ORIGINAL PAPER

S. Chen · J.-Y. Hong · W.-T. Wu

Fed-batch culture of *Bacillus thuringiensis* based on motile intensity

Abstract The operation of a fed-batch culture is more complicated than that of batch or continuous culture. Thus, an appropriate feeding strategy for fed-batch cultures should be carefully designed. In this study, a simple feeding strategy for fed-batch culture of *Bacillus thuringiensis* based on ''motile intensity'' is described. The feeding strategy consisted of two steps: (1) initiating feeding at the peak of motile intensity; (2) terminating feeding at low motile intensity (or non-motility) of the cells. In addition, the motile intensity of *B. thuringiensis* was used to determine the optimum environmental conditions (pH, temperature, and dissolved oxygen) and optimum medium composition. Using this fed-batch strategy, the production of thuringiensin increased 34% compared with batch culture using the same environmental conditions and medium composition. The proposed strategy for fed-batch culture helps to avoid overfeeding of substrate and facilitates on-line control. A comparison of several alternative strategies for fed-batch culture demonstrated that strategies such as glucose-stat and DO-stat result in a lower productivity than that obtained using the motility intensity method.

Keywords *Bacillus thuringiensis* · Fed-batch culture · Motile intensity

Introduction

Bacillus thuringiensis produces seven kinds of bioinsecticides [3,13,20]. One of these, the water-soluble, thermostable metabolite thuringiensin, is a nucleotide ATP analogue that inhibits the production of DNA-dependent RNA polymerase [7]. Thuringiensin is an exotoxin, and has been used to control Colorado potato beetles [16] and several species of mites [12].

While fed-batch culture is a standard technique in fermentation processes, prevention of substrate inhibition is frequently problematic. Various fed-batch strategies, such as glucose-stat, DO-stat and pH-stat, have been used to cultivate *B. thuringiensis* [4,6]. However, in *B. thuringiensis*, the production of thuringiensin is not limited to a single growth phase or metabolic pathway. Thus, a simple fed-batch strategy based on glucose-stat or DO-stat increases cell density but decreases thuringiensin formation.

The motility of bacteria is an important variable during growth and nutrition [5,9,10,11,14,15], and the rotations of the bacterial flagellum and the energy sources for rotation are interrelated [1,2]. While the metabolism-dependent behavior of motility in bacteria has been well studied [17], the relationship between motility and fermentation process has not been exploited. In this report, the motility of *B. thuringiensis* with respect to a fed-batch process was investigated. A quantitative parameter, ''motile intensity'', defined as the mean specific kinetic energy of the bacteria [18], was used to describe the motility of bacteria. In batch culture of *B. thuringiensis*, motile intensity reaches a maximum between late lag phase and early exponential phase. This observation was used to determine the culture time of the inoculum. The optimum inoculum time occurs when motile intensity is maximum [18]. In addition, the motile intensity of *B. thuringiensis* was used to screen media. Optimum medium composition could be determined in a single drop of media by noting the maximum motile intensity of the cells [19].

In the present study, we used motile intensity to design an optimum strategy for fed-batch culture for the production of thuringiensin in *B. thuringiensis*. The strategy consisted of two steps: (1) initiating feeding at maximum motile intensity using an optimum substrate concentration control, (2) terminating feeding at low motile intensity (or non-motility) of the cells. In addi-

S. Chen · J.-Y. Hong · W.-T. Wu (✉)
Department of Chemical Engineering,
National Tsing Hua University,
30043 Hsinchu, Taiwan
E-mail: wtwu@mx.nthu.edu.tw
Fax: +886-3-5728212

tion, the droplet medium-selection method was used to establish optimum environmental conditions and optimum substrate concentration. The results show that the proposed method increases the production of thuringiensin by avoiding overfeeding during growth.

Materials and methods

Strain

Bacillus thuringiensis subst. *darmstadiensis*, HD-199 (provided by Dr. de Barjac, Institute Pasteur, Paris, France) was used in this study.

Cultivation

The pre-culture was carried out in 1-l Erlenmeyer flasks containing 250 ml of medium at 30 °C on a rotary shaker (Firstech, Taiwan) at 200 rpm for 5 h. The main culture was incubated in a 2-l stirred tank fermentor (MD-250, LE Marubishi, Japan) operated at an air flow rate of 3.4 vvm and an agitation rate of 500 rpm. The working volume was 0.88 l. The pH and temperature were varied between 5.0 and 7.0, and between 25 and 37 °C, respectively.

Medium

Cells were cultivated in two different media, a pre-culture medium and a main-culture medium. The pre-culture medium contained (per l distilled water): 5 g yeast extract (Merck, analytical grade); 8 g nutrient broth (Merck, analytical grade), 5 g K_2HPO_4, 5 g KH_2PO_4, 1.5 g $NaNH_4PO_4$, 0.01 g $MnSO_4$ $4H_2O$, 0.01 g Mg SO_4 $7H_2O$, 0.01 g Fe SO_4 $7H_2O$, and 0.01 g $CaCl_2$ $4H_2O$. The main-culture medium contained (per l distilled water): 2 g yeast extract (Merck, analytical grade), 4 g nutrient broth (Merck, analytical grade), 5 g K_2HPO_4, 5 g KH_2PO_4, 1.5 g $NaNH_4PO_4$, 0.01 g $MnSO_4$ $4H_2O$, 0.01 g Mg SO_4 $7H_2O$, 0.01 g Fe SO_4 $7H_2O$, 0.01 g $CaCl_2$ $4H_2O$. The glucose and ammonium sulfate concentrations were varied from 10 to 20 g l^{-1}. The feed consisted of glucose and ammonium sulfate 100 g l^{-1}, each.

Assays

Thuringiensin was assayed by HPLC using an Intertsil 7 ODS-3 column (4.5 mm×15 cm) and following the procedure of Levinson et al. [8] with modifications. The mobile phase was 50 mM KH_2PO_4/ H_3PO_4 (pH 2.8) at 1.0 ml/min.

Image analysis

The movement of *B. thuringiensis* was recorded with a CCD-camera (Panasonic NV-DJ1, Japan) attached to a microscope (Olympus BX40, Japan). The optical magnification of the microscope was 100× (10×10) and the digital magnification of CCD-camera was 10×. The final magnification was thus 100×10.

Prior to microscopy, *B. thuringiensis* cells were diluted to facilitate their observation under the microscope. Measurements were randomly taken in seven sections and each section was filmed for 10 s.

Image analysis was carried out using a computer with an image-capture card. The image from the CCD-camera was saved in the computer with a resolution of 76,800 (320×240) pixels and 256 gray values. The image analysis program was written with WiT software (Logical Vision, Canada. A threshold operation was also applied

for segmentation, which gave a gray correction. Using the image analysis program, the cross-sectional area and the coordinates of the geometric center of each cell in terms of pixels were measured. The data application program, which was written with C + +, allowed determination of the cell cross-sectional area, the shift in the geometric center, and the motile intensity of cells. The shift in the geometric center of a cell was calculated on the basis of two consecutive images taken at a 0.2-s interval.

Motile intensity

The motile intensity I_M is defined as the specific mean kinetic energy of the cells in terms of their cross-sectional area. The kinetic energy was calculated based on the assumption that the density and thickness of living cells are constant. Since the cross-sectional area and biomass of cells have a linear relationship, the assumption is appropriate [18]. The motile intensity is expressed as:

$$I_M = \frac{\sum \frac{1}{2} A_i V_i^2}{\sum A_i} \tag{1}$$

where A_i is the cross-sectional area of the i cell and V_i denotes the moving of geometric center of the i cell. Measurements of the geometric center and the cross-sectional area of the cells were based on the image analysis.

Droplet medium selection

Selection by the droplet medium method was described previously [19]. Briefly, the bacteria are pre-cultured until the motile intensity of the cells is nearly maximal. Subsequently, the cells are inoculated into the testing medium and the motile intensity of the cells is measured. Since the measurements are completed within a few minutes, the oxygen effect for the cells is not a limiting factor. The criterion for selection of the medium is based on the motile intensity value. A low value implies that the medium is not suitable for cultivation of the cells [19]. In order to study the medium composition and environmental conditions such as temperature, pH, and dissolved oxygen (DO), the testing medium was controlled at the desired value. For example, DO was controlled by using air and nitrogen gas to manipulate the DO in the flasks at the desired value.

Results

Environmental conditions and media composition

Figure 1 shows the effect of pH on the motile intensity of the cells and on thuringiensin production after 24 h of culture. Thuringiensin production was measured in batch fermentation in a stirred tank. The optimum pH was around 7.0 for both motile intensity and thuringiensin production. The similarity of the curves of motile intensity and thuringiensin production indicated that motile intensity might be representative of thuringiensin production as well.

The optimum growth temperature based on the motile intensity of the cells and measured by the droplet medium selection technique was 30 °C. The effect of DO levels in the medium on motile intensity is shown in Fig. 2. In this experiment, motile intensity of the bacteria was measured at various DO levels. The minimum DO value of 40% was selected based on the motility intensity of the cells. The optimum conditions of pH 7.0,
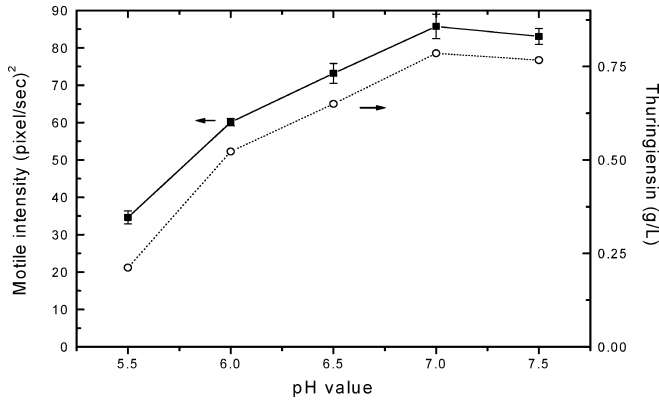
Fig. 1 Effect of the pH of the medium on the motile intensity of *Bacillus thuringiensis* cells after 5 h of preculture, and on thuringiensin production after 24 h of cultivation
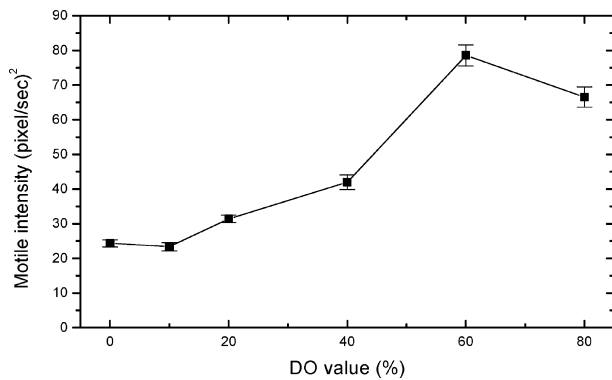

Fig. 2 The effect of the dissolved oxygen concentration of the medium on the motile intensity of *B. thuringiensis* cells as determined by the droplet medium selection method after 5 h of preculture

temperature of 30 °C and a DO of 60% were used in all further experiments.

The optimum medium for growth of *B. thuringiensis* was also determined using the droplet medium selection technique and different concentrations of glucose and $(NH_4)_2SO_4$ (Table 1). Both glucose and $(NH_4)_2SO_4$ were optimum at 15 g/l.

Fed-batch culture based on the motile intensity of *B. thuringiensis*

A typical time-course of motile intensity and the specific glucose consumption rate of *B. thuringiensis* are shown in Fig. 3. Maximum specific glucose consumption occurred at the same time as the maximum motile intensity of the cells. Therefore, the motile intensity of *B. thuringiensis* could serve as an index for predicting the specific glucose consumption rate.

Figure 4 compares a fed-batch fermentation with a batch fermentation in a stirred tank. For the fed-batch fermentation, the glucose concentration was controlled at 15 g/l starting at the moment when the motile inten-

Table 1 Droplet medium design with different carbon and nitrogen sources

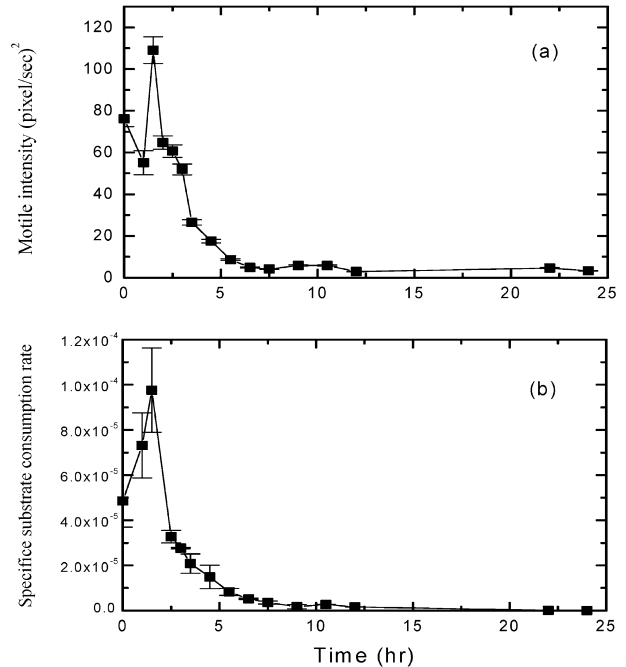| Carbon and nitrogen sources (g/l)	Motile intensity $(pixel/s)^2$
Glucose (10), $(NH_4)_2SO_4$(10)	28.41
Glucose (10), $(NH_4)_2SO_4$(15)	2.23
Glucose (10), $(NH_4)_2SO_4$(20)	1.59
Glucose (15), $(NH_4)_2SO_4$(10)	39.75
Glucose (15), $(NH_4)_2SO_4$(15)	50.43
Glucose (15), $(NH_4)_2SO_4$(20)	9.61
Glucose (20), $(NH_4)_2SO_4$(10)	15.48
Glucose (20), $(NH_4)_2SO_4$(15)	39.46
Glucose (20), $(NH_4)_2SO_4$(20)	46.97

Fig. 3 The time courses of **a** motile intensity and **b** the specific glucose consumption rate during cultivation of *B. thuringiensis*

sity of the cells approached the maximum (~hour 2). The feeding medium was a mixture of glucose and ammonium sulfate (100 g/l, each). Feeding was terminated when the cells reached a low motile intensity (~hour 7). This operation is designated as case A. The motile intensity of the cells increased when the medium was fed. Moreover, the motility time of the cells was also extended. However, the motility time of *B. thuringiensis* could not be further extended after reaching the low motile intensity period even if more substrate was fed into the bioreactor. Figure 3 shows that the specific glucose consumption rate of cells was also weak when the motility was low. Thus, termination of feeding at low motility avoids overfeeding of the substrate. Typical time courses of the cross-sectional area of the cells are shown in Fig. 4b. The cross-sectional area of the cells in case A was almost the same as that of the batch culture before the post-exponential growth phase. Since the cross-sectional area of the cells and the cell concentra-
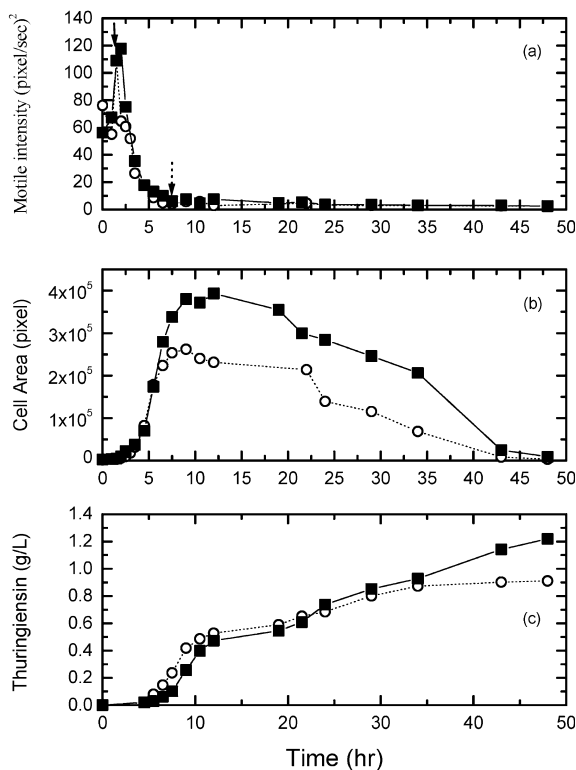
Fig. 4 The typical time courses of **a** motile intensity, **b** the cross-sectional area of the cells, c thuringiensin production during cultivation of *B. thuringiensis*. . ■ Fed-batch culture of case A, ○ batch culture with glucose and ammonium sulfate (15 g/l)

Fig. 5 The typical time courses of **a** motile intensity, **b** the cross-sectional area of the cells, c) thuringiensin production during cultivation of *B. thuringiensis*. . ● Fed-batch culture of case B, ○ batch culture with glucose and ammonium sulfate (15 g/l)

tion have a liner relationship, the maximum cell concentration of case A increased up to 50% compared to the batch culture after the exponential growth phase. The typical time courses of the production of thuringiensin during cultivation are shown in Fig. 4c. In case A, thuringensin production increased by 34% (to 1.129 g/l) compared to the batch culture.

Figure 5 shows the results of an experiment similar to that shown in Fig. 4 but with the feeding time adjusted to later in the motile intensity period. This operation is designated as case B. The time courses of the motile intensity of case B cells and cells of the batch culture during cultivation are shown in Fig. 5a. Compared with cells of case A, the motile intensity of case B cells did not increase significantly when substrate was fed. Furthermore, the motile time of case B cells was not extended. Figure 5b shows the typical time courses of the cross-sectional area of the cells during cultivation. The initial increase of the cross-sectional area of case B cells was almost the same as that of cells from the batch culture. By comparing case A and case B, the initial increase in the cross-sectional area of case B cells was far less than that of case A cells. The time courses of thuringensin production during cultivation are shown in Fig. 5c. Thuringensin production increased by only 10.5% in fed-batch culture compared to the batch culture. A comparison of Figs. 4c and 5c shows that case A cells produced higher amounts of thuringensin than case B
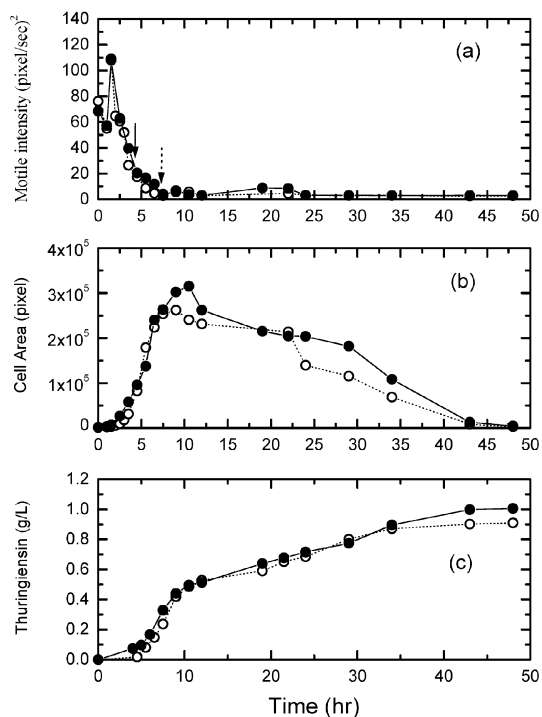
cells. Therefore, the timing of feeding is essential for fed-batch cultures of *B. thuringiensis*, and the motile intensity of the cells may serve as an index for establishing the initial feeding time of these cultures.

Different strategies of fed-batch culture, such as glucose-stat and DO-stat, were also investigated. In the simple glucose-stat fed-batch culture, substrate was fed after glucose was consumed, and the glucose concentration was controlled at 15 g/l. In the DO-stat fed-batch cultures, substrate was fed so as to keep the DO value under 60%. The major difference between these strategies is the timing of initial feeding. Figure 6 shows the typical time courses of thuringiensin production during cultivation. Fed-batch cultures based on the motile intensity of the cells produce the largest amounts of thuringiensin. Other strategies of fed-batch culture reduce thuringiensin production even if the amount of feeding is the same. Thus, a fed-batch culture strategy based on the motile intensity of the cells results in higher productivity than obtained using other strategies of fed-batch culture.

Discussion

Selection by the droplet medium method not only results in selection of optimum medium composition, but also of environmental conditions suitable for cultivation. Furthermore, this method is relatively fast and inexpensive and can thus be readily applied to determining optimal culture conditions..
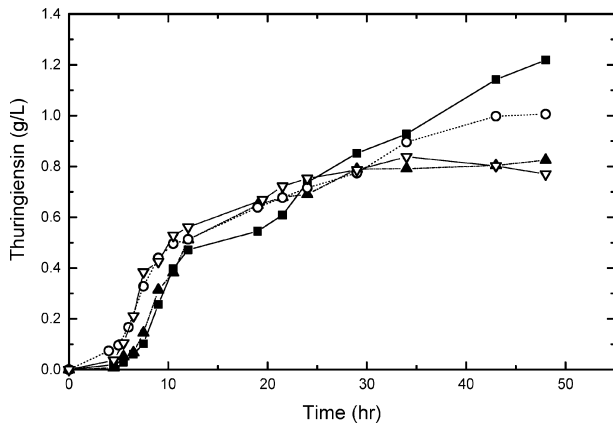
Fig. 6 The time courses of thuringiensin production using different strategies of fed-batch culture. ■ Fed-batch culture of case A, ○ fed-batch culture of case B, ▲ fed- batch culture of glucose stat, ▽ fed-batch culture of dissolved oxygen (DO)-stat

The strategy of fed-batch culture based on the motile intensity of the cells is an economical method for the production of thuringiensin. Based on the motile intensity, the initiation and termination of feeding can be determined. A suitable feeding strategy is the key point in fed-batch cultures. Even though we do not understand the complex metabolic pathways used by the culture, we have found that motility can be used as an indicator to optimize the fed-batch process. Motile intensity acts as an indicator of cellular activity during cultivation, for example, the specific glucose consumption rate of the cells. When motile intensity is high, the specific glucose consumption rate is also high. Consequently, the motile intensity can be used to design a fermentation process based on the motile intensity of cells consisting of the culture time of the inoculum, optimum medium composition, suitable environmental conditions for growth, and fed-batch culture.

Reference

1. Albertini AM, Caramori T, Crabb WD, Scoffone F, Galizzi A (1991) The *fla* A locus of *Bacillus subtilis* is part of a large operon coding for flagellar structures, motility functions, and ATPase-like Polypeptide. J Bacteriol 173:3573–3579
2. Alexandre G, Greer SE, Zhulin IB (2000) Energy taxis is the dominant behavior in Azospirillum brasilense. J Bacterol 182:6042–6048
3. Arcas J, Yantorno O, Ertola R (1987) Effect of high concentration of nutrients *Bacillus thuringiensis* on cultures. Biotechnol Lett 9:105–110
4. Jong JZ, Hsiun DY, Wu WT (1995) Fed-batch culture of *Bacillus thuringiensis* for thuringiensin production in a tower type bioreactor. Biotechnol Bioeng 48:207–213
5. Korber DR, Lawrence JR, Caldwell DE (1994) Effect of motility on surface colonization and reproductive success of *Pseudomonas fluorescens* in dual-dilution continuous culture and batch culture systems. Appl Environ Microbiol 60:1421–1429
6. Lee J, Lee SY, Park S, Middelberg APJ (1999) Control of fed-batch fermentations. Biotechiol Adv 17: 29–48
7. Lecadet MM, Blondel MO, Ribier J (1980) Generalized transduction in Bacillus thuringiensis var Berliner-1715 using bacteriophage-CP-54Ber. J Gen Microbio 121: 203–212
8. Levinson B, Kasyan KJ, Chiu SS (1990. Identification of β-exotoxin production, plasmids encodingβ-exotoxin and a new exotoxin in by HPLC. J Bacteriol 172: 3172–3179
9. Martinez A, Torello S, Kolter R (1999) Sliding motility in Mycobacteria. J Bacteriol 181:7331–7338
10. Mendelson NH, Bourque A, Wilkening K, Anderson KR, Watkins JC (1999) Organized cell swimming motions in *Bacillus subtilis* colonies: patterns of short-lived whirls and jets. J Bacteriol 181:600–609
11. Ormonde P, Hörstedt P, O'Toole R, Milton DL (2000) Role of motility in adherence to and invasion of a fish cell line by *Vibrio anguillarum*. J Bacteriol 182: 2326–2328
12. Paige MR, Cooper RD (1990) Scale-up of beta exotoxin production in fed batch *Bacillus thuringiensis* fermentation. Eur Congr Biotechnol 5th Meeting, pp 146–159
13. Pearson D, Ward OP (1988) Effect of culture conditions on growth and sporulation of *Bacillus thuringiensis* subsp. *israelensis* and development of media for production of the protein crystal endotoxin. Biotechnol Lett 10:451–456
14. Phillips BR, Quinn JA, Goldfine H (1994) Random motility of swimming bacteria: single cells compared to cell populations. AIChE J 2:334–348
15. Romagnoli S, Armitage JP (1999) Roles of chemosensory pathways in transient changes in swimming speed of *Rhodobacter sphaeroldes* induced by changes in photosynthetic electron transport. J Bacteriol 181:34–39
16. Tanigoshi LK, Mayer DF, Babcock HM (1990) Efficacy of the β-exotoxin of *Bacillus thuringiensis* to Lygus hesperus. J Econ Entomol 83: 2200–2206
17. Taylor BL, Zhulin LB (1998) In search of higher energy: metabolism-dependent behavior in bacteria. Mol Microbiol 28:683–690
18. Wu WT, Chen S (2001)An index for determining the culture time of inoculum in cultivation of *Bacillus thuringiensis*. Biotechnol Lett 23:1201–1204
19. Wu WT, Chen S, Hong JY (2001) A droplet medium selection for cultivation of *Bacillus thuringiensis*. Biotechnol Prog 17:778–780
20. Zouari N, Jaoua S (1999) The effect of complex carbon and nitrogen, salt, Tween-80 and acetate on delta-endotoxin production by a *Bacillus thuringiensis* subsp kurstaki. J Ind Microbiol Biotechnol 23:497–502