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Study on Fermentation Kinetics and Optimization of Feeding Strategy for Menaquinone-7 (Vitamin K2) Production by Bacillus Subtilis

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Abstract. Menaquinone-7 (MK-7), a biologically active form of vitamin K2,plays a critical role in preventing osteoporosis and cardiovascular calcification, making it a valuable component in dietary supplements. This study investigated the fermentation process of *Bacillus subtilis* BS018, which produces MK-7, by integrating fermentation kinetics. Based on this, a feeding strategy was developed and validated at the 5 L bioreactor, and fermentation experiments were conducted in a 1 T bioreactor. The selected fermentation kinetic model effectively described the fermentation process of BS018, with kinetic parameter analysis indicating that MK-7 is a non-growth-associated product. In the 5 L bioreactor, the MK-7 yield of the batch supplemented with 10g/L (grams of added glycerol per volume of fermentation broth) glycerol at 24 hours of fermentation was 1.72 times that of the non-fed batch. At the 1-ton bioreactor, the MK-7 yield reached 72.65 mg/L.

Keywords: Vitamin K2, Bacillus subtilis, Kinetic Model, Feeding Strategy.

1. Introduction

Menaquinone-7 (MK-7) is a biologically active form of fat-soluble vitamin K2, serves as a cofactor of γ-carboxylase, participating in the activation of vitamin K-dependent proteins, such as inactive osteocalcin. Vitamin K2 deficiency in humans is associated with the development of diseases such as osteoporosis [1]. Adequate intake of vitamin K2 is beneficial for preventing or alleviating osteoporosis, cardiovascular calcification, cognitive disorders and diabetes [2]. Among vitamin K homologs, MK-7 has a longer half-life in serum and enhanced bioavailability [3], rendering it more advantageous as a dietary supplement.

The microbial synthesis of MK-7 presents significant advantages, including high bioactivity and safety compared to chemical synthesis [4], indicating promising application prospects. The primary microorganisms utilized for MK-7 production include Bacillus subtilis, Bacillus subtilis natto, Bacillus licheniformis, and Flavobacterium [5]. Notably, Bacillus subtilis is generally considered the most promising strain for MK-7 production owing to its non-pathogenicity, rapid growth rate, and relative ease of genetic manipulation [2].

Research methods to enhance MK-7 production by Bacillus subtilis fermentation include mutagenesis breeding, modifying membrane permeability [6-8], using biofilm reactors for MK-7 production [9], optimizing extraction processes, and developing Bacillus subtilis chassis cells [10-11]. However, studies on the fermentation kinetics of Bacillus subtilis for MK-7 production remain unexplored. By integrating fermentation kinetics, establishing kinetic models, and deeply analyzing the biological significance of kinetic parameters in the kinetic models, we can further understand the mechanisms of the fermentation process. This understanding can guide the optimization of fermentation strategies and effectively increase the yield of MK-7.

This study focuses on Bacillus subtilis BS018 [12], a strain developed through molecular modification in our previous work. MK-7 fermentation experiments were conducted in 500 mL

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shake flasks and 5 L bioreactor. By integrating fermentation kinetics, the study emphasizes the fermentation characteristics of the strain in terms of biomass growth and product synthesis. Based on these characteristics, a feeding strategy was formulated and preliminarily validated in a 1-ton bioreactor. This research provides theoretical insights for further increasing MK-7 production and scaling up fermentation processes in the future.

2. Methods

2.1 Microorganisms and Medium

The strain used in this study was Bacillus subtilis BS018, preserved in our laboratory. The seed medium composition: 10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract. The fermentation medium composition: 20.65 g/L glycerol, 20 g/L sucrose, 47.38 g/L soy peptone, 4 g/L yeast extract, 1.88 g/L K2HPO4. The fermentation medium was optimized for BS018 fermentation using response surface methodology in our laboratory (unpublished work).

2.2 Cultivation Methods

Seed culture: The activated strain was inoculated into a 250 mL shake flask containing 50 mL of seed medium and cultured at 37°C, 220 r/min for 12 h to obtain the primary seed culture. Subsequently, 10% (v/v) of the primary seed culture was transferred to fresh seed medium (50 mL/250 mL) and cultured under identical conditions to obtain the secondary seed culture.

Fermentation culture: The secondary seed culture was inoculated into the fermentation medium at a 10% (v/v) inoculation rate. When using shake flasks as fermentation reactors, 500 mL shake flasks were filled with 50 mL of medium and fermented at 40°C, 250 r/min for 6 days. When using fermenters as fermentation reactors, a 5 L bioreactor (Eppendorf, Germany) was filled with 3 L of medium and fermented at 40°C, 400 r/min, and 2 vvm. Additionally, a 1-ton bioreactor (Bailun, China) was filled with 700 L of medium and fermented at 40°C, 120 r/min, and 0.8 vvm.

2.3 Analytical Methods

Biomass determination: Cell density was measured using a UV spectrophotometer at a wavelength of 600 nm. Biomass was calculated based on the linear relationship between dry cell weight (DCW) and OD_{600}, as follows:

$$DCW = 0.35 \times OD_{600}\# \quad (1)$$

Glycerol concentration determination: The supernatant of the fermentation broth, obtained by centrifugation (9000×*g*, 6 min), was diluted to an appropriate concentration, filtered through a 0.22 μm membrane, and analyzed for glycerol concentration using a refractive index detector. The column temperature was set at 50°C, the injection volume was 20 μL, the flow rate was 0.5 mL/min, and the mobile phase was 5 mM H_2SO_4.

MK-7 determination: 2 mL of MK-7 extraction solution (n-hexane: isopropanol = 2:1, *v/v*) was added to 1 mL of fermentation broth, followed by ultrasonication for 30 min and vortexing for 30 min. Then, 1 mL of n-butanol was added to the mixture, vortexed for 30 min, filtered through a 0.22 μm membrane, and transferred to a brown HPLC sample vial for analysis. HPLC (Shimadzu, Japan) was employed for detection, with the column temperature set at 35°C, flow rate at 1 mL/min, injection volume at 20 μL, UV detection wavelength at 248 nm, and mobile phase as methanol: dichloromethane (4:1, *v/v*).

2.4 Fermentation Kinetic Model Construction

2.4.1 Biomass growth

The Logistic equation was initially selected to construct the biomass growth kinetic model. This equation accounts for the effects of both limiting and non-limiting substrates on biomass growth,

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making it superior to the classical Monod equation, which only considers the effect of limiting substrates. The specific expression is as follows:

$$\frac{dX}{dt} = \mu_m X\left(1 - \frac{X}{X_m}\right)\# \quad (2)$$

Where X is the biomass concentration ($g \cdot L^{-1}$), μ_m is the maximum specific growth rate ($g \cdot g^{-1} \cdot h^{-1}$), X_m is the maximum biomass concentration ($g \cdot L^{-1}$), and t is time (h).

Since the MK-7 fermentation cycle of *Bacillus subtilis* is prolonged, and the cells enter the decline phase during the mid-fermentation period without feeding, the Logistic equation was modified to better describe the biomass growth process by considering the effect of cell death [13]:

$$\frac{dX}{dt} = \mu_m X\left(1 - \frac{X}{X_m}\right) + \int_0^t K_0 X(t)dt\ \# \quad (3)$$

Where K_0 is a constant related to cell death.

2.4.2 Product accumulation

The classical Luedeking-Piret equation was selected to construct the product accumulation kinetic model. The specific expression is as follows:

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X\# \quad (4)$$

Where P is the product concentration ($mg \cdot\ L^{-1}$), α is the correlation coefficient between product accumulation and biomass growth, and β is the correlation coefficient between product accumulation and biomass. When α=0 and $\beta \neq 0$, product accumulation is non-growth-related; when $\alpha \neq 0$ and β=0, product accumulation is growth-related; and when $\alpha \neq 0$ and $\beta \neq 0$, product accumulation is partial-growth-related.

The solution of Eq. (2) was obtained by fitting the experimental data to the equation using the Nonlinear Curve Fit tool in Origin, and the optimal parameters were determined through multiple iterations employing the least squares method. The solution of Eq. (3) was obtained by fitting the data using Python in Jupyter Notebook.

3. Results and Discussion

3.1 Analysis of Fermentation Kinetics

The growth process of *Bacillus subtilis* BS018 in a 500 mL shake flask can be divided into three stages: the logarithmic growth phase from 0 to 24 hours, the stationary phase from 24 to 84 hours, and the decline phase after 84 hours, as shown in Fig. 1(A). After 108 hours of fermentation, a slight increase in biomass was observed, which may be attributed to significant evaporation due to the prolonged fermentation period. The weight difference of the 500 mL shake flask between the end and beginning of fermentation was considered as the amount of water evaporated from the system. Assuming the density of water to be 1 g/mL, the experimental data were corrected to obtain Fig. 1(B).

After correcting for evaporation, the cell growth curve more closely follows the classical pattern of logarithmic, stationary, and decline phases. The rate of decrease in biomass slows down at the end of the decline phase, likely because the high evaporation rate in the later stages of fermentation increases the contact area between the liquid surface and air. This enhanced contact is more conducive to the growth of the aerobic bacterium BS018, thus partially offsetting cell death and showing a slowdown in the decrease of biomass at the end of the decline phase.

After 84 hours, the cells enter the decline phase. Therefore, the experimental data from 0 to 84 hours were fitted using Eq. (2), yielding the parameters X_0=0.27, X_m=5.01, μ_m=0.41, R^2=0.99. The fitting results, presented in Fig. 1(C), the Logistic equation can be used to describe the biomass growth process in 0 to 84 hours.

When biomass growth transitions from the logarithmic phase to the stationary phase, MK-7 begins to accumulate. The rate of increase in MK-7 concentration generally decreases over time, reaching a yield of 41.52 mg/L. The experimental data from 0 to 84 hours were fitted using Eq. (4),

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yielding α=0, β=0.090, R^2=0.98. The fitting results are shown in Fig. 1(D), and the MK-7 accumulation during the 0-84 hour period of BS018 fermentation conforms to the non-growth-related type of the Luedeking-Piret equation. This is consistent with the characteristics of MK-7 as a secondary metabolite. After 84 hours, biomass decreases, while MK-7 shows a fluctuating upward trend. This phenomenon possibly due to the deteriorating fermentation environment in the later stages, which stimulating cell respiration. As MK-7 is an important component of the respiratory chain [14], its synthesis is accordingly enhanced.

Regarding substrate consumption, since previous molecular modifications enhanced the glycerol uptake pathway in BS018, glycerol was selected as the key substrate to plot its process curve. It was observed that glycerol was rapidly consumed during the logarithmic growth phase and the early stationary phase, being depleted by 36 hours. It is hypothesized that the main nutrient source for cell maintenance thereafter shifts to the nitrogen source in the medium.

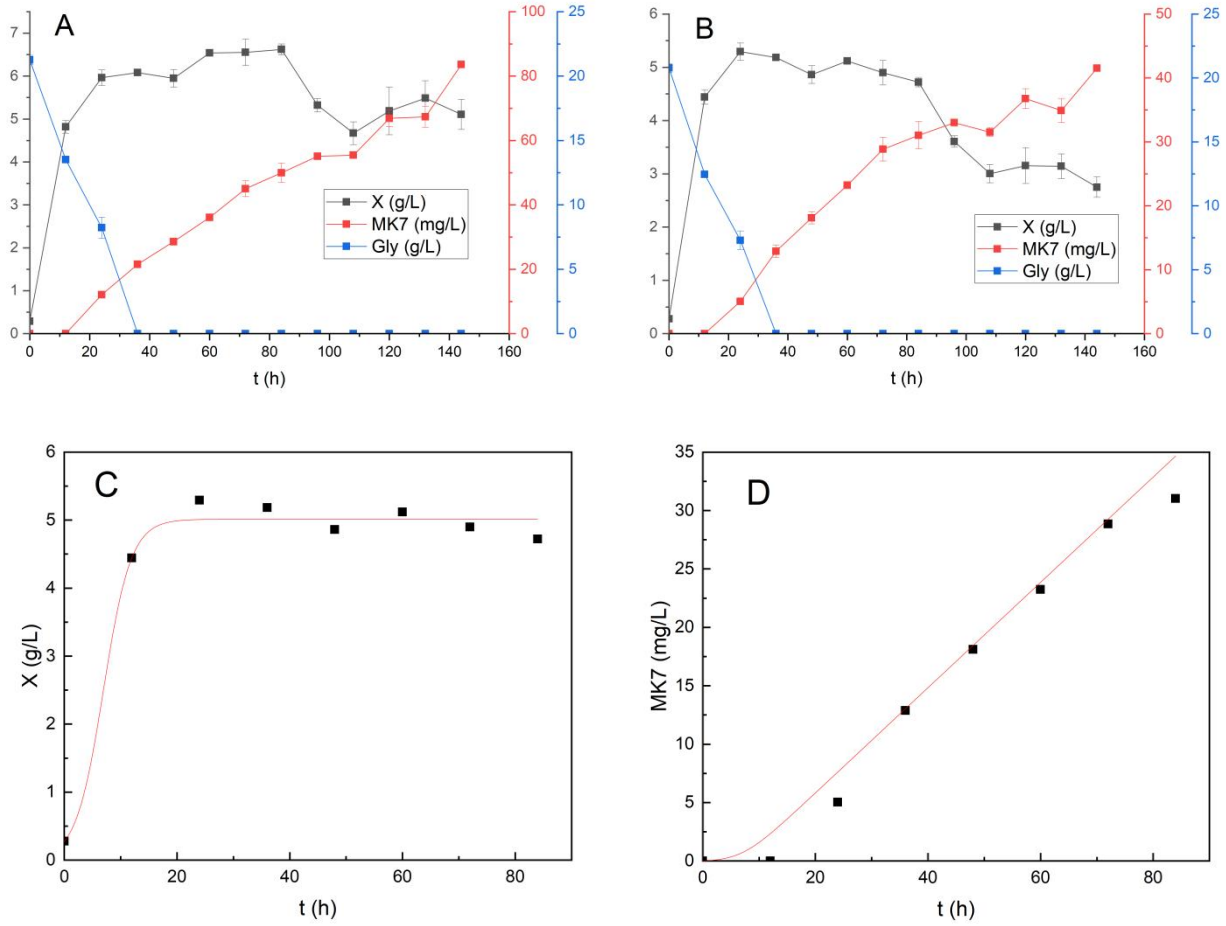

Fig.1 The curves of biomass growth, MK-7 accumulation, and glycerol consumption during the fermentation process in a 500mL shaking flask. (A) Measured values, (B) Corrected values, (C) Fitted results of biomass growth curve, (D) Fitted results of MK7 accumulation curve.

The fermentation scale was expanded, and experiments were conducted in a 5 L bioreactor. Assuming the fermenter to be an ideal cylinder, the volume ratio of the fermentation system at the start to that at the sampling time was calculated based on the change in liquid level height. The experimental data were corrected, with the results shown in Fig. 2. The biomass growth was observed to be in the logarithmic phase from 0-24 hours, began to decline after a 12 hours stationary phase. MK-7 accumulate began at 24 hours, and its production stabilized after 84 hours, reaching a maximum concentration of 21.79 mg/L at 108 hours, while glycerol was exhausted at 48 hours.

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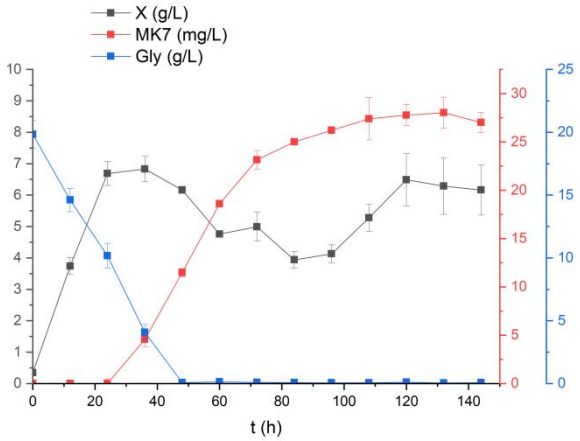

Fig. 2 Curves of biomass growth, MK-7 accumulation, and glycerol consumption during the fermentation process of MK-7 in a 5 L bioreactor.

Due to the noticeable decline in the biomass growth curve within 84 hours, Eq. (3) was selected to fit the data from the 5 L bioreactor. To compare the differences in biomass growth characteristics between the shake flask and the fermenter, Eq. (3) was also used to fit the data from the 500mL shake flask, as shown in Fig. 2. The specific kinetic parameters are listed in Table 1. The maximum biomass (X_m) of *Bacillus subtilis* BS018 in the 5 L bioreactor was higher than that in the 500 mL shake flask, which can be attributed to the greater environmental carrying capacity in the bioreactor. However, the stationary phase of the cells in the bioreactor was shorter, entering the decline phase at 48 hours, with a more pronounced trend of cell death. MK-7 accumulation essentially ceased by 72 hours. Notably, MK-7 primarily accumulated during the stationary phase, and it is proposed to enhance MK-7 production by supplementing glycerol to provide the necessary substrate for biomass maintenance.

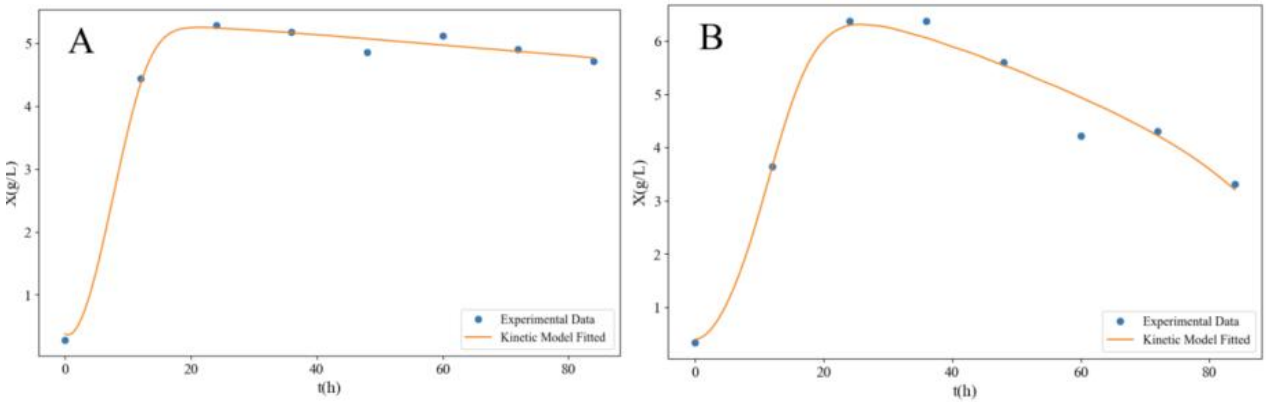

Fig. 3 Fitting results of the modified Logistic equation for fermentation processes (A) 500 mL shake flask (B) 5 L bioreactor

Table 1. Kinetic parameters and R-squared values of MK-7 fermentation bacterial growth in 500 mL shake flask and 5 L bioreactor

Group	μ_m(h^{-1})	X_m(g L^{-1})	K_0(h^{-2})	R^2
shake flask(500mL)	0.37	5.49	-0.00056	0.99
Bioreactor (5L)	0.28	7.24	-0.0016	0.97

3.2 Glycerol Feeding Fermentation in 5 L bioreactor

To increase MK-7 yield, it is essential to increase the biomass in the early stage of fermentation and extend the stable phase. As the fermentation medium has been optimized and it is not advisable to further increase the initial glycerol concentration, glycerol feeding was chosen to be added at 24

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hours. Based on the glycerol consumption process in the non-fed batch fermentation, three batches of glycerol feeding fermentation were conducted, with glycerol added at concentrations of 5 g/L, 10 g/L, and 15 g/L (grams of added glycerol per volume of fermentation broth), respectively. The fermentation process curves are shown in Fig. 4.

Among the batches with glycerol addition, batch-1 had the lowest amount of added glycerol, and the microbial growth trend was similar to that of the non-fed batch, yet achieved relatively higher biomass and MK-7 yield. Batch-3 with the highest glycerol addition, which significantly extended the stable growth phase, however, the increase in MK-7 yield compared to the non-fed fermentation was relatively modest. Batch-2, supplemented with 10 g/L glycerol at 24 hours, achieved the highest MK-7 yield, reaching 37.51 mg/L, representing a 1.72-fold increase compared to the non-fed batch.

Therefore, glycerol feeding is an effective means of increasing MK-7 yield. By maintaining or increasing microbial biomass through feeding, MK-7 accumulation can be prolonged during the later stages of fermentation. However, excessive glycerol addition may redirect the metabolic flux to other pathways rather than the MK-7 synthesis pathway, resulting in lower MK-7 yield.

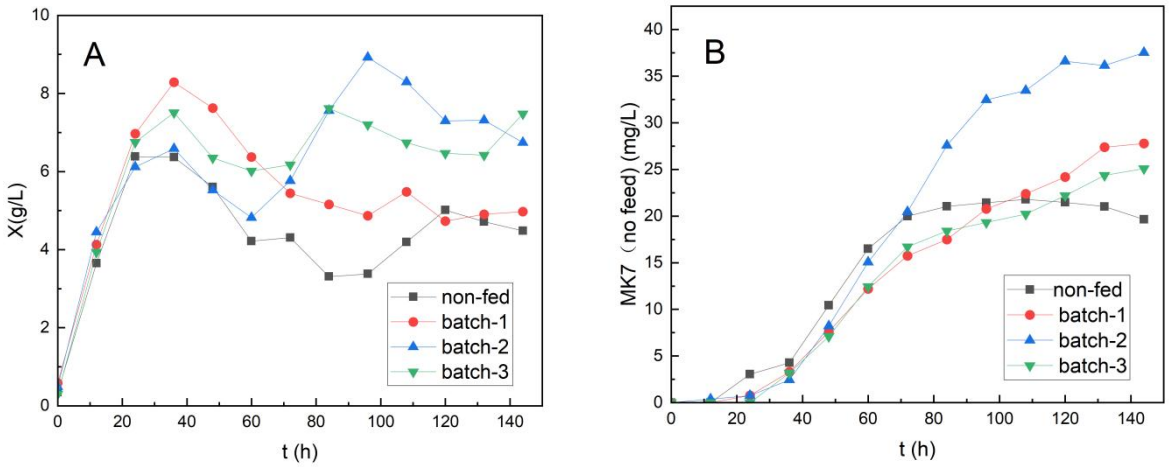

Fig. 4 MK-7 yield of non-fed and fed-batch fermentations in the 5 L bioreactor

3.3 Glycerol Feeding Fermentation in 1 Ton Bioreactor

Two batches of MK-7 fermentation were carried out in the 1-ton fermenter, and the process curves of microbial growth and MK-7 accumulation are shown in the Fig. 5. In the first batch, 10 g/L glycerol was supplemented at 24 hours. The biomass growth entered the stationary phase within 24 hours and persisted until 54 hours, after which a secondary growth phenomenon occurred, with the maximum biomass reaching 18.06 g/L. This may be attributed to the depletion of glycerol, leading the microorganisms to utilize the nitrogen source in the medium for growth. MK-7 was primarily accumulated during the 0–96 hour period, with the curve plateauing after 96 hours and reaching a maximum value of 49.17 mg/L at 114 h.

In the second batch, glycerol was similarly supplemented at 24 hours. Biomass growth entered the stationary phase and persisted until 96 hours. This may be because, compared to the first batch, the biomass was larger when the stationary phase was reached in the second batch, and more substrate were consumed to maintain the biomass, which did not support secondary growth. Given that the MK-7 concentration in the first batch exhibited no significant increase after 96 hours, 10 g/L glycerol was added again at 96 hours in the second batch. After the second glycerol supplementation, the biomass increased, and the MK-7 concentration also rose slightly, reaching a maximum value of 72.65 mg/L at 132 hours, representing a 1.48-fold increase compared to the first batch. This suggests that a multi-time-point feeding strategy could be explored in future studies.

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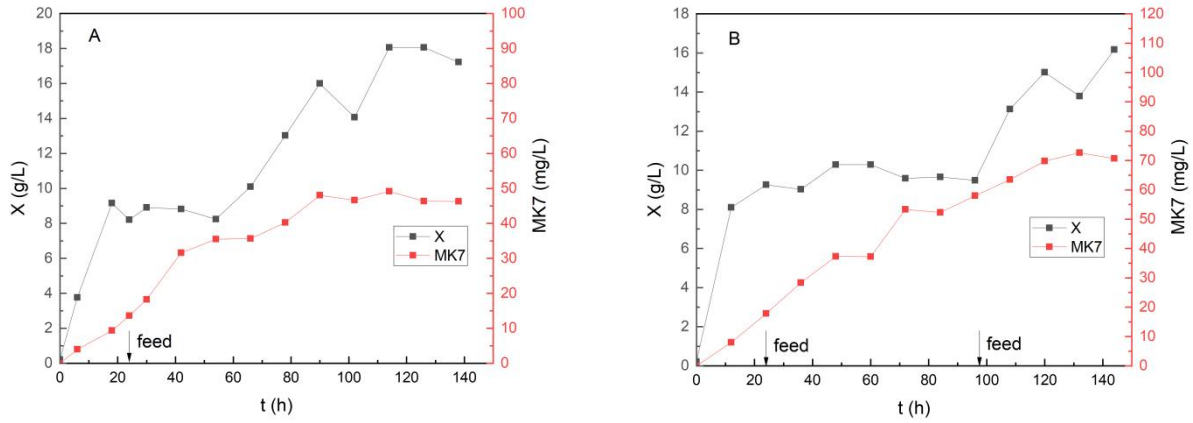

Fig. 5 Process curves of microbial growth and MK-7 accumulation during MK-7 fermentation in the 1-ton bioreactor (A) The first batch (B) The second batch

4. Conclusions

The fermentation characteristics of *Bacillus subtilis* BS018 in producing MK-7 in bioreactor were investigated, integrating fermentation kinetics, and a feeding strategy was developed. The modified Logistic equation, incorporating a microbial death term, can describe the microbial growth process in the non-fed batch fermentation. The non-growth-associated form of the Luedeking-Piret equation effectively modeled the accumulation curve of the secondary metabolite MK7. In the 5 L bioreactor, the feeding strategy of adding 10 g/L (grams of glycerol per liter of fermentation broth) glycerol at 24 hours of fermentation achieved an MK-7 yield of 37.51 mg/L, representing a 1.72-fold increase compared to the non-fed batch. In the 1-ton bioreactor, adding 10 g/L glycerol at both 24 hours and 96 hours of fermentation resulted in an MK-7 yield of 72.65 mg/L.

Acknowledgements

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