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Oxytetracycline production by immobilized *Streptomyces rimosus*

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This study examined whether the production of oxytetracycline by *Streptomyces rimosus* TM-55 (CCRC 960061) would be improved with calcium alginate immobilization in submerged fermentation compared with free cells. Results showed that in 1-mL culture broth, free cells produced 121 to 124 μg of oxytetracycline, whereas immobilized cells produced 153 to 252 μg. Immobilization of the cells retarded the growth rate of *S. rimosus* but increased the length of the growth period and improved the oxytetracycline production. The specific oxytetracycline productivity was 33.3 to 34.2 mg in each gram of free dry cells and was 40.2 to 40.7 mg in immobilized dry cells. The optimum immobilization conditions were alginate 2% and bead diameter of 2.13 mm. Oxytetracycline production increased with increasing inoculum density but decreased with increasing bead diameter. Ethylenediaminetetraacetic acid or monovalent ions could react with calcium in the bead or replace it with sodium ion, thereby reducing the strength of the beads.

Key words: Alginate, cell immobilization, inoculum density, oxytetracycline production, *Streptomyces rimosus*

Oxytetracycline is a broad-spectrum antibiotic produced by *Streptomyces* such as *Streptomyces rimosus*, *Streptomyces capuensis*, *Streptomyces henetus*, and *Streptomyces platensis* in submerged as well as solid-state fermentation [1-4]. The production of oxytetracycline depends on strain, environmental conditions, and culture medium composition [3,5,6]. Immobilization of *Streptomyces*, *Aspergillus*, *Cephalosporium*, *Cladonia*, *Penicillium*, and *Saccharomyces* has been used for the production of enzymes, ethanol, organic acids, alkaloids, and antibiotics [7-13]. Agar, alginate, carrageenan, collagen, gelatin, polyacrylamide, polyelectrolyte, and polyurethane have been used as the supporting materials for gel-entrapping, carrier-binding, and cross-linking immobilization [14].

In previous studies, starch and cellulosic materials were used for tetracycline and oxytetracycline production with mycelium and protoplast in submerged and solid-state fermentation [15-19]. This study compared the production of oxytetracycline by immobilized mycelium of *S. rimosus* in submerged cultivation with free cells.

Materials and Methods

Microorganisms

S. rimosus TM-55 (CCRC 940061) was provided by Cyanamid Taiwan Corporation (Taiwan) for oxytetracycline production. *Bacillus subtilis* ATCC 6633 was obtained from the American Type Culture Collection (Rockville, MD, US) for antimicrobial assay.

Culture media and growth conditions

Streptomycetes were cultivated in a yeast extract-dextrose medium (YD-medium) containing yeast extract 10 g/L, glucose 10 g/L, and agar 18 g/L at pH 7 ± 0.1 at 28°C for 2 days. The mycelia were then transferred to a syntheic medium (S-medium) comprised of glucose 10 g/L, yeast extract 4 g/L, Bacto peptone 4 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KH_2PO_4 4 g/L, and glycine 20 g/L in a 250-mL Erlenmeyer flask at 28°C and 150 rev/min for 1 day till the early stationary phase (absorbance, 4-4.5 at 660 nm). Mycelia were harvested by centrifugation at 1000 x *g* (Model 2010, Kubota, Japan) for 30 min. Part of mycelia (2%, v/v) was transferred to a tryptic-soy broth (TSB) (Merck, Sharp & Dohme, Darmstadt, Germany) containing Bacto dextrose 2.5 g/L, Bacto tryptone (pancreatic digest of casein) 17 g/L, Bacto soytone (pancreatic digest of soybean meal) 3 g/L, NaCl 5 g/L, K_2HPO_4 2.5 g/L, and glycine 20 g/L in a 250-mL Erlenmeyer flask and cultivated at 28°C for 2 days as seed culture.

The oxytetracycline fermentation medium was comprised of soluble starch 20 g/L, $(NH_4)_2SO_4$ 2 g/L, yeast extract 1 g/L, calcium carbonate 6 g/L, and soybean oil 2 mL at pH 7.9 [17]. Oxytetracycline potency was determined with antibiotic agar No. 1

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(Merck, Sharp & Dohme) containing meat extract 1.5 g/L, yeast extract 3 g/L, peptone from casein 4 g/L, peptone from meat 6 g/L, glucose 1 g/L, and agar 15 g/L at pH 6.5 ± 0.1 [20].

Submerged fermentation

Cells were cultivated in an S-medium to the mid-logarithmic phase (absorbance, 2.5-3 at 660 nm). Mycelium suspension 5% (v/v) was inoculated to oxytetracycline fermentation medium 100 mL and cultivated in a 500-mL Erlenmeyer flask at 28°C and rotated at 150 rev/min shaker for 10 days. All experiments were carried out in triplicate, and the mean values and standard deviation were calculated.

Immobilization

An equal volume of mycelial suspension and sodium alginate 4% were gently mixed using a magnetic stirrer for 30 min, and the mixture then passed through a peristaltic pump at 3 mL/min into calcium chloride solution 2% to immobilize the mycelium. After gently stirring for 30 min, the beads were washed several times with sterilized water for oxytetracycline production.

Antibiotic release from the immobilized beads

An equal volume of sodium alginate 4% and oxytetracycline at different concentrations were thoroughly mixed and immobilized at 25°C. The beads with various concentrations of antibiotic were put on antibiotic agar No. 1 with test organism *B. subtilis*, and were incubated at 30°C for 5 days. The diameter of the inhibition zone was measured daily, and the microbial growth on the agar plate was observed.

Adenine nucleotide determination

Cell mass was boiled with 0.02 M Tris buffer at pH 7.6 for 10 min. Adenine nucleotide measurement was made after filtration through a 0.46-μm millipore filter as described by Sparling *et al* [21]. Firefly lantern extract containing luciferin and luciferase (Sigma, US) was dissolved in 0.02 M Tris buffer 5 mL at pH 7.6 and 0.01 M $MgSO_4 \cdot 7H_2O$ 1 mL (dissolved in 0.02 M Tris buffer), and stored at 4°C overnight. Before measurement, the enzyme solution was centrifuged at 3000 x *g* for 10 min, and used within 2 h [22]. Sample or adenine nucleotide standard was mixed with the luciferin-luciferase mixture, and the light emitted was measured with an adenosine triphosphate (ATP) photometer (Turner TD-20e Luminomer, Sunnyvale, CA, US). Adenine nucleotide concentrations were calculated from the standard curve of the authentic compound.

Oxytetracycline measurement

Antimicrobial potency was determined using the paper disk method (diameter, 8 mm; Tokyo Seisakusho, Japan) with *B. subtilis* as the test organism on antibiotic agar No. 1 at 30°C for 18 to 24 h [15,20]. Each milliliter of solution contained 5×10^6 spores of the test organism, and each paper disk contained 35 μL of sample or antibiotic standard. The regression equation was $Y = 0.11682X - 1.67709$ and $r^2 = 0.9962$, where Y is the log concentration of oxytetracycline and X is the diameter of the inhibition zone. For qualitative and quantitative determination of antibiotics, the sample was filtered through a 0.46-μm millipore filter, and determined by a Shimadzu LC-5A liquid chromatograph (Shimadzu, Japan) using a Lichrosphere 60 RP-Select B (5 μm) column with a mixture of methanol, acetonitrile, and oxalic acid at a ratio of 0.8:1.0:3.2 in the mobile phase. The flow rate was 1 mL/min, the antibiotic was detected with an SPD-2A UV detector (Shimadzu) at 350 nm, and the concentration was calculated with a C-R6A integrator (Shimadzu). Authentic oxytetracycline, tetracycline, and chlortetracycline were used as the standards in the range between 1 and 1000 μg/mL.

Other methods

The pH value of culture broth was directly determined using a pH meter (Model 2002, Good, Taiwan). Cell biomass was measured spectrophotometrically (Spectrophotometer U-2000, Hitachi, Japan) at 660 nm. Cells were harvested by centrifugation at 10 000 x *g* for 20 min and washed twice with distilled water. Cell dry weight was determined by drying at 105°C overnight to a constant weight.

Results

Analytical system for tetracyclines

Retention time of the test antibiotics had slightly shifted in the mixtures, but the regression equation between test concentration (X) and peak area (Y) had a linear correlation both alone and in the mixture of 3 antibiotics. The regression equations of the mixture of 3 antibiotics were $Y = (1.38 \times 10^{-4})X - 7.04121$, ($r^2 = 0.99$); $Y = (1.10 \times 10^{-4})X - 2.95187$, ($r^2 = 0.99$); and $Y = (2.51 \times 10^{-4})X + 5.93793$, ($r^2 = 0.99$) for oxytetracycline, tetracycline, and chlortetracycline, respectively. High-performance liquid chromatograph (HPLC) method can thus be used for qualitative and quantitative determination of oxytetracycline, tetracycline, and chlortetracycline in fermentation broth.

Stability of oxytetracycline

Oxytetracycline is an amphoteric compound that reacts with both acids and alkalis. An aqueous solution of oxytetracycline hydrochloride was initially colorless, but became yellowish-brown during storage. The stability of oxytetracycline sharply decreased with increasing storage temperature. Analysis of the regression equation between half-life and storage temperature revealed that the half-life of oxytetracycline was 34, 137, 409, and 2000 h at 33°C, 25°C, 20°C, and 4°C, respectively.

Culture conditions and microbial growth

Culture media

The effects of culture media on cell dry weight and ATP content are illustrated in Fig. 1. The pH value of culture broth decreased initially and then increased gradually during cultivation. The drop in pH value was more significant in S-medium than in TSB-medium.

Cell dry weight of *Streptomyces* increased sharply during the first 12 h of incubation, and then gradually increased during 12 to 60 h and 12 to 48 h of incubation in S- and TSB-medium, respectively. Cells cultivated in S-medium had a longer growth period, and yielded a dry weight around double of that in TSB-medium. In addition, the maximal ATP content was 3.65 μg/mL for 60 h of incubation in S-medium, whereas it was only 1.82 μg/mL in TSB-medium.

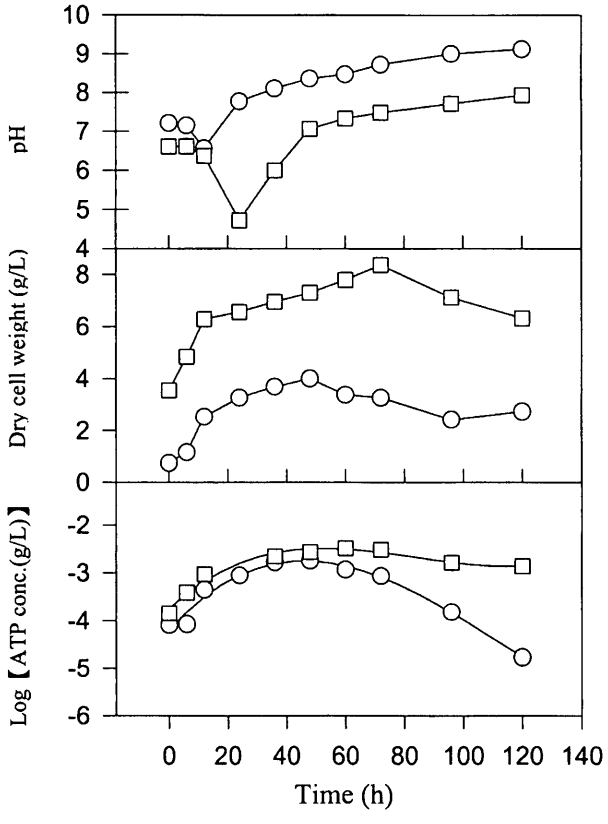

Fig. 1. Growth, pH level, and adenosine triphosphate content of *S. rimosus* in S-medium and in tryptic-soy broth medium.
□ S-medium
○ Tryptic-soy broth medium

A small volume ratio of culture broth-to-vessel volume stimulated the growth of *Streptomyces*. A high aeration rate shortened the culture period for maximal cell production. At the volume ratio of 1:4, the time for maximal growth ($A_{660\,nm}$ = 2.68) was 48 h, whereas the time for maximal growth ($A_{660\,nm}$ = 2.60) was 96 h at the volume ratio of 1:1.5.

Immobilization

Each milliliter of free cell culture broth produced 3.6 to 4 mg of dry cells over 10 days of incubation. In contrast, each milliliter of immobilized cell culture broth supported 2.58 to 2.65 mg of dry cells in the beads, and 1.5 to 1.56 mg of dry cells outside the beads for 16 days incubation, and gave a total dry cell yield of 4.08 to 4.21 mg/mL.

The pH value of free cell culture broth decreased from 7.9 to 6.89 on Day 4, and then gradually increased to 7.3 after 8 days of incubation. In immobilized cells, the pH value decreased from 8 to 7.1 on Day 8, then slightly increased to 7.3 after 10 days of incubation.

Culture broth of free cells was light yellow on the first day of cultivation, and became deep brown on the fourth day. It had absorption peaks between 280 and 410 nm and a shoulder at 230 nm. Culture broth of immobilized cells was light yellow on the second day. The absorption peaks were between 300 and 350 nm, and the shoulder was at 230 nm. The culture broth became earthy yellow after 3 to 4 days of incubation, and had a new absorption peak between 380 and 410 nm. The absorbance above wavelength 400 nm gradually increased during cultivation, and the color became deep brown on the eighth day. The color change was faster in the culture broth of free cells than immobilized cells. The same effects were also observed in the pH value of culture broth and the cell dry weight.

Effect of immobilization conditions on antibiotic production

Immobilization

The effect of cell immobilization on antibiotic production is shown in Fig. 2. Oxytetracycline and chlortetracycline production increased with the duration of cultivation and reached maximal potency on the

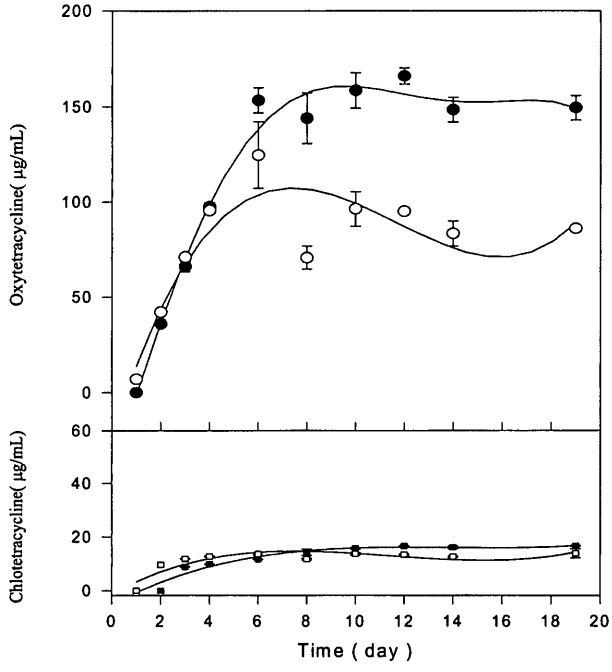

Fig. 2. Antibiotic production in free and immobilized cells of *S. rimosus*.
○ Oxytetracycline production in free cells
● Oxytetracycline production in immobilized cells
□ Chlorotetracycline production in free cells
■ Chlorotetracycline production in immobilized cells

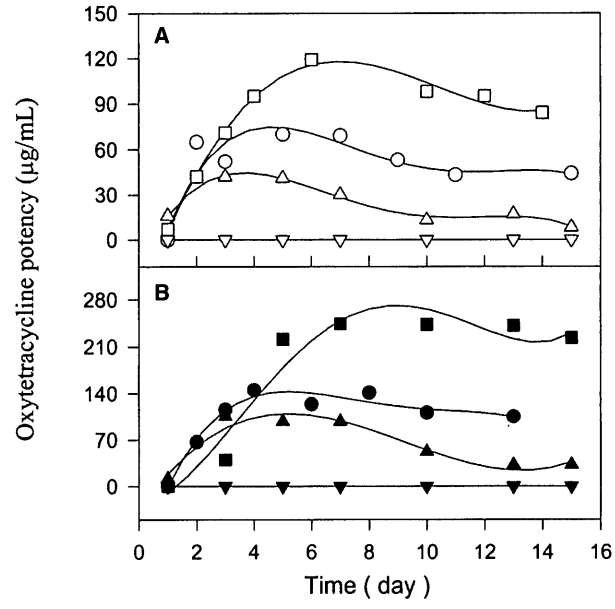

Fig. 3. Effects of incubation temperature on oxytetracycline production. **(A)** Free cells. **(B)** Immobilized cells.
○, ● 28°C
□, ■ 33°C
△, ▲ 40°C
▽, ▼ 50°C

6th day. Each milliliter of free and immobilized cell cultures produced 124 μg and 153 μg of oxytetracycline, respectively. Characteristics of chlortetracycline production in free and immobilized cell cultures were similar to that of oxytetracycline production. Each milliliter of broth yielded 13.6 μg of chlortetracycline in free cell cultures after 6 days of incubation, whereas it yielded 15.7 μg of chlortetracycline in immobilized cells after 10 days of cultivation. For the productivity of oxytetracycline, each gram of dry cells in free form produced 33.3 to 34.2 mg of oxytetracycline, whereas each gram in the immobilized state produced 40.2 to 40.7 mg of oxytetracycline. In contrast, each gram of dry cells in free form produced 3.65 to 3.76 mg of chlortetracycline compared with 4.13 to 4.18 mg in that of the immobilized state.

The effect of the age of mycelium used in the immobilization was not significant. However, each milliliter of immobilized cell culture broth yielded 180 to 210 μg of oxytetracycline after 4 days of incubation with the mycelia at the early stationary phase, late stationary phase, or early decline phase.

Culture temperature

The effect of culture temperature on oxytetracycline production is illustrated in Fig. 3. Both free and immobilized cells had maximal oxytetracycline potency when grown at 33°C, followed by 28°C and 40°C, and produced the lowest amount of oxytetracycline at 50°C. Each milliliter of culture broth produced 121 and 252 μg of oxytetracycline in free and immobilized cells at 33°C, respectively. Oxytetracycline potency was undetectable at 50°C in both treatments.

Concentration of calcium alginate

The best concentration of calcium alginate for mycelium immobilization was 2%. Each milliliter of culture broth yielded 194 ± 12, 157 ± 10, 123 ± 8, and 105 ± 7 μg of oxytetracycline for 5 days of incubation with calcium alginate 2%, 3%, 1%, and 0.5%, respectively. Calcium alginate 5% retarded oxytetracycline production, which reached a maximal value on the seventh day (115 ± 8 μg/mL).

Although copper ion and alginate could form a gel in immobilized mycelium of *S. rimosus*, the stability of the beads was low. During the 24-h cultivation, the color of the immobilized beads changed from light blue to light blue-green as a result of the solubility and the replacement of copper ion by other cations. Oxytetracycline potency in immobilized cells with copper ion was below the detection limit. The diameter of the beads increased by 26.8% (from 2.13 ± 0.2 to 2.7 ±

0.08 mm), 40.8% (from 2.13 ± 0.2 to 3 ± 0.13 mm), and 92.5% (from 2.13 ± 0.2 to 4.1 ± 0.22 mm) in KCl 0.4 M, NaCl 0.4 M, and Na_2 ethylenediaminetetraaceticacid (EDTA) 0.05 M solution, respectively. The beads swelled and the membrane of the beads was severely damaged, resulting in complete leakage in Na_2EDTA 0.1 M because of the chelation of calcium ion with EDTA. The diameter of the beads increased by 40.8% (from 2.13 ± 0.2 to 3.00 ± 0.16 mm) in S-medium and 35.2% (from 2.13 ± 0.2 to 2.88 ± 0.10 mm) in oxytetracycline fermentation medium lacking $CaCO_3$ supplement. In contrast, the diameter of the beads remained constant in oxytetracycline fermentation medium with $CaCO_3$ supplement. Calcium ion in the culture medium was very important for the stability of alginate beads and hence the production of oxytetracycline.

Diameter of beads

The effect of the diameter of the immobilized beads on oxytetracycline production is illustrated in Fig. 4. When beads with smaller diameter were used, the time period for maximal oxytetracycline production was short; but the yield was same at different bead diameters. Each milliliter of culture broth could support 211 ± 13 μg of oxytetracycline with beads of 2.13-mm diameter on the 5th day, and had 201 ± 12 μg of oxytetracycline with beads of 3.26-mm diameter on the 9th day.

Inoculum density

The effect of cell density in immobilized beads on oxytetracycline production is shown in Fig. 5. The effect of inoculum density on oxytetracycline production was not significantly at the early cultivation stage; however,

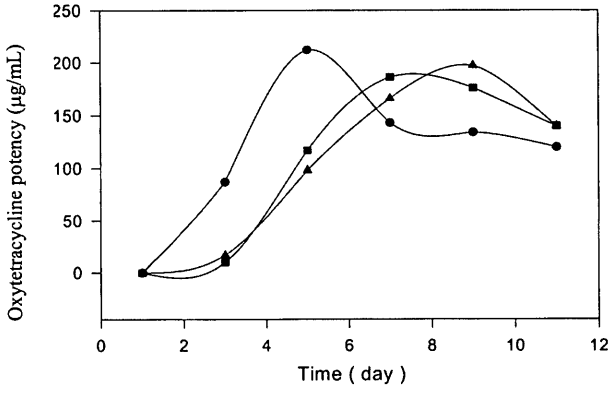

Fig. 4. Effects of the diameter of beads on oxytetracycline production.
● Diameter of 2.13 mm
■ Diameter of 2.46 mm
▲ Diameter of 3.26 mm

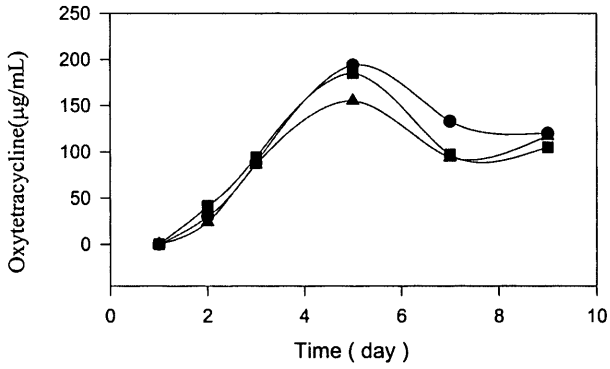

Fig. 5. Effects of inoculum density on oxytetracycline production.
● Fresh mycelium 3.25 mg
■ Fresh mycelium 1.625 mg
▲ Fresh mycelium 1.083 mg

high inoculum density resulted in high oxytetracycline productivity after 5 days of incubation. Each milliliter of culture broth produced 194 ± 12, 185 ± 10, and 155 ± 10 μg of oxytetracycline for 3.25, 1.625, and 1.083 mg of fresh mycelium, respectively.

Oxytetracycline release from immobilized beads

The inhibition zone of immobilized beads increased with concentration of oxytetracycline in the first 3 days of incubation, then gradually decreased from Day 4 onwards. There was a linear correlation between the diameter of the clear zone and the concentration of oxytetracycline in the beads.

Discussion

Antimicrobial activity can be assayed either by spore suspensions or vegetative cell suspensions. For *B. subtilis*, the spore suspension method was more sensitive than the vegetative cell suspension, but the former was unstable during storage at 4°C for bioassay study [20]. The vegetative cell suspension of *B. subtilis* was thus used for bioassay study in the present investigations. Although the standard curve of oxytetracycline shifted slightly in each bioassay experiment because of the different concentrations of test organism, the correlation coefficient (r^2) was always higher than 0.99. Yang and Yuan [2] and Yang and Swei [17] also reported a similar result. To ensure the accuracy and reproducibility of the test sample, a standard curve of test antibiotic should be simultaneously measured in each bioassay experiment.

Oxytetracycline fermentation broth had a major absorption peak at 270 nm, a minor peak at 350 nm, and a shoulder between 250 and 260 nm [23]. Yang

and Ling [15] reported that oxytetracycline had its major absorption peaks at 275 and 353 nm and a shoulder at 248 nm; tetracycline had the major absorption peaks at 275 and 355 nm; and chlortetracycline had the major absorption peaks at 228, 275, and 365 nm. Several researchers have used the absorbances at 350, 355, 360, 365, and 395 nm for the quantitative determination of tetracyclines [15,24-26]. In this study, the peak area at 350 nm and the concentration of antibiotic had a good linear correlation. The absorbance at 350 nm was thus used to measure the concentrations of oxytetracycline and chlortetracycline during cultivation.

Argauer [27] found that oxytetracycline potency decreased during storage, and the half-life of oxytetracycline was around 2 days at 34°C. High concentration and large volume of tested antibiotic reduced the potency decrease during storage. Incubation temperature affected not only aeration and oxygen transport, but also the antibiotic biosynthesis. Marijan *et al* [28] reported that oxytetracycline production in *S. rimosus* had an optimal temperature between 30°C and 33°C, and the production was sharply inhibited when the incubation temperature exceeded 40°C. Yang and Swei [17] indicated that the optimal temperature for oxytetracycline production in solid substrate cultivation of *S. rimosus* was between 25°C and 30°C, and the production has decreased at 37°C. The optimal incubation temperature of *S. rimosus* in submerged culture was slightly higher than that in solid substrate culture, because heat transfers more readily in liquid than in solid substrate.

In this study, the pH value of culture broth decreased initially and then increased gradually. This might have been resulted from the accumulation of organic acid at the early cultivation stage and the final products of primary metabolism that served as the precursors of secondary metabolites at the later stage [29]. To prevent the pH level from dropping during the cultivation, ammonium sulfate was replaced by urea as the sole nitrogen source, or supplemented as a buffer agent in the broth to counter the acid production [2, 30]. Color and pH changes of culture broth were slower in immobilized cells than free cells. Cell growth rate was lower in the immobilized beads than free cells, but the opposite was true for total cell weight and metabolite production. Ogaki *et al* [31] indicated that immobilization of *S. rimosus* with polyurethane reduced the cell growth rate, but increased oxytetracycline production in prolonged cultivation periods. The same phenomenon was also observed in the immobilization of *S. tendae* Tu 901 with calcium alginate to produce tylosin and nikkomycin. The antibiotic production period was only 72 h in free cells, was 96 to 120 h in immobilized cells [32]. Spore immobilization of *S. aureofaciens* with calcium alginate also yielded a 2.5-fold increase of chlortetracycline with an increase in the production period from 48 to 72 h because of the improved pH stability. In the immobilization of *Penicillium chrysogenum* with *k*-carrageenan for penicillin production, it was shown that immobilization did slow down the rate of microbial growth but promoted the expression of antibiotic-producing gene [33]. Mussender *et al* [10] showed that immobilization of *P. chrysogenum* modified the cell physiology and the gene expression. In this study, immobilization of *S. rimosus* also increased the length of the growth period and improved the production of oxytetracycline and the stability of substrate pH.

Adenosine triphosphate content has been used as an index of microbial biomass in fresh water sediment, soil, meat, culture broth, and solid substrate [18,22,34, 35]. This study demonstrated that ATP content and cell dry weight were linearly correlated. Thus, ATP content could be used as an index of microbial activity in both submerged and solid substrate cultivation.

The period of maximal oxytetracycline production was proportional to the diameter of the immobilized beads. The maximal oxytetracycline production of immobilized cells shifted from the fifth to the ninth day when the bead diameter increased from 2.13 to 3.26 mm at the same cell density. Veelken and Pape [32] indicated that antibiotic production decreased with increasing bead diameter, because the gel conformation prevented the exchange of oxygen and substrate between cell and the environment. Mussenden *et al* [10] found that beads with a diameter of 1.5 mm had a 3-fold higher penicillin production in *P. chrysogenum* than beads with a diameter of 3.5 mm. Concentration of calcium alginate affected not only the structure of immobilized beads and the growth of cells, but also the production of oxytetracycline. High concentrations of calcium alginate resulted in tight cross-linking between cells and alginate, which reduced nutrient transport and microbial activity; whereas low concentration of alginate resulted in loose texture between cells and alginate, and facilitated nutrient leakage through stirring treatment during cultivation. In chlortetracycline production with immobilization of *S. aureofaciens*, calcium alginate 3% had the highest potency among the tested concentrations [32]. In this study, oxytetracycline production was reduced by 19.1% and 36.6% when the concentration of calcium alginate changed from 2% to 3% or from 2% to 1%, respectively. The use of alginate 2% resulted in high productivity because

of a moderate texture for cell growth and protection of the structure of beads during cultivation.

During cultivation of immobilized *S. rimosus*, mycelia grew in the beads and synthesized the metabolites. High inoculum density enhanced microbial growth and oxytetracycline production, but the specific productivity of unit immobilized mycelium decreased. Although 3.25 mg/mL of mycelium had a 25.2% higher oxytetracycline production than that of 1.083 mg/mL of mycelium, the specific productivity of unit weight immobilized mycelium of the former was 58.3% lower than that of the latter. Veelken and Pape [32] showed that chlortetracycline production with immobilized spores of *S. aureofaciens* increased with the spore concentrations. They also found that tylosin and nikkomycin production increased with inoculum density of *S. tendae* immobilized mycelium, but the specific productivity of unit weight immobilized mycelium was decreased. Kokubu and Suzuki [7] found that immobilized mycelium 5% of *S. fradiae* had maximal protease activity, whereas immobilization of mycelium 10% or 2.5% did not favor protease production. In this study, although high inoculum density increased the production of metabolites, it reduced the specific productivity of unit weight of cells or mycelia. Thus, both the production and the economic potential must be considered when deciding the optimal inoculum density .

During immobilization of mycelium in the alginate, ample spaces were found in the cross-linking between mycelium and alginate for mycelial growth and metabolite accumulation. When the beads were put on medium, oxytetracycline in the alginate beads was released and inhibited the growth of test organism [36]. There was a positive correlation between the area of inhibition zone and the concentration of antimicrobial agent. The regression equation of the area of the clear zone (X) and the log concentration of oxytetracycline (Y) was $Y = 9.463 \times 10^{-4} X - 0.21247$ and $r^2 = 0.99$. This release equation of oxytetracycline might be used to control the release of antibiotic from the immobilized beads. During cultivation, antimicrobial agent was released from the beads, diffused into the medium, and formed a gradient of concentration. At the early stage, the released concentration of antibiotic was high and hence the microbial growth was inhibited. However, the tested organism resumed its growth when the antibiotic gradually diffused into the medium and decreased in concentration. Multiple layers of microbial growth were thus observed at the interface, resulting in prolonged cultivation.

This study demonstrated that mycelial immobilization of *S. rimosus* reduced the pH change and the microbial growth rate, extended the cell growth period, and enhanced the antibiotic production by 20% to 27.5%. Therefore, this cell immobilization technique may be useful in metabolite productions.

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