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Full Length Research Paper

Optimization of a fed-batch fermentation process for production of bleomycin by *Streptomyces mobaraensis* ATCC 15003

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The objective of this work was to optimize inoculum size and pH for rapid production of bleomycin by *Streptomyces mobaraensis* ATCC 15003 by fed-batch fermentation. In both batch- and fed-batch fermentations, neither production period for bleomycin by *S. mobaraensis* nor the amount of BLM were affected by increasing the inoculum size from regular 10 to 30% (v/v) level. A fed-batch bioreactor not only shortened the lag phase of BLM production from 114 to 60 h, but also fed-batch fermentation enhanced BLM production when combined with an appropriate pH profile with no effect on the amount produced. Due to the substrate inhibition that takes place at high levels of carbon source, fed-batch fermentation was proposed as a better alternative for BLM production. The combined effects of batch and fed-batch fermentation and various pH profiles on BLM production in a bioreactor were evaluated. The tested pH profiles included; (1) a constant pH profile at 6.8 (profile1); (2) a constant pH profile with a period of auto-acidification for 72 h (profile2) and (3) a step-wise pH profile with pH adjustment every 24 h (profile3). When profile 3 was applied, fed-batch fermentation enhanced BLM production in the bioreactor and yielded about two-fold higher BLM concentration than the irrespective batch fermentation. On the other hand, constant pH profile or that which included one period of auto-acidification (profiles 1 and 2) resulted in an insignificantly difference BLM production in fed-batch fermentation. Overall, this study suggested that fed-batch fermentation can be successfully used to enhance BLM production in bioreactor especially with fluctuated pH-profile.

Key words: Bleomycin, *Streptomyces mobaraensis*, rapid production, fed-batch fermentation.

INTRODUCTION

A process which employs microorganisms, animal cells and/or plant cells for the production of materials, is a bioprocess. Most biotechnical products are produced by fermentation. In fermentation, the products are formed by catalysts that catalyze their own synthesis. Enzymes are biological catalysts and antibiotics which are produced as secondary metabolites of enzyme fermentation (Saarela et al., 2003).

Fermentations can be operated in batch, fed-batch or continuous reactors. In batch reactor, all components except gaseous substrates such as oxygen, pH-controlling substances and antifoaming agents, are placed in the reactor in the beginning of the fermentation. During the process there is no input nor output flow. In fed-batch process, nothing is removed from the reactor during the process, but one substrate component is added in order to control the reaction rate by its concentration. They are both input and output flows in a continuous process, but the reaction volume is kept constant. When high substrate level inhibits the growth and/or product formation or when undesired components are produced in parallel with a desired product, the fed-batch operation is shown to be superior to both batch and continuous operations (Chen and Hwang, 1990; Aguirre-Ezkauriatza et al., 2010).

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Two basic approaches to the fed-batch fermentation can be used; the constant volume fed-batch culture and the variable volume fed-batch and fixed volume fed-batch; in this type of fed-batch, the limiting substrate is fed without diluting the culture. The culture volume can also be maintained practically constant by feeding the growth limiting substrate in undiluted form, for example, as a very concentrated liquid. Variable volume fed-batch; as the name implies, is one in which the volume changes with the fermentation time due to the substrate fed. The way this volume changes is dependent on the requirements, limitations and objectives of the operator (Hewitt and Nienow, 2007).

Bleomycin (BLM) belongs to a general group of medicines called antineoplastics. It has been used to treat several types of cancer, including cervix and uterus cancer, head and neck cancer, testicle and penile cancer and certain types of lymphoma. Recently, bleomycin also was used for the treatment of animal cancer (Kelly et al., 2010).

In a previous work (Radwan et al., 2010), a new medium containing date syrup for production of bleomycin by *Streptomyces mobaraensis* ATCC 15003 was developed using response surface methodology. The aim of this work was to compare and optimize the batch and fed-batch fermentation processes for the production of bleomycin from *S. mobaraensis* ATCC 15003 by using the previously developed medium. Since the proper pH profiles may be different for batch and fed-batch-fermentations, the combined effects of fed-batch fermentation and various pH profiles on BLM production in the bioreactor were investigated as well.

MATERIALS AND METHODS

Microbial strains

Lyophilized spore suspension of the *S. mobaraensis* (DSM No. 40903 = ATCC 15003) was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). *Bacillus subtilis* (ATCC 6633) was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA).

Culture medium

10 L culture medium was prepared according to the composition previously described by Radwan et al. (2010).

Experimental design

Three different pH-control profiles (Figure 1) were evaluated in a bioreactor using batch and fed-batch fermentations. These profiles were: (1) pH controlled at 6.8 during the entire fermentation period (profile1), (2) pH controlled at 6.8 from 0 to 72 h and then, allowed to drop freely via auto-acidification from 48 to 72 h before been maintained at constant pH of 6.8 until 336 h (profile 2) and (3) pH controlled using a step-wise profile, in which the pH was maintained at 6.8 until 24 h and then, allowed to drop freely and adjusted back to 6.8 every 24 h until 336 h of fermentation (profile 3). For comparison, batch and fed-batch fermentations in the palnktonic-cell reactor were carried out using the pH profile that performed best for fed-batch fermentation in the bioreactor.

BLM fermentation in bioreactor

BLM fermentations were conducted in a 10 L Bioflo 310 fermentor (New Brunswick Scientific, Edison, NJ, USA). The reactor vessel containing the medium was sterilized by autoclaving at 121°C for 35 min, while sugars solutions (Date syrup and nitrogenous components with mineral salts) were sterilized separately by filtration via membrane filter of 0.45 µ and added to the reactor aseptically. The fermentation temperature was adjusted at 30°C and the agitation speed (150 rpm) and aeration rate (1.5 vvm) were automatically controlled and the dissolved oxygen concentration was maintained at above 40% of air saturation. After inoculation with a 5 day culture of *S. mobaraensis* (1% v/v), at least five fermentation runs were performed to determine the growth and production parameters. The pH was controlled at 6.8 by adding 4 N NaOH. For the fed-batch fermentation, it was performed by the continuous feeding of 50% sterile Date syrup solution at the rate of 0.2 g per hour from 0 to 48 h and 0.1 g Date syrup per hour from 48 to 336 h. The volume of the fermentation culture was not adjusted during the fermentation process and was allowed to be reduced by about 1.5 to 2.3 L/run through evaporation at the mentioned temperature. Antifoam (silicone oil 1:10 v/v) at a concentration of 1:10 (v/v) in water was added after 24 h.

Sample analysis

Samples were collected every 1 or 2 h and analyzed for cell density, sugar consumption and BLM production as previously described by Radwan et al. (2010). The samples were centrifuged (3,800 × g, 4°C) for 15 min and filtered through 0.22 micronfilters (13 mm diameter disc filters, Millipore, Bedford, MA, USA).

Suspended biomass

Suspended-cell density was estimated by dry weight using 1 ml sample dried completely at 60°C till 3 similar successive weights were obtained by a sensitive analytical balance (A&D Company, Tokyo, Japan).

Reducing sugar analysis

The fermentation broth was centrifuged at 3,800 × g at 4°C for 15 min and analyzed with the dinitrosalicylic (DNS) colorimetric method.

Statistical analysis

All treatments were replicated three times. The significant difference of the results was evaluated using the generalized linear model (GLM) (with $p < 0.05$) and Tukey's HSD multiple comparison module of Statgraphics plus version 5.1 (Statpoint Technologies, Inc., Warrenton, VA, USA).

RESULTS

For the fermentation processes, neither the period for production of bleomycin by *S. mobaraensis* nor the amount of BLM were affected by increasing the inoculum

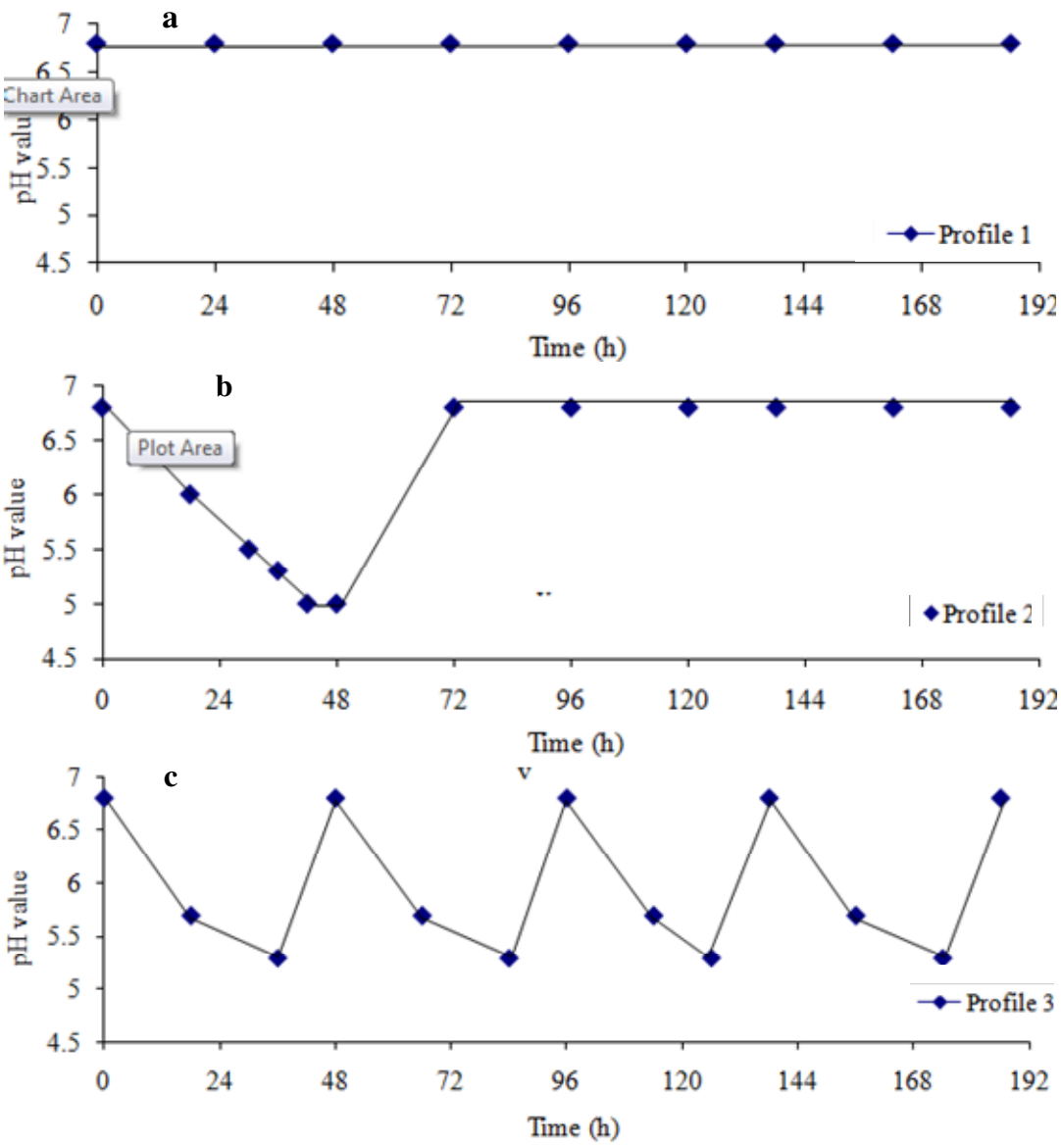

Figure 1. Actual pH profiles of BLM fermentation by *S. mobaraensis* when using: (a) profile 1; (b) profile 2; (c) profile 3.

size from regular 10 to 30% (v/v) level (data not shown).

In this study, fed-batch fermentation was evaluated along with various pH profiles in the bioreactor. Figure 2 shows the amount of biomass (g l^{-1}) in the fermentation broth of both batch and fed batch with profile 1 (constant pH at 6.8). The results clearly indicated that there was no significant difference in terms of the biomass observed between batch and fed-batch cultures or between fermentations with different pH profiles (data not shown). Hence, differences were insignificant; the standard error bars in Figure 2 calculated from the curves obtained by batch and fed-batch runs with the three different pH profiles. The maximum biomass of the fermentation system was 22 g l^{-1} (Figure 2).

In batch fermentation, sugar was depleted after only 120 h whereas, in fed-batch, the sugar decreased gradually even after continues addition of concentrated date syrup solution (Figure 3). It should be noted that, the BLM concentrations also reached its maximum level synchronously at the same time as the depletion of sugars (Figure 4). This evidence of substrate limitation as well as the substrate inhibition present at high sugar level dictated the appropriateness of fed-batch culture for BLM production. Continuous feeding of Date syrup in fed-batch culture prevented the depletion of sugar and eliminated the substrate inhibition by maintaining sugars at a low level (Figure 3).

When profile 1 was used, both biomasses (Figure 3)

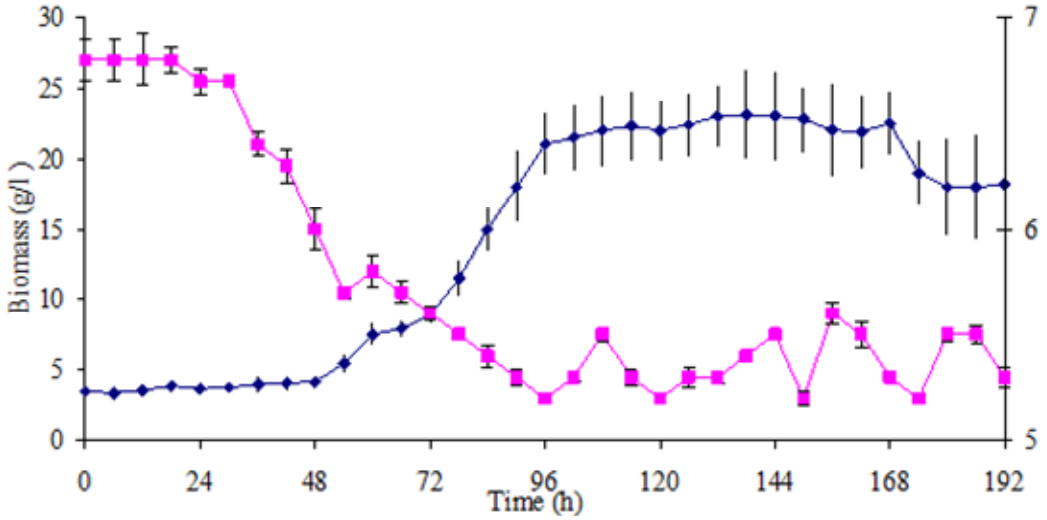

Figure 2. Biomass of *S. mobaraensis* (liquid broth) when free falling pH and pH profiles was used.

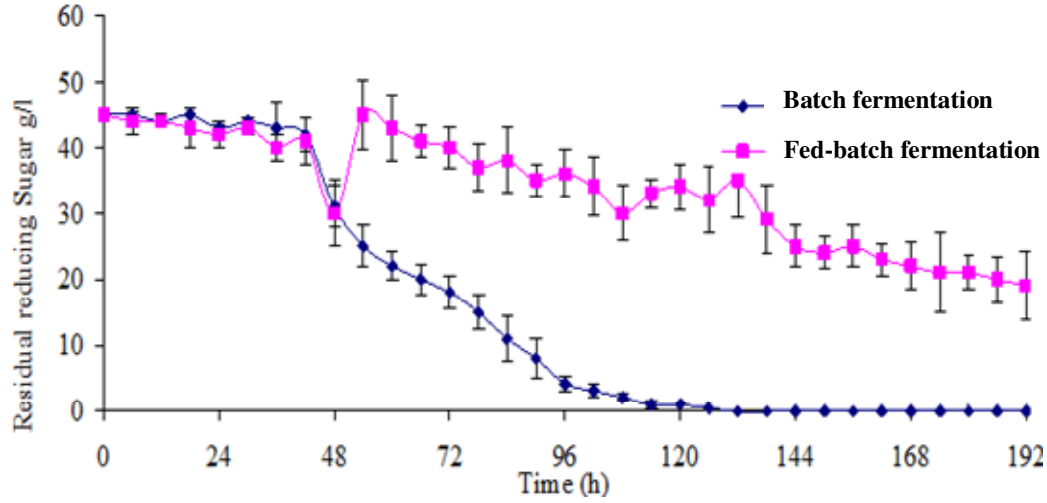

Figure 3. Sugar consumption of *S. mobaraensis* when different culture types pH profile 1 was used.

and BLM concentration (Figure 4) were comparable between batch and fed-batch fermentations until the time that BLM reached its maximum. Therefore, the enhanced BLM production was not a result of higher growth or shifting of metabolism away from acids production, but rather an effect of the fed-batch strategy with its continuous supplementation of sugars. Profile 2, which allows the pH to drop freely via auto acidification after 48 h, was designed to help prevent adsorption of BLM onto the producer cells, as the adsorption was less pronounced in an acidic environment (Yang et al., 1992). According to a previous study (Pongtharangkul and Demirci, 2006), profile 2 significantly enhanced production of microbial products during batch fermentation. Profile 3 was modified from a step-wise pH profile, which was reported to enhance another polypeptide (nisin) production (Cabo et al., 2001; Guerra and Pastrana, 2003 a,b). Since the drop of pH in the early period of fermentation seemed to detrimentally affect growth and second metabolite production (Pongtharangkul and Demirci, 2006), profile 3 was designed so that the pH was maintained at near neutrality (pH = 6.8) up to 24 h before freely dropping via auto acidification. In this way, profile 3 was able to reveal the effect of the fluctuated pH on BLM production.

DISCUSSION

In this study, neither the method of fermentation (batch or fed-batch) nor various pH profiles showed significant difference in terms of biomass of *S. mobaraensis* during the production of BLM. A similar trend was also observed

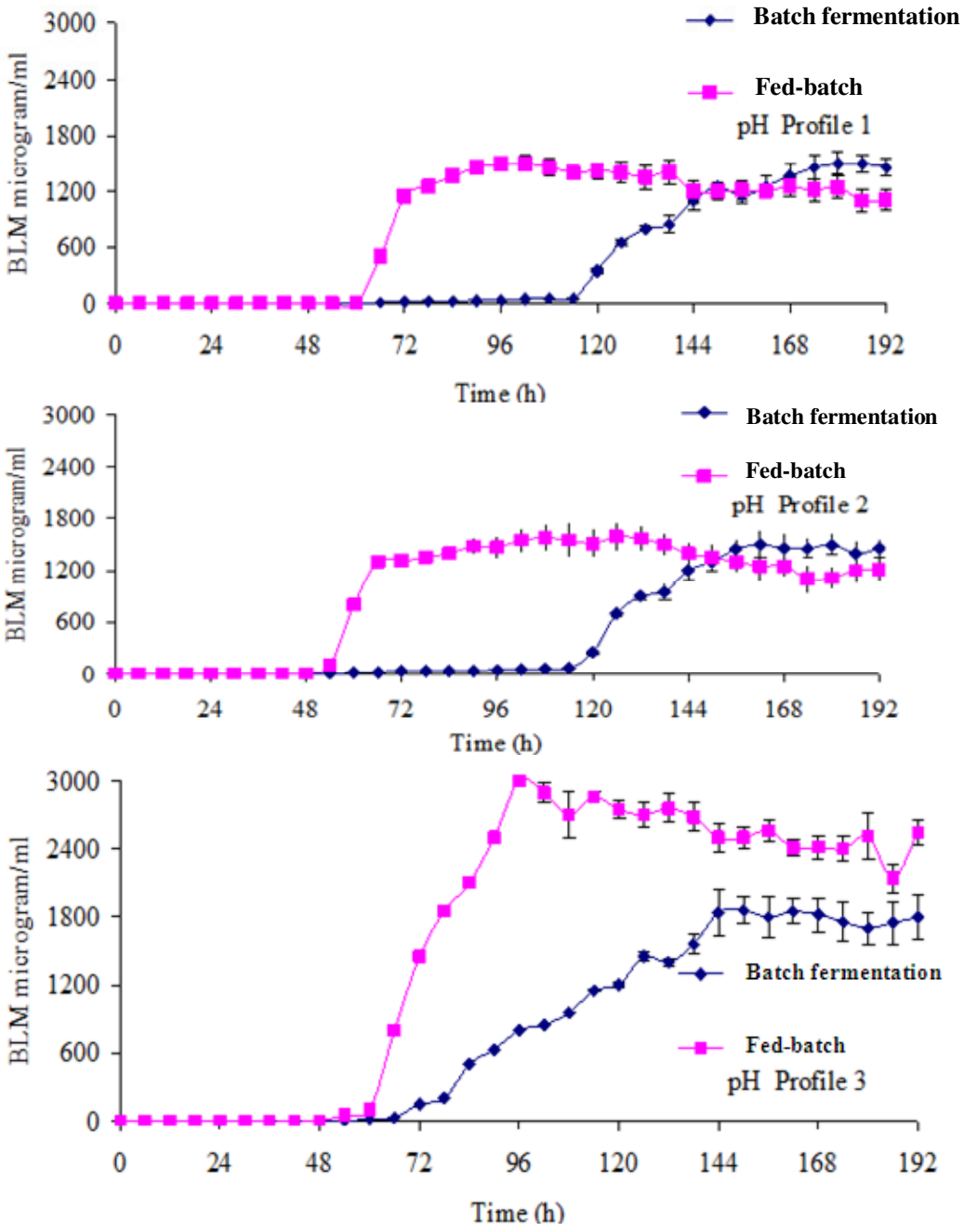

Figure 4. BLM production by batch and fed-batch fermentation with the three pH profiles.

in batch-fermentations for the production of nisin by *Lactococcus lactis* (Pongtharangkul and Demirci, 2006).

The results confirmed the positive influence of the fed-batch culture on BLM production in bioreactors. Continuous feeding of sugars in the fed-batch fermentation enhanced the production of BLM. In order to prevent the dilution of BLM in the culture medium, high concentration of Date syrup (50%) and 4 N NaOH were used. The volume of the culture was reduced during the fermentation by evaporation at the fermentation temperature (30°C). The total volumes of the batch and fed-batch processes were reduced during the fermentation process to 8.5 to 8.2 L. Subsequently, the volume reduction facilitated the concentration of BLM from the culture filtrate.

In fed-batch fermentation, BLM yield was tremendously improved in (1.6 fold) the production of BLM which increased from 26.6 to 66 µg BLM per gram of sugar consumed (profile 3). On the other hand, when pH was maintained at near neutrality (profile 1), the comparable concentration of BLM was attained at 96 h in fed-batch instead of 150 h in batch bioreactor (Figure 4, profile 1).

However, the most efficient nisin production, which is also extra ribosomal poly-peptide, was obtained in fed-batch fermentation with pH controlled according to profile 1 (Pongtharangkul and Demirci, 2006).

Further supplementation of sugar after the BLM level reached its maximum and did not improve BLM production further, but promoted the acidification of the culture medium instead. Although, profile 2 and the step-wise pH profile 3 were reported to enhance nisin production of batch fermentation in both biofilm-reactors (Pongtharangkul and Demirci, 2006) and also in suspended-cell reactors (Cabo et al., 2001), but the toxicity of excess accumulated acidic products in the environment created in both profiles made the profiles highly inappropriate for the fed-batch cultures. Similarly, the application of profile 3 for production of BLM led to the increase in the productivity of the second metabolite. Higher acidity increased the concentration of the non dissociated form of acidic products which can diffuse through the cell membrane (Kashket, 1987) and this is the probable mechanism of BLM diffusion through the cell membrane. BLM outside the cell that is in non-dissociated form is converted into dissociated form as a copper salt due to the near-neutrality environment which enhances salt formation. The elimination of BLM outside the cell leads to the neutralization of the cytoplasm, which can lead to reactivation of metabolism (Mercade et al., 2000) and consequently, the production of BLM. Furthermore, acidic pH helps to prevent adsorption of BLM onto the producer cells, as the adsorption was less pronounced in an acidic environment (Yang et al., 1992).

In conclusion, this study suggested that fed-batch fermentation can be successfully used to enhance BLM production in bioreactors, with a fluctuated pH profile as the most suitable pH profile.

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