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ORIGINAL PAPER

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Optimization of agitation and aeration conditions for maximum virginiamycin production

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Abstract To maximize the productivity of virginiamycin, which is a commercially important antibiotic as an animal feed additive, an empirical approach was employed in the batch culture of *Streptomyces virginiae*. Here, the effects of dissolved oxygen (DO) concentration and agitation speed on the maximum cell concentration at the production phase, as well as on the productivity of virginiamycin, were investigated. To maintain the DO concentration in the fermentor at a certain level, either the agitation speed or the inlet oxygen concentration of the supply gas was manipulated. It was found that increasing the agitation speed had a positive effect on the antibiotic productivity independent of the DO concentration. The optimum DO concentration, agitation speed and addition of an autoregulator, virginiae butanolide C (VB-C), were determined to maximize virginiamycin productivity. The optimal strategy was to start the cultivation at 450 rpm and to continue until the DO concentration reached 80%. After reaching 80%, the DO concentration was maintained at this level by changing the agitation speed, up to a maximum of 800 rpm. The addition of an optimal amount of the autoregulator VB-C in an experiment resulted in the maximal production of virginiamycin M (399 mg/l), which was about 1.8-fold those obtained previously.

Introduction

Many studies on the optimization of antibiotic production in batch and fed-batch cultures have been carried out (Bajpai and Reuss 1981; Mou and Cooney 1983; Lim et al. 1986), most of which were based on a mathematical model. However, empirical studies are also important for industrial process development because generating a rigorous and reliable mathematical model requires considerable time or can be impossible, and several empirically based studies of growth phase have been published.

In previous studies (Yang et al. 1995, 1996), an empirical two-step approach was employed to maximize virginiamycin (M and S) productivity in a batch culture of *Streptomyces virginiae*, based on the fact that virginiamycin production proceeded in non-growth-associated kinetics. Virginiamycin is commercially used as an antibiotic in animal feed. To attain a high cell concentration in the production phase, that is, to extend the growth phase for as long as possible, the optimum composition and concentration of the medium ingredients, particularly the yeast extract concentration, were first investigated. The dissolved oxygen (DO) concentration was maintained at a value higher than 2 mg/l (about 26% of saturation) to increase the productivity during the production phase. In addition, to enhance the productivity, an appropriate amount of an autoregulator, virginiae butanolide C (VB-C), was added. *S. virginiae* produces hormone-like signal metabolites such as virginiae butanolides (VB-A, B, C, D and E) (Yang et al. 1995, 1996), called autoregulators, which exhibit complex functions, such as induction and inhibition, in virginiamycin fermentation. Addition of chemically synthesized VB-C to a batch culture can control the induction time and the amount of virginiamycin produced. The optimum cultivation conditions were found to be an initial yeast extract concentration in the complex medium of 45 g/l, a shot addition of 300 μg/l VB-C 11.5 h after the start of the batch culture, and a DO concentration maintained above 2 mg/l. The maximum concentrations of virginiamycin M and S were about nine times those obtained under non-optimized cultivation conditions, i.e., an initial yeast extract concentration of 7.5 g/l and no VB-C addition.

The DO concentration is also an important parameter to be empirically determined. Oxygen limitation

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concomitant with an increase in the cell concentration in culture is well known to have a detrimental effect on cell activity and to decrease the productivity of antibiotics (Vardar and Lilly 1982). Enhancement of antibiotic productivity by increasing the DO concentration has been shown in penicillin and cephalosporin C fermentations, among others. Yegneswaran and Gray (1991) reported a 2.4-fold increase in cephalosporin C productivity when the DO concentration was maintained at a high level.

In this study, an empirical approach was also employed for determining the optimal agitation speed and DO concentration to maximize virginiamycin (M and S) productivity in a batch culture of *S. virginiae*. Here, the effect of DO concentration and agitation speed on the maximum cell concentration at the production phase, as well as the production activity of virginiamycin, were investigated and the optimum DO concentration, agitation speed and VB-C addition were determined to maximize virginiamycin productivity.

Materials and methods

Strain and media compositions

Streptomyces virginiae MAFF 10-06014 (National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan) was used as the microorganism producing virginiamycin M and S. The culture medium used for virginiamycin production in this laboratory experimental study consisted of 7.5 g/l Bacto-casitone (Difco Laboratories, Detroit, Mich.), 45 g/l yeast extract (Difco), 15 g/l glycerol and 2.5 g/l sodium chloride. The pH was adjusted to 6.5. Components of the medium used to maintain the microorganism and the basic procedures for seed and batch cultures have been described previously (Yang et al. 1995, 1996).

Analysis

Cell concentration was measured as dry cell mass after washing and filtration of the sample. The glycerol concentration was measured enzymatically and the virginiamycin M (VM) and S (VS) concentrations were determined by HPLC. The autoregulator concentration was measured as the VB-C concentration by an improved bioassay method (Yang et al. 1995, 1996). The carbon dioxide concentration in the exhaust gas was monitored as an indicator of the optimal time for VB-C addition. These analytical procedures have been described in detail in our previous papers (Yang et al. 1995, 1996).

DO concentration control

A 5-l jar fermentor (KMJ-5A, Mitsuwa Co., Japan) containing 1.5 l medium was usually operated at 28 °C, 450 rpm, and at a pH of 6.4–6.6. A rather small liquid volume was employed for keeping the same experimental conditions in a wide range of cell concentration. Control of the DO concentration was achieved by two methods. One (method 1) was to adjust the DO concentration mainly by manipulating the agitation speed but also by using the on/off flow control of the pure oxygen supply in addition to regulating the air flow to the fermentor when adjustment by agitation speed did not result in a sufficiently high DO concentration. As shown in Fig. 1, the DO concentration decreased with cell growth as the fermentation proceeded. When the DO concentration reached a set value, the agitation speed could be manipulated to maintain the DO concentration. When the agitation speed reached the maximum value, usually set at 1000 rpm, and could not maintain the DO concentration at a set value, the on/off flow control of pure oxygen was additionally employed. By contrast, in method 2 the pure oxygen supply was predominantly used. That is, the on/off flow control of the pure oxygen supply was used in addition to the air flow to the fermentor and the agitation speed control, when the maximum supply of pure oxygen did not give the desired DO concentration. In both cases, the total inlet gas (air + pure oxygen) flow was kept constant at 1 l/min. The same gas flow rate guaranteed that the degree of mixing by bubbling did not change. The minimum and maximum agitation speeds were 450 rpm and 1000 rpm respectively. Method 1 was used unless otherwise stated.

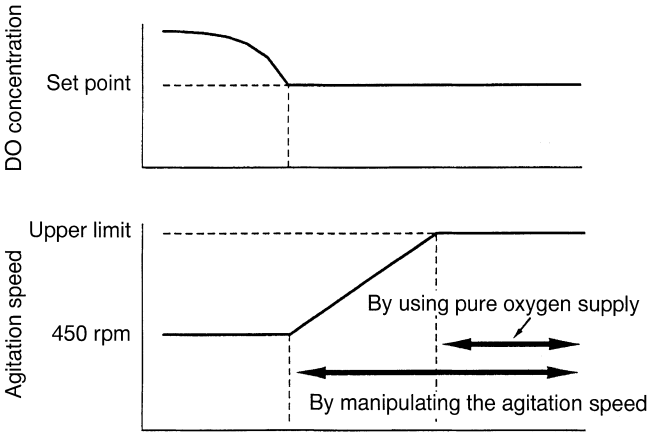

Fig. 1 Schematic diagram of dissolved oxygen (*DO*) concentration controlled by method 1

Results

Effect of DO concentration on virginiamycin productivity

Method 1 could maintain the DO concentration at 50% of the saturation level in a batch virginiamycin fermentation, as shown in Fig. 2. Manipulation of the DO concentration started after it reached 50% of the saturation level. That is, in the beginning of the batch culture the DO concentration was higher than 50%. As is characteristic of virginiamycin fermentations, around 1–2 h before the start of virginiamycin production (at 10 h), the autoregulator VB began to be produced. During the virginiamycin production phase, (after 11–12 h), the specific growth rate was much lower than the growth rate from the start of batch culture and for up to 12 h.

As reported previously, *S. virginiae* produces VM and VS, which act synergistically on microorganisms as antibiotics, but the ratio of VM to VS was found to be almost the same in all experiments in this study. Moreover, because the amount of VS produced was only about 10% that of VM, we focused mainly on VM production in subsequent experiments.

Table 1 shows the effect of DO concentration on the cell growth and VM productivity. Here, the DO con-

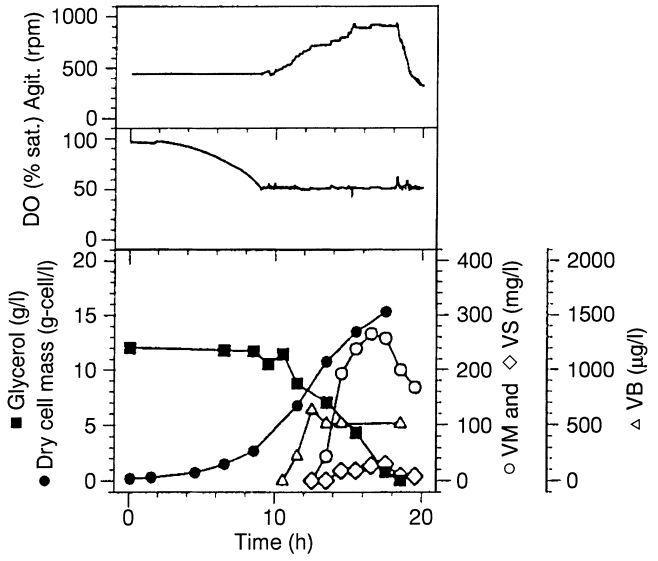

Fig. 2 Time course of virginiamycin fermentation in a batch culture of *Streptomyces virginiae* where the DO concentration was controlled at 50% by method 1. *VM* virginamycin M, *VS* virginiamycin S, *VB* virginiae butanolides

centration was controlled by method 1. As can be seen in the table, the optimum DO concentration (25%–50%) must be maintained to obtain the maximum cell concentration. Agitation speed control started earlier when the required DO concentration was higher (data not shown). To maintain the DO concentration at 80%, the maximum agitation speed had to be used and an additional control was achieved by using the pure oxygen supply. When the DO concentration is too low, it limits the cell growth whereas with a high DO concentration, the higher agitation speed used may cause a decrease in the growth rate. However, for maximizing the productivity of VM, a high DO concentration was desired. Maintaining the DO concentration at 80% resulted in the production of approximately 300 mg/l VM, which was about 1.4 times higher than the maximum value previously obtained (Yang et al. 1996).

Table 1 Cell growth and productivity of virginiamycin (*VM*) at various dissolved oxygen (*DO*) concentrations controlled by method 1. *VB* autoregulator: virginiae butanolides

Parameter	Value at a DO concentration of:			
	10%	25%	50%	80%
VM (mg/l)	91	141	266	296
VS (mg/l)	10	21	30	26
VB (μg/l)	300	280	634	741
VM/cell (mg/g cells)	7.6	10.2	18.7	27.0
VB/cell (μg/g cells)	37.4	32.9	74.7	76.6
Cell concentration at the maximum VM (g/l)	12.0	13.8	14.2	11.0

Positive effect of agitation speed on the productivity

In this study (Table 1), the DO concentration was maintained mainly by adjusting the agitation speed. This means that a high DO concentration corresponds to a high agitation speed. The question therefore arises: which parameter, a high DO concentration or a high agitation speed, is responsible for the high virginiamycin productivity? To clarify this, the following experiment was performed. The adjustment of the agitation speed change was similar, within 100 rpm, to those shown in Fig. 1, but the DO concentration was lower than 50%, i.e., 25%. This was achieved by mixing N_2 gas in the air flow to the fermentor.

As can be seen in Fig. 3, the agitation speeds in both cases were almost the same, whereas the DO concentrations were 50% and 25%. In addition, the effect of identical DO concentrations but different agitation speeds was compared to determine the effect of agitation speed on the productivity of virginiamycin.

Figure 4 compares the effect of agitation speed and DO concentration on virginiamycin productivity. The columns *A* represent the results of the experiment in which the DO concentration was controlled at 25% by adjusting the agitation speed. The columns *B* represent the results of the experiment in which the DO concentration was controlled at 25% by the method used to obtain the results shown in Fig. 3 (solid line). The col-

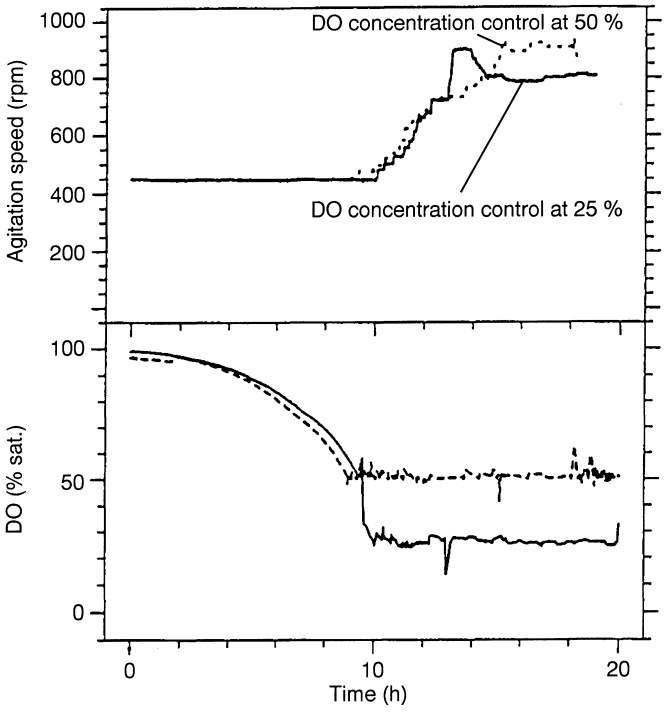

Fig. 3 Almost the same time courses of agitation speeds for two different experiments in which the DO concentrations were adjusted at different values. —— Data from the experiment in which the DO concentration was controlled at 25%, using a mixed air supply with nitrogen gas; - - - data from the experiment in which the DO concentration was controlled at 50% using normal air

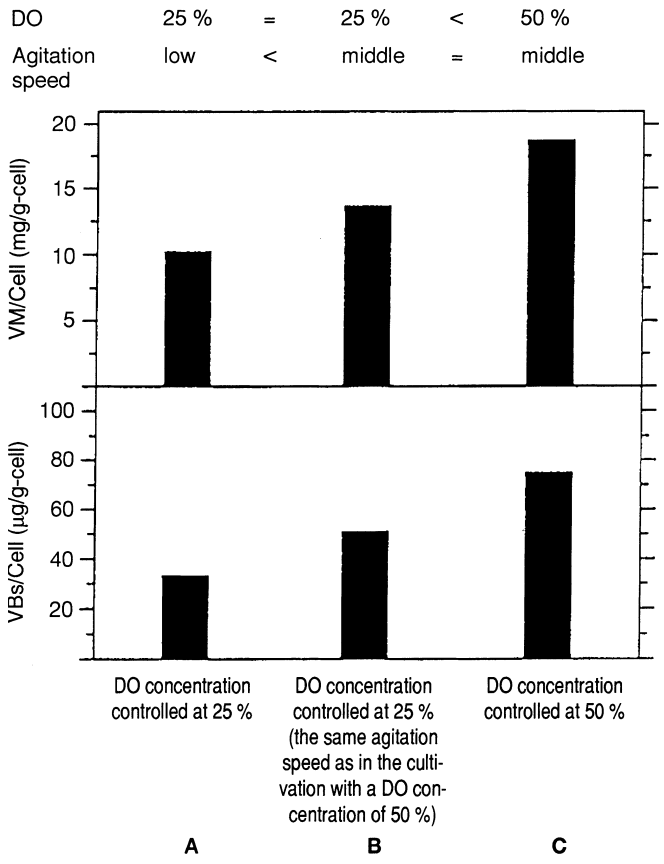

Fig. 4 The effect of DO concentration and agitation speed on VM and VB productivities. The maximum VM concentration is 120 mg/l in *B*. The data *A* and *C* are the same as those of DO = 25% and 50% respectively in Table 1

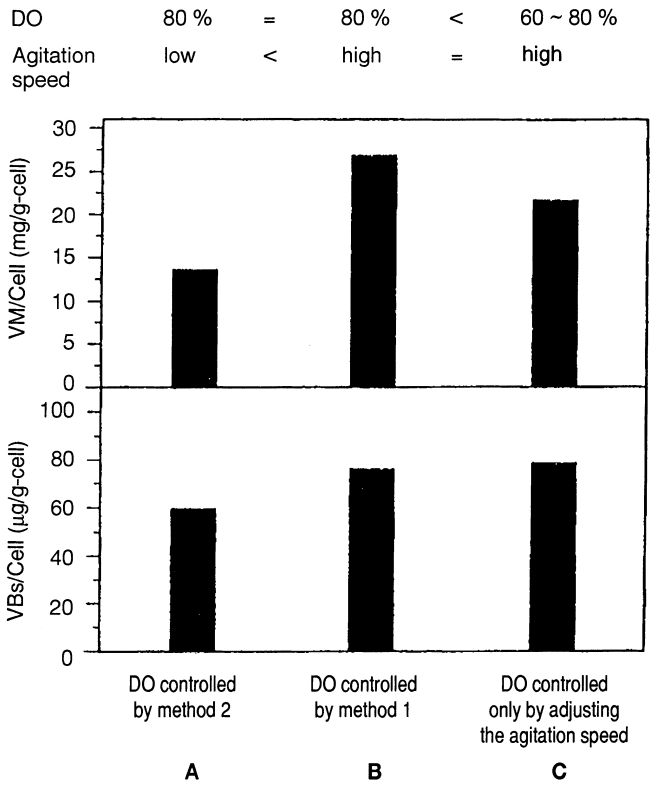

Fig. 5 The effect of DO concentration and agitation speed on VM and VB productivities. Maximum VM concentrations are; **A** 167 mg/l, **B** 296 mg/l, **C** 276 mg/l. The data of columns **B** are the same as those of DO = 80% in Table 1

umns *C* represent the results obtained when the DO concentration was controlled at 50% by adjusting the agitation speed (broken line in Fig. 3). When the data from *A* and *B* are compared, the effect of agitation speed can be evaluated because the DO concentration was the same. Moreover, when you compare the data from *B* and *C*, the effect of DO concentration can be evaluated because the agitation speed profile was almost the same in both cases. From Fig. 4 it can be seen that both agitation speed and DO concentration in the fermentor have significant positive effects on the productivity of virginiamycin and the autoregulator. A positive effect of DO concentration on productivity may not be a new finding but a positive effect of agitation speed on the productivity that is independent of the DO concentration has not been clearly shown previously.

The same results were observed at high DO concentration and agitation speed and are shown in Fig. 5. The columns *A* represent results obtained in the experiment in which the DO concentration was controlled by method 2 (where a change in the additional agitation speed from 450 rpm to 650 rpm was needed during production phase). The columns *B* represent results obtained by method 1. The columns *C* represent results obtained in the experiment in which the DO concentration was controlled only by adjusting agitation speed, and in the latter half of this experiment the DO concentration could not be maintained at 80% but only between 60% and 80%. It is concluded that both agitation speed and DO concentration affect virginiamycin productivity.

Optimal agitation speed

In general, high agitation speeds damage microorganisms and, as a result, the cell concentration and virginiamycin productivity do not increase. Figure 6 shows the results of such an experiment. If the agitation speed was maintained at 1000 rpm from the beginning of the culture, the cell growth was significantly decreased. As a result, the virginiamycin productivity was also significantly decreased (data not shown). However, the agitation speed will enhance virginiamycin productivity if it is increased just in the production phase, as already mentioned.

It was shown that not only the DO concentration but also the agitation speed enhances virginiamycin productivity. However, as already mentioned, very high agitation speeds decrease the cell growth rate. The cell concentration during the production of virginiamycin was related to the productivity of VM and VS. Thus, there may be an optimum agitation speed. In the

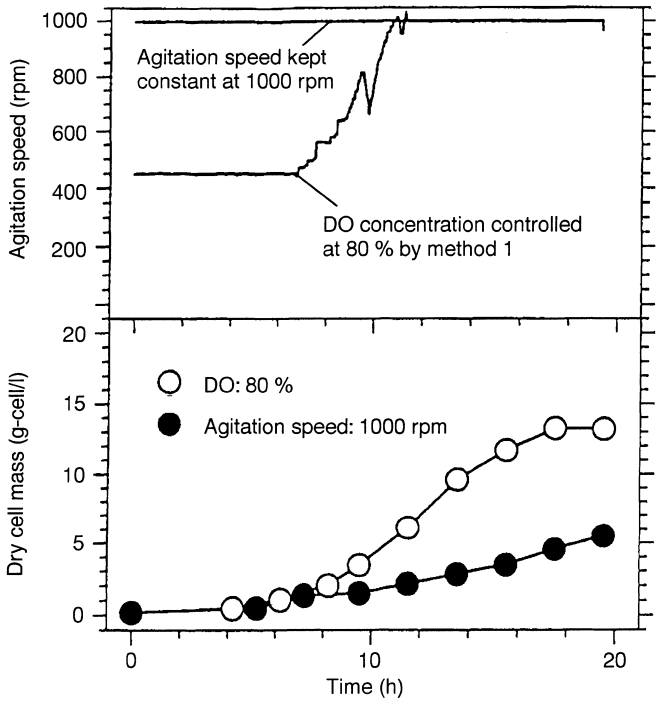

Fig. 6 The influence of high agitation speed on cell growth at the beginning of the batch culture

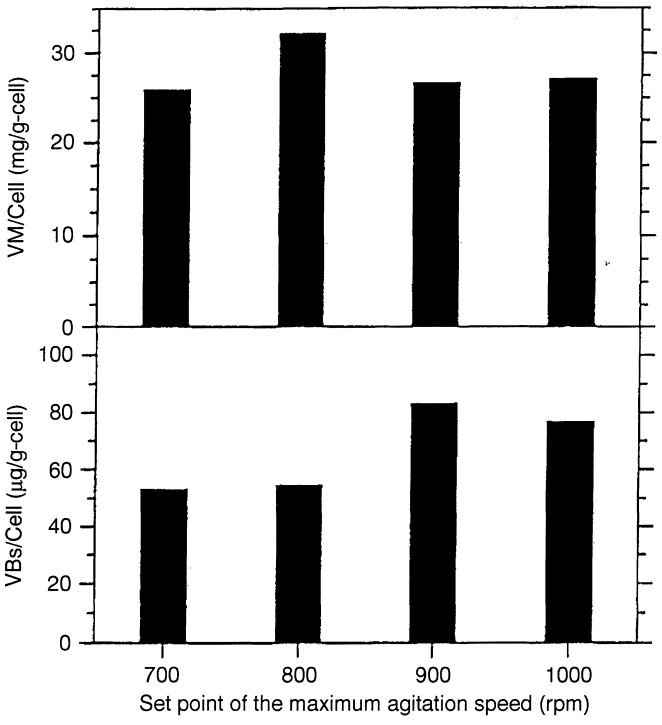

Fig. 7 The effect of the maximum agitation speed on VM and VB productivities where DO concentration was controlled at 80% by method 1. The maximum VM concentrations are (700 rpm) 274 mg/l, (800 rpm) 334 mg/l, (900 rpm) 320 mg/l, (1000 rpm) 296 mg/l (as in Table 1)

experiments described, 1000 rpm was the maximum agitation speed employed in method 1. The effect of this maximum agitation speed on virginiamycin productivity was therefore investigated and the results are shown in Fig. 7. The data on VM productivity were obtained in batch experiments in which the DO concentration was controlled at 80% by method 1 and in which the maximum agitation speed was set at levels between 700 rpm to 1000 rpm. From the figure, the optimum agitation speed can be seen to be 800 rpm. Of course, maintaining an agitation speed as high as 800 rpm from the start of batch culture was not beneficial for cell growth and virginiamycin productivity. Thus, the optimal strategy was to start the culture at 450 rpm and continue until the DO concentration reached 80%. Then, to maintain the DO concentration at this level, method 1 was used with the maximum agitation speed set at 800 rpm.

As well as controlling the DO concentration by method 1, the effect of adding an appropriate amount of the autoregulator VB-C at an appropriate time was investigated. In a previous study, it was found that the optimum auto-regulator concentration per cell (mg/g cells), shown in Fig. 8, was around 73 mg/g cells. The amount of VB was evaluated as the sum of the added VB and the VB produced by the microorganisms. This criterion of an optimum auto-regulator concentration of 73 mg/g cells was also employed here. Figure 8 shows the relationship between the amount of VB (added and produced) per cell and VM productivity. The highest productivity of VM and the maximum production of VM were attained at the desired VB concentration per cell. Finally, upon addition of VB-C, the maximum amount of VM produced was 399 mg/l, which is about 1.8 times the values obtained previously.

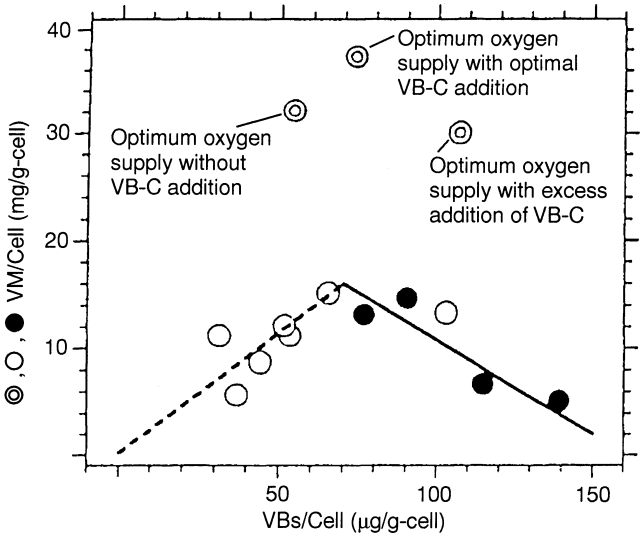

Fig. 8 The relationship between VB and VM productivities. Maximum VM productivity was obtained by VB-C addition and optimum oxygen supply. ◎ DO concentration controlled by method 1 (this study), ○ DO concentration controlled by method 2 (previous study), ● DO concentration not controlled and with a constant agitation speed of 400 rpm (previous study)

Discussion

The autoregulator is now understood to be part of the peptide for signal transduction, which regulates the onset of virginiamycin production. However, the function of the autoregulator is complicated, as its overaddition inhibits virginiamycin production. It was found that the agitation speed affects virginiamycin productivity independently of the DO level. The reason is not yet clear but we speculate that it is due to micromixing occurring around cells, related to the autoregulator attachment to the cell. An appropriate mixing may enhance the attachemnt of the autoregulator, VB, to the cell. The reason for the positive effect of agitation will be investigated in a future study, but we can consider the possible candidates such as (1) a general respiratory and metabolic activity change, including enzyme activity or enzyme formation related to VM and VB biosynthesis, and (2) biodegradation of VM and VB.

The reason why there was an optimum autoregulator concentration per cell (Fig. 8) is also still not clear. Too much VB may stimulate the self-defence mechanism against VM, and an appropriate amount of VB per cell at an appropriate period of cultivation may enhance the VM production. This is also left to a future study.

Finally, mention should be made of the production yields for the different concentrations of yeast extract. The yields based on the DO concentration in each case did not differ greatly. The product concentration and productivity are dependent on DO and agitation conditions. This means that the distribution of metabolic flux was not changed by these conditions.

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