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Effects of Dissolved Oxygen Level on Avermectin B_{1a} Production by *Streptomyces avermitilis* in Computer-Controlled Bioreactor Cultures

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Abstract In order to investigate the effect of dissolved oxygen (DO) level on AVM B_{1a} production by a high yielding mutant of *Streptomyces avermitilis*, five sets of bioreactor cultures were performed under variously controlled DO levels. Using an online computer control system, the agitation speed and aeration rate were automatically controlled in an adaptive manner, responding timely to the oxygen requirement of the producer microorganism. In the two cultures of DO limitation, the onset of AVM B_{1a} biosynthesis was observed to casually coincide with the fermentation time when oxygen-limited conditions were overcome by the producing microorganism. In contrast, this phenomenon did not occur in the parallel fermentations with DO levels controlled at around 30% and 40% throughout the entire fermentation period, showing an almost growth-associated mode of AVM B_{1a} production: AVM B_{1a} biosynthesis under the environments of high DO levels started much earlier than the corresponding oxygen-limited cultures, leading to a significant enhancement of AVM B_{1a} production during the exponential stage. Consequently, approximately 6-fold and 9-fold increases in the final AVM B_{1a} production were obtained in 30% and 40% DO-controlled fermentations, respectively, especially when compared with the culture of severe DO limitation (the culture with 0% DO level during the exponential phase). The production yield ($Y_{p/x}$), volumetric production rate (Qp), and specific production rate ($\bar{q}_p$) of the 40% DO-controlled culture were observed to be 14%, 15%, and 15% higher, respectively, than those of the parallel cultures that were performed under an excessive agitation speed (350 rpm) and aeration rate (1 vvm) to maintain sufficiently high DO levels throughout the entire fermentation period. These results suggest that high shear damage of the high-yielding strain due to an excessive agitation speed is the primary reason for the reduction of the AVM B_{1a} biosynthetic capability of the producer. As for the cell growth, exponential growth patterns during the initial 3 days were observed in the fermentations of sufficient DO levels, whereas almost linear patterns of cell growth were observed in the other two cultures of DO limitation during the identical period, resulting in apparently lower amounts of DCW. These results led us to conclude that maintenance of optimum DO levels, but not too high to cause potential shear damage on the producer, was crucial not only for the cell growth, but also for the enhanced production of AVM B_{1a} by the filamentous mycelial cells of *Streptomyces avermitilis*.

Key words: *Streptomyces avermitilis*, avermectin B_{1a}, dissolved oxygen, computer-controlled fermentation

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Avermectins (AVMs) are oleandrose disaccharide derivatives of 16-membered pentacyclic lactones produced as secondary metabolites by filamentous mycelial cells of *Streptomyces avermitilis*[28]. It has been reported that eight congeners of AVMs of similar structure (A_{1a}, A_{1b}, A_{2a}, A_{2b}, B_{1a}, B_{1b}, B_{2a}, B_{2b}) are biosynthesized by the producer, of which AVM B_{1a} has the most powerful anthelmintic and insecticidal activities[14]. Therefore, it is used as an efficient agent in the field of agriculture and animal health. In addition, AVM B_{1a} is currently used as a precursor for ivermectin, a semisynthetic avermectin, which is widely used in veterinary and agricultural fields for the treatment of diseases caused by nematodes and arthropods [2]. Recently, it has been proved that AVM B_{1a} is synthesized via a polyketide pathway with 7 acetate units, 5 propionate units, and a branched chain of fatty acid unit [3].

Secondary metabolites such as AVM B_{1a} are usually formed under obligate aerobic conditions. It is well known that the solubility of oxygen in aqueous solutions under

1 atm of air is extremely low, which is in the order of 10 parts per million (ppm) near the ambient temperature, resulting in only a small reservoir in solution available for the producing cells [6]. In particular, gas-liquid oxygen mass transfer rates have been shown to become significantly diminished during mycelial growth in submerged cultures, owing to the three-dimensional structure of the filamentous organisms, which imparts very high non-Newtonian viscosities to the fermentation broth [8]. An additional limitation is that oxygen must travel a long, difficult path before cells have access to it [7]. As a consequence, the availability of oxygen for the microbial use depends not only on the solubility, but also on the mass transfer rates of this gas in the fermentation broth. Since synthesis of secondary metabolites is generally considered to depend heavily on the energy generated through primary metabolism [16], it is quite natural to find that many published reports emphasize the importance of control of oxygen supply rate to the bioreactor system in secondary metabolite fermentations [20, 24]. It has been shown that oxygen supply to the bioreactor is an important variable in antibiotic production, both in terms of the range of dissolved oxygen (DO) concentration and the timing of changes in the DO level [9]. For example, a three-fold reduction of cephalosporin production with *S. clavuligerus* was observed by Yegneswaran and Gray [27], when DO levels were reduced to less than 75% of air saturation. In penicillin fermentation, DeTilly *et al.* [4] demonstrated that a decrease of dissolved oxygen to less than 10% of air saturation during the early growth phase of *Penicillium chrysogenum* cells resulted in a very negative effect on penicillin production, compared with a similar reduction during the later stage of fermentation.

Despite the detailed knowledge of the biosynthetic gene clusters and the enzymes involved in the biosynthetic pathway of polyketide compounds including AVM B_{1a} [10, 17, 13], there has been little information on the fermentation physiology of the producer microorganism and engineering aspects involved in the enhanced production of this secondary metabolite. The objective of this work was to investigate the effects of different dissolved oxygen (DO) levels on the cell proliferation and AVM B_{1a} production by a high yielding mutant of *Streptomyces avermitilis* that was obtained through an efficient rational screening program in our laboratory. For this purpose, several bioreactor cultures were carried out under various levels of DO, using an online computer-controlled fermentation system, through simultaneous adjustment of agitation speed and aeration rate according to the oxygen requirement of the producer microorganism. Additionally, key fermentation parameters such as specific AVM B_{1a} production rate and production yield coefficients were quantified and compared with each other in variously DO-controlled fermentations.

MATERIALS AND METHODS

Culture Media

A solid agar medium and two kinds of liquid media were used in this study. The growth medium (GM) which was able to support high cell growth, was a complex medium composed of soluble starch (30 g/l), yeast extract (15 g/l), corn steep liquor (5 g/l), and KH_2PO_4 (0.4 g/l). The other was a production medium (PM) optimized statistically using the response surface method (RSM) in our laboratory, consisting of soluble starch (60 g/l), soybean meal (10 g/l), skim milk (15 g/l), KH_2PO_4 (0.5 g/l), and PEG 2000 (2.5 g/l). Composition of the agar solid medium containing 20 g/l of agar, used mainly for a large production of spores as inoculum into the growth culture, was identical to that of GM.

Strain and Cultivation

The microorganism used in this study was *Streptomyces avermitilis* ENP88-207, which is a AVM B_{1a} high-yielding mutant improved through a rational screening strategy in our laboratory, and has the features of resistance to various antimetabolites such as *O*-methyl threonine and/or *p*-fluorophenoxy acetic acid [23]. For the preparation of inoculum in bioreactor cultures, 5 ml of spore suspension (1×10^7 spores/ml) scraped from solid agar slant using aliquots of glycerol solution was transferred to 100 ml of sterile growth medium (GM) (5% v/v) in a 500-ml Erlenmeyer flask. The pH of GM was initially adjusted to 7.2 with KOH (1 N) before sterilization. The flask was then incubated on a rotary shaker at 28°C and 250 rpm for 2 days. At this stage, cell mass was approximately 5 g of DCW/l. This culture was then used for the inoculation of production culture at 10% (v/v) in shake flasks (various working volumes in 250-ml flasks) and/or in the main bioreactors (3-l working volume in 5-l fermentor). To minimize the variation of the activity of the high-yielding mutant, the following preservation method was adopted: 5 ml of 1.5-day-old liquid culture, which was cultivated in GM, was transferred to a 30-ml vial containing a sterile mixture of 1 ml of glycerol and 4 ml of distilled water. After thoroughly mixing the solution, it was stored in a −121°C freezer.

Measurement of Cell Growth, and Recovery and Analysis of AVM B_{1a}

Even though a small portion exists in the culture broth, AVM B_{1a} is an intracellular product, and therefore, an extraction procedure is needed to recover it. For the shake-flask culture, three 10-ml-each samples were taken from homogenized fermentation broth, which was collected from each flask sacrificed. For the cell culture in bioreactor, approximately 30 ml of whole-broth sample was obtained from the fermentor at every sampling time, and homogenized.

From the homogenized fermentation broth, one or two 10-ml each of sample(s) were taken for cell mass determination, and the other 10 ml of the homogenized sample was used for AVM B_{1a} analysis by HPLC. Homogenization of the fermentation broth was necessary, because this strain was found to form various pellets during the fermentation. Otherwise, it was found to be quite difficult to obtain a reproducible result. The samples were centrifuged for 5 min at 15,000 rpm, and then the supernatant was decanted and kept in a freezer at -20°C for the analysis of AVM B_{1a}. The compacted precipitated sample was washed three times with 10 ml of distilled water to completely remove the residual nutrients. Then, the washed pellet was dried at 85°C for 24 h to obtain constant dry cell weight (DCW). The centrifuge employed in this analysis was an MF80 from Hanil Instruments (South Korea). The sample was thawed and extracted by adding an equal volume of methanol. The above sample was then incubated at 27°C in a rotary shaker at 250 rpm for 24 h. After extraction of AVM B_{1a}, the culture/solvent mixture was separated by the centrifuge at 10,000 rpm for 10 min. After separating the supernatant, 1 ml of the supernatant was then filtered through a 0.2-mm microfilter (Millipore, Bedford, MA, U.S.A.) and transferred to a microtube for HPLC injection. Authentic AVM B_{1a} standard was generously provided by Dae-Sung Microbiological Labs. Co., Ltd (Seoul, South Korea), and the following conditions were used for the analysis of AVM B_{1a} by HPLC:

Column: YMC-Pack ODS-A reverse-phase column (4.6×250 mm) (YMC Co., Japan);

Mobile phase: 85 % methanol;

Detector: M 720 UV detector (Young-Lin Instrument Co., S. Korea), 246 nm;

Pump & Flow rate: M930 (Young-Lin Instrument Co., S. Korea), 1.2 ml/min.

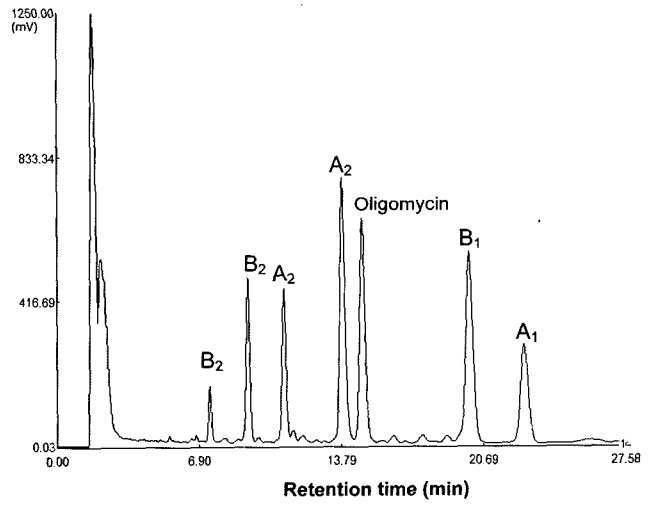

Fig. 1. HPLC histogram for avermectins (AVMs).

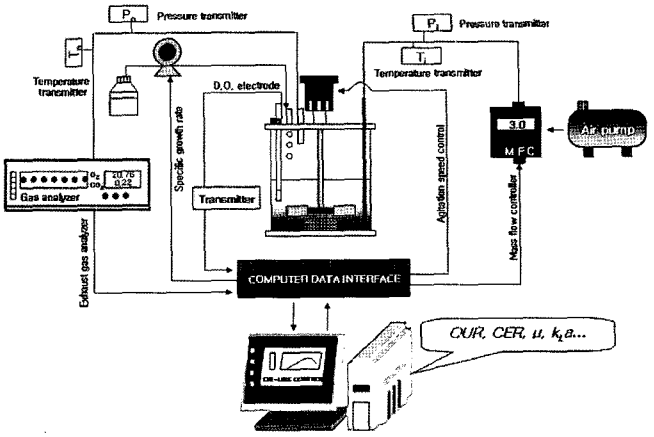

Fig. 2. Representative diagram of DO-controlled fermentation system.

Fig. 1 shows HPLC chromatogram for 8 components of the avermectin family extracted from the sample of *Streptomyces avermitilis* bioreactor culture.

Fermentation Equipment for the Automatic Control of Dissolved Oxygen Level

In order to elucidate morphological and physiological differences among the fermentations carried out under various sets of DO-control modes, a computer-controlled bioprocess system was developed in our laboratory by using a 5-l stirred-tank fermentor (Kobiotech Co., Ltd., Korea) with 3-l working volume (Fig. 2). For facilitated transfer of dissolved oxygen into the fermentation broth, a 6-flat-blade disc turbine impeller and a 12-hole ring sparger, as modified for the cultivation of filamentous *Streptomyces* cells, were used for all the experiments. In the culture medium, DO concentration was accurately regulated at a set point by simultaneously adjusting the agitation speed of the impeller and aeration rate through a mass flow controller, GFC17 (Aalborg Instrument & Controls, Inc., Orangeburg, NY, U.S.A.). For initial calibration of DO concentration, DO level of the fermentation broth was adjusted to 100% before the inoculation, using air as the inlet gas at the fermentation temperature and pressure. An electric zero was used for the calibration at 0% saturation. This electric zero calibration was confirmed by exchanging air with a pure nitrogen feed. A polarographic type DO probe (Mettler Toledo, Inc., Columbus, OH, U.S.A.) with replaceable membrane, which is connected to a DO analyzer of the computer-controlled fermentor system, was used. DO concentration, agitation speed, and aeration rate were continuously recorded via the computer interface system during the whole fermentation period. Specific operating conditions for the respective culture are provided separately in the Results and Discussions section together with experimental results.

RESULTS AND DISCUSSIONS

Effect of DO on AVM B_{1a} Production in Shake-Flask Cultures

Many studies have shown that the microbial physiology of filamentous *Streptomycetes* was significantly influenced by dissolved oxygen (DO) level in suspended cultures [5, 11, 19, 24], and some of the above studies also suggested that the critical DO level for *Streptomycetes* should be greater than 50% of the saturated DO value. Therefore, in order to investigate the effects of DO on cell growth and AVM B_{1a} production by *S. avermitilis*, 250-ml shake-flask fermentations with various culture volumes (from 10 to 130 ml) were preliminarily carried out with inoculated amount of 10% (v/v) cells into the flasks. All the fermentations were performed at fixed agitation speed of 220 rpm for 7 days. The morphology of the producer microorganism was found to be compact pellet-form, regardless of the culture volume, whose diameter was in the range between 0.5 mm and 1 mm. As described in Fig. 3, however, AVM B_{1a} production decreased dramatically when the culture volume in a 250-ml shake flask was more than 50 ml. Cell growth showed almost similar pattern to that of AVM B_{1a} biosynthesis, although the extent of reduction due to the culture volume was not significant. The result suggests that optimum supply of DO is crucial for the enhancement of AVM B_{1a} production, since the cells in a larger working volume are exposed to more severe oxygen-limited conditions in shake-flask fermentations, when performed under identical agitation speed.

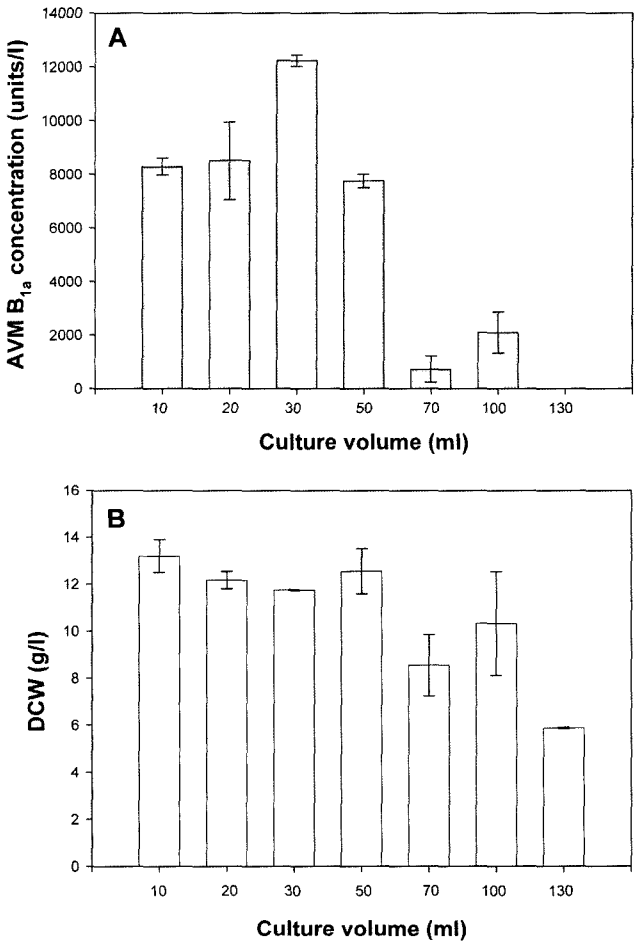

Fig. 3. (**A**) AVM B_{1a} production and (**B**) dry cell weight according to culture volume, in 250-ml shake-flask fermentations (the inoculation size was 10 % (v/v) in all conditions).

Comparison of Fermentation Physiology and AVM B_{1a} Production in Various DO-Controlled Bioreactor Fermentations

In order to investigate in detail the effect of DO level on AVM B_{1a} production by the high-yielding mutant, several bioreactor cultures were performed under various DO environments. The computer-controlled fermentation system developed for the automatic control of DO had already been described in detail (Fig. 2). A 12-hole sparger and a 6-flat-disc turbine impeller were employed in all the 5-l bioreactor fermentations. Time-course profiles of DO, agitation speed, and aeration rate in the fermentations performed under the DO-limited and 30% DO-controlled environments are presented in Fig. 4. In the case of DO-limited culture, where agitation speed and aeration rate were kept constant at 250 rpm and 1 vvm, respectively, DO became almost exhausted as early as at around 10 h (Fig. 4A), whereas DO concentration in the 30% DO-controlled culture could be maintained above 30% of air saturation throughout the whole fermentation period via simultaneous regulation of agitation speed and aeration rate (Fig. 4B). It can be seen in Fig. 4B that both the agitation speed and the aeration rate increased rapidly up to the maximum operating set-values of 270 rpm and 2.0 vvm, respectively, during the early exponential phase in order to prevent DO levels from falling below 30%, and then declined gradually before turning to the minimum set-values of 150 rpm and 1.0 vvm at the beginning of the stationary phase. Despite the low agitation speed and aeration rate, the DO level started to increase at around 96 h, most probably due to a reduced oxygen uptake rate (OUR) of the producing microorganism caused by the lack of essential nutrients other than DO, reaching nearly air saturation value at the end of the fermentation. Such metabolic changes of the producer could also be confirmed by the rapid rise of pH (around 144 h as demonstrated in Fig. 4A), probably resulting from the change-over of carbon source metabolism from the easily utilizable sugars to the proteinous carbon sources, such as skim milk and/or soybean meal, which were supplemented to the production medium. In order to find out the fermentation stage at which DO was most efficiently utilized for AVM B_{1a} biosynthesis, another set of DO-controlled culture was

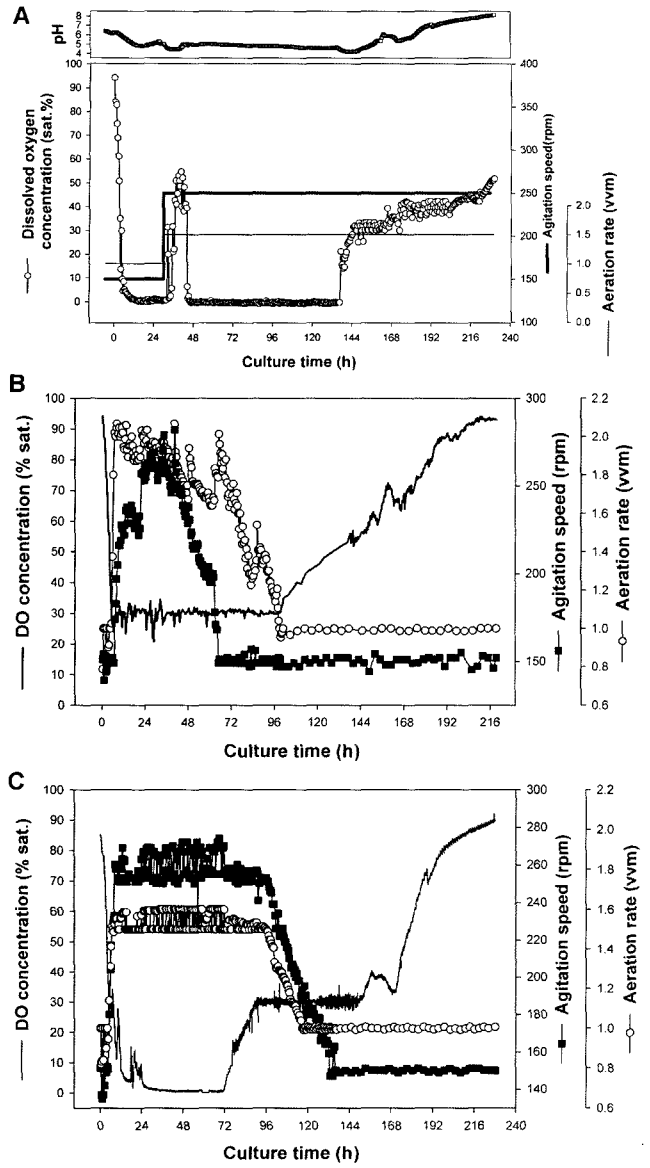

Fig. 4. Temporal profiles of DO concentration, agitation speed, and aeration rate in fermentation performed under the conditions of (**A**) DO noncontrolled, (**B**) 30% DO controlled at early exponential phase, and (**C**) 30% DO controlled at late exponential phase.

carried out under severe DO limitation during the early exponential phase, and then under about 30% DO regulation during the late exponential phase (*i.e.*, from around 96 h). As seen in Fig. 4C, almost the same trends of agitation speed, aeration rate, and DO profile as those in the previous 30% DO-controlled fermentation were observed, except that approximately two more days of maximum agitation speed and aeration rate were needed in order to sustain DO concentrations around a 30% saturation value during the late exponential phase (*i.e.*, until 150 h of fermentation).

As for the cell growth (Fig. 5B), the amounts of DCW at the start of each fermentation were relatively high, ranging from 6 to 7.5 g/l. This was most probably due to the contribution of the insoluble nutrients contained in the production medium to DCW. Most of the insoluble nutrients were found to result from soybean meal, a very important carbon and nitrogen source in AVM B_{1a} production medium, which turned out to be dissolved completely at around day 3. Therefore, it is highly likely that the dotted lines in Fig. 5B represent the actual dry cell weight. In the 30% DO-controlled fermentation, an approximately 40% higher amount of maximum DCW was obtained than the parallel DO-limited culture. The exponential growth pattern during the initial 3 days of the 30% DO-controlled culture should be noted. On the contrary, an almost linear pattern of cell growth was noted from day 2 until day 7 in the fermentation without DO-control, apparently due to severe DO limitations. In the case of fermentation where DO was regulated at around 30% only during the late exponential phase, cell growth was also severely limited during the early exponential phase, as expected, followed by slow linear growth

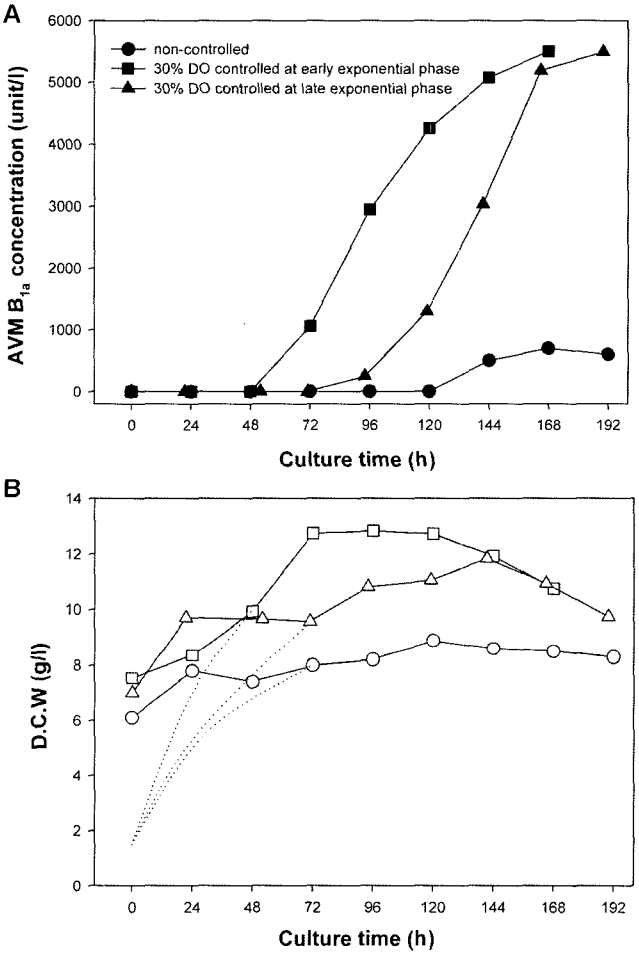

Fig. 5. Temporal profiles of (**A**) AVM B_{1a} production and (**B**) dry cell weight in fermentations performed under the conditions of non-controlled, 30% DO controlled at early exponential phase, and 30% DO controlled at late exponential phase, in 5-l bioreactors.

from day 3 to day 6, finally reaching the maximum cell concentration of 11.5 g/l at day 6.

Results to compare both AVM B_{1a} biosynthesis and cell growth versus DO level are described in Fig. 5. Notably, in the two DO-limited fermentations (*i.e.*, the culture with DO-control at around 30% only at the later stage of exponential phase, and the culture with no DO-control), the starting points of AVM B_{1a} biosynthesis were found to casually coincide with the fermentation time when the DO-limited conditions were overcome by the reduced oxygen uptake rate (OUR) of the producing microorganisms (*i.e.*, at around 72 h and 130 h, respectively, as inferred from Figs. 4A and 4C) (Fig. 5A). These phenomena, however, could not be observed in the 30% DO-controlled fermentation throughout the whole period, where nearly growth-associated mode of AVM B_{1a} biosynthesis was displayed: Namely, it can be seen in this case that AVM B_{1a} biosynthesis started 1.5 days and 2 days earlier, respectively, than the two parallel oxygen-limited cultures, hence leading to a significant enhancement of AVM B_{1a} production during the exponential phase. As a result, an about 6-fold increase in the final AVM B_{1a} production was obtained in the culture of 30% DO-control, as compared with the DO-uncontrolled fermentation (maximum AVM B_{1a} concentrations of 5,500 units/l *vs.* 900 units/l at 168 h). These results illustrate that DO levels had great influences on both the cell growth and, as will be discussed in more detail in the later section, the secondary metabolism for the biosynthesis of AVM B_{1a}, in agreement with the results from the flask experiments above. Similarly to our results, Pfefferle *et al.* [18] observed that reduction of DO concentration in *Streptosporangium* cultures significantly delayed the starting point of rhodomycinone biosynthesis in their batch bioreactor fermentation. Rollins *et al.* [20] also reported that an increase in the DO level during batch fermentations enhanced cephamycin C production by *Streptomyces clavuligerus* almost three-fold compared with a DO-uncontrolled fermentation. Subsequently, the same group [21] examined the response of the key enzymes, which are involved in the biosynthesis of cephamycin, to the changes in oxygen availability during batch fermentations, and found that oxygen had a greater derepressive effect on the specific activity of the DAOCS (deacetoxy cephalosporin C synthetase) enzyme (2.3-fold) than other biosynthetic enzymes, thus implicating DAOCS as one of the key regulatory enzymes in the cephamycin C biosynthetic pathway [21].

Determination of Optimum DO Level through Examination of Fermentation Physiology under Sufficiently High DO Environments

In order to further optimize DO level, we investigated fermentation physiology of the high-yielding mutant under sufficiently high DO environments. Operating conditions of bioreactor such as agitation speed and aeration rate were chosen carefully, so that DO levels of the fermentation broth remained far above the critical DO level, therby never acting as a growth-limiting nutrient. Time-course profiles of DO, agitation speed, and aeration rate in the cultures performed under 40% DO-control conditions as well as sufficiently high shear environments (*i.e.*, fixed rates of as high as 350 rpm and 1 vvm) over the whole fermentation period are presented in Fig. 6. In Fig. 6A for the 40% DO-control culture, it can be seen that both the agitation speed and aeration rate responded timely to the oxygen requirement of the producing microorganism, with DO level maintained at around 40% of air saturation until day 6: That is, agitation speed as well as aeration rate increased rapidly, reaching the highest set-values of 300 rpm and 2.0 vvm within 1 day, subsequently declining steadily to the minimum set-values of 150 rpm and 1.0 vvm at around day 7. In the case of a comparable culture (Fig. 6B) performed under the highest agitation speed of 350 rpm about 10 h after the start of fermentation with a constant aeration rate of 1 vvm, DO levels were sustained, as expected, at above 50% during almost the entire fermentation time.

It is noteworthy that, in both fermentations, AVM B_{1a} started to be biosynthesized as early as at day 2, most

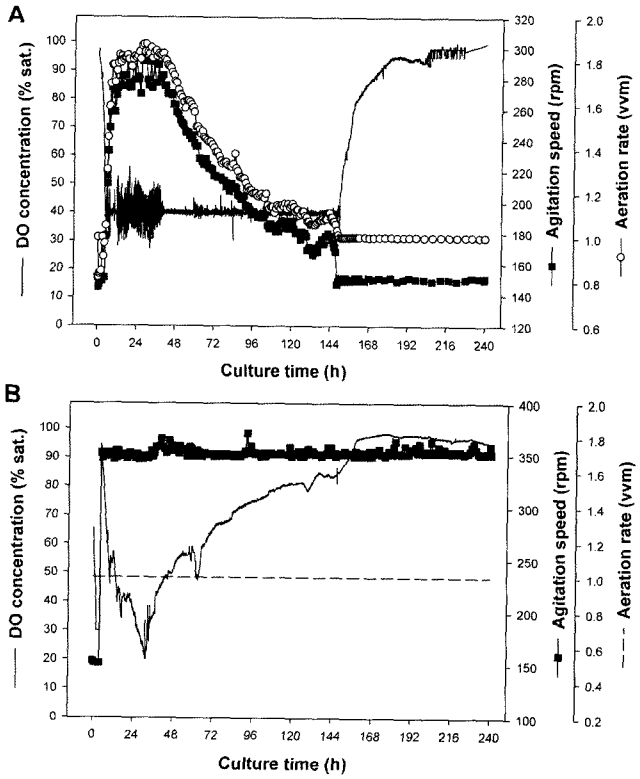

Fig. 6. Temporal profiles of DO concentration, agitation speed, and aeration rate in fermentation under the conditions of (**A**) 40% DO controlled and (**B**) DO controlled at high level, in 5-l bioreactors.

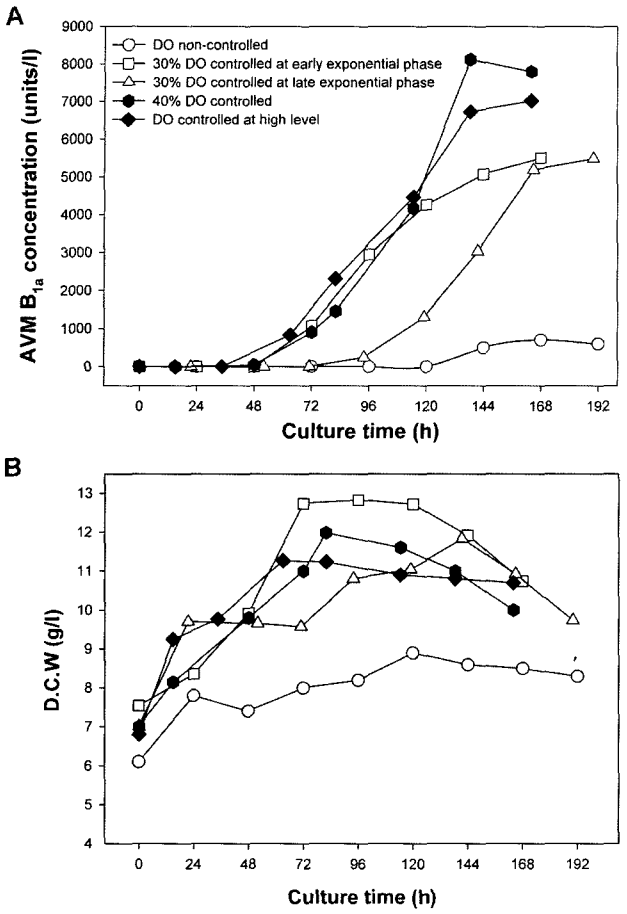

Fig. 7. Comparison of (**A**) AVM B_{1a} production and (**B**) dry cell weight, according to various fermentation conditions in 5-l bioreactors.

probably due to no apparent DO limitations during the actively growing phase (Fig. 7B). A similar phenomenon had also been observed in the 30% DO-controlled fermentation (Fig. 5A). The biosynthetic patterns of AVM B_{1a} for the two cultures were almost identical, producing maximum amounts of 8,000 and 7,200 units/l, respectively. The 1.1-fold increase of AVM B_{1a} production by the 40% DO-controlled culture, compared with the parallel fermentation of the highest agitation speed, should be noted in this result. With respect to this phenomenon, more details will be discussed later by referring to Table 1. Also notable in Fig. 7A were 1.5-fold and 9-fold increases of AVM B_{1a} production by the 40% DO-controlled culture as compared with the cultures of 30% DO-control and severe DO limitation (almost 0% DO level), respectively. As for the cell growth (Fig. 7B), exponential growth patterns were observed during the initial 3 days in the 3 sets of fermentations with no DO limitations during the actively growing stage, whereas a almost linear pattern of cell growth in the other two cultures of DO limitation was manifested during the identical period over the exponential phase, resulting in apparently lower amount of DCW at the 4th day (37% and 25% reduction, respectively, in comparison with the DCW obtained in the 30% DO-controlled fermentation). These results imply that maintenance of optimum DO level was crucial not only for the cell growth, but also for the enhanced production of AVM B_{1a} by the filamentous mycelial cells of *Streptomyces avermitilis*.

Comparison of Fermentation Parameters in Various DO-Controlled Bioreactor Fermentations

Summarized in Table 1 are key fermentation parameters for the bioreactor cultures performed under various DO-control modes, as specified above. Profound differences in the fermentation physiology can be seen, when the specific AVM B_{1a} production rate ($\bar{q}_p$) of each system is compared. The value of $\bar{q}_p$ for the culture of 40% DO-control was approximately 8.9-fold and 1.8-fold higher, respectively, than those for the parallel cultures of DO-limitation (*i.e.*, the severe DO-limited culture and the 30% DO-controlled culture at the later stage of fermentation). A strong, positive effect of optimal maintenance of DO level on AVM B_{1a} production could also be inferred by referring to the $Y_{p/x}$ (AVM B_{1a} yield on DCW) and Q_p (volumetric production rate), as described in Table 1. At this moment, however, it should be emphasized that $Y_{p/x}$, Q_p, and $\bar{q}_p$ in the 40% DO-controlled culture were 14%, 15%, and 15% higher, respectively, than those of the parallel culture performed under the excessive agitation speed (350 rpm) for the maintenance of sufficiently higher DO levels over the whole fermentation period. High shear environments to

Table 1. Fermentative parameters relevant to AVM B_{1a} production in various fermentation conditions in 5-l bioreactors.

	Maximum AVM B_{1a} concentration (units/l)	DCW at maximum AVM B_{1a} concentration (g/l)	$Y_{p/x}$	Q_p	$\bar{q}_p$
A	900	8.5	105.8	5.3	1.07
B	5,500	10.5	523.8	32.7	5.45
C	5,487	9.74	563.3	28.87	5.14
D	8,125	10.8	752.3	58.87	9.57
E	7,021	10.7	656.1	50.87	8.34

A, DO noncontrolled; B, 30% DO controlled at early exponential phase; C, 30% DO controlled at late exponential phase; D, 40% DO controlled; E, DO controlled at high level; $Y_{p/x}$, AVM B_{1a} production yield (units AVM B_{1a}/g DCW); Q_p, volumetric AVM B_{1a} production (units AVM B_{1a}/l/h); $\bar{q}_p$ (h^{-1}), average specific AVM B_{1a} production rate (h^{-1}).

inflict shear damage on the high-yielding strain appeared to be the primary reason for the reduction in the AVM B_{1a} biosynthetic capability of the producing microorganism. In summary, these comparative results could be interpreted in terms of producer's fermentation physiology as follows: (i) In the optimally DO-controlled fermentations, especially when DO levels were regulated at around 40% during the exponential phase of the fermentation, a greater portion of the available carbon source appeared to be utilized for the AVM B_{1a} biosynthesis rather than for the cell growth, the producer starting to biosynthesize AVM B_{1a} around the mid-point of the exponential stage (*i.e.*, at a point in the physiological state of the microorganism when AVM B_{1a} synthetases were derepressed) (Fig. 7). (ii) Conversely, in the DO-limited cultures, the suppression of AVM B_{1a} production during the same period indicates a fundamentally different physiological state of the producer, revealing that the enzymes involved in the biosynthesis of AVM B_{1a} might be fully repressed, possibly due to preferential utilization of the available carbon source(s) for the primary metabolism rather than for the secondary metabolic functions.

It has often been reported that oxygen demand of producer microorganism in a very large-scale cultivation sharply increases as cell mass increases. In such case, as the agitation speed increases in order to keep DO levels at a desired set point, shear stress concurrently becomes large, thus influencing the physiology as well as morphology of the mycelial-forming strains such as filamentous fungi and *Streptomycetes* [26, 25, 12]. Roubos and his coworkers [22] showed that *Streptomycetes* were very sensitive to the potential shear stress when they were grown in batch cultures under the environment of high agitation speeds. Morphological changes and resulting cell lysis were observed to occur at the high shear rates, thus not only leading to reduced overall yield in the biosynthesis of secondary metabolites and increased product degradation, but also negatively influencing the broth filtering in the downstream bioprocess [22]. Several approaches to solve the problems caused by limited supply of DO in microbial bioreactor cultures have been undertaken by many researchers. One of the notable approaches was to develop genetically engineered producers through insertion of a bacterial hemoglobin gene into them, thus conferring on them an enhanced capability of utilizing DO as efficiently as possible. In their experiment, Brunker *et al.* [1] observed that erythromycin production by the mutant strain of *Saccharopolyspora erythraea*, which was transfected with the *Vitreoscilla* hemoglobin (*VHb*) gene, was 60% higher than that in the parallel non-transfected strain. Magnolo *et al.* [15] also found that the introduction of the *VHb* gene into the cells of *Streptomyces coelicolor* led to a 10-fold increase in the specific yield of actinorhodin, when compared with the control culture of non-transfected cells, and also resulted in much less sensitivity of the producer microorganism to air supply.

Based on the fact that the secondary metabolic functions involved in the biosynthesis of AVM B_{1a} were very active in the *S. avermitilis* cultures under the optimal DO levels, we undertook the following approach and tried to improve the oxygen mass transfer capability of the stirred tank bioreactor system. Thus, new types of impeller and sparger were developed in our laboratory, which proved to be especially suitable for the culture of the shear-sensitive mycelial cells of *Streptomyces avermitilis*, and they will be presented in a subsequent article, placing special emphasis on the enhanced oxygen transfer features with resulting increase of the AVM B_{1a} productivity.

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