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REVIEW

Dynamics of Morphological and Physiological Differentiation in the Actinomycetes Group and Quantitative Analysis of the Differentiations

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Key words: *Streptomyces exfoliatus*, leupeptin, leupeptin inactivating enzyme, trypsin-like protease, aerial spore, metalloprotease, submerged spore, morphological differentiation, *Streptomyces albidoflavus* SMF301, characterization, taxonomy, kinetics of sporulation, pyrolysis mass spectrometry, artificial neural networks, *Nocardia mediterranei*, rifamycin, yield shear stress, morphological factor

Streptomycetes are gram-positive bacteria with unusual morphological differentiation characteristics, forming a substrate mycelium on solid medium and an aerial mycelium which differentiates into arthrospores. The bacteria are important because of their unique metabolism that produces various antibiotics (physiological differentiation). Both morphological and physiological differentiations are induced by a shift-down of essential nutrients such as carbon, nitrogen, and phosphate. The effects of nutrient limitation on morphological differentiation have been determined mostly in agar cultures. Since the concentrations of nutrients and products change significantly with culture time and the location of the growing site in the solid culture, the effects of a specific nutrient or product cannot be clearly distinguished.

Species of *Streptomyces* sporulating in submerged cultures would provide advantages over the solid cultures for the determination of the specific responses of the strains upon the environmental changes. Parameters presenting the state of differentiation of *Streptomyces* spp. are thought to be very useful for the quantitative evaluation of fermentation process. In this review, characteristics of spores formed in solid culture and those in submerged culture are compared and methods for the quantitative analysis of morphological and physiological differentiations are illustrated.

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Characteristics of Submerged Spores and Aerial Spores

A minority of *Streptomyces* can sporulate readily in submerged culture [14]. They are *Streptomyces griseus* [33], *Streptomyces viridochromogenes* [40], *Streptomyces roseosporus* [28], and *Streptomyces venezuelae* [21]. Changes in nucleotide pools during sporulation of *Streptomyces griseus* in submerged culture have been analyzed [51] and cloning of DNA involved in sporulation of *S. griseus* has been reported [2].

One soil isolant, *Streptomyces albidoflavus* SMF301, formed spores abundantly in submerged culture [55, 57]. The characteristics of spores formed in solid and submerged culture of *S. albidoflavus* SMF301 were compared [42] (Fig. 1). Cysteine content was particularly higher in submerged spores compared to aerial spores, while profiles of other major amino acids were very similar. The unsaturated fatty acid content in submerged spores of *S. albidoflavus* SMF301 was remarkably high; it is noteworthy that unsaturated fatty acids have not been detected previously in *Streptomyces* [44]. Furthermore, the *ai* (anti-iso)-C_{14}, *ai*-C_{15}, *ai*-C_{16}, and *ai*-C_{17} contents in the submerged spore were also much higher compared to those in aerial spores. The major fatty acid in aerial spores was *n*-C_{18} (61.74%), whereas in submerged spores it was *ai*-C_{16} (33.68%) [42].

The major and minor menaquinones in aerial spores of *S. albidoflavus* SMF301 are MK-9(H_4) and MK-9(H_6), respectively while those in submerged spores are MK-9(H_6) and MK-9(H_4), respectively [42]. It was reported that menaquinone profiles change during cell morphogenesis in *Streptomyces* spp. However, it was thought that

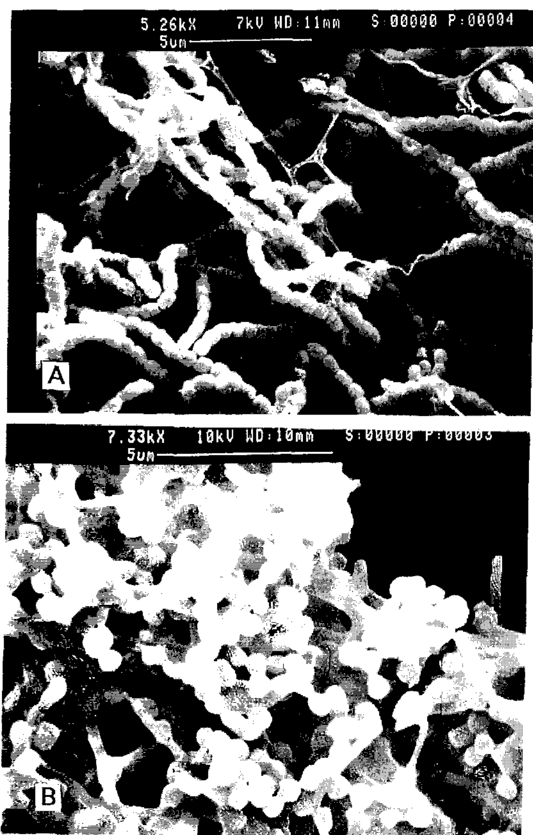

Fig. 1. Scanning electron photomicrographs of spores of *S. albidoflavus* SMF301.

A, aerial spores formed on solid culture using rich agar medium for 7 days at 28°C; B, submerged spores formed in submerged culture using chemically defined medium for 7 days at 28°C.

MK-9(H_6) and MK-9(H_8) are the typical pattern of menaquinones in the mycelium of *Streptomyces* spp. [41].

The cellular content of Ca and Mg were higher in submerged spores of *S. albidoflavus* SMF301. The content of trehalose in aerial spores was higher than that in the submerged spores of *S. albidoflavus* SMF301. The resistance of aerial spores to lysozyme digestion, mild acid treatment, heating and desiccation was higher than that of submerged spores, but the submerged spores were more resistant to sonication [42]. Trehalose is the major carbohydrate in *Streptomyces* spores [3, 17]. The resistance of actinomycete spores to heat and desiccation are linked closely to their content of trehalose, Ca, and Mg [16, 33, 46, 56].

Proteases and Protease Inhibitors Play Important Roles in the Morphological Differentiation of *Streptomyces* spp.

The secretion of extracellular proteolytic enzymes in streptomycetes often temporarily coincides with the onset of secondary metabolism or the formation of aerial mycelia and spores [3, 20]. The growth of aerial mycelium and the formation of spores is supported by the utilization of degraded substrate mycelium, as the aerial mycelium has limited access to other sources of nourishment for its growth and development [8, 9, 27, 48, 49, 60].

Physiological and morphological differentiation in streptomycetes are closely regulated by nitrogen nutrition [58]. Besides the roles of extracellular proteases in the assimilation of external proteinaceous nitrogen sources, serine protease expressed in stationary-phase cultures of *Streptomyces peucetius* and *Streptomyces lactamdurans* has been reported to regulate the turnover metabolism associated with the formation of secondary metabolites and morphogenesis [19, 20].

Recently, it was found that trypsin-like protease (TLP) was produced together with aerial mycelium formation in *Streptomyces aburaviensis*, *S. albidoflavus* SMF301, *Streptomyces coelicolor* A3(2), *Streptomyces exfoliatus*, *Streptomyces limosus*, *Streptomyces microflavus*, *Streptomyces roseus*, *Streptomyces felleus*, *S. griseus*, *Streptomyces lavendulae*, *Streptomyces rimosus*, and *Streptomyces rochei*, indicating that aerial mycelium formation is closely related to TLP activity or TLP production [35]. TLP (32 kDa) from *S. albidoflavus* SMF301 showed a similar pH optimum (7.5) and thermal instability to those of other TLPs from *Streptomyces*, although its molecular weight was higher than those of other TLPs reported from *Streptomyces* spp.: 19 kDa for *S. griseus* [59], 19 kDa for *Streptomyces moderatus* [67], 21.4 kDa for *Streptomyces erythreus* [29], 22~25 kDa for *Streptomyces paromomycinus* [11], 28 kDa for *S. rimosus* [53], and 31.5 kDa for *Streptomyces* 771 [52].

In addition to TLP (32 kDa), CTP and MTP (18 kDa), several extracellular proteases were detected in a submerged batch culture of *S. albidoflavus* SMF301 using Na-caseinate as the sole nitrogen source [30]. The optimum pH and temperature were 10 and 40°C for TLP and were 8 and 55°C for MTP. TLP was stable at alkaline pH (6~9) and unstable above 45°C while MTP was stable at alkaline pH and unstable above 80°C [31]. The production of the proteases was found to be very closely associated with the morphological differentiation; mycelium growth was linked with the production of CTP, while submerged spore formation was accompanied by the production of TLP and MTP. The defective production of TLP and MTP in a bald mutant suggests that TLP might play a role in the formation of thickened mycelium or branching, and that MTP might participate in the formation of submerged spores from the thickened mycelium [30] (Fig. 2).

Although streptomycetes produce a variety of proteases, protease inhibitors are also widely distributed in streptomycetes [1]. A typical low molecular weight serine/cysteine protease inhibitor, leupeptin, has been isolated from various strains of *Streptomyces* spp. [1, 12,

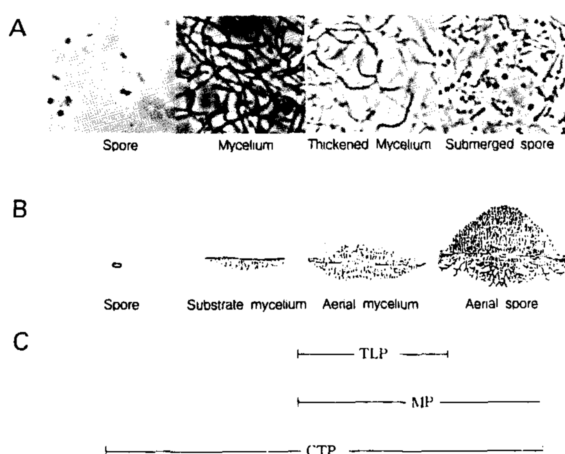

Fig. 2. Morphological differentiation of *S. albidoflavus* SMF13.
A, in submerged; B, solid cultures; C, production phase of CTP, TLP, and MTP.

34, 45]. Even larger molecules of proteinaceous protease inhibitors have also been identified from *Streptomyces* spp. and *Streptoverticillium* sp.

S. exfoliatus SMF13 was shown to produce leupeptin, CTP, leupeptin inactivating enzyme (LIE), and TLP extracellularly [36–38]. The activity of TLP was specifically inhibited by leupeptin. Production of leupeptin was closely associated with growth. However, leupeptin was inactivated by LIE when growth reached stationary phase in submerged cultures, or when the aerial mycelium started to form on surface cultures. After the stationary phase, autolysis of mycelium in submerged cultures was apparently retarded by the addition of leupeptin; on surface cultures, aerial mycelium formation was clearly retarded by the addition of leupeptin [36, 37].

As a good comparision, in three *bld* mutants isolated from UV-mutagenesis of *S. exfoliatus* SMF13, mycelium autolysis did not occur, and neither LIE nor TLP was produced although leupeptin was produced. Production of both LIE and TLP was restored in a spontaneous spo^+ revertant of a *bld* mutant. In contrast, two *whi* mutants sequentially produced leupeptin, LIE, and TLP. TLP effectively hydrolyzed the mycelial protein extract of *S. exfoliatus* SMF13, but the hydrolytic activity was inhibited by leupeptin. Therefore, the physiological roles of CTP, TLP, leupeptin and LIE are thought to be as follows: CTP participates primarily in the utilization of a proteinaceous nitrogen source; TLP functions as an essential enzyme involved in the metabolism of mycelial protein; leupeptin inhibits the activity of TLP, and LIE inactivates autogenous leupeptin in *S. exfoliatus* SMF13. The cascade of regulatory activities of the compounds, which are produced sequentially during mycelium development, may provide selective advantages in adverse culture conditions [36–39] (Fig. 3).

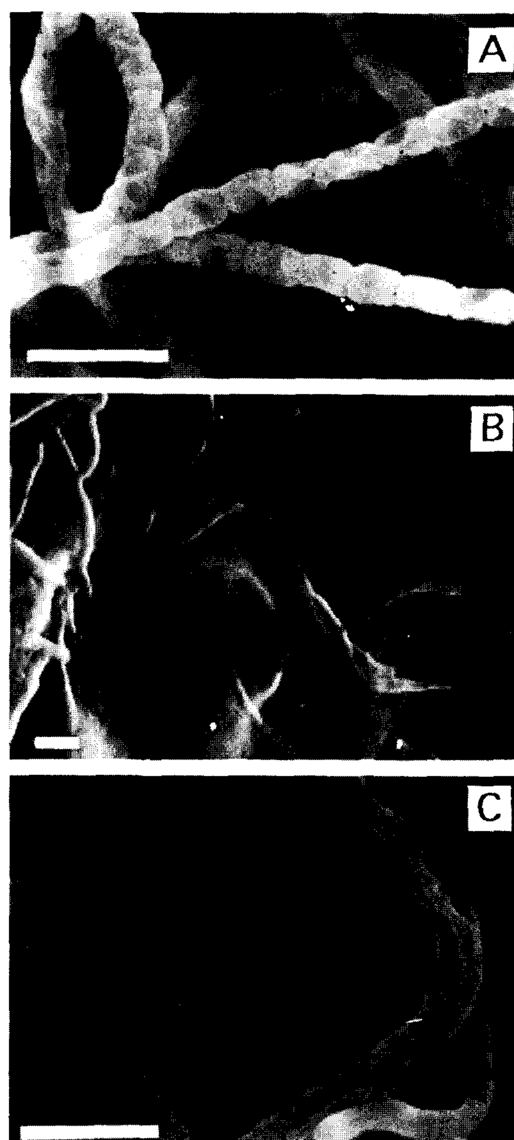

Fig. 3. Scanning electron micrographs of strains of *S. exfoliatus* on synthetic agar medium after incubation for 10 days.
A, wild-type strain SMF13; B, *bld* mutants SMF13B1; C, *whi* mutant SMF13W1.

Kinetics of Nutrients Utilization and Morphological Differentiation in *Streptomyces* spp.

Limitation of glucose was reported to have a variety of effects on the differentiation in *Streptomyces* spp. Sporulation of several species was repressed by a high glucose concentration. This has been attributed to acid accumulation and changes in culture pH, and not by high concentrations of glucose [2, 14]. However, sporulation of *S. venezuelae* in a submerged culture was repressed by high concentrations of glucose, that was not merely a secondary result of acid accumulation [21]. The results are consistent with the observation that cellular differentiation in surface

cultures was inhibited by high concentrations of glucose [10]. Sporulation of *S. griseus* in the submerged culture was initiated when the ammonium ion was starved [33]. An excess of nitrogen source was inhibitory to submerged spore formation in *S. venezuelae* [21].

Quantitative analysis revealed that sporulation of *S. albidoflavus* SMF301 in a submerged culture was significantly affected by the culture pH; spore formation was not repressed by a glucose concentration up to 80 g l^{-1} when the culture pH was maintained at 7.0. Kinetic parameters calculated from batch and chemostat cultures showed that the specific submerged spore formation rate (q_{spo}) was inversely related to the specific mycelium growth rate (μ). The optimum growth rate for submerged spore formation was 0.05 h^{-1} where the maximum value of q_{spo} (1.0×10^6 spores g^{-1} h^{-1}) was obtained. Spore formation was simultaneous with the endogenous consumption of mycelium, the turnover rate of biomass (a) was 0.029 h^{-1} and 5.6×10^6 spores were formed from 1 g of mycelium [54].

Sporulation of *S. albidoflavus* SMF301 in a submerged culture was significantly repressed by an excess of ammonium ions or inorganic phosphate, but enhanced by the starvation of ammonium ions or inorganic phosphate, indicating that starvation of the nitrogen source or phosphate effects a shift from mycelium growth to submerged spore formation [54]. High C/N (nitrogen limitation) and low C/N (carbon limitation) were beneficial to spore formation, TLP and MTP synthesis. Specific nutrient uptake rates (q_{glu}, q_{amn}, and q_{pho}) affected sporulation and protease production; q_{spo}, q_{TLP}, and q_{MTP} were high at low q_{glu}, q_{amn}, and q_{pho}, and the mycelium growth rate and q_{CTP} production were high at high q_{glu}, q_{amn}, and q_{pho}. This indicated that a limitation of any of the essential nutrients (glucose, nitrogen, or phosphate) triggers morphological differentiation in *S. albidoflavus* [32]. q_{CTP} production was optimum at 0.1 h^{-1}, but q_{TLP} and q_{MTP} were optimum at 0.025 h^{-1}.

Leupeptin accumulated during mycelial growth was maintained throughout the stationary phase and LIE was not produced in the glucose-excess condition. However, leupeptin was inactivated by LIE produced in a condition of glucose exhaustion and mycelium declination. The production of leupeptin was related to mycelial growth; optimum levels of leupeptin production were attained in cultures grown on glucose-excess, phosphate-limited, and casamino acids media. However, leupeptin-inactivating enzyme (LIE) was produced in the cultures grown on glucose-limited, phosphate-excess, and Na-caseinate medium where mycelium degradation was present. LIE was one of the most important factors affecting leupeptin productivity. Optimum production of leupeptin was attained by phosphate-limited continuous cultivation, which did not permit LIE production. The maximum productivity was 0.24 g l^{-1} h^{-1} [36, 37].

PyMS and ANN as New Quantitative Analytical Methods for the Prediction of Morphological and Physiological Differentiation in *Streptomyces* spp.

Streptomyces spp. produce various kinds of secondary metabolites, and the onset of antibiotics (physiological differentiation) in various actinomycetes is closely associated with mycelium morphological changes [5, 7, 15]. If quick and precise methods for the determination of differentiation were available, they would be very useful for the successful control of fermentation. The combination of PyMS and ANNs has proven to be a powerful method, and new areas of application are being developed [13, 18, 22-26, 50].

Cells of *S. albidoflavus* taken from batch culture at various growth stages have been analysed by PyMS. The PyMS spectra are complex, and vary with the growth phase. Since the pyrolysis mass spectra can reveal the cellular composition of cells or profiles of molecules in the cells, the variation in the PyMS spectra implies that the molecules involved in growth and sporulation may be varied. Normalized PyMS data were analyzed using multivariate statistics techniques. As a result, the mycelium growth state can be clearly distinguished from the spore state. ANNs were trained on PyMS data to predict the differentiation state using two different algorithms: backpropagation and a radial basis function classifier. Both the backpropagation and radial basis classifier succeeded in separating the differentiation state and identified the transient state [31]. These results suggest that the combination of PyMS and ANNs is a promising method to monitor the morphological differentiation process quantitatively in *Streptomyces* (Fig. 4).

This method was applied to the analysis of clavulanic production using *Streptomyces clavuligerus* in the chemostats. Clavulanic acid production showed to be inversely related to growth rate. The specific rate of clavulanic acid production (q_{cla}) was optimum at 0.025 h^{-1} while mycelium growth yields ($Y_{x/glu}$) were maximum at 0.1 h^{-1}. Since morphological differentiation varies upon the specific growth rate, the degree of morphological differentiation of *S. clavuligerus* in various chemostat cultures was analysed by PyMS, data of which was trained with the radial basis classifier. The relation between PyMS data and clavulanic acid production rate were compared, showing that morphological differentiation and clavulanic acid production in *S. clavuligerus* was successfully predicted by PyMs and ANN (Fig. 5).

The Morphological Factor Is a Good Parameter Predicting Morphological and Physiological Differentiation Processes

The onset of the antibiotic in actinomycetes was closely related to the mycelium morphological changes. However,

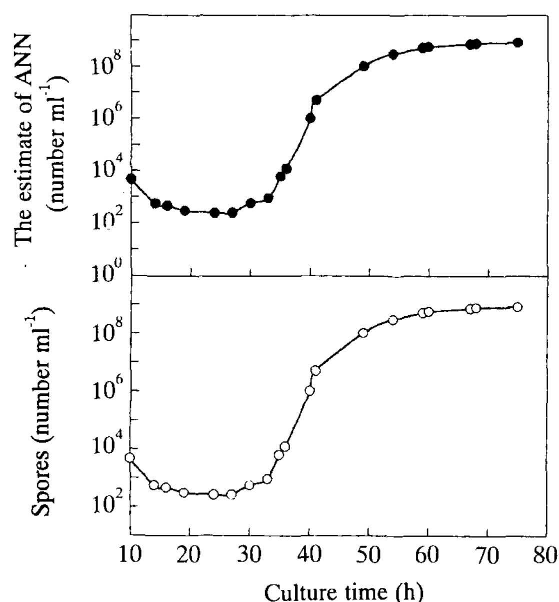

Fig. 4. The quantitative determination of morphological differentiation in *S. albidoflavus* SMF13 by ANN.
(○), real estimates of spore number; (●), estimates by ANN.

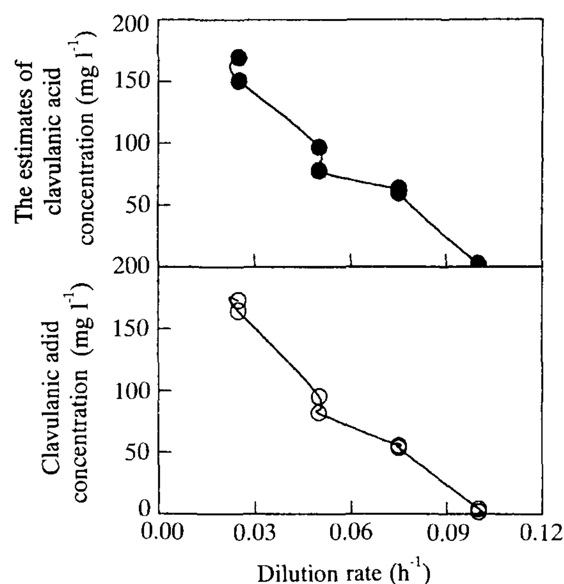

Fig. 5. Quantitative determination of physiological differentiation in *S. clavuligerus* by ANN.
(○), real estimates of clavulanic acid; (●), estimates by ANN.

the highly branched mycelia caused an increase in the viscosity of the culture broth and subsequently interfered with proper mixing and aeration.

The relationship between mycelium morphological changes and rifamycin B production using *Amycolatopsis mediterranei* were evaluated in conjunction with the rheological properties of the culture broth. In terms of rheological properties, the culture broth at the trophophase demonstrated Newtonian fluid behaviour but changed at the idiophase to a non-Newtonian fluid, following Casson plastic behaviour [43].

Culture conditions directly affected the characteristics of mycelium morphology, which in turn determined the rheological properties of the culture broth. The values of yield shear stress (τ_o) and morphological factor (δ^*) were varied according to the degree of mycelium differentiation, indicating that δ^* could be considered as a quantitative parameter representing the mycelium differentiation. The production of rifamycin B continued as long as the yield shear stress (τ_o) or the morphological factor (δ^*) of the culture broth increased to or maintained at their maximum values. The rheological parameters could be controlled by changing the medium component. For instance, by replacing $(NH_4)_2SO_4$ with KNO_3, the yield shear stress (τ_o) and the mycelium morphology factor (δ^*) in the culture could be maintained for extended periods to give rise to higher productivity of rifamycin. The specific rifamycin B production rate (q_{rif}) increased as the yield shear stress (τ_o) increased, and maintained a maximum value while the value of stress (τ_o) was maintained. The specific rifamycin B production rate (q_{rif}) was closely related to the yield shear stress (τ_o) and the morphological factor (δ^*) [43]. Therefore, it was suggested that the rheological parameters could be used as important parameters in monitoring the production of antibiotics using the Actinomycetes group [43].

Abbreviations: ANN, Artificial neural network; CTP, Chymotrypsin-like protease; LIE, Leupeptin inactivating enzyme; MK, Menaquinone; MTP, Metalloprotease; PyMS, Pyrolysis mass-spectrometry; TLP, Trypsin-like protease; μ, specific growth rate; q_{TLP}, specific rate of trypsin-like protease production; q_{MTP}, specific rate of metalloprotease production; q_{CTP}, specific rate of chymotrypsin-like protease production; q_{glu}, specific glucose uptake rate; q_{amn}, specific ammonium uptake rate; q_{pho}, specific phosphate uptake rate; q_{SPO}, specific spore production rate; $Y_{x/glu}$, mycelium growth yield

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REFERENCES

1. Aoyagi, T., S. Miyata, M. Nanbo, F. Kojima, M. Matsuzaki, M. Ishizuka, T. Takeuchi, and H. Umezawa.

1969. Biological activities of leupeptins. *J. Antibiot.* **22**: 558–568.

2. Babcock, M. J. and K. E. Kendrick. 1988. Cloning of DNA involved in sporulation of *Streptomyces griseus*. *J. Bacteriol.* **170**: 2802–2808.
3. Bascarán, V., C. Hardisson, and A. F. Braña. 1990. Regulation of extracellular protease production in *Streptomyces clavuligerus*. *Appl. Microbiol. Biotechnol.* **34**: 208–213.
4. Brana, A. F., C. Mendez, L. A. Diaz, M. B. Manzanal, and C. Hardisson. 1986. Glycogen and trehalose accumulation during colony development in *Streptomyces antibioticus*. *J. Gen. Microbiol.* **132**: 1319–1326.
5. Champness, W. C. 1988. New loci required for *Streptomyces coelicolor* morphological and physiological differentiation. *J. Bacteriol.* **170**: 1168–1174.
6. Chandrasekaran, S. and S. C. Dhar. 1987. Multiple proteases from *Streptomyces moderatus* II. Isolation and purification of five extracellular proteases. *Arch. Biochem. Biophys.* **257**: 402–408.
7. Chater, K. F. 1984. Morphological and physiological differentiation in *Streptomyces*, pp. 89–115. *In* R. Losick and L. Shapiro (ed.), *Microbial development.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
8. Chater, K. F. 1989. Multilevel regulation of *Streptomyces differentiation. Trends Genet.* **5**: 372–376.
9. Chater, K. F. 1993. Genetics of differentiation in *Streptomyces. Ann. Rev. Microbiol.* **47**: 685–713.
10. Chater, K. F., E. J. Lawlor, C. Mendez, C. J. Bruton, N. K. Davis, K. Plaskitt, E. P. Guthrie, B. L. Daly, H. A. Baylis, and K. V. Trong. 1988. Gene expression during *Streptomyces* development, pp. 64–70. *In* Y. Okami (ed.), *Biology of Actinomycetes '88. Proceedings of the 7th International Symposium for Biology of Actinomycetes.* Tokyo, Japan Scientific Societies Press.
11. Chauvet, J., J. Dostal, and R. Acher. 1976. Isolation of a trypsin-like enzyme from *Streptomyces paromomycinus* (paromotrypsin) by affinity adsorption through kunitz inhibitor-sepharose. *Int. J. Peptide. Protein. Res.* **8**: 45–55.
12. Chi, C. W., H. Z. Liu, C. Y. Liu, B. A. K. Chibber, and F. J. Castellino. 1989. The inhibition of the enzymatic activity of blood coagulation and fibrinolytic serine proteases by a new leupeptin-like inhibitor, and its structural analogues, isolated from *Streptomyces griseus*. *J. Antibiot.* **42**: 1506–1512.
13. Chun, J., E. Atalan, A. C. Ward, and M. Goodfellow. 1993. Artificial neural network analysis pyrolysis mass spectrometry data in the identification of Streptomyces stains. *FEMS Microbiol. lett.* **107**: 321–326.
14. Daza, A., J. F. Martin, A. Dominuez, and J. A. Gil. 1989. Sporulation of several species of *Streptomyces* in submerged culture after nutritional downshift. *J. Gen. Microbiol.* **135**: 2483–2491.
15. Demain, A. L. 1982 Catabolic regulation in industrial microbiology, pp. 3–20. *In* V. Krumphanzi, B. Sikya and Z. Vanek (ed.), *Overproduction of microbial products.* Academic Press, NY.
16. Dworkin, M. 1985. Streptomyces, pp. 85-104. *In* D. Martin (ed.), *Developmental biology of the bacteria.* The Benjamin/Cummings Publishing Co. Inc.
17. Ensign, J. C. 1978. Formation properties and germination of Actinomycete spores. *Ann. Rev. Microbiol.* **32**: 185–219.
18. Freeman, R., R. Goodacre, P. R. Sisson, J. G. Magee, A. C. Ward, and N. F. Lightfoot. 1994. Rapid identification of species within the *Mycobacterium tuberculosis* complex by artificial neural network analysis of pyrolysis mass spectra. *J. Med. Microbiol.* **40**: 170–173.
19. Gibb, G. D. and W. R. Strohl. 1988. Physiological regulation of protease activity in *Streptomyces peucetius. Can. J. Microbiol.* **34**: 187–190.
20. Ginther, C. L. 1978. Sporulation and the production of serine protease and cephamycin C by *Streptomyces lactamdurans. Antimicrob. Agents Chemother.* **15**: 522–526.
21. Glazebrook, M. A., J. L. Doull, C. Stuttard, and L. C. Vining. 1990. Sporulation of *Streptomyces venezuelae* in submerged cultures. *J. Gen. Microbiol.* **136**: 581–588.
22. Goodacre, R., D. B. Kell, and G. Bianchi. 1992. Neural networks and olive oil. *Nature* **359**: 594.
23. Goodacre, R. and D. B. Kell. 1993a. Rapid and quantitative analysis of bioprocesses using pyrolysis mass spectrometry and neural networks: application to indole production. *Anal. Chim. Act.* **279**: 17–26.
24. Goodacre, R. and D. B. Kell. 1993b. Rapid assessment of the adulteration of virgin olive oils by other seed oils using pyrolysis mass spectrometry and artificial neural networks. *J. Sci. Food Agric.* **63**: 297–307.
25. Goodacre, R., S. Trew, C. Wrigley-Jones, M. J. Neal, J. Maddok, T. W. Ottley, N. Porter, and D. B. Kell. 1994. Rapid screening for metabolite overproduction in fermentor broths, using pyrolysis mass spectrometer with multivariate calibration and artificial neural networks. *Biotechnol. Bioeng.* **44**: 1205–1216.
26. Goodacre, R., S. Trew, C. Wrigley-Jones, G. Saunders, M. J. Neal, N. Porter, and D. B. Kell. 1995. Rapid and quantitative analysis of metabolites in fermentor broths using pyrolysis mass spectrometry with supervised learning: application to the screening of *Penicillum chrysogenum* fermentations for the overproduction of penicillins. *Anal. Chim. Act.* **313**: 25–43.
27. Granozzi, C., R. Billeta, R. Passantino, M. Sollazzo, and A. M. Puglia. 1990. A breakdown in macromolecular synthesis preceding differentiation in *Streptomyces coelicolor* A3(2). *J. Gen. Microbiol.* **136**: 713–718.
28. Huber, F. M., R. L. Piper, and F. P. Mertz. 1987. Sporulation of *Streptomyces roseosporus* in submerged culture. *J. Ind. Microbiol.* **2**: 235–241.
29. Inoue, H., A. Sasaki, and N. Yoshida. 1972. An anionic trypsin-like enzyme from *Streptomyces erythreus*: chracterizaton. *Biochim. Biophys. Acta* **284**: 451–460.
30. Kang, S. G., I. S. Kim, Y. T. Rho, and K. J. Lee. 1995a. Production dynamics of extracellular proteases accompanying morphological differentiation of *Streptomyces albidoflavus* SMF301. *Microbiology* **141**: 3095–3103.

31. Kang, S. G., I. S. Kim, J. G. Ryu, Y. T. Rho, and K. J. Lee. 1995b. Purification and characterization of trypsin like protease and metalloprotease in *Streptomyces albidoflavus* SMF301. *J. Microbiol.* **33**: 307–314.
32. Kang, S. G. and K. J. Lee. 1997. Kinetic analysis of morphological differentiation and protease production in *Streptomyces albidoflavus* SMF301. *Microbiology* **143**: 2709–2714.
33. Kendrick, K. E. and J. C. Ensign. 1983. Sporulation of *Streptomyces griseus* in submerged culture. *J. Bacteriol.* **155**: 357–366.
34. Kim, I. S., Y. T. Han, K. D. Barrow, and K. J. Lee. 1993. The structure of protease inhibitors produced by *Streptomyces exfoliatus* SMF13. *Kor. J. Microbiol.* **31**: 326–334.
35. Kim, I. S., S. G. Kang, and K. J. Lee. 1995. Physiological importance of trypsin like protease during morphological differentiation of Streptomycetes. *J. Microbiol.* **33**: 315–321.
36. Kim, I. S. and K. J. Lee. 1995a. Physiological roles of leupeptin and extracellular proteases in mycelium development of *Streptomyces exfoliatus* SMF13. *Microbiology* **141**: 1017–1025.
37. Kim, I. S. and K. J. Lee. 1995b. Regulation of production of leupeptin, leupeptin inactivating enzyme and trypsin like protease in *Streptomyces exfoliatus* SMF13. *J. Ferment. Bioeng.* **80**: 434–439.
38. Kim, I. S. and K. J. Lee. 1995c. Kinetic study on the production and degradation of leupeptin in *Streptomyces exfoliatus* SMF13. *J. Biotech.* **80**: 434–439.
39. Kim, I. S. and K. J. Lee. 1996. Trypsin like protease of *Streptomyces exfolitus* SMF13, a potential agent in mycelial differentiation. *Microbiology* **142**: 1797–1806.
40. Koepsel, R. and J. C. Ensign. 1984. Microcycle sporulation of *Streptomyces viridochromogenes. Arch. Microbiol.* **140**: 9–14.
41. Lechevalier, H. A. and M. P. Lechevalier. 1970. The chemotaxonomy of actinomycetes, pp. 227–291. *In* Dietz and Thayer (ed.) *Actinomycete taxonomy*, special publication 6. Society for Industrial Microbiology, Arlington, VA, U.S.A.
42. Lee, K. J. and Y. T. Rho. 1993. Characteristics of spores formed by solid and submerged cultures of *Streptomyces albidoflavus* SMF301. *J. Gen. Microbiol.* **139**: 3131–3137.
43. Lee, K. J. and Y. T. Rho. 1994. Quantitative analysis of mycelium morphological characteristics and rifamycin B production using *Nocardia mediterranei. J. Biotech.* **36**: 239–245.
44. Locci, R. 1989. *Streptomyces* and related genera, pp. 2451–2508. *In* S. T. Williams, M. E. Sharpe & J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, Vol. 4. Williams & Wilkins, Baltimore, U.S.A.
45. Maeda, K., K. Kawamura, S. I. Kondo, T. Aoyagi, T. Takeuchi, and H. Umezawa. 1971. The structure and activity of leupeptins and related analogs. *J. Antibiot.* **24**: 402–404.
46. McBride, M. J. and J. C. Ensign. 1987a. Effect of intracellular trehalose content on *Streptomyces griseus* spores. *J. Bacteriol.* **169**: 4995–5001.
47. McBride, M. J. and J. C. Ensign. 1987b. Metabolism of endogenous trehalose by *Streptomyces griseus* spores and by spores or cells of other Actinomycetes. *J. Bacteriol.* **169**: 5002–5007.
48. Mendez, C., A. F. Brana, M. B. Manzanal, and C. Hardisson. 1985. Role of substrate mycelium in colony development in *Streptomyces. Can. J. Microbiol.* **31**: 446–450.
49. Miguelez, E. M., M. Garcia, C. Hardisson, and M. B. Manzanal. 1994. Autoradiographic study of hyphal growth during aerial mycelium formation in *Streptomyces antibioticus. J. Bacteriol.* **176**: 2105–2107.
50. Montague, G. and J. Morris. 1994. Neural networks contributions in biotechnology. *Trend. Biotech.* **12**: 312–323.
51. Ochi, K. 1987. Changes in nucleotide pools sporulation of *Streptomyces griseus* in submerged culture. *J. Gen. Microbiol.* **133**: 2787–2795.
52. Palubinskas, V. I., N. B. Yankevich, K. K. Yanulaitene, V. S. Vesa, V. G. Bendikene, A. V. Maksimenko, M. P. Torchilin, E. V. Ilyina, V. N. Smirnov, L. N. Krestyanoe, Y. E. Bartoshevich, and R. C. Zabirova. 1984. Trypsin-like enzyme from *Streptomyces* 771. Purification and properties of native and immobilized enzyme. *Appl. Biochem. Biotechnol.* **9**: 231–241.
53. Renko, M., L. J. Vitale, M. Kokalj, and M. Pokorny. 1989. *Streptonyces rimosus* extracellular proteases 4. Trypsin-like proteinase. *Appl. Microbiol. Biotechnol.* **31**: 38–44.
54. Rho, Y. T. and K. J. Lee. 1994. Kinetic studies on mycelium growth and spore formation in submerged cultures of *Streptomyces albidoflavus* SMF301. *J. Gen. Microbiol.* **140**: 2061–2065.
55. Rho, Y. T., H. T. Kim, K. H. Oh, H. I. Kang, A. C. Ward, M. Goodfellow, Y. C. Hah, and K. J. Lee. 1992. Numerical identification of a *Streptomyces* strain producing spores in submerged culture. *Kor. J. Microbiol.* **30**: 278–285.
56. Salas, J. A., J. A. Guijarro, and C. Hardisson. 1983. High calcium content in *Streptomyces* spores and its release as an early event during spore germination. *J. Bacteriol.* **155**: 1316–1323.
57. Shin, H. S. and K. J. Lee. 1986. Regulation of extracellular alkaline protease biosynthesis in a strain of *Streptomyces* spp. *Kor. J. Microbiol.* **24**: 32–37.
58. Shapiro, S. 1989. Nitrogen assimilation in actinomycetes and the influence of nitrogen nutrition on actinomycete secondary metabolism, pp. 149–153. *In* S. Shapiro (ed.), *Regulation of secondary metabolism in Actinomycetes*, CRC Press. Boca Raton, FL, U.S.A.
59. Trop, M. and Y. Birk. 1968. The trypsin-like enzyme from *Streptomyces griseus* (Pronase). *Biochem. J.* **109**: 475–476.
60. Wildermath, H. 1970. Development and organization of the aerial mycelium in *Streptomyces coelicolor. J. Gen. Microbiol.* **60**: 43–50.