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Physiological Characteristics of Immobilized *Streptomyces* Cells in Continuous Cultures at Different Dilution Rates

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Abstract Physiological characteristics such as specific productivity, morphology of *Streptomyces* cells immobilized on celite beads, and operational stability at different dilution rates were investigated in continuous immobilized-cell cultures for the production of kasugamycin. At a dilution rate (D) of 0.05 h^{-1}, a relatively high specific productivity was attained and the loss of cell-loaded beads was negligible. At D=0.1 h^{-1}, a higher specific productivity and cell concentration could be obtained, resulting in a significantly improved volumetric kasugamycin productivity. However, no stable operation could be maintained due to a significant loss of cell-loaded beads from the reactor that was caused by their fluffy morphology developed in the later stage. At D=0.2 h^{-1}, the production of kasugamycin and cell growth were observed to be severely inhibited by the high concentration of residual maltose.

Key words: Immobilized *Streptomyces* cells, continuous culture, kasugamycin, dilution rates

Streptomyces strains have been playing an important role in the pharmaceutical industry, because a large number of pharmaceuticals are produced through a secondary metabolism of *Streptomyces* [6]. Therefore, it is essential to develop a highly productive fermentation process for the production of secondary metabolites by using *Streptomyces*. It is generally known that the secondary metabolism of *Streptomyces* cells is triggered by the depletion of certain key nutrients such as glucose or maltose and/or by reducing the specific growth rate of the cells [7]. In such a situation, investigating the effects of nutrition is very important to establish an optimal feeding strategy and thus to obtain a high productivity in a fed-batch or continuous culture.

The use of continuous culture is essential in investigating the physiological characteristics of cells under different conditions, since the concentrations of key nutrients which greatly affect the production of the target secondary metabolite can be easily controlled and maintained at a desired level in a continuous culture [19]. In addition to its contribution as an experimental tool, a continuous culture can provide higher productivity than batch or fed-batch cultures, especially in the production of secondary metabolites which are susceptible to catabolic repression or inhibition [5]. However, a simple suspended-cell continuous fermentation has a major drawback. At a dilution rate higher than the maximum specific growth rate, washing out of the cells will occur. When the dilution rate is lower than the maximum specific growth rate but still high enough to maintain a significant level of the carbon source concentration, for example having a significant specific growth rate, the specific productivity will be extremely low, therefore, the production of the target secondary metabolite will be seriously hampered [4]. Even when the specific productivity is high enough in an operation at a low dilution rate below a critical level, the productivity of the target product can still be quite low due to a very low cell concentration which is generally observed in suspended-cell continuous cultures. An effective way of avoiding such a difficulty inherent to simple suspended-cell cultures is the application of a continuous immobilized-cell culture. In this type of culture, the growth phase and the production phase can be easily decoupled and thus optimized separately. Such an operational flexibility allows a high cell concentration and a high specific productivity to be achieved simultaneously, which results in a very high productivity. More detailed

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accounts of immobilized-cell cultures are well described elsewhere [2, 4, 20].

In a high-cell-density cultivation of a mold such as a *Streptomyces* strain, the morphology of the cells is a very important factor affecting the productivity [9]. Usually, pellet form is preferred to filamentous morphology, which causes an excessively high viscosity of broth and consequently causes a serious limitation of oxygen transfer. In an immobilized-cell culture using a bead type support material, as used in the present study, most of the cells exist in pellets consisting of the cells and the support.

Recently, an immobilized-cell reactor system was developed by us, and the feasibility of a long-term continuous operation of the reactor system was explored for the production of kasugamycin by employing *Streptomyces kasugaensis*. It was demonstrated a stable long-term continuous operation with a high productivity was possible. The effects of increased feed medium concentration on the physiology of the producer microorganism were also investigated [11].

In the present work, we investigated the effects of the dilution rate on physiological characteristics such as the specific productivity, morphology of *Streptomyces* cells immobilized on celite beads, and operational stability in continuous immobilized-cell cultures.

MATERIALS AND METHODS

Microorganism and Immobilization Method

Streptomyces kasugaensis (KCCM 11390) was used. The support used for cell immobilization was Celite grade 560 (Celite Co. Lontoc, CA, U.S.A.), made up of beads ranging from 100 to 500 mm in diameter. The pretreatment of celite beads and the whole immobilization procedure were exactly the same as described earlier [11, 16].

Medium and Culture Conditions

The composition of solid sporulation medium and the procedure of cell immobilization are described in detail elsewhere [11].

A synthetic medium was used for the production of kasugamycin, with a composition of 30 g/l maltose, 10 g/l glycine, 1.4 g/l K_2HPO_4, 1 g/l NaCl, 0.1 g/l $CaCl_2$, 2 g/l PEG, and 20 ml/l trace mineral salt solution [11].

Continuous Immobilized-Cell Culture

The schematic diagram of the system employed in this study is presented elsewhere [11]. A 3.4 liter top-driven stirred tank fermentor (Kobiotech Co., Ltd., Inchon, Korea) was used and the working volume was 2.0 liter. An immobilized-cell separator was used to allow an efficient separation of cell-loaded beads by gravity-mediated settling and/or fluid flows inside [15]. Temperature was maintained at 29°C and pH was controlled with 2 N H_2SO_4 or 2 N NaOH solutions. Dissolved oxygen (DO) concentration was maintained over 20% of air saturation by manipulating the agitation speed at an aeration rate of 1–2 vvm. An oxygen-enriched air (oxygen, 50%) was sparged when DO decreased to below the set point even at the maximum agitation speed of 430 rpm. The maximum agitation speed was preset to protect the cell-immobilized beads from excessive attrition.

Analytical Methods

Maltose concentration was measured by the dinitrosalicylic acid (DNS) method. Kasugamycin concentration in the supernatant was measured by an isocratic HPLC method [11, 12]. Cell and bead concentrations, and cell loading were measured in the same manner as previously described [11].

RESULTS AND DISCUSSION

A series of continuous cultures were carried out at three different dilution rates (D) of 0.05 h^{-1}, 0.1 h^{-1}, and 0.2 h^{-1}.

As the start-up procedure, batch culture was started at pH 7.0 after spore immobilization, and the pH was then shifted to pH 4.0 at 139 h of cultivation. Forty-eight

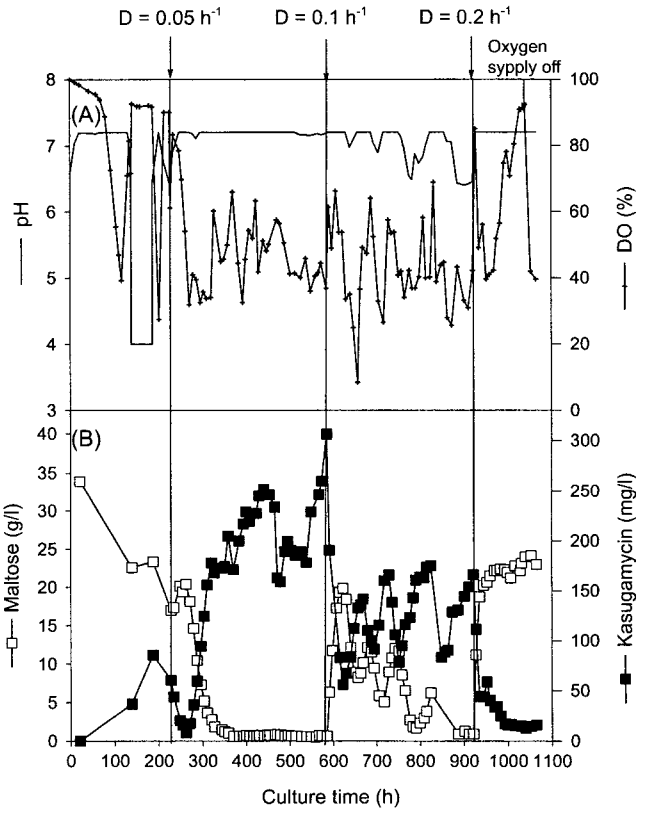

Fig. 1. Time profiles of continuous immobilized-cell culture.
(A), pH and DO concentration; (B), maltose and kasugamycin concentrations.

hours later, the pH was shifted back to pH 7.0 and then maintained until the end of the culture [Fig. 1(A)]. The purpose of this pH switching was to induce kasugamycin production as previously described [12].

Continuous feeding was started after 228 h of batch fermentation. As shown in Fig. 1(B), the kasugamycin concentration decreased while the residual maltose concentration increased after the continuous feeding had been started at a dilution rate of 0.05 h^{-1}. The kasugamycin concentration started to increase at 271 h with a decrease in the maltose concentration. The maltose concentration in the perfusate became nil at 385 h. The kasugamycin concentration reached 252 mg/l and then fell down to 159 mg/l at 478 h. After that, it rose again, when the dilution rate was changed to 0.1 h^{-1}, showing a oscillatory pattern to reach 306 mg/l at 585 h. This kind of oscillation was also observed in the continuous production of penicillin G when an immobilized *Penicillium chrysogeum* was used [8]. Pirt *et al.* [19] reported that the steady state is a self-regulating one where temporary disturbances of steady-state conditions in a continuous suspension culture often occurs, especially when the specific growth rate of the biomass is less than the maximum rate, however, they failed to give a systematic explanation on this phenomenon.

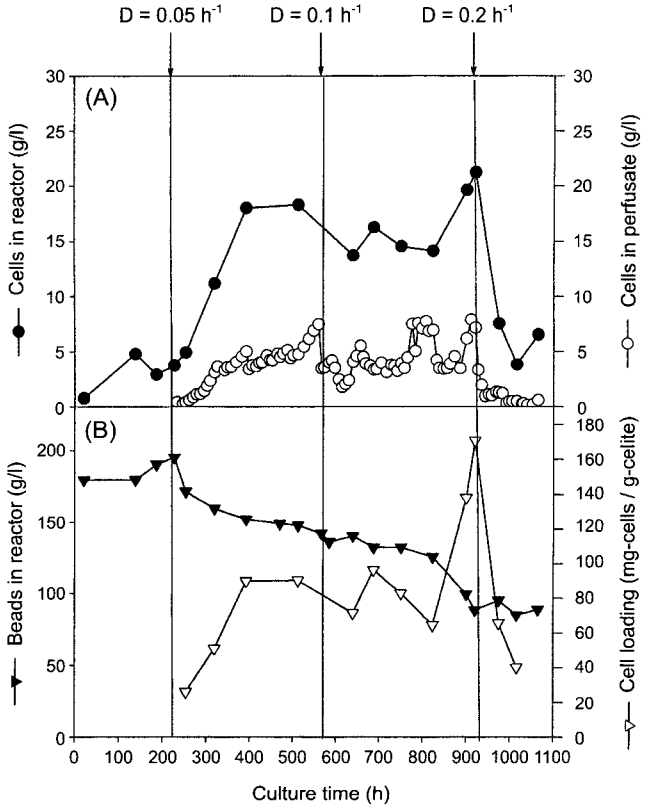

Fig. 2. Time profiles of continuous immobilized-cell culture. (A), cell concentrations in reactor and perfusate; (B), cell loading and bead concentration in reactor.

Fig. 2(A) shows the profiles of cell concentrations in the reactor and in the perfusate. The total concentration of immobilized and suspended cells in the reactor was observed to be 18 g/l at 393 h, remaining almost constant afterwards. The cell concentration in the perfusate gradually increased to 7.0 g/l and then dropped to 4 g/l, when the dilution rate was changed from 0.05 h^{-1} to 0.1 h^{-1}. The bead concentration in the reactor was observed to be 136 g/l at 585 h, which corresponded to a bead loss of 36 g/l in the course of continuous run at D=0.05 h^{-1} [Fig. 2(B)]. During this period, separation of cell-immobilized beads in the effluent and their recycling to the reactor was done smoothly without any difficulty, due to the good performance shown by the immobilized-cell separator. Therefore, the bead loss of 36 g/l from the reactor was considered to be mostly caused by sampling. A maximum cell loading of 90 mg-cells/g-celite was observed at 393 h [Fig. 2(B)].

Also, when the dilution rate was increased to 0.1 h^{-1} at 585 h, it was observed that kasugamycin was produced in a cyclic pattern in the range of 78–175 mg/l. The maltose concentration gradually decreased, also showing a cyclic pattern to reach 0.9 g/l at 886 h. It is also worthy to note that the opposite changes in the kasugamycin and maltose concentrations were observed, and this phenomenon could be explained by catabolic repression or inhibition that is a general concept of secondary metabolism [17]. The cell concentration in the reactor remained at about 14 g/l from 640 h and then significantly increased after 824 h with an active consumption of maltose [Fig. 2(A)]. A cell concentration of 19.6 g/l was obtained at 901 h. In contrast to the increase in the reactor, the cell concentration in the perfusate was maintained at 3.5–7.5 g/l, which was practically no greater than before. However, a concomitant increase in the cell loading was observed. The cell loading increased to reach approximately 170 mg-cells/g-celite at 901 h just before the dilution rate was switched to 0.2 h^{-1}. Doubling the dilution rate from 0.05 h^{-1} to 0.1 h^{-1} was considered to have relieved nutrient limitation and thus have resulted in the increase in the cell loading and cell concentration in the reactor [2]. We expected that the increment in cell concentration in the reactor would be proportional to that in the cell loading. However, contrary to our expectation, only a 1.2-fold increase in the cell concentration in the reactor was observed despite a 1.9-fold increase in the cell loading with the doubling of the dilution rate. As shown in Fig. 2(B), the major reason for this seemed to be the significant loss of cell-immobilized beads from the reactor. The bead concentration in the reactor remained almost constant for about 240 h after switching the dilution rate to 0.1 h^{-1} at 585 h. However, poor settling of cell-immobilized bead particles was observed at 824 h in the immobilized-cell separator and thus a large amount of bead particles began to be lost through the effluent, thus resulting in a bead concentration of 88.4 g/l

in the reactor at 921 h. The bead concentration at the end of operation at D=0.1 h^{-1} corresponded to only about 65% of that when the dilution rate was switched to D=0.1 h^{-1}.

It was considered that bead settling is affected by several factors such as broth viscosity, the amount of cell loading on beads, and the morphology of cell-immobilized bead particles. A poor solid-liquid separation was encountered due to high broth viscosity that was caused by free mycelia cells [2]. In this study, however, this could not offer a plausible explanation. Compared with the operation at D= 0.05 h^{-1}, a larger amount of bead particles was lost from the reactor in the operation at D=0.1 h^{-1}, although the free cell concentration in both cases was in the same range (data not shown). Furthermore, it was noted that a serious bead loss was accompanied by the increase in cell loading during the later period of operation. As seen in Fig. 3 showing the immobilized cells sampled at 921 h, the surface mycelia had a rather fluffy morphology due to excessive growth. Thus, it is evident that excessive growth of immobilized cells due to sufficient supply of nutrients can result in an increase in cell loading and a fluffy bead morphology. It can also be seen in Fig. 3 that cell loading was not uniform among the beads, in agreement with a previous observation by Arcuri *et al.* [1]. In such a situation, beads loaded with more cells can be easily removed from the reactor, because of the higher drag forces and lower bead density. This argument may support the hypothesis that the serious bead loss through the separator at the later stage of the operation at D=0.1 h^{-1} was mainly caused by the fluffy morphology of the cell-immobilized beads, which was associated with the increase in cell loading. It is, therefore, suggested that control of the morphology of immobilized cells is crucial for assuring a stable continuous culture operation to occur. In studies on immobilize-cell cultures in a fluidized-bed bioreactor, Kim *et al.* [13] and Oh *et al.* [18] observed that bioparticles developed in a rich medium resulted in a fluffy loose morphology and eventually led to a poor operation of the bioreactor. They suggested that cell-loaded beads developed under a phosphate-limiting condition had an optimum size as well as a smooth and compact morphology, which allowed stable operation to take place. In this aspect, further study is needed for investigating the relationship existing between the morphology of cell-immobilized beads and the performance of the separator at various concentrations of the key nutrients such as carbon, nitrogen, and phosphate sources.

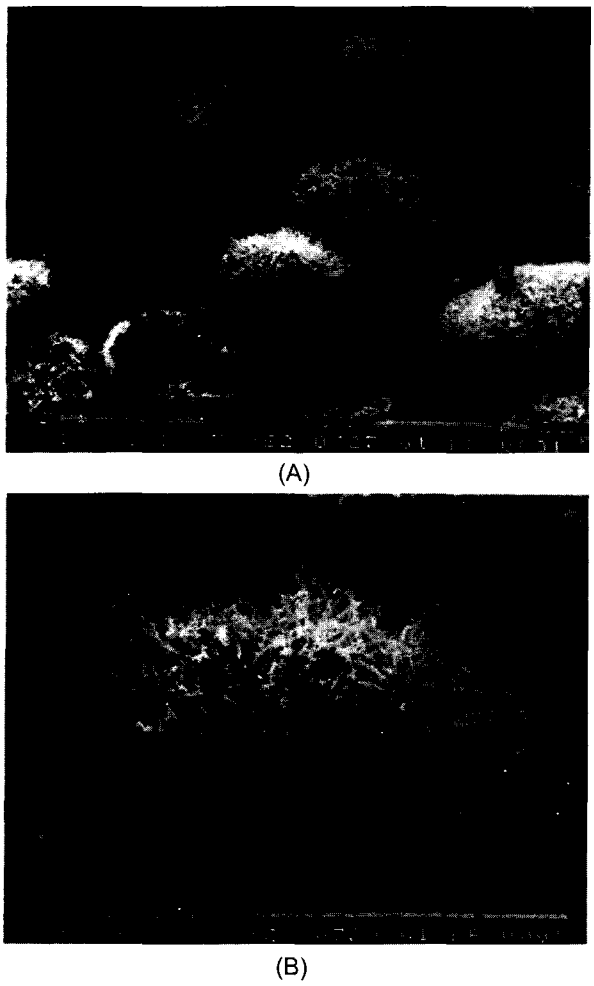

Fig. 3. Scanning electron micrographs of cell-immobilized beads sampled at 921 h.
(A), cell-loaded beads (×300, bar=0.1 mm). (B), cells immobilized on the bead surface (×965, bar=0.1 mm).

Despite the operational difficulties due to the bead loss at D=0.1 h^{-1}, the experiment was continued to observe changes occurring at a higher dilution rate. The dilution rate was increased to 0.2 h^{-1} at 921 h. Upon increasing the dilution rate, kasugamycin concentration significantly decreased to about 15 mg/l. The maltose concentration increased up to 22 g/l [Fig. 1(B)]. The cell concentration in the perfusate was nil. The cell concentration in the reactor significantly decreased to 6.5 g/l at 1,065 h. The bead concentration in the reactor was maintained at 88 g/l with practically no bead loss during the operation. As shown in Fig. 4, for a cell-loaded bead sampled at 1,065 h, a bundle of hyphal filaments was seen within the pore matrix without projecting outwards from the bead surface. This morphology contributed to an effective retention of cell-loaded beads in the reactor without any loss. However, the cell loading was observed to decrease, which made the cell concentration in the reactor also decrease. The decreased cell loading at D=0.2 h^{-1} was evident by the data in Figs. 3 and 4. These results suggest that at D=0.2 h^{-1}, the maltose concentration was maintained at a level high enough to inhibit its consumption by the cells and their growth as well as kasugamycin production. In addition, detachment of cell from the beads was evident.

Figure 5 shows the time profile of volumetric kasugamycin productivity. In the culture at D=0.05 h^{-1}, it was in the

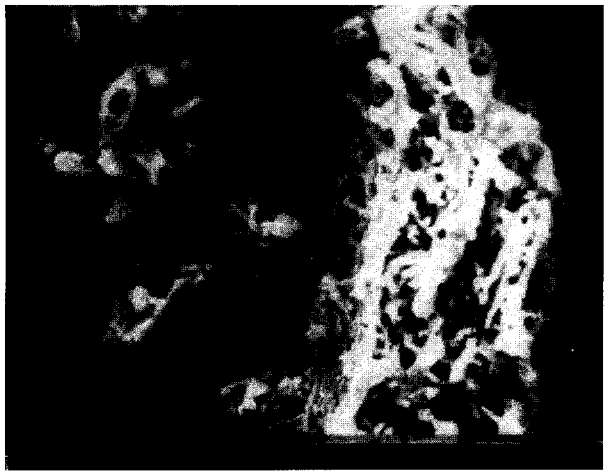

Fig. 4. Scanning electron micrographs of cell-immobilized beads sampled at 1,065 h (×2,500, bar=10 mm).

range of 7.8–12.3 mg/l/h. After switching the dilution rate to D=0.1 h^{-1}, it increased slightly to 8.3–17.3 mg/l/h, but rapidly decreased to 3.2 mg/l/h with the switching to D= 0.2 h^{-1}. Table 1 summarizes the productivity data at these three dilution rates. The average volumetric kasugamycin productivity ($Q_{p,avg.}$) was calculated by using a statistical analysis of the data shown in Fig. 5. The average cell concentration in the reactor ($X_{avg.}$) increased 1.2-fold and the average specific kasugamycin productivity ($q_{p,avg.}$) increased 1.1-fold upon doubling the dilution rate from 0.05 h^{-1} to 0.1 h^{-1}. As a result, the average volumetric kasugamycin productivity increased 1.3-fold. These data clearly demonstrate that the increase in volumetric productivity was mainly due to the increase in cell concentration in the reactor. Arcuri *et al.* [2] reported a similar result in a study of continuous production of thienamycin by using immobilized

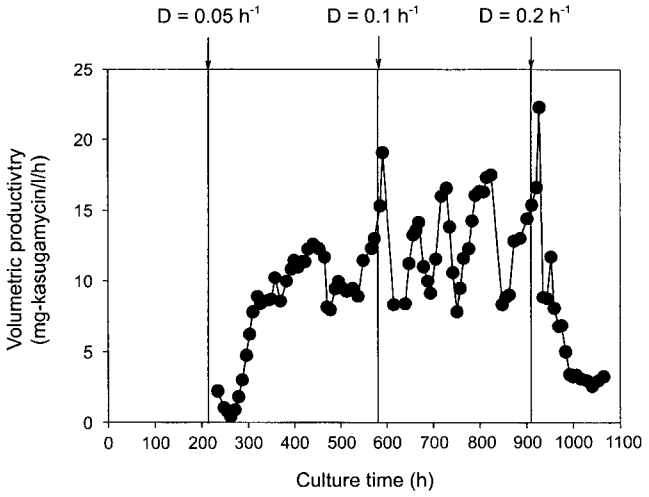

Fig. 5. Volumetric kasugamycin productivity in continuous immobilized-cell cultures.

Table 1. Productivity data from continuous immobilized-cell cultures.

Dilution rate (h^{-1})	$Q_{p,avg}$ (mg/l/h)	X_{avg} (g/l)	$q_{p,avg}$ (mg/g-cell/h)
0.05	10.2 (±1.53)	18.3	0.56
0.10	13.0 (±2.95)	21.2	0.61
0.20	3.1 (±0.26)	6.5	0.48

Streptomyces cells. Therefore, it is suggested that even a higher volumetric kasugamycin productivity would have been achieved with no loss of immobilized cells in the operation at D=0.1 h^{-1}. Increasing the dilution rate to 0.2 h^{-1} resulted in a much lower specific productivity of 0.48 mg/g-cell/h. The decrease in the specific productivity can be explained by catabolic repression or inhibition due to high maltose concentration (22 g/l), as generally known [17]. Umezawa and Yagi [21] reported that the yield of kasugamycin in suspended-cell batch cultures was found to decrease when the initial concentration of maltose was 60–80 g/l. It has been demonstrated that the production of a variety of secondary metabolites (e.g., rebeccamycin, avermectin) was repressed in the presence of a sugar such as glucose at high levels [10, 14]. Good yield of antibiotic was obtained in a complex medium when only this carbon source was supplied as a limiting nutrient by continuous feeding [3]. A further study is needed in order to find the effects of the dilution rate with feeding media containing lower concentrations of maltose than those used in the present study.

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References

1. Arcuri, E. J., J. R. Nichols, T. S. Brix, V. G. Santamarina, B. C. Buckland, and S. W. Drew. 1983. Thienamycin production by immobilized cells of *Streptomyces cattleya* in a bubble column. *Biotechnol. Bioeng.* **25:** 2399–2411.
2. Arcuri, E. J., G. Slaff, and R. Greasham. 1986. Continuous production of thienamycin in immobilized cell systems. *Biotechnol. Bioeng.* **28:** 842–849.
3. Boeck, L. D., D. M. Berry, F. P. Mertz, and R. W. A. Wetzel. 1992. A complex of novel growth-promoting thiopeptide antibiotics produced by a strain of *Streptomyces gardneri*. Taxonomy and fermentation studies. *J. Antibiotics* **45:** 1222–1230.
4. Constantinides, A. and N. Mehta. 1991. Periodic operation of immobilized live cell bioreactor for the production of candicidin. *Biotechnol. Bioeng.* **37:** 1010–1020.

5. Dalili, M. and P. C. Chau. 1988. Production of actinomycin D with immobilized *Streptomyces parvullus* under nitrogen and carbon starvation conditions. *Biotechnol. Lett.* **10:** 331–336.
6. Demain, A. L. 1999. Pharmaceutically active secondary metabolites of microorganisms. *Appl. Microbiol. Biotechnol.* **52:** 455–463.
7. Doull, J. L. and L. C. Vining. 1990. Nutritional control of actinorhodin production by *Streptomyces coelicolor A3*(2): Suppressive effects of nitrogen and phosphate. *Appl. Microbiol. Biotechnol.* **32:** 449–454.
8. Flanagan, W. P., H. E. Klei, W. D. Sundstrom, and C. W. Lawton. 1990. Optimization of a pellicular biocatalyst for penicillin production by *Penicillium chrysogenum. Biotechnol. Bioeng.* **36:** 608–616.
9. Gbewonyo, K. and D. I. C. Wang. 1983. Confining mycelial growth to porous microbeads: A novel technique to alter the morphology of non-Newtonian mycelial cultures. *Biotechnol. Bioeng.* **25:** 967–983.
10. Ikeda, H., H. Kotaki, H. Tanaka, and S. Omura. 1988. Involvement of glucose catabolism in avermectin production by *Streptomyces avermitilis. Antimicrob. Agents Chemother.* **32:** 282–284.
11. Kim, C. J., Y. K. Chang, G.-T. Chun, Y. H. Jeong, and S. J. Lee. 2001. Continuous culture of immobilized *Streptomyces* cells for kasugamycin production. *Biotechnol. Prog.* **17:** 453–461.
12. Kim, C. J., G.-T. Chun, and Y. K. Chang. 2000. Enhancement of kasugamycin production by pH shock in batch cultures of *Streptomyces kasugaensis. Biotechnol. Prog.* **16:** 548–552.
13. Kim, J. H., D. K. Oh, S. K. Park, Y. H. Park, and D. A. Wallis. 1986. Production of penicillin in a fluidized-bed bioreactor using a carrier-supported mycelial growth. *Biotechnol. Bioeng.* **28:** 1838–1844.
14. Lam, K. S., J. Mattei, and S. Forenza. 1989. Carbon catabolite regulation of rebeccamycin production in *Saccharothrix aerocolonigenes. J. Ind. Microbiol.* **4:** 105–108.
15. Lee, T. H., G.-T. Chun, and Y. K. Chang. 1999. Development of a cell-loaded biosupport separator for continuous immobilized-cell perfusion culture. *Biotechnol. Prog.* **15:** 267–272.
16. Lee, T. H., G.-T. Chun, and Y. K. Chang. 1997. Development of sporulation/immobilization method and its application for the continuous production of cyclosporin A by *Tolypocladium inflatum. Biotechnol. Prog.* **13:** 546–550.
17. Martin, J. F. and A. L. Demain. 1980. Control of antibiotic biosynthesis. *Microbiol. Rev.* **44:** 230–251.
18. Oh, D. K., C. K. Hyun, J. H. Kim, and Y. H. Park. 1988. Production of penicillin in a fluidized-bed bioreactor: Control of cell growth and penicillin production by phosphate limitation. *Biotechnol. Bioeng.* **32:** 569–573.
19. Pirt, S. J., eds. 1975. Chemostat culture, pp. 29–41. In *Principles of Microbe and Cell Cultivation.* John Wiley & Sons, New York, U.S.A.
20. Sarra, M., C. Casas, and F. Godia. 1997. Continuous production of a hybrid antibiotic by *Streptomyces lividans* TK21 pellets in a three-phase fluidized-bed bioreactor. *Biotechnol. Bioeng.* **53:** 601–610.
21. Umezawa, H. and Y. Yagi. 1984. High yielding preparation of kasugamycin. Japanese Patent. J58170494. 07.10.83.