藥物食品檢驗局調查研究年報. 14: 68-78. 1996.
Ann. Rept. NLFD Taiwan R.O.C. 14: 68-78. 1996.

Morphogenesis, Biomass and Oxytetracycline Production of *Streptomyces rimosus* in Submerged Cultivation

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The minimal concentration of adenosine triphosphate (ATP) which could be detected with spectrophotometry, HPLC and luciferin-luciferase methods was 1.0 μM, 3.3 μM and 100 nM, respectively. In submerged cultivation, most *Streptomyces rimosus* TM-55 was in hyphae fragment form at 65 h, became short-rod mycelia at 166 h, and lysed at 504 h incubation. The ATP content had maximal value at 24 h, then gradually decreased during cultivation. The oxytetracycline potency increased as incubation occurred, had maximal potency 178.9 μg/ml at 166 h, and then gradually decreased. Morphogenesis was very important in oxytetracycline production in submerged cultivation of *Streptomyces*; short-rod mycelia had high oxytetracycline production.

Key words: *Morphogenesis, Streptomyces rimosus, oxytetracycline potency, ATP content, submerged cultivation.*

INTRODUCTION

Morphogenesis of microbes involves chemical composition of cell[1], enzyme production[2], protease secretion[3], solvent production[4], and antibiotic production[5-7]. It is affected by environmental conditions and gene regulations[2,8].

Microbial biomass in submerged cultivation can be determined by microscopic cell count, plate count, turbidity, nitrogen determination, dry weight, oxygen uptake, carbon dioxide release, fatty acid, ATP and chitin contents[9-11]. ATP content has been used as the index of microbial biomass in fresh water, marine[12], sewage[13], soil[14], meat, milk[15] and sweet potato residue[16].

Tetracycline is a broad-spectrum antibiotic used in a variety of infections caused by gram-positive and gram-negative bacteria, rickettsiae, chlamydiae, coccidia, amoebae, balantidia and mycoplasmas[17]. It can be imported and the derivatives then prepared locally.

In previous studies[18-22], starchy and cellulosic materials were used as the substrate for tetracycline and oxytetracycline production with solid state fermentation. This work focused on the relationships among morphogenesis, ATP content, cell growth and oxytetracycline production of *Streptomyces rimosus* in submerged cultivation.

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Received: December 19, 1994
Accepted: January 15, 1995

原載於中華微免雜誌. 1995. 28(1): 21-31.

MATERIALS AND METHODS

Test organisms

Streptomyces rimosus TM-55 was obtained from Dr. Thomas H. H. Ku, Cyanamid Taiwan Corporation, and used for oxytetracycline production. *Bacillus cereus* var. *mycoides* ATCC 11778 was used for antimicrobial assay.

Culture media and culture conditions

The oxytetracycline-producing organism was cultivated at 28°C in a slant containing (g/l) soluble starch, 10; yeast extract, 1; beef extract, 1; tryptone, 2; $FeSO_4 \cdot 7H_2O$, 0.1; and agar, 20 at pH 7.2.

Submerged culture contained (g/l) soluble starch, 20; corn steep liquor, 10; $(NH_4)_2SO_4$, 6; $CaCO_3$, 8; NaCl, 5; and soy bean oil, 2 at pH 6.8 to 7.2. Each ml of medium was inoculated with 1.0×10^6 conidia and shaken, then cultured at 250 rpm at 28°C for 7 to 10 days.

Protein content

Total nitrogen was determined by a modified Kjeldahl method[23]. Protein content was calculated as the value of 6.25 times of total nitrogen, and cell pellet was used as the index for cell biomass.

Extraction of adenosine nucleotides

Cells were harvested by centrifugation at 6,000 × *g* for 30 min, and boiled with 10 vol of 0.02 M Tris buffer, pH 7.6, for 10 min. The filtrate was used for adenosine nucleotide determination[24].

Determination of adenosine nucleotides

Spectrophotometric method

Adenosine nucleotides were determined by spectrophotometer at absorbances of 250 nm, 254 nm, 260 nm, and 270 nm. Authentic ATP, ADP, and AMP were used as the standard in the range from 1.0 to 100 μM.

Luciferin-luciferase method

Firefly lantern extract containing luciferin and luciferase (Sigma, USA) was dissolved in 5 ml of 0.02 M Tris buffer, pH 7.6, and 1 ml of 0.01 M $MgSO_4 \cdot 7H_2O$ (dissolved in Tris buffer), and stored at 4°C overnight. Before measurement, the enzyme solution was centrifugated at 3,000 × *g* for 10 min. The enzyme solution must be used within 2 h[25]. Sample or adenosine nucleotide standard was mixed with luciferin-luciferase mixture, and measured by ATP photometer (Turner TD-20e Luminomer, USA). Adenosine nucleotide was calculated from the standard curve of the authentic compound.

HPLC method

The sample was filtered with 0.46 μm Millipore filter, and adenosine nucleotide was determined by LC-5A Liquid Chromatograph (Shimadzu, Japan) with column Lichrospher 60 RP-Select B (5 μm) and eluted with the mixture of 0.2 M NaH_2PO_4 and 0.1 M $NaClO_4$ at pH 5.0 or 0.06 M K_2HPO_4 and 0.04 M KH_2PO_4 at pH 6.0 as the mobile phase at 0.8 ml/min. Adenosine nucleotide was detected with UV detector at wavelength 270 nm with 1.28 AuFs. Authentic adenosine nucleotide was used as the standard in the range from 3.3 to 333 μM.

Quantitative determination of antibiotics

After fermentation, the antimicrobial

activity of culture broth was determined by bioassay and HPLC methods.

Bioassay method

Paper disc method (diameter 8 mm, Toyo Seisakusho Co., Japan) using *B. cereus* var. *mycoides* as the test organism in antibiotic medium I (Difco Laboratory, USA) at 30°C was used for bioassay of antimicrobial activity. Total oxytetracycline equivalent potency was calculated from the clear zone of the standard curve of oxytetracycline (Sigma, USA) in the range from 1.0 μg/ml to 10 mg/ml.

HPLC method

Culture broth was filtered with 0.46 μm Millipore filter, and antimicrobial activity was determined by Shimadzu LC-5A Chromatograph. The operation conditions were with column Lichrospher 60 RP-Select B (5 μm) and the mixture of methanol : acetonitrile : water : 0.2 M phosphate buffer, pH 2.5, at ratios of 10 : 20 : 60 : 10 as the mobile phase. The flow rate was 0.8 ml/min, and the antibiotic was detected with UV detector at wavelength 350 nm with 2.56 AuFs. Authentic oxytetracycline was used as the standard in the range from 1 μg/ml to 100 μg/ml.

Observation under scanning electron microscope

Morphogenesis of the test organism at different culture periods was observed under Hitachi S-550 Scanning Electron Microscope (Hitachi, Japan) at 20 KV with gold metal shadowing.

Cell dry weight

Culture broth was centrifugated at 3,000 × *g* for 10 min, and cell pellet was dried at 105°C for 10 h to a constant weight.

Energy charge (EC)

The mole fraction of adenosine 5′-triphosphate (ATP) plus half the mole fraction of adenosine 5′-diphosphate (ADP) was the parameter for measurement of the amount of metabolically available energy stored in the adenylate pool[26]. EC was calculated by the following equation:

$$EC = \frac{[ATP] + 1/2[ADP]}{[ATP] + [ADP] + [AMP]}$$

where [ATP], [ADP], and [AMP] are the mole concentrations of ATP, ADP, and AMP, respectively.

RESULTS

Measurement of adenosine nucleotides

ATP, ADP, and AMP have significant absorption at wavelength 260 nm. There were linear relations between the absorbances at 250 nm, 254 nm, 260 nm, and 270 nm and the concentration of ATP, ADP, and AMP from 1.0 to 100 μM. The minimal concentration of ATP which could be detected with spectrophotometry was 1.0 μM. Since the UV absorption spectra of ATP, ADP, and AMP were very similar, therefore, UV spectrophotometric method could not differentiate ATP, ADP, and AMP. However, HPLC method could separate ATP, ADP, and AMP with the mixtures of methanol (concentration was less than 10%), 0.2 M NaH_2PO_4 and 0.1 M $NaClO_4$ at pH 5.0 as the mobile phase, the retention time was 4.5 min, 5.2 min, and 8.3 min, respectively. While the mixture of 0.06 M K_2HPO_4 and 0.04

M KH_2PO_4 at pH 6.0 was used as the mobile phase, the retention time was 4.3 min, 5.0 min, and 8.1 min, respectively. There was also a linear relation between the peak area and the concentration of adenylates in the range from 33.3 nM to 333 μM. When the concentration of adenylates was 33.3 nM, the peak could be detected in the record only, but no peak was shown in the integrator.

There was also a linear relation between the amount of luminescence and the concentration of ATP in the range from 180 pM to 1.0 μM with ATP disodium and magnesium sulfate or ATP anhydrous as the authentic compound. Although the luciferin-luciferase method could detect minimal concentration of adenylates at 100 pM, the interference of the presence of ADP and AMP was significant at the 10 nM level. The amount of luminescence of ADP and AMP were only 3.64% and 2.27% of that of ATP at 100 nM. However, the values increased to 22.78% and 19.53% at 10 nM level.

Morphogenesis, biomass and oxytetracycline production in submerged culture

ATP content, cell protein, and cell dry weight in submerged culture of *S. rimosus* are shown in Figs. 1 and 2. ATP content had the maximal value, 7.45 nM/mg cell protein, at 24 h incubation; this decreased as time went on, and remained constant after 166 h incubation. Cell growth had the log phase before 65 h, the stationary phase between 65 and 166 h and the decline phase after more than 166 h incubation. In the early log phase, the ATP content was very consistent with the cell protein. The same phenomenon was also found in the ADP and the AMP contents, which had maximal values, 860 pM/mg cell protein and 130 pM/mg cell protein, at 24 h, respectively. In 24 h cultivation, most of the cells were in mycelial form of 0.42 × 7.00 μm (Fig. 3a), the size was larger than at other culture stages. Adenosine nucleotide content, growth rate and energy charge had the highest value, while the pH had minimal value. After 65 h incubation, most of cells were in mycelial fragments of the size 0.35 × 3.57 μm (Fig. 3b), and the pH gradually increased, while the cell protein, cell dry weight, growth rate and adenosine nucleotide content decreased. The size of the mycelial fragment was almost the same during the cultivation from 65 h to 112 h (Figs. 3c and 3d). After 166 h incubation, the cells were in the late stationary phase and became short-rod mycelial fragments. The size of the mycelial fragment slightly decreased, and some spores were found (Figs. 3e and 3f). For 480 h cultivation, the cells were in the decline phase, most of the mycelia had been autolyzed (Fig. 3g), and cell dry weight and growth rate sharply decreased. Some conidia were formed, and adenosine nucleotide content was low (Table 1).

Oxytetracycline potency could be detected in the late log phase, was at maximal value in the middle of the stationary phase, and then decreased in the decline phase (Fig. 4). Each ml of medium produced 178.9 μg of total oxytetracycline equivalent potency with bioassay method or 160 μg of oxytetracycline with HPLC method. Oxytetracycline potency determined with bioassay method was the same as that measured by the HPLC method. This result indicated that oxytetracycline was the only antibiotic produced in

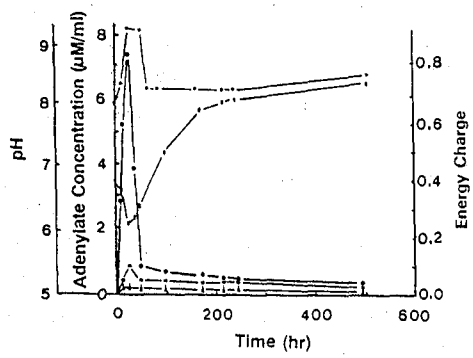

Fig. 1. Time course of adenylate content, energy charge, and pH value of *S. rimosus* TM-55 in submerged cultivation. Each ml of medium was inoculated with 1.0 × 10^6 conidia and shaken cultured at 250 rpm at 28°C for 504 hr.
● — ● ATP content
▬ — ▬ ADP content
▲ — ▲ AMP content
▮ — ▮ pH value
★ — ★ Energy charge

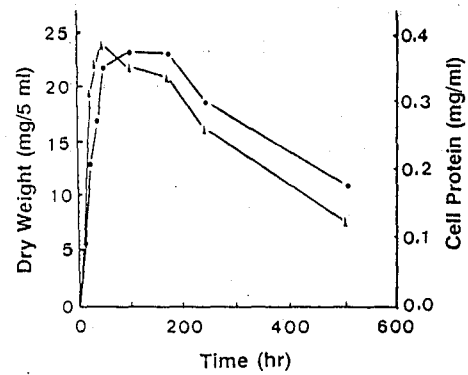

Fig. 2. Time course of cell protein and cell dry weight of *S. rimosus* TM-55 in submerged cultivation. Each ml of medium was inoculated with 1.0 × 10^6 conidia and shaken cultured at 250 rpm at 28°C for 504 hr.
● — ● Cell protein
▲ — ▲ Cell dry weight

S. rimosus.

In addition, the culture broth had pigment with light absorption between 350 nm and 450 nm and had a maximal absorption peak at 385 nm. The pigment increased with incubation (Fig. 5). This pigment might be one of the secondary metabolites of *S. rimosus*. The production of the pigment will be studied in the future.

It is clear that antibiotic production of *S. rimosus* in the submerged culture might be connected with the morphogenesis, short-rod mycelial fragment had high oxytetracycline production.

DISCUSSION

Estimation of the microbial population in the culture is very important for the production of enzyme and antibiotic with *Streptomyces*. The plate count method, turbidity measurement and cell number provide some technical problems in *Streptomyces* cultivation. ATP content was about 0.4% of the dry cell[26] and could be used as the index of biomass of microbes in water and soil[27,28]. The nucleotides of adenosine had chromophores with absorption between wavelength 240 nm and 270 nm. There were linear relations between the absorbance and the concentration of ATP, ADP and AMP from 1.0 to 100 μM by the spectrophotometric method, between the peak area and the concentration of ATP, ADP and AMP from 33.3 nM to 333 μM by the HPLC method, and between the luminescence and the concentration of ATP from 100 nM to 10 μM in the luciferin-luciferase method. The sensitivity of the luciferin-luciferase method for ATP measurement

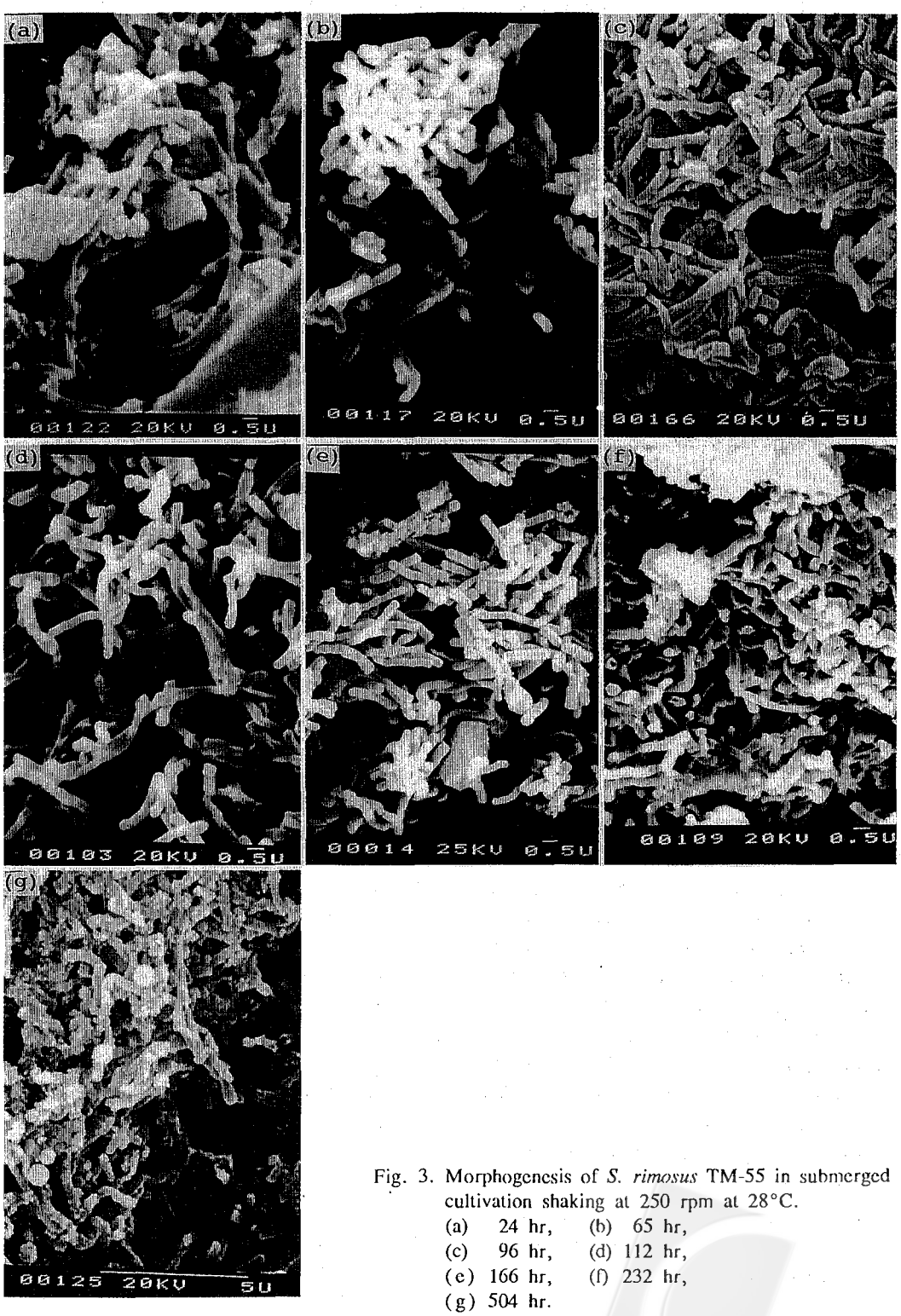

Fig. 3. Morphogenesis of *S. rimosus* TM-55 in submerged cultivation shaking at 250 rpm at 28°C.
(a) 24 hr, (b) 65 hr,
(c) 96 hr, (d) 112 hr,
(e) 166 hr, (f) 232 hr,
(g) 504 hr.

Table 1. Cell size, biomass, ATP, and oxytetracycline potency in submerged cultivation*

Item	Incubation time (h)						
	0	24	65	112	166	208	480
Mycelia (μM)	0.40 × 6.55	0.42 × 7.00	—	—	—	—	—
Mycelial fragment (μm)	—	—	0.35 × 3.57	0.34 × 3.57	0.35 × 2.57	0.33 × 2.13	0.31 × 2.57
Spore (μm)	—	—	0.35 × 1.14	0.36 × 1.00	0.39 × 1.29	0.50 × 0.88	0.71 × 0.71
Cell protein (mg/ml)	0.02	0.20	0.28	0.38	0.37	0.35	0.17
Cell dry weight (mg/5ml)	1.10	19.2	24.6	21.7	21.5	15.6	7.2
Adenylate nucleotide (nM/mg cell protein)							
ATP	0.01	7.45	3.80	0.83	0.80	0.75	0.70
ADP	0.005	0.86	0.45	0.50	0.52	0.53	0.43
AMP	0.003	0.13	0.12	0.11	0.10	0.09	0.05
EC ratio**	0.69	0.93	0.92	0.75	0.74	0.76	0.77
pH	6.8	6.1	6.3	7.3	7.6	7.8	8.2
Oxytetracycline (μg/ml)	0.0	0.0	42.0	142.5	178.9	162.3	100.5

* Average of triplicates

** $EC = \frac{[ATP] + 1/2[ADP]}{[ATP] + [ADP] + [AMP]}$

was the highest, but the HPLC method could determine ATP, ADP and AMP simultaneously. The specificity of luciferin-luciferase method for ATP determination was high [29]. The luminescence of ADP and AMP was only 3.64%, and 2.27% of that of ATP at 100 nM. Since the ADP and AMP contents were low in the biological materials, therefore the luciferin-luciferase method for ATP measurement could be used for biomass determination in submerged cultivation of *Streptomyces*.

ATP content is a potential index of microbial biomass[15], and energy charge is a useful indicator of the energetic state of cells[30]. In this study, it was found that ATP content of the substrate was highly consistent with cell protein in the early log phase. At 24 h incubation, ATP content and energy charge had maximal values, while pH value had the minimal. Energy charge of *S. rimosus* in submerged culture was between 0.74 and 0.93. These results are very consistent with energy charge 0.87 in *Acetobacter aceti*[31], 0.87 in *Bacillus brevis*[32], 0.89 in *B. cereus*[33], 0.87 to 0.96 in *Citrobacter freundii*[34],

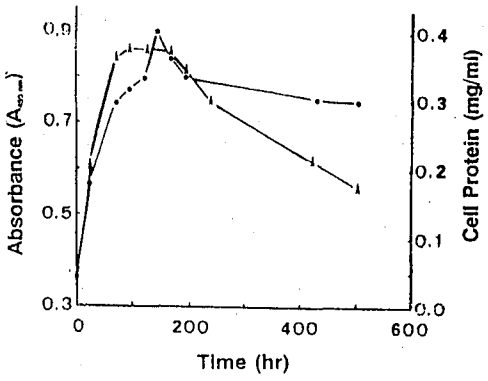

Fig. 4. Time course of oxytetracycline production of *S. rimosus* TM-55 in submerged cultivation. Each ml of medium was inoculated with 1.0 × 10^6 conidia and shaken cultured at 250 rpm at 28°C for 480 hr.
● — ● Bioassay method
▲ — ▲ HPLC method

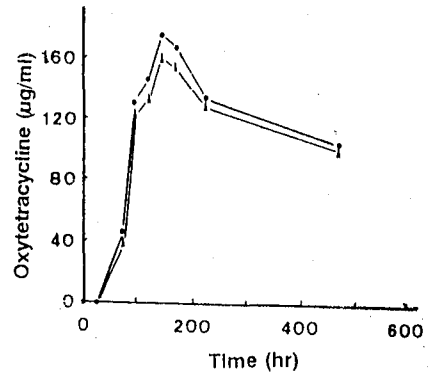

Fig. 5. Pigment formation with absorbance at 450 nm and cell protein of *S. rimosus* TM-55 in submerged cultivation. Each ml of medium was inoculated with 1.0 × 10^6 conidia and shaken cultured at 250 rpm at 28°C for 480 hr.
● — ● Absorbance
▲ — ▲ Cell protein

0.76 to 0.79 in *Clostridium kluyveri*[35], 0.76 to 0.85 in *Escherichia coli*[36-38], 0.97 in *Pseudomonas aeruginosa*[33], 0.92 in *Staphylococcus aureus*[33], and 0.8 to 0.9 in *Saccharomyces cerevisiae* grown aerobically[39]. While the value was higher than energy charge 0.15 to 0.42 in the mono-culture or the co-culture of amylolytic fungi[16], or 0.5 in *S. cerevisiae* grown anaerobically with glucose-limited culture, and 0.39 in *S. cerevisiae* suspended in a starvation salt medium and aerated[39]. Energy charge was high in the log phase, and decreased in the stationary and decline phases. This might result from the high metabolic activity in the log phase, and the limiting of nutrient and the accumulation of organic acid to reduce the cell activity in the stationary and decline phases.

Morphogenesis of *Streptomyces* in oxytetracycline production was very important. The initiation of biosynthesis usually is restricted to a specific developmental stage, or to specialized cells within the organism. In *Penicillium brevicompactum*, mycophenolic acid, brevianamide A, asperphenamate and ergosterol are coordinately synthesized with the development of aerial hyphae[40]. Sarkar and Paulus[41] also reported that the function of secondry metabolites was differentiation. In this study, it was also found that short-rod mycelial fragment in the stationary phase had high oxytetracycline production capacity. Wang[7] and Matzumura and Imanaka[6] also showed that cephalosporin C was produced by *Cephalosporium acremonium* with swollen mycelium. Therefore, short-rod mycelial fragment of *S. rimosus* might be good for antibiotic production.

ACKNOWLEDGEMENTS

The authors thank the National

Science Council of the Republic of China for its financial support via NSC 82-0409-B002-195.

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Streptomyces rimosus 在浸沉培養時之形態化育、生質量與地靈黴素生產

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以分光光度儀、HPLC 和螢光素一螢光素酶法測定 ATP 時，其最低之檢出濃度分別爲 1.0 μM，3.3 μM 和 100 nM。在浸沉培養 65 小時時，大部分之 *Streptomyces rimosus* TM-55 以菌絲片斷存在，培養 168 小時則形成短桿狀菌絲，而培養 504 小時則有部分菌絲被分解。培養 24 小時時其 ATP 含量最高，而後隨培養過程而逐漸降低。地靈黴素產量亦隨培養過程而增加，於 166 小時達最高力價 178.9 μg/ml 而後逐漸下降。形態化育在 *Streptomyces* 浸沉培養生產地靈黴素時佔很重要之角色，短菌絲片段較有利於抗生素生產。