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# Large-Scale Fermentation for the Production of Teicoplanin From a Mutant of *Actinoplanes teichomyceticus*

**LEE, JAE-CHAN, JUNG-WON MIN, DONG-JIN PARK, KWANG HEE SON, KI-HONG YOON[1], HAE-RYONG PARK[2], YOUNG-SOO PARK[3], MU-GIL KWON[3], JUNG-MIN LEE, AND CHANG-JIN KIM***

*Korea Research Institute of Bioscience & Biotechnology, P.O. Box 115, Yusong, Daejon 305-600, Korea*
*[1]School of Food Science & Biotechnology, Woosong University, Daejon 300-718, Korea*
*[2]Division of Food Science and Biotechnology, Kyungnam University, Masan 631-701, Korea*
*[3]Central Research Center, Kunwha Pharmaceutical Co., Kongju 314-821, Korea*

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**Abstract** Mutation and its pilot-scale fermentation were conducted for the production of teicoplanin from *Actinoplanes teichomyceticus*. The fermentation medium was optimized by replacement and Plackett-Burman experimental design. A maximum production of 1,500 mg/l teicoplanin was obtained by pilot-scale fermentation in an optimized medium containing (g/l): 30 g maltodextrin, 5 g glucose, 5 g yeast extract, 5 g soybean meal, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.1 g $CaCl_2 \cdot 2H_2O$, and 50 g Diaion HP-20. The production of teicoplanin was improved 3-fold from the parental strain by mutation, media optimization, and fermentation, and laboratory-scale fermentation was successfully demonstrated in a pilot-scale fermenter for the industrial production of teicoplanin.

**Key words:** *Actinoplanes teichomyceticus*, fermentation, mutagenesis, mutation, teicoplanin

Teicoplanin is a glycopeptide antibiotic used for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections and considered to be the last-resort antibiotic against this pathogen [3]. Mutation and fermentation skills for the high production of teicoplanin have been improved, and it has recently been reported that a valine analogue-resistant mutant produced 1,800 mg/l teicoplanin with 0.5-fold higher yield than the parental strain [2, 4, 6, 13]. In molecular biology, the teicoplanin gene cluster participating in teicoplanin biosynthesis, regulation, resistance, and export has been isolated and characterized [7, 14]. In this report, we describe the isolation of a mutant, media optimization, and its scale-up fermentation for the industrial production of teicoplanin.

*Corresponding author
Phone: 82-42-860-4332; Fax: 82-42-860-4595;
E-mail: changjin@kribb.re.kr

## Materials and Methods

### Microorganisms and Media

The *Actinoplanes teichomyceticus* MSL 1510 used in the study was derived from the parental strain *Acp. teichomyceticus* MSL 2211 [9], a teicoplanin-producing mutant of wild-type strain *Acp. teichomyceticus* ATCC 31121 [11]. Strains were maintained on Bennett's agar medium containing: 10 g/l glucose, 1 g/l Bacto yeast extract (Difco), 2 g/l Bacto peptone (Difco), 1 g/l beef extract, and 15 g/l agar in distilled water, pH 7.0. The seed medium contained: 3 g/l glucose, 3 g/l yeast extract, 5 g/l peptone, 3 g/l malt extract, and 2 g/l beef extract. Diaion HP-20 (Mitsubishi Chemical Industries Limited, Tokyo, Japan), as an adsorbent resin, was used in fermentations to eliminate toxic effects on growth and reduce feedback repression of product, and prepared as described previously [8, 10].

### Mutagenic Treatment and Overlay Assay

Spore suspensions from *Acp. teichomyceticus* MSL 2211 were irradiated with photons of energies between 70 eV and 1.5 keV generated by LIGA beamline (Deep Etch X-ray lithography, Pohang Light Source in Pohang, Korea) in the chamber designed with copper-reflection mirror [5] and gamma-irradiated for various time periods corresponding to doses from 0 to 0.2 mrad ($10^6$ radiation). Immediately after the irradiation, plates with spores were kept at 4°C for 1 h to prevent back-mutation, and overlay assay on Luria-Bertani agar (Miller, Difco) seeded with *Bacillus subtilis* ATCC 6633 was followed as described previously [9].

### Analytical Methods

The Plackett-Burman experimental design [12] was used to evaluate the effects of commercial nitrogen sources on

teicoplanin production. The eleven variations listed in Table 3 represent the eight nutritional components and three dummies. Measured antibiotic potency is also shown in Table 3. The significance of each variable on teicoplanin production was determined by applying the Student's *t*-test [12], as shown in Table 3. Recovery of teicoplanin and assay of antibiotic potency were performed as described previously [8]. Dry mycelia weight was determined by heating washed mycelia at 80°C for 24 h, and residual sugar was determined with anthrone reagent for total sugar [1].

### Fermentation

The seed culture, prepared as described above, was grown on a rotary shaker at 28°C for 30 h and then inoculated at 10% (v/v) into the fermenter. Batch cultures were grown in a 5-l stirred jar fermenter (KF-5 L, KoBioTech, Korea) containing 3-l of optimized production medium that contained: 30 g/l maltodextrin (DE 14-20), 5 g/l glucose, 5 g/l yeast extract, 5 g/l soybean meal, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l NaCl, 0.1 g/l $CaCl_2 \cdot 2H_2O$, and 50 g/l Diaion HP-20. Aeration was 1 vvm and agitation was controlled at 300 rpm. pH was not controlled. Pilot-scale batch fermentation was carried out in a 500-l tank fermenter (KoBioTech, Korea) with 300-l working volume at 28°C. Thirty liters of seed culture was inoculated from the seed tank to the main tank fermenter, and pH was controlled automatically at 7 by adding 3 mol/l NaOH or 2 mol/l HCl. Aeration was controlled below 1 vvm stepwise and the agitation under 80 rpm stepwise.

## RESULTS

### Strain Selection

*Acp. teichomyceticus* MSL 2211 was subjected to LIGA beamline and gamma-ray mutagenesis. A number of colonies obtained through whitebeam of LIGA beamline treatment of the parent strain were preliminarily screened, and the spores of selected colonies were gamma irradiated. Irradiation with whitebeam of LIGA beamline was carried out with photons of the energies between 70 eV and 1.5 keV for 10, 20, 30, 60, and 90 min and the death rate was below 70%, and gamma-ray irradiation conducted was with the doses of 0, 0.05, 0.07, 0.1, 0.15, and 0.2 mrad and the death rate was 0, 69, 78, 84, 93, and 97%, respectively. We collected 3,000 colonies mutagenized with LIGA beamline, and an increased productivity of 450 mg/l teicoplanin was observed in one mutant. The selected strain was irradiated with gamma-ray, and a mutant, *Acp. teichomyceticus* MSL 1510, which was selected among 8,000 colonies, showed the highest productivity of 1,000 mg/l teicoplanin (Table 1). The production of teicoplanin was improved 2-fold over the parental strain that produces 500 mg/l teicoplanin [9] (Table 1). Morphologically, 20–25 μm size of spores were changed into 5–8 μm, and the pink aerial mass color was changed into orange (Table 1). However, the growth of the mutant was still sensitive at 15 mg/l teicoplanin.

**Table 1.** The morphological characteristics of wild-type and mutants.

| Characteristics | ATCC 31121 | MSL 2211 | MSL 1510 |
|---|---|---|---|
| Chain | Sporangium | Sporangium | Sporangium |
| Spore surface | Warty | Warty | Warty |
| Size (μm) | 5–7 | 20–25 | 5–8 |
| Aerial mass color | Brown | Pink | Orange |
| Reverse color | Yellow | Yellow-pink | Yellow |
| Antibiotic potency (mg $l^{-1}$) | 20–100 | 65–500 | 1,000–1,500 |

### Media Optimization

With an industrial exploitation in mind, we compared the productivity of teicoplanin in the presence of several inexpensive carbon and nitrogen sources and at the various ratios of carbon/nitrogen sources. Maltodextrin (DE 14-20) gave the best result as a carbon source and showed high production of teicoplanin at the higher DE (dextrose equivalence) number (Table 2). Maltodextrin is a good carbon source for the replacement of mannose, which is an expensive carbon source of parent strain [9]. Statistical data displayed in Table 3, based on the application of the Student's *t*-test [12], revealed that soybean meal and fish meal were highly stimulatory for the production of teicoplanin, and pharmamedia did not affect the production of teicoplanin.

**Table 2.** Effect of different carbon sources on teicoplanin production.

| Carbon sources* | Antibiotic potency (mg $l^{-1}$)[c‡] |
|---|---|
| Glucose | 790 |
| Galactose | 803 |
| Fructose | 641 |
| Mannose | 1,046 |
| Manitol | 834 |
| Xylose | 734 |
| Lactose | 615 |
| Sucrose | 821 |
| Maltose | 945 |
| Dextrin (<DE 5[†]) | 1,100 |
| Maltodextrin (DE 11) | 1,125 |
| Maltodextrin (DE 11-14) | 1,180 |
| Maltodextrin (DE 14-20) | 1,210 |
| Starch | 1,033 |

*Each carbon source was added to production medium containing 5 g/l yeast extract to give a final concentration of 3% (w/v).

[†]DE (dextrose equivalence) is a measure of reducing power, compared to a dextrose standard of 100 (%).

[‡]The cultures were shaken at 150 rpm on a rotary shaker at 28°C for 120 h. Antibiotic potency was analyzed by HPLC. Data represent an average of three replicates.

**Table 3.** The twelve trials of Plackett-Burman experimental design matrix for evaluation of the effect of nitrogen sources on teicoplanin production.

| Trial No. | Variable | | | | | | | | | | | Antibiotic potency (mg $l^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Gal | Suc | Man | SBM | FM | Dex | Phar | Mal | $D_1$ | $D_2$ | $D_3$ | |
| 1 | + | – | + | + | + | – | – | – | + | – | + | 1,008 |
| 2 | – | + | + | + | – | – | + | + | + | – | – | 1,125 |
| 3 | + | + | + | – | – | – | + | – | – | + | + | 870 |
| 4 | + | + | – | – | – | + | – | + | + | – | + | 983 |
| 5 | + | – | – | – | + | – | + | + | + | + | – | 990 |
| 6 | – | – | – | + | – | + | + | – | + | + | + | 1,177 |
| 7 | – | – | + | – | + | + | + | + | – | – | + | 1,130 |
| 8 | – | + | – | + | + | – | – | + | – | + | + | 1,217 |
| 9 | + | – | + | + | – | + | – | + | – | + | – | 1,300 |
| 10 | – | + | + | – | + | + | – | – | + | + | – | 1,297 |
| 11 | + | + | – | + | + | + | + | – | – | – | – | 1,092 |
| 12 | – | – | – | – | – | – | – | – | – | – | – | 950 |
| Con. (%) | 1.0 | 1.0 | 1.0 | 0.5 | 0.5 | 1.0 | 0.5 | 1.0 | | | | |
| *t*-value | -1.34 | -0.64 | -0.34 | 1.38 | 1.01 | 1.51 | -1.05 | 1.02 | 0.69 | -0.01 | -1.05 | |

The basal medium contained 1.0% glucose, 0.5% yeast extract, 0.15% asparagines, 0.05% $MgSO_4$, 0.01% NaCl, 0.01% $CaCl_2$, and 5.0% Diaion HP-20. The cultures were shaken at 150 rpm on a rotary shaker at 28°C for 120 h. Antibiotic potency was analyzed by HPLC. Data represent an average of three replicates.

+, – indicate adding and not adding, respectively.

Gal, galactose; Suc, sucrose; Man, mannose; SBM, soybean meal; FM, fish meal; Dex, dextrin; Phar, pharmamedia; Mal, maltose; D, dummy.

Yeast extract was an essential nitrogen source, but industrial yeast extract was not suitable for the production of teicoplanin (data not shown). Therefore, soybean meal was selected as a nitrogen source, and the modified medium comprised of 30 g/l maltodextrin (DE 14-20), 5 g/l glucose, 5 g/l yeast extract, 5 g/l soybean meal, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l NaCl, 0.1 g/l $CaCl_2 \cdot 2H_2O$, and 50 g/l Diaion HP-20. The optimal ratio of carbon and nitrogen source was 3:1 (%, w/w).

## Pilot-Scale Fermentation

In order to compare with *Acp. teichomyceticus* MSL 2211 and *Acp. teichomyceticus* ATCC 31121, fermentation of *Acp. teichomyceticus* MSL 1510 was carried out in a 5-l jar fermenter using 3 l of optimized production medium (Fig. 1). Fermentation patterns were similar to those of *Acp. teichomyceticus* MSL 2211 with higher cell mass and teicoplanin production. The formation of teicoplanin reached 1,350 mg/l. For the industrial exploitation, scale-up fermentation was carried out in a 500-l fermenter using an optimized production medium. As shown in Fig. 2, the key fermentation parameters, such as teicoplanin productivity, cell mass, and sugar consumption, were almost identical to those obtained in the 5-1 jar fermenter when aeration and rpm were stepwise controlled. In particular, cell growth was controlled by decreasing dissolved oxygen (DO) to 24 h, and rpm was stepwisely increased afterward. The synthesis of teicoplanin started when the growth phase had ended, and it reached to 1,500 mg/l teicoplanin. The productivity of teicoplanin was increased 3-fold higher than the parent strain, and it was overall 75-fold higher than the wild-type strain, *Acp. teichomyceticus* ATCC 31121 [11].

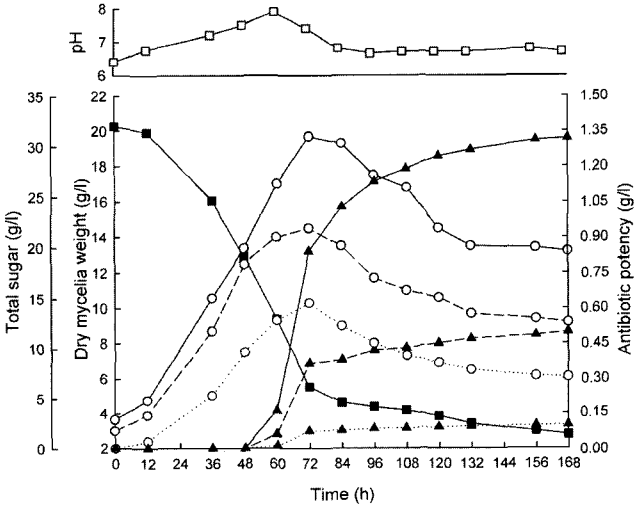


**Fig. 1.** Laboratory-scale (5-l) batch fermentation profiles of *Acp. teichomyceticus* MSL 1510, MSL 2211, and ATCC 31121. (○) Dry mycelia weight, (□) pH, (▲) antibiotic potency, (■) total sugar. Solid line represents *Acp. teichomyceticus* MSL 1510, dashed line *Acp. teichomyceticus* MSL 2211, and dotted line *Acp. teichomyceticus* ATCC 31121. *Acp. teichomyceticus* was cultured at 3 l of production medium in a 5-l jar fermenter. Fermentation was continued at 28°C for 168 h with an aeration rate of 1 vvm and 150 rpm. Fifteen ml of fermentation broth was sampled and analyzed for the content of teicoplanin, cell growth, total sugar, and pH. The profile of sugar consumption and pH of *Acp. teichomyceticus* ATCC 31121 and MSL 2211 showed patterns similar to MSL 1510.

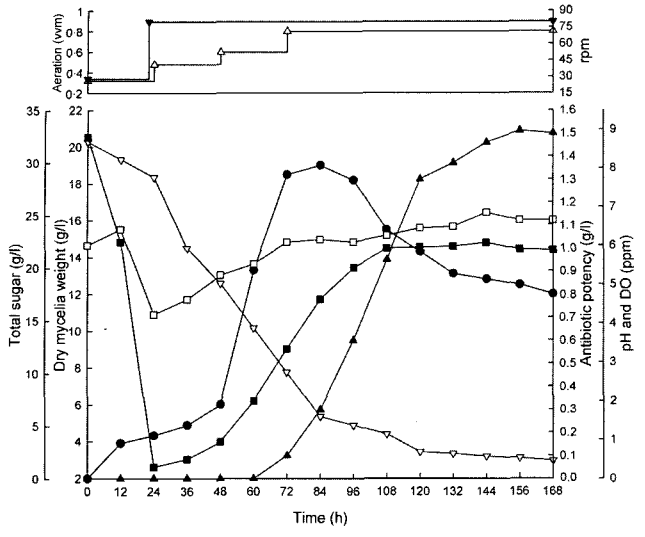


**Fig. 2.** Pilot-scale (500-l) fermentation profiles of *Acp. teichomyceticus* MSL 1510 at 28°C.
Fermentation broth (50 ml) was sampled and analyzed for the content of teicoplanin, cell growth, total sugar, and pH. (●) Dry mycelia weight, (▲) antibiotic potency, (■) dissolved oxygen, (▽) total sugar, (□) pH, (▼) rpm, (△) aeration.

## DISCUSSION

The production of teicoplanin was improved by mutation, medium optimization, and fermentation, and the productivity was improved 2-, 2.7-, and 3-fold over the parent strain, respectively. Gamma-ray was the most powerful mutagenesis, and it has been suggested that the higher cell mass and production of teicoplanin are induced by the mutation of genes participating in teicoplanin biosynthesis in the *tcp* gene cluster, except genes participating in resistance [14]. In the two mutagenesis treatments, the growth of the mutant was still sensitive at 15 mg/l teicoplanin, and 5% (w/v) Diaion HP-20 was added to the liquid culture broth at the time of culture inoculation to eliminate toxic effects on growth and reduce feedback repression of the product. In medium optimization, mannose was replaced by maltodextrin (DE 14-20): It is considered that the higher the DE, the greater the extent of starch depolymerization, resulting in a smaller average polymer size for easy uptake. Yeast extract was essential for the production of teicoplanin by *Acp. teichomyceticus*, and soybean meal could replace yeast extract to some degree. Additionally, we found it possible to increase the production of teicoplanin by trapping phosphates (data not shown). When 0.5% (w/v) kaolin was added, teicoplanin production was increased in the flask culture, and it is quite likely that the concentration of phosphate could repress the formation of teicoplanin. However, the effect of phosphate was not observed in the scale-up fermentation and it remains to be investigated. Fermentation was successfully demonstrated in pilot-scale with optimized carbon and nitrogen sources. Inoculum volume, dissolved oxygen, internal pressure, and the initial state of the mycelium within 24 h were the key factors for the fermentation of *Acp. teichomyceticus* MSL 1510. Diaion HP-20 and better environmental conditions such as agitation with shear force of impeller helped the recovery of teicoplanin considerably. Further improvement in teicoplanin production could also be considered by two-step fermentation, including the mass-cell production stage and teicoplanin production stage, by controlling phosphate regulation.

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