bullet 2H_2O$, 0.28 g NaOH, 15g agar and 2 mL trace elements solution. The trace elements solution contains (per liter distilled water): 40 mg $ZnCl_2$, 200 mg $FeCl_3 \cdot 6H_2O$, 10 mg $CuCl_2 \cdot 2H_2O$, 10 mg $MnCl_2 \cdot 4H_2O$, 10 mg $Na_2B_4O_7 \cdot 10H_2O$, 10 mg $(NH_4)_6Mo_2O_4 \cdot 4H_2O$ [11,12].
The oxytetracycline quantities of the regenerated mutants were determined to screen the high yielding strains.

2.3 Genome shuffling and screening

Five relatively high oxytetracycline yielding mutants obtained in NTG mutagenesis screening were used as parental strains for genome shuffling. The parental strains were divided into two groups randomly, one group has three strains and another has two strains. The protoplast of each strain was prepared as described above and mixed by approximately the same turbidity to form the group combination. One group was inactivated by ultraviolet (15W UV lamp, irradiation for 30min with stirring), and another group was inactivated by heat (70°C water bath for 35 min). Equal volume (1mL) of inactivated protoplasts from different populations were mixed and then centrifuged at 1,000 × g for 7 min. The pellet was re-suspended in 40% polyethylene glycol 4000 (PEG 4000) in P buffer and immediately suspend the protoplast by pipetting rapidly, and incubated for 3 to 5 min at room temperature. The fused protoplasts were precipitated by centrifugation at 1,000 × g for 7 min, washed and re-suspended with P buffer. For regeneration of recombinants, mixed the suspension with 40°C of R2 medium rapidly and regenerated on R5 plate medium at 28°C for 4 days. Spores from the regenerated cells were pooled and used to inoculate a culture for the production of protoplasts. Protoplasts from this mixed culture were divided into two groups, inactivated by ultraviolet or heat treatment, fused and regenerated. This process was repeated five additional times[14-19].

Totally, after six rounds of recursive protoplast fusion, the oxytetracycline quantities of the recombinants were determined to screen the high yielding strains.

2.4 Analytical methods

The mutants and the recombinants were preliminary screened by direct fermentation in liquid medium using test tube (16mm×160mm) by comparing with the initial strain *Streptomyces rimosus* JH. The medium contains (per liter distilled water): 15g starch, 15g dextrin, 10g peanut powder, 2g yeast extract, 4g $(NH_4)_2SO_4$, 4g $CaCO_3$, 0.3g KH_2PO_4, l5g NaCl, the pH was monitored and adjusted to 7.0. They were fermented at 28°C and 200r/min for 7 days, centrifuged at 3,000 × g and measured the OD268.1 of 100 times diluted supernatant. The OD268.1 value was assumed to indicate the amounts of the secreted oxytetracycline extracellular because the maximum absorbance of it was observed under the experimental conditions. And the oxytetracycline extracellular is positive correlated with the total tilter well.

The relative high yielding mutants (or recombinants) were further screened by flask fermentation and determination of the total tilter of the oxytetracycline. The seed for fermentation was obtained by culturing the strain for 1 day at 28°C and 200 r/min using the same medium to preliminary screening. After check for purity, the seed was inoculated into fermentation medium by 10% (v/v), culturing for 8 days at 28°C and 200 r/min. The fermentation medium contains (per liter distilled water): 60g starch, 20g maltose, 30g peanut powder, 2g yeast extract, 9g $(NH4)_2SO_4$, 8g $CaCO_3$, 0.3g KH_2PO_4, 3g NaCl, the pH was monitored and adjusted to 7.0[6]. At the end point, the pH of the fermentation broth was adjusted to pH 2.0 with oxalic acid, and then add 3.0% sodium ferrocyanide and 3.0% $ZnSO_4$ (w/v) to the broth. The treated fermentation broth was centrifuged at 3,000×g for 10 min, and the oxytetracycline amount was determined by ultraviolet spectrophotometry method according to the described procedure [20, 21].

III. Results and Discussion

3.1 Protoplast preparation, NTG mutagenesis and screening of higher oxytetracycline producing mutants

Successful formation of protoplasts from several different strains has been demonstrated previously [11-15]. We found that even the fresh mycelia of routine culture of *Streptomyces rimosus* JH was difficult to form protoplast, so the addition of glycine in culture medium YM and the lysozyme concentration for protoplast formation were optimized. When 2.0% glycine was supplemented in the medium and 2.0 mg/mL lysozyme was used, the highest protoplast formation rate could reach within 2h. After treated with NTG and regenerate, 326 mutants were obtained, of which 12.9% (42) are negative mutants, 87.1% (284) are positive mutants. Among the positive mutants, 155 strains had the yielding of 10-20% more than that of the initial strain *S. rimosus* JH, and 71, 35, 18, 4 and 1 strain showed 20-30%, 30-40%, 40-50% and >50% more than strain JH, respectively(Figure 1) . Strain 3016 has the highest oxytetracycline yield of all the mutants, which showed 53.8% higher titer than that of the initial strain *S. rimosus* JH (2510U/mL). Five higher-oxytetracycline production mutants of were selected as parental strains for genome shuffling (Table1), which distributed among mutants with different increased levels.

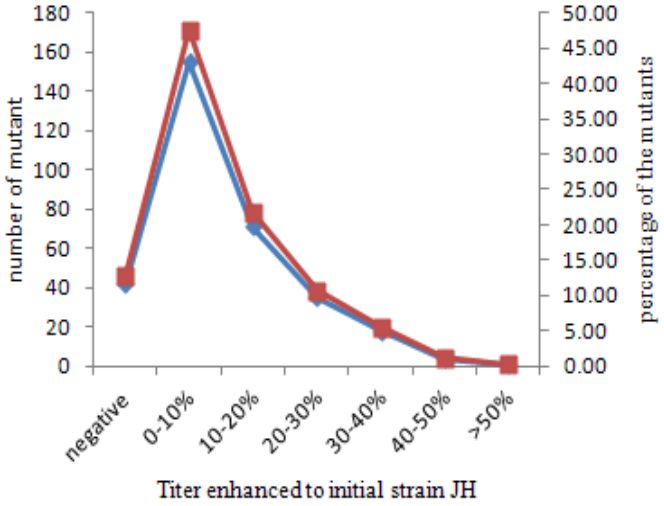

Figure 1 Enhanced production proportion distribution of the NTG mutagenesis mutants comparing to the initial strain *S. rimosus* JH

number of mutant percentage of the mutants

Table 1 The high Oxytetracycline-producing mutants selected as parental strains for genome shuffling

Strain	Yield (U/mL)
2016	2690
1017	2900
4016	3070
4018	3240
3016	3860

3.2 Genome shuffling for improvement of oxytetracycline production

In this study, genome shuffling technology to improve oxytetracycline productivity was well performed. After continuous six rounds genome fusion, 288 recombinants were subjected to two rounds screening. Through preliminary screening in test tube scale fermentation and secondary screening in shaking flask scale fermentation, 37(12.8%) recombinants showed more than 100% improved than the highest titer of the parental strain 3016 (3860 U/mL). The result indicates that after six continuous rounds of protoplasts fusion process, the oxytetracycline yield of the recombinants had great be enhanced. Especially five oxytetracycline over-producing recombinants were screened out, which enhanced at least 221.2% in titer than the initial strain *S. rimosus* JH(Figure 2, Table 2), and 108.8% than the highest parental strain 3016. For the best recombinant 26173, the oxytetracycline titer in shaking flask scale fermentation reached 11040 U/mL, which was 186% higher than the highest titer of the parental strain 3016 and about 339.8% higher than that of the initial strain *S. rimosus* JH. By comparing the effect of NTG mutagenesis, it's easy to find that genome shuffling is more efficient than traditional NTG mutagenesis technique for improving oxytetracycline productivity of *S. rimosus.*

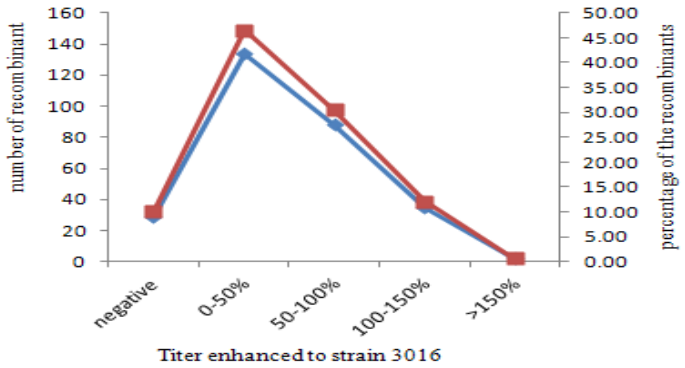

Figure 2 Enhanced production proportion distribution of the recombinants comparing to the strain 3016

number of recombinant percentage of the recombinants

Table 2 Oxytetracycline production of the five best recombinants after genome shuffling by six round of recursive protoplast fusion

Strain	Yield (U/mL)	Enhanced proportion to JH (%)
Initial strain JH	2510	--
3016	3860	53.8
26146	8063	221.2
26140	8125	223.7
26045	9530	279.7
26157	9706	286.7
26173	11040	339.8

3.3 The genetic stability of the oxytetracycline over-producing strain 26173

To check the genetic stability of the strain 26173, we continuous passage cultured it for 10 generations within two months and determined the oxytetracycline production of every other generation in shaking-flask scale. We got the average product titer of 11090±267 U/mL. The results clearly showed that this strain is genetically stable in oxytetracycline production. Although this strain still has a chasm between the industry strains, it showed very stable productivity in fermentation, and it could be used for further shuffling to improve the oxytetracycline productivity.

IV. Conclusion

In this project, the initial low-oxytetracycline-producing strain *Streptomyces rimosus* JH was subjected to NTG mutagenesis and further for genome shuffling. The oxytetracycline titer was improved dramaticly by more than 3.3 times to the initial strain at the end. Although the classical microbial mutation breeding approaches have been successfully used in the development of oxytetracycline producing strains, we can concluded that genome shuffling is a efficient process that can obtained oxytetracycline over-producing and genetic stable strains.

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